# STUDIES ON RABBIT LIVER PHOSPHOGLUCOMUTASE

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by

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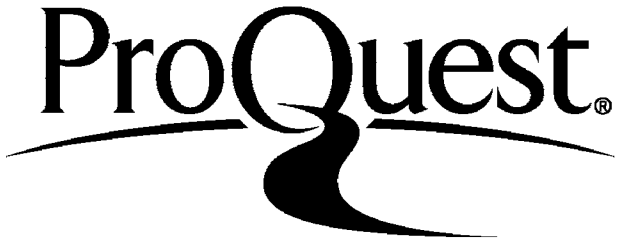

ABSTRACT

Phosphoglucomutases from different sources exhibit a variety of kinetic behaviour though it is probable they all have a common phosphoenzyme mechanism. The variations in behaviour arise partly from an intrinsic diphosphatase activity, which, even when present as a minor component, can affect the initial velocity patterns. Only isotopic induced-transport tests distinguish unequivocally between possible mechanisms. Rabbit liver phosphoglucomutase has a substantial amount of an isoenzyme, which appears to be associated with a high intrinsic diphosphatase activity. This activity may be involved in modulation of the glucose-1,6-diP level in the liver and hence in the regulation of carbohydrate metabolism.

A procedure for the isolation of comparatively undegraded phosphoglucomutase (mol. wt. 67,600) from rabbit liver with high specific activity is described. The purified preparation showed a low diphosphatase activity. Kinetic studies, using a sensitive fluorimetric assay at ionic strength 0.05 mol $l^{-1}$ and $Mg^{++}$ free = 2.0 mM, give a parallel line initial velocity pattern. This behaviour is consistent with the phosphoenzyme mechanism. The liver enzyme indicated a higher $K_m$ for glucose-1,6-diP than many other phosphoglucomutases. Induced transport tests using $^{14}C$- and $^{32}P$-labelled substrates showed that the enzyme can only have a phosphoenzyme mechanism. The isolation of $^{32}P$-labelled phosphoenzyme which rapidly exchanged more

than 95% of its $^{32}P$-label with substrates confirmed that this phosphoenzyme is a true kinetic intermediate in the mutase reaction. Labelled phosphoenzyme contained 0.45-0.6 mol $^{32}P$ $mol^{-1}$ enzyme and its half-life was 47.5 h at 30 $^{o}C$. Evidence is presented that the phosphate is bound to a serine residue of the enzyme.

It is concluded that the low diphosphatase activity associated with the enzyme does not significantly modulate the level of glucose-1,6-diP in liver. Moreover, other factors must be responsible for the failure to isolate a completely phosphorylated phosphoenzyme. Although this phosphoglucomutase has a relatively high $K_m$ for glucose-1,6-diP, the known fluctuations in the level of glucose-1,6-diP in the liver are unlikely to affect phosphoglucomutase activity.

ABBREVIATIONS

| | |
|---|---|
| PGM | Phosphoglucomutase |
| G6PDH | Glucose-6-phosphate dehydrogenase |
| G1P | α-D-glucose-1-phosphate |
| G6P | α-D-glucose-6-phosphate |
| G1P6P | α-D-glucose-1,6-diphosphate |
| F6P | α-D-fructose-6-phosphate |
| F1P6P | α-D-fructose-1,6-diphosphate |
| F2P6P | β-fructose-2,6-diphosphate |
| 3-PGA | D-glycerate-3-phosphate |
| 1,3-PGA | D-glycerate-1,3-diphosphate |
| 2,3-PGA | D-glycerate-2,3-diphosphate |
| PEP | Phosphoenolpyruvate |
| GmP | Glucose monophosphate |
| UDP | Uridine diphosphate |
| UTP | Uridine triphosphate |
| GDP | Guanosine diphosphate |
| GTP | Guanosine triphosphate |
| dTDP | Deoxythymidine diphosphate |
| Tris | 2-amino-2-(hydroxymethyl)propane-1,3-diol |
| Mes | 2-(N-morpholino)ethane-sulphonic acid |
| $NAD^+$ | β-Nicotinamide-adenine dinucleotide |
| NADH | β-Nicotinamide-adenine dinucleotide (reduced) |
| $NADP^+$ | β-Nicotinamide-adenine dinucleotide phosphate |
| NADPH | β-Nicotinamide-adenine dinucleotide phosphate (reduced) |
| EDTA | Ethylenediaminetetra-acetic acid |
| PMSF | Phenylmethylsulphonyl fluoride |

| | |
|---|---|
| DTT | Dithiothreitol |
| SDS | Sodium dodecyl sulphate |
| TEMED | N,N,N',N'-tetramethylethylenediamine |
| NBT | Nitro blue tetrazolium |
| PMS | Phenazine methosulphate |

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# CHAPTER ONE

# INTRODUCTION

## 1.1 HISTORICAL SURVEY

### 1.1.1 INTRODUCTION

Phosphoglucomutase (α-D-glucose-1,6-diphosphate:α-D-glucose-1-phosphate phosphotransferase, E.C. 2.7.5.1.) catalyses the reversible transfer of a phosphate group between the 1- and 6-positions of glucose monophosphates and thereby provides a link between glycogen metabolism and glycolysis. Cori and Cori (1936, 1937) first discovered the existence of this enzyme in muscle during studies of glycogen metabolism. They observed that glycogen breakdown resulted in accumulation of a previously unknown phosphate ester in addition to the well known phosphate ester G6P. This new phosphate ester was later identified as G1P (Cori et al., 1937) and the enzyme which was responsible for its conversion to G6P was characterized.

Phosphoglucomutase (PGM) has since been identified and isolated from various sources. A comprehensive review has been published in which the knowledge of the enzyme up to 1972 is summarized (Ray and Peck, 1972). Apart from the sources mentioned in the above review, PGMs from beef liver (Chiba et al., 1976), and Bacillus subtilis (Maino and Young, 1974a) have also been isolated and investigated. The PGM from rabbit muscle has been most intensively investigated and this enzyme is used as a reference for the study of rabbit liver PGM presented in this thesis. All PGMs have been shown to require a divalent cation and G1P6P for their activity. In addition to these requirements, most PGMs require a metal-chelating agent for maximum activity, since heavy metal ions inhibit the enzyme (1.1.5).

TABLE 1.1: Molecular weights of phosphoglucomutases

| Source | Molecular weight | Reference |
|---|---|---|
| Rabbit muscle | 62,000 | Yankeelov *et al.*, 1964<br>Harshman and Six, 1969 |
| Human muscle | 60,000 | Joshi and Handler, 1969 |
| Flounder muscle | 63,000 | Hashimoto and Handler, 1966 |
| Shark muscle | 63,000 | Hashimoto and Handler, 1966 |
| Beef liver | 64,000 | Chiba *et al.*, 1976 |
| Yeast (*S.cerevisia*) | 69,000 | Hirose *et al.*, 1970 |
| *E.coli* | 62,000-65,000 | Joshi and Handler, 1964 |
| *M.lysodeikticus* | 58,000 | Hanabusa *et al.*, 1966 |
| *B.cereus* | 63,000 | Hanabusa *et al.*, 1966 |
| Potato tuber | 63,000 | Pressey, 1957 |
| Jack bean | 63,000 | Cardini, 1951 |
| *B.subtilis* (dimer) | 130,000 | Maino and Young, 1974b |
| (monomer) | 60,000 | |

### 1.1.2 MOLECULAR WEIGHT AND SUBUNIT STRUCTURE

The molecular weight of rabbit muscle PGM has been reported to be in the range 62,000-67,000. The apparent molecular weight is 64,900 by sedimentation equilibrium analysis, 60,000-70,000 by gel filtration (Harshman and Six, 1969) and 65,000-67,000 by summation of integral residues (Sloane et al., 1964). The most frequently reported value for the molecular weight is 62,000 (see Filmer and Koshland, 1963; Yankeelov et al., 1964).

PGM from rabbit muscle was believed to consist of only a single polypeptide chain (Ray et al., 1972). This conclusion was based on finger-print maps of tryptic digests (Joshi et al., 1967), on the finding of a single metal-binding site on the enzyme (Ray and Peck, 1972), a single phosphorylatable serine residue (Milstein and Milstein, 1968), and one N-terminal amino acid, lysine (Ray and Peck, 1972). However, Duckworth and Sanwal observed in 1972 that the dephosphorylated form of rabbit muscle PGM can be dissociated, in the presence of SDS, into two subunits of identical molecular weight 32,000 and that these subunits have different N-terminal residues (lysine and valine). However, dissociation did not occur in the presence of guanidine HCl. They suggested that the rabbit muscle enzyme is a very stable dimer. Joshi and Lane (1978) contradicted this suggestion and showed that both phosphorylated and dephosphorylated forms of the rabbit muscle enzyme, isolated without the heat treatment step, are stable in SDS at room temperature for several days and thus demonstrated that PGM is a monomer. They observed that even the enzyme isolated

TABLE 1.2: pH optima of phosphoglucomutases

| Source | pH optimum | Reference |
|---|---|---|
| Rabbit muscle | 7.5 | Jagannathan and Luck, 1949 |
| Rabbit liver | 7.5 | Handler et al., 1965 |
| Beef liver | 7.5 | Chiba et al., 1976 |
| Human muscle Form 1 | 7.8 | Joshi and Handler, 1969 |
| Form 2 | 7.5 | |
| Flounder muscle | 7.8 | Hashimoto and Handler, 1966 |
| Shark muscle | 7.6 | Hashimoto and Handler, 1966 |
| Yeast (S.cerevisiae) | 7.5 | McCoy and Najjar, 1959 |
| E.coli | 8.5-9.0 | Joshi and Handler, 1964 |
| M.lysodeikticus | 8.5 | Hanabusa et al., 1966 |
| B.cereus | 8.3 | Hanabusa et al., 1966 |
| B.subtilis | 7.5-8.6 | Maino and Young, 1974a |
| Spinach leaves | 7.8-7.9 | Mühlback and Schnarrenberger, 1978 |

using a heat treatment, appeared as a monomer. They suggested that the appearance of multiple bands after SDS denaturation (as in the Duckworth and Sanwal experiments) could be due to artifacts generated by contaminating proteases. Joshi and Lane (1978) also separated a comparatively undegraded form of rabbit muscle enzyme of slightly higher molecular weight (64,500), using milder conditions in the isolation procedure.

The enzyme isolated from Bacillus subtilis is the only PGM which has been shown to exist as a dimer. The dimer, with a molecular weight of 130,000, has at least one active monomer of molecular weight 60,000 (Maino and Young, 1974b).

PGMs isolated from other sources have molecular weights similar to that of the rabbit muscle enzyme (see Table 1.1).

### 1.1.3 pH OPTIMUM

The pH optima of all PGMs appear to be in the range of 7.4 to 9.0 (see Table 1.2). Jagannathan and Luck (1949a) reported that the pH optimum of rabbit muscle PGM was 7.5. When $V_{max}^{app}$ was plotted against pH, a pH range of 6.5-8.0 was obtained for this enzyme (Ray, 1969). A value of pH 7.4 has been commonly used for rabbit muscle PGM (see Ray and Roscelli, 1964a, 1964b; Britton and Clarke, 1968).

The pH optima of PGMs are summarised in Table 1.2.

### 1.1.4 EFFECTS OF METAL IONS

PGM from rabbit muscle requires a divalent metal ion for its catalytic activity (Milstein, 1961a). Cori et al. (1938)

first discovered and Najjar (1948), Stickland (1949) and Jagannathan and Luck (1949) later confirmed that no significant activity is present in the absence of added divalent cations. Milstein (1961a) demonstrated a small activity in the absence of added metal ion, but this finding was probably due to traces of metal ions; the activity of the enzyme can be reduced almost to zero by the addition of EDTA, owing to chelation and effective removal of the metal ions. In the absence of divalent metal ions, the activity is reported to be $10^{-5}$ of that obtained in the presence of $Mg^{++}$ (Ray and Peck, 1972).

$Mg^{++}$ is the most efficient divalent ion activator of rabbit muscle PGM. Activity of the enzyme rises to a maximum at between 2-4 mM $Mg^{++}$. The results of steady state kinetic studies of Ray et al. (1966) at saturating G1P6P with varying $Mg^{++}$ and G1P, and at saturating G1P with varying $Mg^{++}$ and G1P6P show that $Mg^{++}$ complexes of either G1P or G1P6P are not the substrates for muscle PGM. Hence, above 8 mM $Mg^{++}$, inhibition of PGM occurs owing to accumulation of Mg-G1P and especially Mg-G1P6P complexes.

Milstein (1961a) first showed the existence of an enzyme-magnesium complex in the absence as well as in the presence of substrate and reported the dissociation constant of the enzyme-Mg complex as 0.5 mM. Subsequently Ray et al. (1966) showed that $Mg^{++}$ is bound by the enzyme in both its phosphorylated and dephosphorylated forms as well as by the binary enzyme-substrate complex. They reported that for the phospho and dephospho forms of the enzyme, the $Mg^{++}$ dissociation

constants are 0.025 and 0.2 mM respectively. Ray and Roscelli (1966b) suggested the possibility that the phosphate group is directly involved in binding of $Mg^{++}$ to the enzyme. The role of $Mg^{++}$ in the PGM reaction was considered by Ray and Roscelli (1966b) and Ray et al. (1966). The equilibrium isotope-exchange pattern which they obtained showed that $Mg^{++}$ cannot suppress the dissociation of G1P, G6P and G1P6P from the central complex, nor can G1P, G6P and G1P6P suppress the dissocation of $Mg^{++}$ from this complex. The rate constant for $Mg^{++}$ release from the central complex was 0.004% of the catalytic rate. These results show that a substantial quantity of $Mg^{++}$ remains attached to the enzyme during the whole of the enzymic reaction cycle, so that in deriving kinetic equations for the reaction the enzyme may always be considered to be an enzyme-magnesium complex.

In addition to $Mg^{++}$, a variety of other divalent metal ions elicit an extensive activation relative to the apparent rate in the absence of added metals, though less efficiently than $Mg^{++}$ (Milstein, 1961a), even though most of the ions bind more tenaciously to the enzyme than does $Mg^{++}$ (Ray, 1969). The relative efficiencies of metal activation of the rabbit muscle PGM at pH 7.4 and at 30 $^{o}C$ are: $Mg^{++}$, 100; $Ni^{++}$, 70; $Mn^{++}$, 15; $Co^{++}$, 5; $Cd^{++}$, 0.8; $Ca^{++}$, <0.5; $Zn^{++}$, 0.3 (Ray, 1969; Ray and Peck, 1972). The dissociation constants of the enzyme complex with the most efficient activator, $Mg^{++}$, and the least efficient activator, $Zn^{++}$, are $10^{-5}$ M and $10^{-11}$ M respectively (Ray, 1967).

The efficiency of a given metal ion appears to vary with the rate of dissociation of that metal ion from the enzyme complex; the higher the dissociation constant, the higher the efficiency of the enzyme (Ray and Peck, 1972). In the presence of $Mg^{++}$, other metal ions act as inhibitors. Thus the least efficient activator $Zn^{++}$ is the most effective inhibitor of the PGM. $Zn^{++}$ competes with $Mg^{++}$ for a binding site on both the free enzyme and the free enzyme-substrate complex (Ray, 1967). $Mn^{++}$ also probably binds to the $Mg^{++}$ site of the enzyme and inhibits enzyme to a lower extent than does $Zn^{++}$. $Cu^{++}$, on the other hand, which is a trace contaminant of aqueous solutions, does not activate the PGM in the absence of $Mg^{++}$, but inhibits strongly through binding at sites other than that occupied by $Mg^{++}$ (Long and Ray, 1970). The dissociation constant of $Cu^{++}$ is appreciably smaller than that of $Zn^{++}$ and this ion inhibits rabbit muscle PGM more strongly than does $Zn^{++}$ (Milstein, 1961c). Similarly $Be^{++}$ is also not an activator of PGM; it inhibits by binding at $Mg^{++}$ sites (Hashimoto et al., 1967a), but other metal ions must be removed before $Be^{++}$ inhibition is observed (Ray and Peck, 1972). Jagannathan and Luck (1949a) observed only slight inhibition of rabbit muscle PGM by $Hg^{++}$, whereas Sutherland (1949) reported extensive inhibition by both $Hg^{++}$ and $Ag^{+}$.

$Mg^{++}$ is also the most efficient activator for all other PGMs isolated so far except the enzyme from jack beans which functions more efficiently with $Mn^{++}$ than with $Mg^{++}$ (Yang and Miller, 1963). The optimum $Mg^{++}$ concentration of all PGMs is in the range 0-5 mM; a value of 1 mM has been commonly reported for PGM from a variety of sources. Most of the

work related to the effect of other metal ions has been carried out on rabbit muscle PGM. Little information is available about this behaviour with the other PGMs. McCoy and Najjar (1959) reported that the least efficient activator for rabbit muscle PGM, $Zn^{++}$, is as efficient as $Mg^{++}$ for yeast PGM, but the activation of this enzyme with $Ni^{++}$ and $Mn^{++}$ is similar to that with rabbit muscle enzyme. The beef liver enzyme behaves in a similar way to the rabbit muscle enzyme (Chiba et al., 1976). Activations of PGM from E.coli by $Mn^{++}$ and $Ni^{++}$ were reported to be 10% and 20% respectively as effective as that by $Mg^{++}$ (Joshi and Handler, 1964). $Hg^{++}$ does not inhibit the E.coli enzyme (Joshi and Handler, 1964) and at $10^{-5}$ M concentration it inhibits by only 50% the activity of PGM from B.cereus (Hanabusa et al., 1966).

### 1.1.5 CHELATING AGENT REQUIREMENT

Lehmann (1939) first observed the activation of PGM in crude extracts by glutathione. Najjar (1948) later reported the requirement of cysteine for the activity of pure crystalline rabbit muscle PGM. Jagannathan and Luck (1949a) showed the activation of rabbit muscle PGM by the addition of sulphite and serum albumin, whilst Sutherland (1949) observed activation with histidine, free insulin, 8-hydroxyquinoline, diphenylthiocarbazone and histamine. Milstein (1961b) and Robinson et al.(1965) reported that rabbit muscle PGM requires the presence of a chelating agent with $Mg^{++}$ for maximum activity. The role of the chelating agent was suggested by Milstein (1961b) to be a consequence of the

binding of heavy metals. He also pointed out that cysteine and histidine are better activators of PGM than is EDTA, although EDTA binds more powerfully to the metal ions. The rate of removal of metal ions by EDTA from the enzyme-metal complex is directly related to the enzyme-metal dissociation constant (Ray, 1967). EDTA acts efficiently in removing free metal ions including $Mg^{++}$ which is required for enzyme activity. In contrast, histidine, imidazole and cysteine do not bind $Mg^{++}$ significantly and hence more enzyme is available as the active enzyme-magnesium complex. Furthermore, removal of metal ions from the enzyme is facilitated by these chelators as they compete with the enzyme for the metal and hence once the chelator replaces the enzyme at a co-ordination point of the metal, reassociation is prevented. Thus the metal-ligand complex is the species that dissociates from the enzyme, rather than the metal ion itself (see Ray and Peck, 1972).

The presence of substrate in the assay mixture also affects the dissociation rate of metals. It has been shown for $Zn^{++}$ that binding of G1P to the rabbit muscle phosphoenzyme reduces the rate of $Zn^{++}$ dissociation by a factor of 50 (Ray, 1967). Thus maximum activity in rabbit muscle enzyme is obtained by incubating the enzyme prior to assay with $Mg^{++}$ and a chelating agent in the absence or in the presence of very low concentrations of substrates (Ray and Roscelli, 1966a). However, the presence of $Mg^{++}$ and a chelating agent is also necessary in the assay system. Ray (1967) showed that 0.1 imidazole, 1 mM EDTA at pH 7.5, are highly effective in removing bound metals from rabbit muscle PGM with respect to both rate and extent of removal.

Rabbit muscle PGM activity is stimulated about 5-fold through preincubation with either histidine or imidazole and $Mg^{++}$ in appropriate conditions (Harshman et al., 1965; Robinson et al., 1965). Flounder and Shark muscle enzymes are also activated by this preincubation treatment, though to a smaller extent (1.5-fold) than rabbit muscle enzyme (Hashimoto and Handler, 1965). However, human muscle PGM, as purified by Joshi and Handler (1969), showed lack of stimulation by preincubation. The following explanation for low activation and lack of activation of PGM activity by this treatment has been proposed. Hashimoto and Handler (1965) suggested that the low activation of the enzymes from flounder and shark muscle is probably due to the presence of enzymes in partially phosphorylated forms. Metal ions have a higher binding affinity for the phosphoenzyme than for the dephosphoenzyme. Phosphorylation of the enzyme is believed to induce a conformational change by the addition of the two negative charges to the protein; it has been shown that phosphate ions covalently bound to protein possess high affinity for $Ca^{++}$ and $Mg^{++}$ (cf. Barany et al., 1980). Ray and Peck (1972) also mention that the $Mg^{++}$ binds more tenaciously to the phosphoenzyme than to the dephosphoenzyme. Thus the removal of an inhibitory metal ion would be more difficult with the phosphoenzyme. For human muscle enzyme, which did not require the preincubation step, Joshi and Handler (1969) suggested that the removal of inhibitory ions occurred during purification.

It is also likely that the level of hormones at the time of an animal's death influences the percentage of the enzyme

in the active enzyme-magnesium form. Hashimoto et al. (1967b) showed that an injection of adrenalin before killing the animal increases the requirement for rat liver and muscle enzymes to be preincubated for maximum activity, but the injection of insulin decreases it. Peck and Ray (1971) were able to show that in the muscle of normal rabbits $Mg^{++}$ and $Zn^{++}$ are significantly bound to PGM in vivo and 35% of the enzyme has bound $Mg^{++}$ (active form) and the remainder has $Zn^{++}$ (inactive form). They also observed that insulin shock in starved animals increases the fraction of PGM in the $Mg^{++}$ form from 35 ± 10% to 83 ± 8%. These results indicate that the presence of insulin results in conversion of the inactive form of the enzyme ($Zn^{++}$ bound) to the active ($Mg^{++}$ bound) so that the preincubation of the enzyme with $Mg^{++}$ and chelators does not result in such a large increase in activity. Insulin possibly releases the bound $Mg^{++}$ and increases the $Mg^{++}/Zn^{++}$ ratio to convert $Zn^{++}$ bound enzyme to $Mg^{++}$ bound (Peck and Ray, 1971). However it also chelates $Zn^{++}$ strongly (Sutherland, 1949) and this effect may also be involved. Adrenalin probably has the reverse effect to that of insulin.

None of the known bacterial enzymes have been shown to require preincubation with chelating agent and $Mg^{++}$. PGM from E.coli and M.lysodeikticus require cysteine in the assay mixture for maximum activity, but the enzyme from B.cereus does not (Hanabusa et al., 1966). It has been suggested that the cysteine requirement of the E.coli and M.lysodeikticus enzymes is probably related to the sensitivity of their activity to sulphydryl groups and not to protection against the inhibitory metal ions (Ray and Peck, 1972).

Unlike all other PGMs for which $Zn^{++}$ is an inhibitor, $Zn^{++}$ is an activator of the yeast enzyme (McCoy and Najjar, 1959). Preincubation of yeast enzyme with chelating agent has an inhibitory effect, whereas simultaneous addition of $Mg^{++}$ and chelating agent to the assay mixture activates the enzyme 1.7-fold (Hirose et al., 1970). Activation of this enzyme with chelating agent is not related to protection against $Zn^{++}$, but protection against other metal ions is necessary. Furthermore, citrate has been shown to be a weak inhibitor (see 1.1.6) of PGMs due to formation of a complex with $Mg^{++}$ (Zwarenstein et al., 1967) but the addition of 5 mM citrate in the absence of other chelating agents activates the yeast enzyme 1.25-fold (Hirose et al., 1970). This again, is probably due to chelation of heavy metal ions.

### 1.1.6 EFFECTS OF ANIONS

Many anions are inhibitors of PGM and most of these are competitive with G1P6P (Ray and Peck, 1972). $SO_4^{2-}$ and $Cl^-$ have also been shown to compete with G1P (Ray and Roscelli, 1966a; Ray et al., 1966). Inhibition competitive to G1P is caused by binding to the phosphorylated form of the enzyme and that competitive to G1P6P is brought about by binding of inhibitor to the dephosphorylated form (Ray and Peck, 1972). Polyvalent anions are more potent inhibitors of phosphoglucomutase than the monovalent ones. It was shown by Kovács and Bot (1975) that sulphate inhibits rabbit muscle enzyme more powerfully than chloride and that this enzyme is inhibited by pyrophosphate twice as potently as by orthophosphate.

The effects of various anionic metabolites on PGM from rabbit muscle and beef liver have been examined. 1,3-diPGA, at low concentration activates the rabbit muscle PGM but at $10^{-6}$ M or above, it inhibits, in the presence or absence of G1P6P (Alpers, 1968). The kinetic constants $K_i$ and $K_m$ have been calculated to be the same ($5 \times 10^{-6}$ M); this shows that this metabolite binds at the cofactor-binding site on the enzyme. The activation of PGM in the absence of G1P6P is due to phosphorylation of the nonphosphorylated form and the inhibitory effect is due to competition with G1P6P and displacement of this cofactor from the enzyme (Alpers, 1968). F1P6P also activates the PGM in the absence of G1P6P and it completely phosphorylates the dephospho-enzyme (Lowry and Panonneau, 1969). The $K_m$ for F1P6P is 45 times larger (2.7 μM) than that for G1P6P (0.06 μM). In the presence of G1P6P, F1P6P is an inhibitor for the rabbit muscle enzyme with a $K_i$ of between 5 and 10 μM. The similarities in $K_m$ and $K_i$ values confirm that a single site is involved in the binding of inhibitor and cofactor. Inhibition of rabbit muscle PGM by 3-PGA ($K_i$ = 500 μM), 2,3-diPGA ($K_i$ = 50 μM) and of beef liver PGM by F1P6P ($K_i$ = 60 μM) 1,3-diPGA ($K_i$ = 20 μM), 2,3-diPGA ($K_i$ = 38 μM) and PEP ($K_i$ = 360 μM) (Chiba et al., 1976) have also been reported and these inhibitions are competitive with G1P6P. None of these anions would significantly inhibit animal PGM in vivo as the concentration of G1P6P is normally much in excess of its $K_m$.

Inhibition by various nucleotides has been demonstrated. Kovács and Bot (1965) reported that ATP, ADP, AMP, UTP and

UDP are competitive with G1P6P. Duckworth et al. (1973) observed that GDP, acetyl CoA, GTP and dTDP are non competitive or almost competitive with G1P6P, but are uncompetitive with G1P. They also pointed out that the dephosphorylated form of the enzyme has a greater affinity for nucleotide inhibitors than the phosphorylated form. The E.coli enzyme is somewhat more sensitive to nucleotide inhibition than the rabbit muscle enzyme (Duckworth et al., 1973). This could arise from the fact that the rabbit muscle enzyme is isolated in the completely phosphorylated form whereas the E.coli enzyme is isolated as the completely dephosphorylated form. The inhibition of rabbit liver enzyme would be expected to be greater than that of rabbit muscle PGM since it is not in the completely phosphorylated form (see 1.1.7).

Anions which are metal chelators can inhibit the enzyme in two ways. They can bind $Mg^{++}$ and thereby reduce the availability of this essential cation and secondly they can bind to the enzyme as anions usually in competition with G1P6P. Inhibition by ATP and citrate is of this type (Kovács and Bot 1965; Zwarenstein et al., 1967). In the presence of $Mg^{++}$, ATP and citrate exist as magnesium complexes and compete with G1P6P for a binding site (Beitner et al., 1975; Kovács and Bot, 1965). The ATP inhibition has been shown to be partially relieved by increasing the concentration of G1P6P or $Mg^{++}$. Total abolition of ATP inhibition is achieved by addition of both $Mg^{++}$ and G1P6P in excess (Kovacs and Bot, 1965). Inhibition by citrate is low in comparison to that by ATP and total removal of its inhibition (at 2.5 mM citrate) may be achieved by increasing the concentration of G1P6P

only (Beitner et al., 1975). This suggests that citrate is a less powerful inhibitor than ATP, possibly owing to its lower negative charge.

High concentrations of substrates also inhibit the enzyme. Both G1P and G1P6P inhibition have been reported for rabbit muscle ($K_{i(G1P)}$ = 0.5 mM; $K_{i(G1P6P)}$ = 0.7 mM), flounder muscle ($K_{i(G1P)}$ = 1.0 mM; $K_{i(G1P6P)}$ = 0.7 mM) and shark muscle ($K_{i(G1P)}$ = 1.2 mM; $K_{i(G1P6P)}$ = 0.5 mM) PGMs (Ray et al., 1966; Hashimoto and Handler, 1966). Hanabusa et al. (1966) showed that inhibition by G1P6P is pronounced with E.coli PGM and inhibition by G1P with M.lysodeikticus PGM. With the beef liver enzyme, inhibition only by G1P ($K_{i(G1P)}$ = 5.2 mM) is observed (Chiba et al., 1976). G1P inhibits competitively with G1P6P because G1P binds in a dead-end manner to the dephosphorylated form of the enzyme. Similarly G1P6P inhibits competitively with G1P by dead-end binding to the phosphorylated form of the enzyme (Ray and Peck, 1972).

### 1.1.7 HETEROGENEITY OF PHOSPHOGLUCOMUTASE

Many enzymes have been shown to exist in multiple forms. All multiple forms of an enzyme catalyze the same reaction but they may have different mechanisms of action or kinetic properties (Harris and Hopkinson, 1976). Enzymes performing the same biochemical function may occur in alternative forms not only in different species but also in different organs of the same animal, and even in different parts of the same cells (Wilkinson, 1969). Organisms commonly synthesize many of their enzymes in several different molecular forms to fulfil specialized metabolic requirements.

TABLE 1.3: Separation of phosphoglucomutases by column chromatography

(Joshi et al., 1967)

| Source | Chromatography System | Phosphoglucomutase peak (%) Phosphoenzyme 1 (Form I) | Phosphoenzyme 2 (Form II) | Dephosphoenzyme 3 (Form II) | Dephosphoenzyme 4 (Form I) |
|---|---|---|---|---|---|
| Rabbit (NZW) muscle | CM-Sephadex | 20 | 70 | 10 | 0 |
| | CM-cellulose | 20 | 70 | 10 | 0 |
| Rabbit (NZW) liver | CM-Sephadex | 6 | 54 | 11 | 29 |
| Rabbit muscle[x] | CM-Sephadex | 80 | 15 | 5 | 0 |
| Rabbit liver[x] | CM-Sephadex | 92 | 8 | 0 | 0 |
| Human muscle | CM-Sephadex | 80 | 14 | 6 | 0 |
| Rat muscle (adult) | CM-Sephadex | 64 | 24 | 12 | 0 |
| Rat liver (adult) | CM-Sephadex | 92 | 8 | 1 | 0 |
| Rat liver (newborn) | CM-Sephadex | 96-97 | 3-4 | 0 | 0 |
| Flounder muscle | DEAE-cellulose | 99 | 0 | 0 | 0 |
| Flounder muscle | CM-Sephadex[†] | 99.5 | 0.5 | 0 | 0 |
| Sweet potato | DEAE-Sephadex | 1-2 | 60 | 20 | 18 |
| White potato | DEAE-Sephadex | 30-40 | | 60-70 | |
| Yeast | CM-cellulose | 10 | 85 | 5 | 0 |
| Yeast | CM-cellulose* | 5 | 95 | 0 | 0 |
| E.coli | CM-Sephadex | 99.9 | 0 | 0 | 0 |

x Rabbit strain unknown but other than New Zealand White
† Hashimoto and Handler (1966)
* Tsoi and Douglas (1964)
NZW = New Zealand White

It has been well established that phosphoglucomutase exists in multiple forms. The enzymes from human muscle (Hopkinson and Harris, 1965, 1966), rabbit muscle (Dawson and Mitchell, 1969; Joshi et al., 1967; Scopes, 1968; Duckworth and Sanwal, 1972), rabbit liver (Joshi et al, 1967), rat muscle and adipose tissue (Beitner et al., 1975), flounder muscle, E.coli (Joshi et al., 1967) yeast (Tsoi and Douglas, 1964), spinach leaves (Mühlback et al., 1978) and potato tuber (Kahl et al., 1973) all show heterogeneity.

Dawson and Mitchell (1969) proposed three possible origins for heterogeneity in PGMs. Heterogeneity may arise from the existence of the enzyme in phospho and dephospho forms (see also 1.1.8). These forms may be separated by CM-cellulose chromatography. Yankeelov et al. (1964) first separated rabbit muscle PGM in phospho and dephospho forms by this method. Joshi et al. (1967) separated PGM from various sources using ion-exchange column chromatography (see Table 1.3). PGM activity was eluted with a salt gradient in 2-4 peaks depending upon the source of the enzyme. The first eluted peak was designated as form I, and peak 2 as form II. Peaks 3 and 4 were the dephosphorylated froms of forms II and I respectively. In muscle from human, flounder, rat and rabbit (unknown strain) and in liver from rat and rabbit (unknown strain), a major part of the activity was found to be attributable to form I. Form II enzyme activity dominated in only New Zealand White (NZW) rabbit muscle and liver, sweet potato and yeast. Dephosphorylated form I (peak 4) appeared only in NZW rabbit liver and sweet potato. Joshi et al. (1967) showed by amino acid analysis and finger

print maps that the two separable forms (I and II) of PGM appeared to be different in composition. The isoenzyme distribution of the NZW muscle and liver is markedly different from that of the rabbit of unknown strain (see Table 1.3). The significance of this is not clear. However, as NZW rabbits were used for the present study, this isoenzyme pattern only will be considered. Chromatographic separation of phosphoenzyme forms from rabbit muscle and liver, human muscle and yeast shows that the percentage of the enzyme present in the phosphoenzyme form is comparable with the phosphate contents of freshly isolated enzymes (see 1.1.8). Furthermore, these results indicate that the quantities of the two forms of PGM found in rabbit muscle (I, 80%; II, 20%) and rabbit liver (I, 65%; II, 35%) are different, so that there appears to be a difference in their isoenzyme distribution. Of these isoenzymes, form I in the liver appears to hydrolyse readily to yield the dephospho form. Thus, it is possible that rabbit liver PGM has an intrinsic diphosphatase activity which may be responsible for its isolation in a partially phosphorylated form.

Heterogeneity may also arise from the isoenzymes generated by separate gene loci. With PGM, 3 such gene loci ($PGM_1$, $PGM_2$ and $PGM_3$) have been identified which are responsible for generating three separate zones of isoenzymes (Hopkinson and Harris, 1966, 1968). At each locus multiple alleles occur and they determine a characteristic set of 3 to 4 isoenzymes. $PGM_1$ locus isoenzymes from most human tissue contribute 85-95% of total PGM activity and $PGM_2$ and $PGM_3$ loci isoenzymes about 2-15% and 1-2% respectively

(McAlpine et al., 1970b). These three zones of isoenzymes have been found to differ in their electrophoretic mobilities (Spencer et al., 1964; Hopkinson and Harris, 1966, 1968), molecular weights (Monn, 1969; McAlpine et al., 1970c), thermostabilities (McAlpine et al., 1970a), pH optima (Quick et al., 1974) and levels of phosphoribomutase activity (Quick et al., 1972). However, the isoenzymes determined by a single locus only appear to differ in their electrophoretic mobilities. The PGM activity in each set of isoenzymes differs from tissue to tissue, so that, for example, human liver $PGM_1$, $PGM_2$ and $PGM_3$ loci isoenzymes contain approximately 96.7%, 2.4% and 1.0% of the total PGM respectively, whereas in human muscle $PGM_3$ locus isoenzymes have not been located and $PGM_1$ and $PGM_2$ contribute 94.3% and 5.6% of the PGM activity.

Heterogeneity may also be due to the presence of different conformers of the same isoenzyme. Kitto et al. (1966) showed that a number of electrophoretically distinct malic dehydrogenase isoenzymes are similar in their amino acid compositions and catalytic characteristics and are quite similar in their reactions with specific antibodies. The isoenzymes have distinct three-dimensional conformations which are inter-convertible by urea denaturation or by iodination. Dawson and Mitchell (1969) separated multiple forms of phosphoglucomutase from various mammalian tissues into 4, 5 or 6 equally spaced bands on starch gel. They also used a DEAE-cellulose column to separate the isoenzymes but could not compare the results of the two techniques. All isoenzymes obtained from one source were found to be similar

in their pH optima, heat stabilities and catalytic activities. No differences were found between the isoenzyme patterns after dialysis of samples against G1P (to yield dephosphorylated form) or G1P6P (to yield phosphorylated form). However, differences in the electrophoretic mobilities of the isoenzymes were observed after addition of p-mercuribenzoate or partial iodination. It was therefore suggested that the isoenzymes, which are separable on starch gel as well as on DEAE-cellulose, are inter-convertible conformational isomers (conformers) of PGM with identical polypeptide structure. This type of heterogeneity arises mainly from disulphide bridging or modification of sulphydryl groups. However, the different conformers of a single polypeptide chain could also be the result of changes in various types of electrostatic bonding.

The results of Dawson and Mitchell (1969) indicate that this heterogeneity in PGM can largely be eliminated by protecting the sulphydryl groups of the enzyme during isoenzyme separation. If the sulphydryl groups are so protected, the heterogeneity of PGM would be expected to be due only to the presence of phospho and dephospho forms and to derivation from different gene loci. However, it should be noted that $PGM_1$ locus isoenzymes dominate in most mammalian tissues (85-95% of total PGM activity). The main source of heterogeneity would therefore be due to gene locus $PGM_1$ isoenzymes and the presence of phospho and dephospho forms.

### 1.1.8 PHOSPHO AND DEPHOSPHO FORMS

The presence of phosphate bound to rabbit muscle PGM was first demonstrated by Jagannathan and Luck (1949b). They showed that this phosphate may readily be exchanged with phosphate in the substrate and thus must be involved in the reaction cycle of the enzyme. Anderson and Jolles (1957) and Kennedy and Koshland (1957) reported that phosphate is covalently bound to a serine residue of rabbit muscle PGM. Subsequently Milstein and Sanger (1961) showed the presence of a single serine residue in the rabbit muscle enzyme to which the phosphate was bound. The sequence of the active site of the enzyme around the serine phosphate appeared to be Thr-Ala-SerP-His-Asp. This sequence was later confirmed by Milstein and Milstein (1968). The same sequence has also been reported for PGM from rat liver and yeast (Milstein, 1961d), flounder muscle (Hashimoto and Handler, 1966), E.coli (Handler et al., 1965) and M.lysodeikticus (Milstein et al., 1973).

The existence of PGM in both phospho and dephospho forms was recognised by Najjar and Pullman (1954). These forms appear to differ in some of their properties. The dephospho-enzyme is more sensitive to heat (Najjar, 1962; Alpers and Lam, 1969), and some of its sulphydryl groups react more readily with p-hydroxymercuribenzoate than do the corresponding groups in the phosphoenzyme (Bocchini et al., 1967). The optical activity of these two forms is also different. Bocchini et al. (1967) provided evidence that phospho and dephospho enzymes differ in their three dimensional conformations.

TABLE 1.4: Phosphate contents of freshly isolated phosphoglucomutases

| Source | mol P $mol^{-1}$ enzyme | Reference |
|---|---|---|
| Rabbit muscle | 1.0 | Yankeelov *et al.*, 1964<br>Sidbury and Najjar, 1967 |
| Human muscle | 1.0 | Joshi and Handler, 1969 |
| Flounder muscle | 0.3-0.5 | Hashimoto and Handler, 1966 |
| Shark muscle | 0.4-0.6 | Hashimoto and Handler, 1966 |
| Rabbit liver (NZW) | 0.5-0.7 | Handler *et al.*, 1965 |
| Yeast (*S.cerevisiae*) | 1.0 | McCoy and Najjar, 1959 |
| *E.coli* | 0 | Joshi and Handler, 1966 |
| *M.lysodeikticus* | 0.04 | Hanabusa *et al.*, 1966 |
| *B.cereus* | 0 | Hanabusa *et al.*, 1966 |

PGMs may be isolated as fully phosphorylated forms, partially phosphorylated forms or completely non-phosphorylated forms, depending upon the source of the enzyme. When freshly isolated, PGMs from rabbit muscle (Yankeelov et al., 1964) human muscle (Joshi and Handler, 1969) and yeast (McCoy and Najjar, 1959) contain an equivalent amount of phosphate. Enzyme from flounder and shark muscle (Hashimoto and Handler, 1966) and rabbit liver (Handler et al., 1965) are isolated in partially phosphorylated forms and contain less than 0.7 mol P per mol enzyme (see Table 1.4). The enzymes from E.coli (Joshi and Handler, 1964), M.lysodeikticus and B.cereus (Hanabusa et al., 1966) are devoid of phosphate. However, labelled phosphoenzymes have been prepared from the former two sources (Joshi and Handler, 1964; Clarke et al., 1974). These data of phosphate of freshly isolated enzymes from rabbit muscle and liver, human muscle and yeast are comparable with those of phosphoenzymes separated chromatographically by Joshi et al. (1967) (see Table 1.3). Chromatographic separation (Table 1.3) of 99.9% of the enzyme as a phosphoenzyme from E.coli and flounder muscle suggests that these enzymes can also be isolated in completely phosphorylated forms. However, the rabbit liver PGM has been found to contain only 0.5-0.7 mol P per mol enzyme and the separation on CM-Sephadex gave only 60% of the enzyme in the phospho form (Table 1.3). This suggests that the separation of this enzyme in completely phosphorylated form may not be possible and that its phosphoenzyme may be unstable.

The rate of phosphate transfer from rabbit muscle phosphoenzyme to water is $3 \times 10^{-8}$ $s^{-1}$ in the absence of substrate

and under optimum reaction conditions. Thus the rate of transfer of phosphate from the enzyme to the 6-position of glucose is $3 \times 10^{10}$ faster ($1000 s^{-1}$) than that to water. This rate enhancement represents a "substrate-induced rate effect" (Ray and Long, 1976). The presence of other substrate-like compounds can also accelerate the transfer of phosphate from the enzyme. For example, xylose-1-phosphate increases the transfer of phosphate to water by about $2 \times 10^5$ fold, because of the similarities in the structure of G1P and this substance. For steric reasons, no hydroxy group in the xylose molecule can accept phosphate from the enzyme (Ray et al., 1976). Even in the absence of an activator like xylose-1-phosphate, the rate of phosphate transfer to water is about 300 times faster than the extrapolated value for the hydrolysis of the serine phosphate at pH 7.5, suggesting that the enzyme itself catalyses the hydrolytic reaction (Ray et al., 1976). For the flounder and shark muscle enzymes the rate of transfer of phosphate to water in the absence of any sugar phosphate activator is much more rapid than that observed for rabbit muscle PGM, so that these enzymes appear to act as specific diphosphatases (Hashimoto and Handler, 1966). Thus, it is possible that in rabbit liver the low concentration of phosphoenzyme is due to an instability associated with its function as a diphosphatase.

### 1.1.9 KINETICS AND REACTION MECHANISM

Early work on the reaction mechanism of PGM was conducted on the rabbit muscle enzyme. Leloir et al. (1948) showed

that this enzyme requires G1P6P for the efficient conversion of G1P to G6P. Najjar and Pullman (1954) isolated a phosphoenzyme and showed that the rabbit muscle enzyme exists in both phosphorylated and dephosphorylated forms. Phosphoenzyme reacts with glucose monophosphate but not with glucose diphosphate, whilst the dephospho form reacts with glucose diphosphate but not with glucose monophosphate. Najjar and Pullman (1954) proposed a mechanism in which both phosphorylated and dephosphorylated forms were involved (ping-pong mechanism). The overall reaction requires two steps (scheme 1):

$$EP + G1P \rightleftharpoons E + G1P6P \qquad (1)$$

$$E + G1P6P \rightleftharpoons EP + G6P \qquad (2)$$

EP: phosphoenzyme

E: dephosphoenzyme

Najjar and Pullman (1954) also pointed out that the EP which combines with G1P may be different from that which combines with G6P. This mechanism should give a parallel-line initial-velocity pattern.

An alternative one step sequential addition mechanism was suggested by Cleland (1963) for the PGM reaction based on Bodansky's (1961) substrate-velocity measurements (scheme 2):

$$E + G1P + G1P6P \rightleftharpoons E + G1P6P + G6P \qquad (3)$$

In this mechanism the dephosphoenzyme facilitates a direct transfer of phosphate between G1P6P and either G1P or G6P. This reaction sequence should produce an intersecting-line initial-velocity pattern.

Subsequently Ray and Roscelli (1964a, 1964b) investigated the kinetics of rabbit muscle phosphoglucomutase in order to discriminate between the alternative mechanisms. They obtained a parallel-line pattern and their results indicated the involvement of both phosphorylated and dephosphorylated forms of the enzyme in a ping-pong like pathway similar to that previously suggested by Najjar and Pullman (1954). They also suggested that free G1P6P is neither an obligatory nor an important reaction intermediate, since treatment of $^{32}$P-phosphoenzyme with excess of G1P and G1P6P resulted in the appearance of label primarily in the G6P fraction. The reaction sequence of Najjar and Pullman (1954) must therefore be modified as shown below (scheme 3):

$$EP + G1P \rightleftharpoons \begin{bmatrix} EP\cdot G1P \\ E\cdot G1P6P \\ EP\cdot G6P \end{bmatrix} \rightleftharpoons EP + G6P$$

$$E + G1P6P \qquad \text{——————} \quad (4)$$

Assuming that the E.G1P6P is an obligatory intermediate and is in equilbrium with EP-monophosphates, this reaction sequence can be rationalized if the release of G1P6P from the central complex is slower than that of G6P. The rate of release of G6P must be 20 times the rate of release of G1P6P to account for the experimental results. In this reaction sequence the dissociation and association of G1P6P need not occur in each catalytic cycle as required by the scheme of Najjar and Pullman (scheme 1) and the EP-G1P complex must isomerize into EP-G6P. The function of G1P6P is to maintain the enzyme in the active phospho enzyme form. This mechanism should also give a parallel-line initial-velocity pattern.

The kinetic behaviour of PGM from flounder and shark muscle (Hashimoto and Handler, 1966), beef liver (Chiba et al., 1976), E.coli, B.cereus, M.lysodeikticus (Hanabusa et al., 1966) and yeast have also been investigated. Flounder and shark muscle showed a parallel-line initial-velocity pattern characteristic of the ping-pong mechanism. However, the $K_m$'s of G1P and G1P6P were greater than for the rabbit muscle enzyme. A diphosphatase activity was shown to be associated with these two enzymes and the phosphoenzymes were found to be comparatively unstable phosphoenzymes. Hashimoto and Handler (1966) confirmed the observations of Ray and Roscelli (1964a, 1964b) that incorporation of $^{32}P$ into G1P6P was extremely slow from labelled enzyme and that the results suggested that dissociation of G1P6P from the central complex was even slower. However, in the preparation of the phosphoenzyme, they found 1 mol of G1P6P bound to 1 mol of enzyme. Because of the difficulty in reconciling this finding with the phosphoenzyme mechanism, they suggested that the reactions of flounder and shark muscle PGMs might occur via Cleland's sequential mechanism (scheme 2) and the phosphoenzyme might represent an artifact of the isolation procedure.

Beef liver PGM also exhibited the parallel-line initial-velocity pattern characterstic of the ping-pong mechanism (Chiba et al., 1976). Similarly E.coli PGM gave a parallel-line initial-velocity pattern and the enzyme forms a phosphoenzyme. However, this initial-velocity pattern was found to be distorted by a marked competitive inhibition of G1P6P by G1P. The interpretation of the pattern was therefore uncertain.

Hanabusa et al. (1966) failed to isolate a phosphoenzyme from B.cereus and M.lysodeikticus and could not detect any glucose monophosphate formation when G1P6P was added to substrate quantities of these enzymes, as would be expected if phosphoenzyme formation occurred. They obtained converging rather than parallel lines in initial-velocity plots and took this to mean that both substrate and cofactor had to be present simultaneously on the enzyme molecule. They proposed, therefore, that these enzymes catalyzed an intramolecular transfer by a mechanism similar to that proposed by Cleland (scheme 2). However, a phosphoenzyme mechanism for the latter enzyme has subsequently been established by induced-transport tests (see later).

With yeast PGM, conflicting results have been reported. An intersecting-line initial-velocity pattern characteristic of a sequential mechanism was obtained by Hirose et al. (1970) and a random sequential mechanism was proposed. In contrast, the existence of a phosphoenzyme was demonstrated by showing the incorporation of $^{32}P$-label from [$^{32}P$] G6P in the presence of G1P6P (Kennedy and Koshland, 1957; Yankeelov et al., 1964). However, the kinetic competence of the phosphoenzyme mechanism was not demonstrated.

It was propsed by Cleland (1963) that the mechanism of enzyme action can be distinguished from initial-velocity patterns. A ping-pong mechanism gives a parallel-line initial-velocity pattern whereas a sequential mechanism exhibits intersecting-line pattern. For phosphomutases this kinetic distinction between the two mechanisms is, however, unreliable. An enzyme with a phosphoenzyme mechanism may produce an intersecting line pattern if the

phosphoenzyme is unstable and the rate of the phosphorylation of the enzyme is comparable with the rate of hydrolysis of the phosphoenzyme. PGM from animal sources has some diphosphatase activity (see 1.1.9) and, even if this is small compared with the mutase activity, it could be responsible for the production of an intersecting-line initial-velocity pattern. Similarly, the sequential mechanism, which should give an intersecting-line initial-velocity pattern, can, when realistic mechanisms are considered, give rise to parallel-line patterns (Clarke and Britton, unpublished results). PGMs from rabbit muscle (Ray and Roscelli 1964a, 1964b), flounder and shark muscle (Hashimoto and Handler, 1966), human muscle (Joshi and Handler, 1969), beef liver (Chiba et al., 1976), rabbit liver (Joshi et al., 1967), E.coli (Joshi and Handler, 1964), M.lysodeikticus (Clarke et al., 1974) and yeast (McCoy and Najjar, 1959) all exist as phosphoenzymes but some of these appear to hydrolyse relative rapidly. These are the phosphoenzymes from flounder and shark muscle (Hashimoto and Handler, 1966) and rabbit liver (see 1.1.8). The phosphoenzyme from B.cereus probably also hydrolyzes spontaneously though its existence has yet to be confirmed. Among these enzymes only those from yeast, M.lysodeikticus and B.cereus produce intersecting-line initial-velocity patterns, even though the phosphoenzyme of yeast seems to be stable (see 1.1.8).

It appears, therefore, that there is not a 1:1 correspondence between the existence of phosphoenzymes and the form of the initial-velocity pattern. Even when phosphoenzymes have been isolated it is necessary to demonstrate their kinetic competence before assigning a phosphoenzyme mechanism to the enzyme concerned.

Britton and Clarke (1968) introduced induced-transport tests to establish the mechanism of reaction of PGM. These tests depend upon the ratio of the fluxes from G1P to G6P and from G6P to G1P when measured using $^{32}P$-labelled substrates or $^{14}C$-labelled substrates. These authors proposed three possible reaction mechanisms for PGMs and showed that induced transport distinguishes unequivocally between them. These mechanisms are:

1. Phosphoenzyme mechanism (Mechanism 1)

$$\begin{matrix} G1P + EP_1 \\ \uparrow\downarrow \\ G6P + EP_2 \end{matrix} \rightleftharpoons E.G1P6P \rightleftharpoons E + G1P6P \qquad (5)$$

This phosphoenzyme mechanism is similar to scheme 1 and should give a parallel-line initial-velocity pattern. In this mechanism phosphate from G1P is transferred to the enzyme whilst phosphate attached to the enzyme is transferred to the 6-position of the substrate. Thus two reaction cycles are needed to transfer phosphate from G1P to G6P. $^{14}C$ glucose is transferred directly from substrate to product by each catalytic cycle. It is possible in this phosphoenzyme mechanism that the intermediate is either a true phosphoenzyme or an enzyme-G1P6P complex performing the role of phosphoenzyme. The latter possibility was referred to as Mechanism 1[1], and this should also give a parallel-line initial-velocity pattern.

2. Intramolecular transfer mechanism (Mechanism 2)

$$\begin{matrix} G1P + E \\ | \\ 6 \end{matrix} \rightleftharpoons \overset{6}{E.G1P} \rightleftharpoons \begin{matrix} E + G1P \\ \quad | \\ \quad 1 \end{matrix} \qquad (6)$$

This mechanism shows the direct transfer of phosphate from the 1-position to the 6-position. The role of G1P6P is to maintain the enzyme in an active configuration. It does not, however, participate directly in the reaction and labelling must enter G1P6P via side reactions. One reaction cycle is required for transfer of both $^{32}P$ and $^{14}C$ from G1P to G6P.

3. Intermolecular transfer mechanism (Mechanism 3)

$$E + G1P6P \rightleftharpoons \left[E.G1P6P\right]_1 \quad (7)$$

$$G1P + \left[E.G1P6P\right]_1 \rightleftharpoons E.G1P6P.G1P \rightleftharpoons \left[E.G1P6P\right]_2 + G6P \quad (8)$$

$$\left[E.G1P6P\right]_2 \rightleftharpoons \left[E.G1P6P\right]_1 \quad (9)$$

This mechanism is similar to scheme 2 proposed by Cleland (1963) and shows the indirect transfer of phosphate involving G1P6P. Two reaction cycles are required to transfer $^{14}C$-label from G1P to G6P and three to transfer $^{32}P$-label. In this mechanism G1P6P directly participates in the reaction. In the presence of excess G1P6P, the dissociation of enzyme-G1P6P can be ignored.

In all of the above three mechanisms, the active form of the enzyme may exist in two isomeric forms.

Britton and Clarke (1968) confirmed that the rabbit muscle PGM has a phosphoenzyme mechanism as proposed by Najjar and Pullman (1954) and Ray and Roscelli (1964a, 1964b). The mechanism of M.lysodeikticus PGM has also been investigated by the induced-transport technique (Clarke and Britton, 1974). The enzyme was previously believed to have a sequential

addition mechanism (Hanabusa et al., 1966) but these tests supported a phosphoenzyme mechanism. Evidence has also been provided that the enzyme has a stable phosphoenzyme and that phosphate is attached to a serine with a sequence around the serine similar to that in the rabbit muscle enzyme (Clarke et al., 1974; Milstein et al., 1973). That the phosphoenzyme is a true kinetic intermediate of the PGM from the M.lysodeikticus reactions was confirmed by incubation of $^{32}$P-labelled enzyme with G6P and G1P6P and the observation of rapid exchange of label into the G6P fraction (Clarke et al., 1974). These results are in contrast to the observation of Hanabusa et al. (1966). Though it is possible that small errors in the determination of initial velocities at low concentrations of G1P and G1P6P could have led to the production of the apparently converging-line initial-velocity pattern observed by Hanabusa et al. (1966).

Available data suggest a general phosphoenzyme mechanism for all PGMs, but this has only been unequivocally established for the enzymes from rabbit muscle and M.lysodeikticus with the aid of the induced-transport tests. Conflicting results for the kinetic behaviour of the enzyme and the mechanism of action have been reported for the enzymes from yeast, M.lysodeikticus (see above) and Neurospora crassa (in Britton and Clarke, 1968). Enzyme from wild forms of N.crassa gives an intersecting-line initial-velocity pattern whereas that from a mutated strain produces a parallel-line pattern, though enzymes from a single species would be expected to show similar patterns. These data suggest an initial-velocity pattern could lead to an incorrect assignment of mechanism, especially if the enzyme has a high diphosphatase activity as is possible for rabbit liver PGM.

### 1.1.10 FUNCTION OF G1P6P AND CONTROL OF ITS LEVEL IN TISSUES

G1P6P is required for activity by all PGMs isolated up to the present (see 1.1.9). In addition, it acts as a potent regulator for several key enzymes in carbohydrate metabolism (Beitner , 1979 ). In particular G1P6P is a strong activator for phosphofructokinase from skeletal muscle, brain,red blood cells, heart and adipose tissue. It is also a potent inhibitor of both hexokinases type I (predominant form in brain) and type II (predominant form in muscle). G1P6P also stimulates pyruvate kinase from liver and red blood cells (Koster et al., 1972). Furthermore, it exerts an inhibitory effect on the activity of liver fructose-1,6-diphosphatase, one of the key enzymes in gluconeogenesis (Marcus, 1976). 6-phosphogluconate dehydrogenase, the first enzyme of the pentose phosphate pathway, is also inhibited (Beitner and Nordenberg, 1979).

Although Passonneau et al. (1969) reported that the concentration of G1P6P decreases in the rat brain under ischemia, it does not appear to change significantly in liver or muscle during starvation or electrical stimulation. Later Beitner and coworkers showed that the concentration of G1P6P in muscle fluctuates under different physiological and hormonal conditions. For example, during growth the concentration of G1P6P increases from 6 to 18 μM in rat muscle (Beitner et al., 1978) and after adrenalin shock increases from 6.9 to 9.5 μM in rat diaphragm muscle (Beitner, Heberman and Nordenber, 1978). In starvation its concentration decreases from 6.9 to 3.7 μM in rat diaphragm muscle and in Muscular

Dystrophy from 41.5 to 18.7 μM in rat muscle. Thus concentration of G1P6P fluctuates from 50% to 300% of its normal value under various conditions.

The level of G1P6P in all tissues except perhaps in brain appear to correspond to the physiological levels required for regulation of the key enzymes of glycolysis rather than for PGM activity. In all cases studied the concentration of G1P6P appears to be greatly in excess of its $K_m$, so that PGM activity should be invariant.

The level of G1P6P will depend upon the relative rates of synthesis and degradation. Several mechanisms have been proposed to account for its synthesis. These are:

1. $$2\ \text{G1P} \xrightleftharpoons{\text{dismutase}} \text{G1P6P} + \text{glucose}$$

The synthesis by this reaction was reported in E.coli (Leloir, 1949) and rabbit muscle (Sidbury et al., 1956), but the glucose-1-phosphate dismutase reaction has still to be confirmed (Passonneau et al., 1969). The reaction, however, is found not to be responsible for synthesis of G1P6P in beef liver (Ueda et al., 1976).

2. $$\text{G1P} + \text{ATP} \xrightarrow{\text{PFK}} \text{G1P6P} + \text{ADP}$$

This reaction is catalysed by phosphofructokinase (PFK) as a side reaction and has been observed in rabbit muscle (Eyer et al., 1971), yeast (Paladini et al., 1949) and rat liver (Levey and Alpers, 1965) but not in beef liver (Ueda et al., 1976).

3. $$\text{G1P} + \text{1,3-diPGA} \xrightarrow[\text{synthetase}]{\text{PGM or G1P6P}} \text{G1P6P} + \text{3-PGA}$$

PGMs from rabbit muscle, beef liver (Hirose et al., 1976) and mouse brain (Rose et al., 1957) appear to catalyse this reaction. This G1P6P synthesis activity can be separated from the major peak of PGM activity from mouse brain (Rose et al., 1975) and beef liver (Ueda et al., 1976) on a DEAE-cellulose or DEAE-Sephadex. However, the PGM activity still remains in the G1P6P synthesis activity peak and the enzyme concerned was named G1P6P synthetase by Rose et al. (1975). It was suggested that this was one of the PGM isoenzymes which has a high ratio of G1P6P synthesis activity to mutase activity.

4. $$\text{G1P} + \text{F1P6P} \underset{}{\overset{\text{PGM}}{\rightleftharpoons}} \text{G1P6P} + \text{F6P}$$

Purified PGM from yeast (Hirose et al., 1972), beef liver (Hirose et al., 1976) and partially purified enzyme from muscle (Passonneau et al., 1969) have found to catalyse synthesis by this reaction. The ratio of G1P6P synthesis to mutase activity is $1.5 \times 10^{-4}$ in rat muscle and $1.1 \times 10^{-4}$ in beef liver. Hirose et al. (1976) showed that the ratio of G1P6P synthesis and mutase activities does not change during purification of PGM from beef liver; synthesis of G1P6P by this reaction depends solely on PGM activity.

Biosynthesis of G1P6P appears to be possible by all four of the above reactions, but the way in which the concentration of G1P6P is regulated in different tissues has not been established. Neither has it been established which reaction is the main pathway of the synthesis. However, from the results of Ueda et al. (1976) it appears that most of the G1P6P synthesis in beef liver occurs by reaction 4.

Degradation of G1P6P must also be a controlling factor in the level of G1P6P in tissues. Beitner and Cohen (1980) reported that diphosphatase activity undergoes changes in muscle under various conditions which are inversely related to the level of G1P6P. This activity is partly due to a specific diphosphatase which has been reported in various rat tissues (Hashimoto and Yoshikawa, 1967). The diphosphatase activity is highest in rat liver but very low in muscle. Diphosphatase activity has also been detected in beef liver by Ueda et al. (1976), who showed that the enzyme is localised in the mitochondrial and microsomal fractions of the cell homogenate. Their results are consistent with the findings of Hashimoto and Yoshikawa (1967) that diphosphatase activity is much richer in the sediment than in the supernatant fraction after centrifugation of ox liver homogenate at 10,000 g. However, the experiments also show that some diphosphatase activity, approximately 17% in beef liver (Ueda et al., 1976) and 12% in ox liver (Hashimoto and Yoshikowa, 1967), occurs in the soluble fraction. This activity is distinguishable from acid phosphatase and glucose-6-phosphatase in beef liver (Ueda et al., 1976). It is, however, possible that PGM is also responsible for diphosphatase activity in tissues where the phosphoenzyme is unstable, such as flounder and shark muscle (Hashimoto and Handler, 1966) and rabbit liver (1.1.7 and 1.1.8). Thus, the possibilities are that a rabbit liver PGM diphosphatase activity may be partly responsible for the control of G1P6P in this tissue and hence in the control of the carbohydrate metabolism.

## 1.2 AIMS OF THIS STUDY

PGMs from different sources exhibit a variety of kinetic behaviour, though it is possible that they have a common mechanism. PGM requiring G1P6P as a cofactor may transfer phosphate from substrate to product via a phosphoenzyme or via a cofactor as in the sequential mechanism. The former mechanism is characterized by a parallel-line initial-velocity pattern and the latter by an intersecting line. The kinetic distinction between the mechanisms is, however, unreliable, since phosphoenzyme instability may result in an intersecting-line pattern. Similarly an enzyme with sequential mechanism could also produce a parallel-line pattern under certain conditions (see 1.1.9). Only isotopic induced-transport tests distinguish unequivocally between possible mechanisms.

Anomalies in kinetic evidence could also arise as a consequence of enzyme degradation during isolation. Thus isolation of phosphorylated flounder muscle enzyme by ion-exchange chromatography (Table 1.3) indicates that the phosphoenzyme may be stable in contrast to the earlier observation when only 0.3-0.5% was found in each molecule of enzyme, which suggested that the phosphoenzyme was unstable. Consequently the level of diphosphatase activity associated with a mutase preparation may depend upon a variable degradation of the enzyme during isolation rather than on its fundamental properties. In the case of rabbit muscle enzyme, careful isolation procedures lead to an enzyme with a slightly increased molecular weight of 65,000 instead of 62,000 (see 1.2).

The multiple forms could also be responsible for the discrepancy observed with this enzyme, if these forms have different mechanisms of action.

Rabbit liver PGM was chosen for study as it has a substantial amount of an isoenzyme which appears to be associated with a high intrinsic diphosphatase activity (see 1.1.8). This could account for the failure to isolate a completely phosphorylated enzyme. The isoenzyme appears to be different to that found in rabbit muscle. Differences in isoenzyme composition between the enzymes from these two sources and the finding of a diphosphatase activity in the PGM from rabbit liver suggest that an isoenzyme from this source may have an alternative mechanism of action.

Evaluation of the diphosphatase activity associated with PGM is of interest as this could be a factor in the control of G1P6P levels in the liver and thus may be involved in the regulation of carbohydrate metabolism in this tissue.

The investigation took the following form:

1. A method was adopted to isolate an unmodified and undegraded PGM from rabbit liver in order to maintain its original properties for investigations <u>in vitro</u>.
2. PGM isoenzymes from rabbit liver and rabbit muscle were separated and compared.
3. Some physical and chemical properties of rabbit liver PGM were examined for comparison with the well-investigated rabbit muscle enzyme.
4. Diphosphatase activity in the liver and such activity associated with PGM were measured and attempts were made

to explain the effect of any diphosphatase activity on PGM and on the level of G1P6P in liver tissue.

5. $^{32}P$-labelled phosphoenzyme was prepared in order to check the stability of the phosphoenzyme and the nature of the site of phosphate attachment. The viability of the phosphoenzyme as a kinetic intermediate was also considered.
6. Kinetic studies were carried out at low concentrations of substrates, using a sensitive fluorimetric coupled dehydrogenase assay at constant $Mg^{++}$ concentration and ionic strength, to evaluate kinetic constants needed in radiochemical studies and to determine the form of the initial-velocity pattern.
7. The mechanism of action of rabbit liver PGM was investigated by means of induced-transport tests.

# CHAPTER TWO

# METHODS

## 2.1 MATERIALS AND APPARATUS

### 2.1.1 MATERIALS

| | Source |
|---|---|
| Ammonium sulphate (specially low in heavy metals) | (BDH) |
| 2-(N-Morpholino) ethanesulphonic acid (MES) | (HW) |
| Glucose-1,6-diphosphate tetracyclohexylammonium salt | (BM) |
| Imidazole | (SIGMA) |
| D-glyceraldehyde-3-phosphate diethylacetal | (BM) |
| Phenylmethylsulphonylfluoride | (SIGMA). |
| Decon-90 (phosphate free) | (BDH) |
| Triton X-100 (scintillation grade) | (KL) |
| 2,5-diphenyloxazol (PPO) | (HW) |
| 1,4 Di[2-(5-phenyloxazolyl)benzene] (POPOP) | (KL) |
| Thixotropic gel powder (Cab-O-Sil) | (PIC) |
| Acrylamide (specially purified for electrophoresis) | (BDH) |
| NN'-Methylenebisacrylamide (specially purified for electrophoresis) | (BDH) |
| Glycine (chromatographically homogenous) | (BDH) |
| Phenazine methosulphate | (SIGMA) |
| Nitro blue tetrazolium | (SIGMA) |
| Coomassie brilliant blue G-250 | (SF) |
| Cytochrome c from horse heart | (KL) |
| Sodium dodecyl sulphate (specially pure) | (BDH) |
| Low molecular weight calibration kit | (PFC) |

| | |
|---|---|
| Cellulose phosphate P 11 | (WL) |
| Diethylaminoethyl cellulose DE 32 | (WL) |
| DEAE-cellulose A-50 | (PFC) |
| Sephadex G-100 | (PFC) |
| Polygram cel 300 PEI | (MNC) |

BDH: British Drug House, Poole, Dorset, England

BM: Boehringer Mannheim GMBH Biochemica, West Germany

HW: Hopkin & Williams, Chadwell Heath, Essex, England

KL: Koch-Light Laboratories, Colnbrook, Bucks, England

PFC: Pharmacia Fine Chemicals, Uppsala, Sweden

PIC: Packard Instrument Co. Inc., Downers Grove, Illinois, U.S.A.

SF: Serva Feinbiochemica, Heidelberg

SIGMA: Sigma Chemical Co., St Louis, Missouri, U.S.A.

MNC: Macherey-Nagel & Co., Duran, West Germany

WL: Whatman Ltd., Springfield Mill, Maidstone, Kent

Reagents of special category are mentioned above. All other reagents were of analytical grade and obtained from BDH. Enzymes were obtained from Boehringer Mannheim GMBH Biochemica, West Germany and radioactive compounds were supplied by the Radiochemical Centre, Amersham, U.K.

### 2.1.2 APPARATUS

Spectrophotometric studies

Pye Unicam SP 1800 ultraviolet spectrophotometer.

Fluorimetric studies

Perkin-Elmer fluorescence spectrometer, model 3000.

Liquid scintillation counting

Packard Tri-Carb liquid scintillation spectrometer, model 3375.

Radioactive scanning

Panax RCMS 2 chromatography scanner in conjunction with a Panax PX series modular control unit (see 2.5.1.4 for details).

Scanning of protein bands in the tube gels

Pye Unicam SP 8-100 ultraviolet spectrophotometer fitted with 790826 densitometer.

## 2.2 ASSAY METHODS

### 2.2.1 ENZYME ASSAYS

#### 2.2.1.1 RABBIT LIVER PHOSPHOGLUCOMUTASE

##### 2.2.1.1.1 SPECTROPHOTOMETRIC ASSAY

Rabbit liver phosphoglucomutase activity was assayed by a modification of the spectrophotometric method used by Mulhausen et al. (1970), with the following reaction sequence:

$$\text{G1P} \xrightarrow[\left(\begin{smallmatrix}\text{G1P6P}\\ \text{Mg}^{++}\end{smallmatrix}\right)]{\text{PGM}} \text{G6P}$$

$$\text{G6P} + \text{NADP}^{+} \xrightarrow{\text{G6PDH}} \text{6-P gluconate} + \text{NADPH} + \text{H}^{+}$$

G6PDH: glucose-6-phosphate dehydrogenase (E.C.1.1.1.49)

NADPH and NADH have an absorption peak at 340 nm; the oxidised forms do not absorb in this region. By using the above coupled enzyme reaction and by monitoring the absorbance at 340 nm, the rate of enzymic conversion of G1P to G6P in the presence of excess G6PDH can be followed directly with the spectrophotometer. In the steady state, the reaction rate of phosphoglucomutase is given by the rate of formation of NADPH provided that G6PDH concentration exceeds that of phosphoglucomutase by a factor of at least 50 times.

Reagents

A reaction mixture contained:

40 mM histidine
12 mM Tris-HCl Buffer, pH 7.6 (30 °C)
2 mM $MgCl_2$
5 µM EDTA
1 mM G1P
2 µM G1P6P
475 µM $NADP^{+}$

in a total volume of 2.995 ml.

Assay Enzyme

0.7 unit G6PDH

Procedure

The reaction mixture was incubated with G6PDH at 30 °C for 10 min in a final volume of 2.995 ml. A suitable quantity of the phosphoglucomutase (less than $10^{-2}$ unit) in a small volume (2-5 µl) was added to start the reaction. The rate of reaction was estimated from the change of absorbance at 340 nm, assuming a extinction coefficient for NADPH of 6.22 $mM^{-1}$ $cm^{-1}$ (in Dawson et al., 1969). The enzyme activity at 30 °C was expressed as µmol substrate transformed per minute (units) and specific activity as units per mg protein.

2.2.1.1.2 FLUORIMETRIC ASSAY: MEASUREMENT OF KINETIC PARAMETERS

Fluorimetric measurements of rabbit liver phosphoglucomutase activity were carried out by assaying enzyme at low concentrations of substrate and cofactor using a coupled dehydrogenase reaction. Fluorimetric techniques for determination of enzyme activity were introduced by Lowry in 1957. The fluorimetric method is more sensitive than the spectrophotometric method and it is therefore particularly suitable for assaying the enzyme activity at low substrate concentrations or low enzyme concentration in coupled dehydrogenase reactions where a small quantity of reduced $NADP^+$ is produced. Use of this technique gives an increase in sensitivity of up to 100-fold in comparison with absorption photometry (Trautschold and Löffler, 1965).

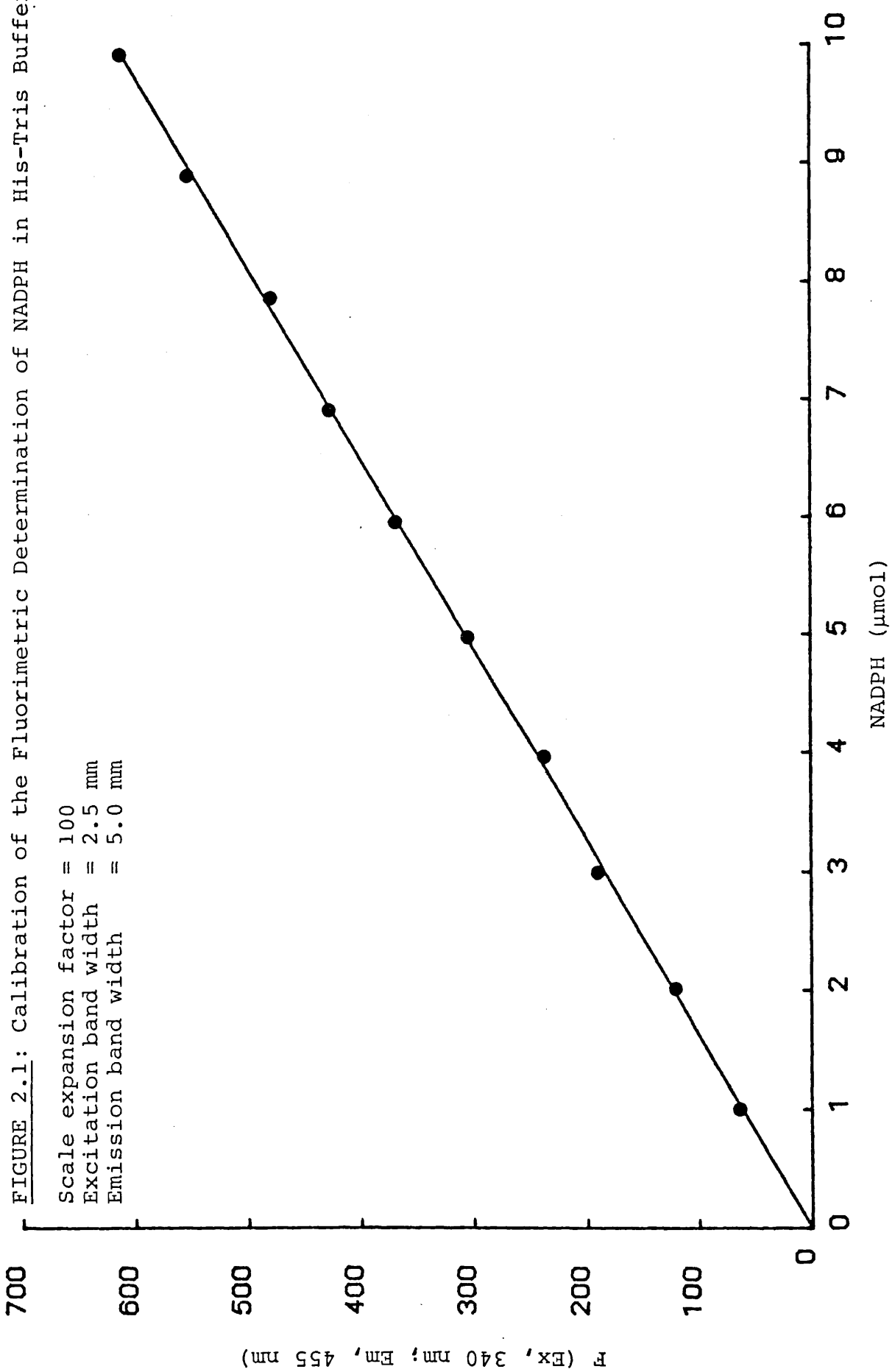



FIGURE 2.1: Calibration of the Fluorimetric Determination of NADPH in His-Tris Buffer, pH 7.6

Principle

The reduced forms of $NADP^+$ and $NAD^+$ absorb light in the near u.v. region at 340 nm and emit energy as a blue fluorescent band at 455 nm. NADPH (or NADH) can be measured in this region in the presence of $NADP^+$ (or $NAD^+$) since the oxidised form does not fluoresce under these conditions (Williamson and Corkey, 1969).

Phosphoglucomutase activity can be measured by using the coupled dehydrogenase reaction (see 2.2.1.1.1) and monitoring the increase in fluorescence due to reduction of $NADP^+$ at 455 nm (excitation, 340 nm). The rate of enzymic conversion of G1P to G6P will be the same as the rate of formation of NADPH in the presence of excess G6PDH.

Cleaning of glassware

The glassware used for fluorimetric assay was specially cleaned to remove any dust and fluorescent particles. This was achieved by boiling the glassware in 5 M HCl and subsequently rinsing with distilled water, boiling in distilled water and finally rinsing with double distilled water.

Standardisation of NADPH (Fig. 2.1)

The fluorescence of NADPH in the enzymic reaction does not provide an absolute measure of its concentration. Therefore a calibration curve of NADPH concentration (0-10 µmol) related to fluorescence change at emission 455 nm and excitation 340 nm (emission slit width, 5mm; excitation slit width, 2.5 mm) was constructed. NADPH solutions of various concentrations were prepared in the buffer used for phosphoglucomutase assay

(40 mM histidine, 12 mM Tris-HCl, pH 7.6) and the concentrations of these solutions were standardised in the spectrophotometer at 340 nm (extinction coefficient of NADPH = 6.22 $mM^{-1}$ $cm^{-1}$).

Reagents

The reaction mixture contained His-Tris buffer, pH 7.6 (40 mM histidine, 12 mM Tris), 2 mM $Mg(NO_3)_2$, 4-100 µM G1P, 0.05-1.0 µM G1P6P and 0.8 units of ammonium sulphate-free G6PDH in a total volume of 2.995 ml. The ionic strength of the solution at the various concentrations of G1P and G1P6P was maintained at 0.05 mol $l^{-1}$ by the addition of appropriate quantities of 200 mM $KNO_3$. Specially purified G1P was used in this assay (see below).

Purification of G1P

G1P, obtained from BDH, contains G1P6P as an impurity and it was purified according to the method of Bartlett (1959) and Ray and Roscelli (1964a) by eluting from a Dowex-1-formate column in pyridine-formic acid buffer, pH 3.0, as described below.

A Dowex-1-formate column (1.8 x 25 cm) was prepared from Dowex-1-Cl (80% cross linked, 20-50 mesh) as follows. Dowex-1-Cl was first converted to the hydroxide form by washing with 2M NaOH. The eluate was tested for removal of $Cl^-$ ions from the column with acidified $AgNO_3$. Subsequently the column was converted to the formate form by washing with 4 M formic acid.

2 g G1P was dissolved in 20-30 ml distilled water and applied to the Dowex-1-formate column. G1P was eluted with a linear gradient of 0-0.75 M formic acid-pyridine buffer, pH 3.0. 2 $\ell$ of buffer and a flow rate of 60 ml $h^{-1}$ were used to elute G1P. 5 ml fractions were collected and G1P was detected in each fraction as described in 2.2.6.

The fractions containing G1P were combined and freeze-dried to reduce the volume. A small volume of viscous fluid was obtained after freeze-drying and subsequently dissolved in 40 ml distilled water. The pH of the solution was adjusted to 7 with 5 M NaOH. Excess barium acetate (x2 molar excess over G1P) was then added to the G1P solution and it was left to stand for 4 h at 0 $^{o}$C. This was followed by the addition of 4 vol. ethanol. The precipitate formed was collected by centrifugation and dissolved in a small volume of water. The barium salt of G1P was then changed to the sodium form by adding excess Dowex-1-$Na^{+}$. The resultant solution of the disodium salt of G1P was decolourised with activated charcoal (Norit NK) and stored at -20 $^{o}$C.

A yield of 50% was obtained in the final solution of G1P. It was shown to be free from G1P6P since no rabbit muscle phosphoglucomutase activity was observed in the absence of added G1P6P.

Procedure

Phosphoglucomutase and glucose-6-phosphate dehydrogenase were centrifuged separately at 100,000 g for 1 h to remove most of the ammonium sulphate before kinetic assays. The pellets obtained after centrifugation were dissolved in His-Tris-HCl buffer, pH 7.6 (40 mM histidine, 12 mM Tris)

containing 2 mM $Mg(NO_3)_2$.

Kinetic assays were carried out as follows. The reaction mixture (see reagents) containing 0.8 units of ammonium sulphate-free G6PDH was incubated for 10 min at 30 °C in a total volume of 2.995 ml. The reaction was started by the addition of 0.44 µg (5 µl) PGM diluted with His-Tris-HCl buffer, pH 7.6 (40 mM histidine, 12 mM Tris) containing 2 mM $Mg(NO_3)_2$ and preincubated at 30 °C for 20 min. The production of NADPH was followed by monitoring the increase of fluorescence at 455 nm (excitation 340 nm) using the Perkin-Elmer fluorescence spectrometer. The scale-expansion setting and the emission and excitation slit width were the same as used for the NADPH calibration curve; the rate of reaction was calculated with the aid of this calibration curve (Fig. 2.1).

### 2.2.1.2 PHOSPHOGLYCERATE MUTASE (E.C.2.7.5.3)

Phosphoglycerate mutase activity was estimated spectrophotometrically by a coupled enzyme assay method.

$$\text{glycerate-3-P} \xrightarrow[\text{(rate-limiting)}]{\overset{\text{2,3-di PGA}}{\text{PGAM}}} \text{glycerate-2-P}$$

$$\text{glycerate-2-P} \xrightarrow{\text{ENOLASE}} \text{phosphoenolpyruvate} + H_2O$$

$$\text{phosphoenolpyruvate} \xrightarrow[+\text{ ADP}]{\text{PK}} \text{pyruvate} + \text{ATP}$$

$$\text{pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{L-lactate} + NAD^+$$

PGAM: phosphoglycerate mutase (E.C.2.7.5.3)

PK: pyruvate kinase (E.C.2.7.1.40)

LDH: lactate dehydrogenase (E.C.1.1.1.27).

Reagents

The reaction mixture contained:

18.5 mM Tris-HCl, pH 7.4 (30 °C)
3.4 mM $MgCl_2$
19.0 mM KCl
2.2 mM glycerate-3-phosphate
0.002 mM glycerate- 2,3-diphosphate
0.74 mM ADP
0.2 mM NADH in a total volume of 2.6 ml.

Assay Enzyme

0.16 units (2 µl) enolase
8.00 units (4 µl) pyruvate kinase
6.00 units (4 µl) lactate dehydrogenase

Procedure

2.6 ml reaction mixture was incubated with assay enzyme for 10 min at 30 °C in a final volume of 2.7 ml. A small volume (2-5 µl) of phosphoglycerate mutase solution was added to the reaction mixture. The reaction rate was followed on the spectrophotometer by measuring the change in absorbance at 340 nm. The phosphoglycerate mutase activity was calculated assuming a millimolar absorbance for NADH of 6.22 $mM^{-1}$ $cm^{-1}$.

### 2.2.1.3 PHOSPHOGLUCOSE ISOMERASE (E.C.5.3.1.9)

A coupled enzyme assay method was used to estimate phosphoglucose isomerase activity as follows.

$$F6P \xrightarrow[\text{(rate-limiting)}]{PGI} G6P$$

$$G6P + NADP^+ \xrightarrow{G6PDH} 6\text{-P gluconate} + NADPH + H^+$$

PGI: phosphoglucose isomerase (E.C.5.3.1.9)

Reagents

The reaction mixture contained:

| | | |
|---|---|---|
| 100 | mM Tris-HCl, pH 8.0 (30 °C) | |
| 5 | mM $MgCl_2$ | |
| 10 | mM F6P | |
| 0.457 | mM $NADP^+$ | in a volume of 2.99 ml. |

Assay enzyme

0.7 units G6PDH

Procedure

5 µl G6PDH was added to 2.99 ml of reaction mixture which was then incubated for 10 min at 30 °C. The reaction was started by the addition of a small volume (2-10 µl) of a solution containing PGI. The rate of reaction was followed by observing the change in absorbance at 340 nm from which the isomerase activity was calculated.

### 2.2.1.4 HEXOKINASE (E.C.2.7.1.1)

Hexokinase activity was measured spectrophotometrically with the following coupled enzyme reactions:

$$D\text{-Glucose} + ATP \xrightarrow[\text{(rate-limiting)}]{HK} G6P + ADP$$

$$G6P + NADP^+ \xrightarrow{G6PDH} 6\text{-P-gluconate} + NADPH + H^+$$

HK: hexokinase

Reagents

The reaction mixture contained:

| | | |
|---|---|---|
| 100 | mM | Tris-HCl, pH 8.0 (30 $^{o}$C) |
| 5 | mM | $MgCl_2$ |
| 10 | mM | ATP |
| 10 | mM | D-glucose |
| 0.457 | mM | $NADP^+$ |

in a volume of 2.99 ml.

Assay enzyme

0.7 units G6PDH

Procedure

To 2.99 ml of reaction mixture was added 5 µl G6PDH. After incubation at 30 $^{o}$C for 10 min, a small volume (2-5 µl) of a solution containing hexokinase activity was added and the rate of change of absorbance at 340 nm was measured.

2.2.1.5 GLUCOSE-6-PHOSPHATE DEHYDROGENASE (E.C.1.1.1.49)

G6PDH activity was estimated spectrophotometrically by measuring the rate of formation of NADPH.

$$\text{D-G6P} + NADP^+ \xrightarrow{\text{G6PDH}} \text{6-P gluconate} + NADPH + H^+$$

Reagents

The reaction mixture contained:

| | | |
|---|---|---|
| 100 | mM | Tris-HCl, pH 8.0 (30 $^{o}$C) |
| 5 | mM | $MgCl_2$ |
| 1 | mM | G6P |
| 0.455 | mM | $NADP^+$ |

in a volume of 2.99 ml.

Procedure

2.99 ml reaction mixture was incubated at 30 $^{o}$C for 10 min.

The reaction was started by the addition of the enzyme in a small volume (2-5 µl). The rate of reaction was calculated from the change in absorbance at 340 nm with time.

### 2.2.1.6 GLUCOSE-6-PHOSPHATASE (E.C.3.1.3.9)

Glucose-6-phosphatase activity was estimated by observing the rate of formation of $P_i$.

$$G6P + H_2O \xrightarrow{G6Pase} Glucose + P_i$$

The inorganic phosphate liberated was determined as a blue phosphomolybdate complex as described in 2.2.5.

Reagents

The reaction mixture contained:

100 mM sodium cacodylate buffer, pH 6.5
10 mM G6P in a total volume of 990 µl.

Procedure

990 µl of reaction mixture was incubated for 10 min, at 30 °C and then the reaction was started by the addition of 10 µl enzyme solution. 100 µl samples were withdrawn at timed intervals and added to the tubes containing 1.9 ml distilled water and 0.2 ml 60% perchloric acid to stop the reaction. Inorganic phosphate present in each sample was estimated by Allen's method (2.2.5) and the rate of reaction determined.

### 2.2.2 FLUORIMETRIC ASSAY OF GLUCOSE-1,6-DIPHOSPHATASE ACTIVITY

Glucose-1,6-diphosphatase activity in crude extracts of rabbit

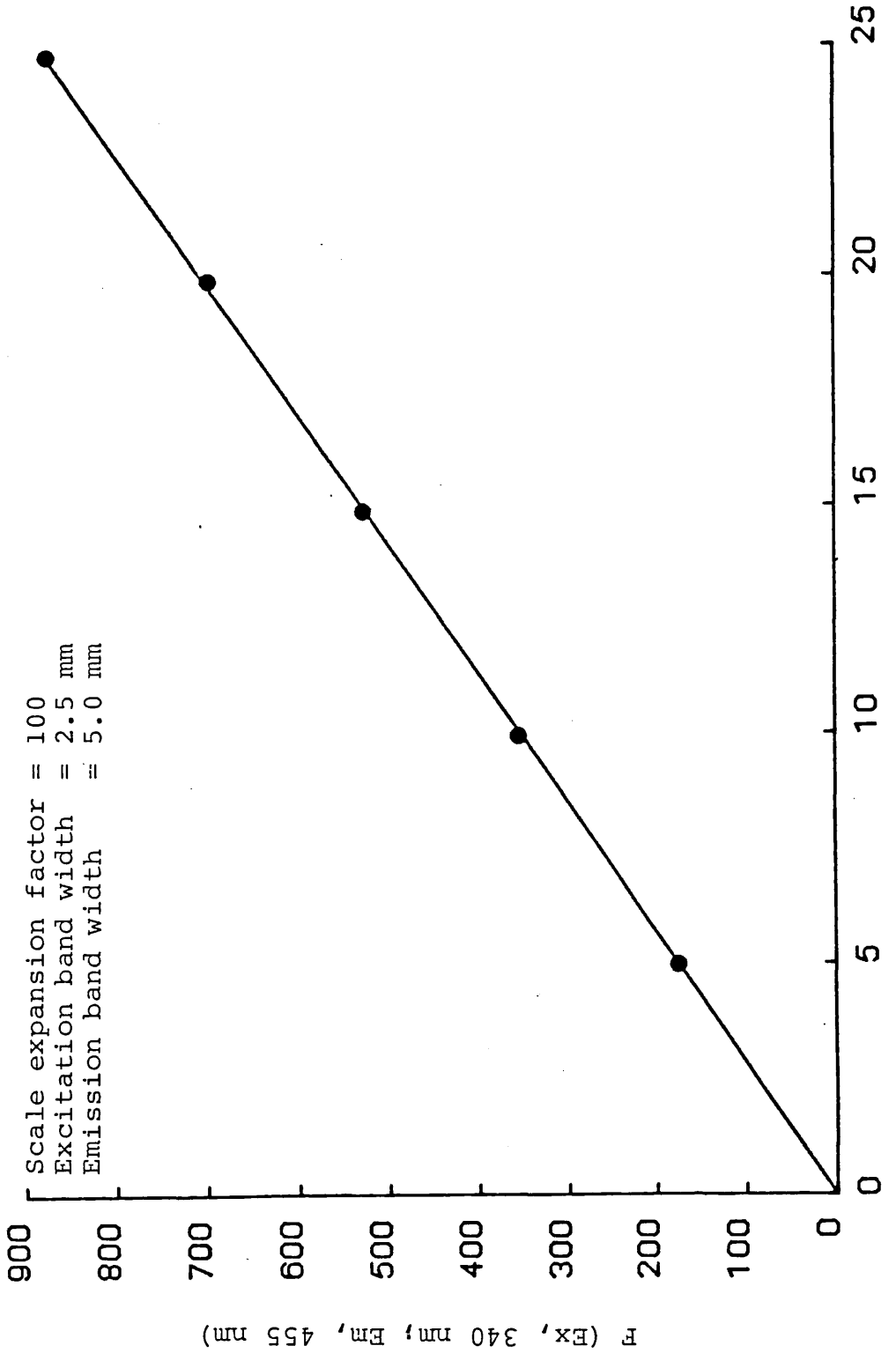



FIGURE 2.2: Calibration of the Fluorimetric Determination of NADPH in Tris-HCl Buffer, pH 7.6

liver and rabbit muscle was determined fluorimetrically with the following coupled reactions:

$$\text{G1P6P} \xrightarrow[\text{diphosphatase}]{\text{Glucose 1,6-}} \underbrace{\text{G1P + G6P}}_{\text{in equilibrium proportions}} + P_i$$

$$\text{G6P} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{6-P gluconate} + \text{NADPH} + H^+$$

The rate of enzymic conversion of G1P6P to G1P and G6P in the presence of excess G6PDH can be followed by monitoring the increase in flourescence at 455 nm (excitation wavelength =340 nm) due to NADPH production. Details of the procedure are described in 2.2.1.1.2.

Reagents

The reaction mixture contained:

33 mM Tris-HCl, pH 7.6 (30 °C)
5 mM $MgCl_2$
10 mM G1P6P
475 µM $NADP^+$ in a total volume of 2.985 ml.

Assay enzyme

0.7 units G6PDH

Standard curve (Fig. 2.2)

0-25 nmol NADPH in 33 mM Tris-HCl, pH 7.6 was used for the calibration curve. The concentration of the stock NADPH solution was determined from its absorbance at 340 nm.

Preparation of sample

Measurement of glucose-1,6-diphosphatase activity in crude extracts of rabbit liver and rabbit muscle was carried out as follows. Tissues were homogenised with 2 vol (w/v)

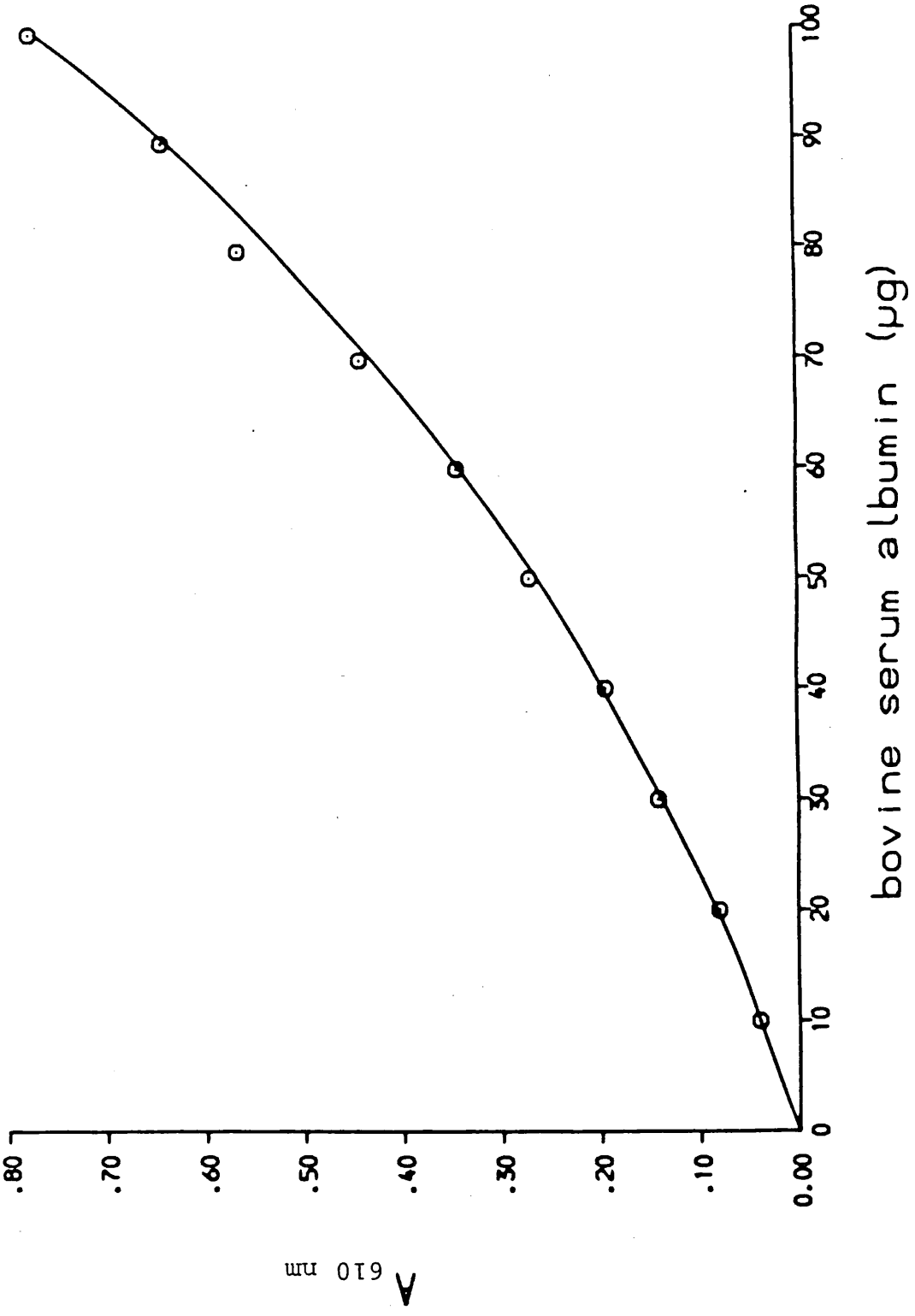



FIGURE 2.3: Calibration Curve for Protein Determination by Bromophenol Blue Method

10 mM Tris-HCl, pH 7.0, containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The homogenates were clarified by centrifugation at 25,000 g for 10 min. 10 µl samples of supernatant were taken for the enzyme assay.

Procedure

The reaction mixture was incubated with 0.7 unit G6PDH at 30 $^{o}$C for 10 min. in a total volume of 2.99 ml. A small volume of the sample to be assayed (10 µl) was added to the reaction mixture and the change in fluorescence was followed on the fluorescence spectrometer at 455 nm (ex. 340 nm). The excitation and emission slit width were 2.5 mm and 5.0 mm respectively. The rate of reaction was determined by using the calibration curve of NADPH concentration versus fluorescence (Fig. 2.2).

### 2.2.3 PROTEIN ASSAY

#### 2.2.3.1 BROMOPHENOL BLUE METHOD (Flores, 1978)

Bromophenol blue is yellow below pH 3.0; it absorbs maximally at 440 nm. When protein binds to the bromophenol blue in acid solution, the absorption at 440 nm decreases and a new peak appears at 610 nm. The absorbance of the protein-bromophenol blue complex at 610 nm under acidic conditions is almost linear over a range of 10-80 µg protein.

Standard line (Fig. 2.3)

Bovine serum albumin (BSA) was dried in a vacuum dessicator. 0-100 µg of dried BSA per estimation was used to produce the standard line relating absorbance at 610 nm to µg protein.

Reagents

Bromophenol blue solution - 7.5 mg bromophenol blue was dissolved in 15 ml 95% ethanol and 2.5 ml glacial acetic acid. The volume was made up to 100 ml with distilled water. This solution was freshly prepared each week.

Procedure

To 0.1 ml of each protein sample was added 0.9 ml bromophenol blue solution. The absorbance at 610 nm was measured against a blank containing 0.1 ml distilled water and 0.9 ml bromophenol blue solution.

### 2.2.3.2 ULTRAVIOLET ABSORPTION METHOD

For rapid determination of protein concentration, the ultraviolet absorption method was used in final preparative stages of the enzyme. This method has been used by Warburg and Christian (1941) as a rapid and fairly sensitive measure of protein concentration.

Most proteins have an absorption maximum at 275-280 nm due to presence of tyrosine, tryptophan or phenylalanine residues. The aromatic amino acid content of various proteins tends to lie within reasonably narrow limits (E. Layne, 1957). The absorbance at 280 nm of a 1% solution of an average protein (1 cm light path) is approximately 10. Absorbance at 280 nm can be used for rough measurements of protein concentration between 0.1 and 0.5 mg $ml^{-1}$ in the absence of other substances which absorb at this wavelenth (e.g. nucleotides).

Procedure

1 ml of the protein sample was transferred to a 1cm quartz cuvette (of small volume) and the absorbance was measured at 280 nm against a water blank.

## 2.2.4 PHOSPHOGLUCOMUTASE PROTEIN ASSAY

The total enzyme protein was estimated using a value of 7.7 for the absorbance at 278 nm of 1% (w/v) solution of phosphoglucomutase in 1 cm light-path cell (Najjar, 1948).

Procedure

30 µl of a solution of pure phosphoglucomutase was added to 970 µl distilled water in a 1 ml quartz cuvette (1 cm path) and the absorbance was measured at 278 nm against a water blank in the spectrophotometer. Enzyme concentrations were calculated using the value for the extinction coefficient given above.

## 2.2.5 ESTIMATION OF INORGANIC PHOSPHATE

Inorganic phosphate was estimated by the method of Allen (1940). Inorganic phosphate reacts with molybdate in an acid medium to form a phosphomolybdate complex, which turns blue in the presence of a reducing agent. The reaction is carried out in the presence of perchloric acid and used a solution of 2,4-diaminophenol hydrochloride (Amidol) in sodium metabisulphite as the reducing agent. The intensity of the blue colour of phosphomolybdate complex is proportional to the amount of inorganic phosphate present over a wide range and the absorbance at 600 nm is stable for 30 min after the reaction.

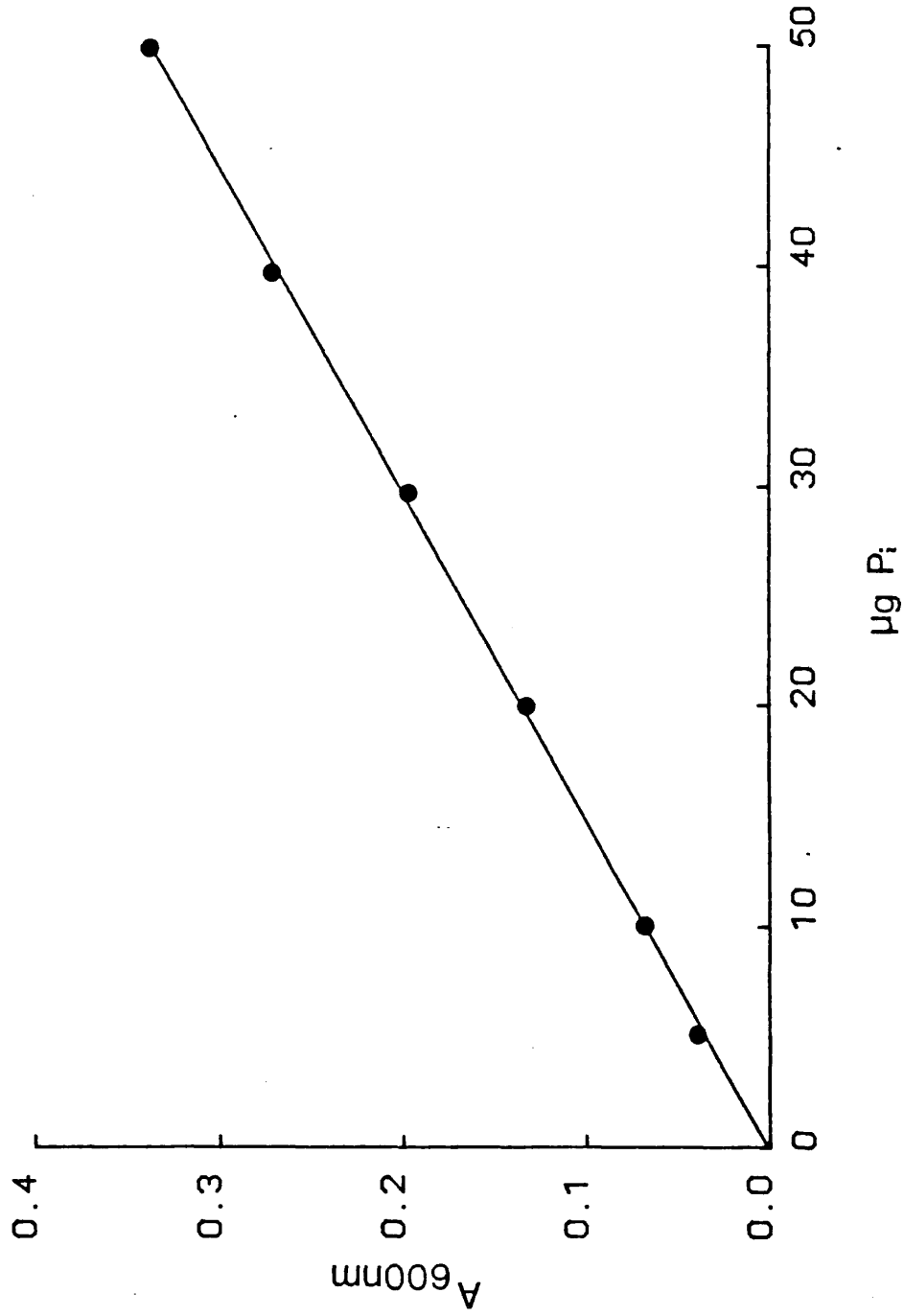



FIGURE 2.4: Calibration of the Spectrophotometric Determination of Inorganic Phosphate by Allen's Method

Standard line (Fig. 2.4)

This was obtained with solutions of 0-50 μg $ml^{-1}$ $KH_2PO_4$.

Reagents

60% perchloric acid

8.3% ammonium molybdate

Amidol reagent:

1 g 2,4-diaminophenol hydrochloride (Amidol)

2 g sodium metabisulphite in 100 ml distilled water

Cleaning of glassware

For reproducible estimation all glassware must be cleaned to remove adsorbed phosphate. Tubes were soaked in 5% Decon 90 (phosphate-free) overnight and then thoroughly rinsed with double distilled water before use.

Procedure

To a 0.1 sample were added in the following order, 1.9 ml double distilled water, 0.2 ml 60% perchloric acid, 0.2 ml Amidol reagent and 0.1 ml 8.3% ammonium molybdate, all at room temperature. The mixture was agitated with a whirli-mixer after each addition and the final solution left to stand for 10 min. Standards and blanks were run concurrently. The absorbance was measured at 600 nm.

## 2.2.6 ESTIMATION OF GLUCOSE-1-PHOSPHATE

The concentration of G1P in the presence of G6P was determined by a modified method of Bartlett (1959) as described by Ray and Roscelli (1964a). In this method G1P was hydrolysed and the phosphate liberated estimated as a blue phosphomolybdate complex.

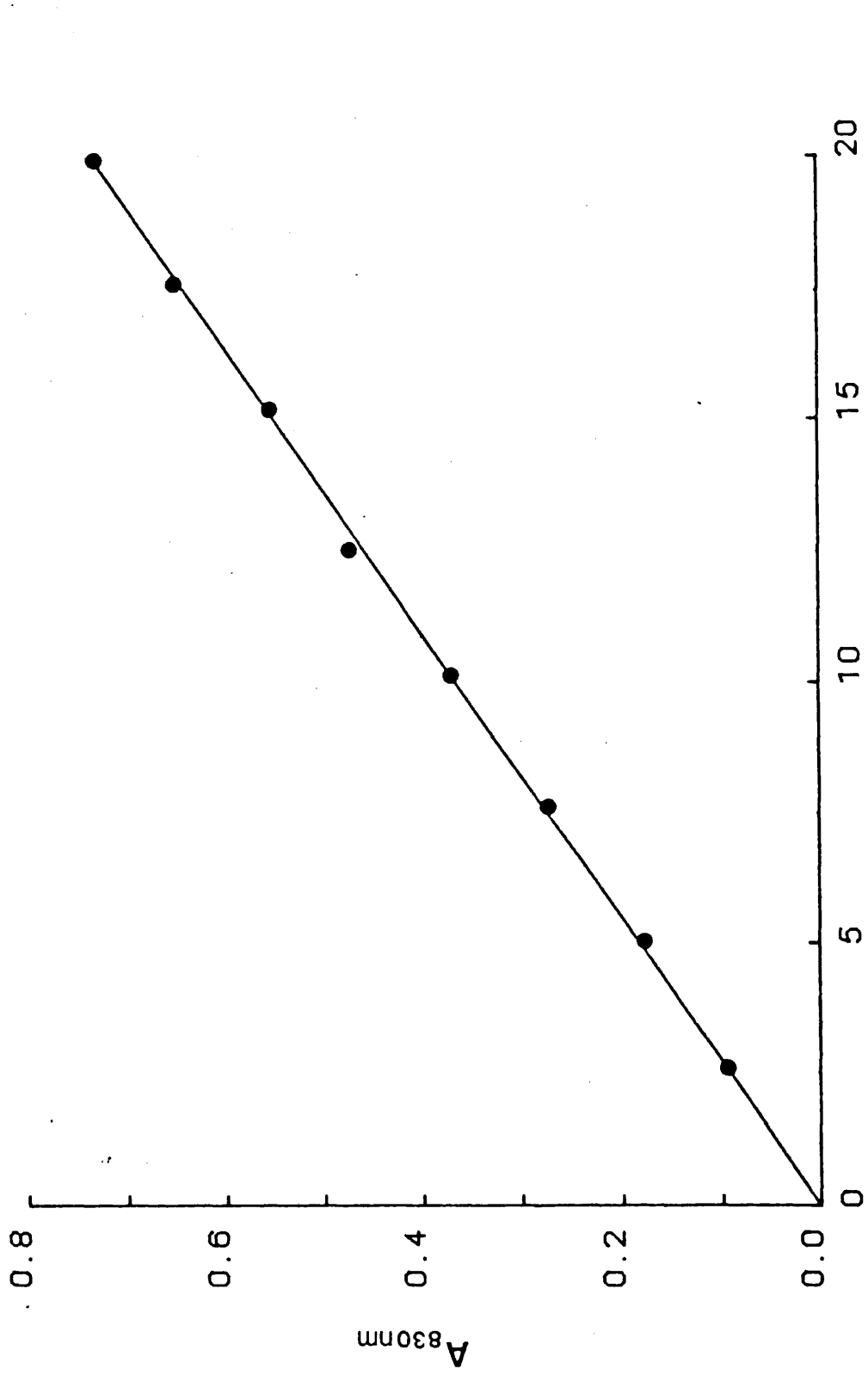



FIGURE 2.5: Calibration of the Spectrophotometric Determination of G1P

G1P is acid and heat labile and has a half life of approximately 1 min in 1 M acid at 100 $^{o}$C (Leloir and Cardini, 1941). G1P hydrolysis is complete under the conditions used for this experiment whilst the hydrolysis of G6P is negligible (Clarke, 1972). The liberated phosphate reacts with molybdate in the presence of acid and produces a phosphomolybdate complex which is subsequently reduced to a blue colour in the presence of Fiske/Subbarow reagent. The advantages of this method are (1) the processes of G1P hydrolysis and colour development occur concurrently; (2) there is a linear relationship between the colour density and amount of G1P originally present; (3) other substances have little effect on the colour development.

Standard line (Fig. 2.5)

A standard curve for absorbance at 830 nm against G1P concentration was obtained over a range of G1P concentrations (0-20 mM).

Reagents

0.18% ammonium molybdate in M-$H_2SO_4$

Fiske/Subbarow Reagent:

2.49 g anhydrous sodium sulphite

12 g sodium metabisulphite

0.2 g 1-amino-2-naphthol-4-sulphonic acid

dissolved in water and made up to 100 ml.

Procedure

0.1 ml samples were added to 15 ml conical stoppered centrifuge tubes containing 1 ml 0.18% ammonium molybdate in

M-$H_2SO_4$ followed by 0.2 ml Fiske/Subbarow reagent. The solutions were thoroughly mixed and the tubes heated to 100 $^{o}$C for 10 min in a boiling water bath then rapidly cooled to room temperature. The standards and blank were prepared concurrently. The intensity of the blue colour was measured at 830 nm.

## 2.2.7 ESTIMATION OF GLYCERALDEHYDE-3-PHOSPHATE

Glyceraldehyde-3-phosphate was estimated spectrophotometrically using the following enzyme reaction:

$$\text{GAP} \xrightarrow{\text{TIM}} \text{DAP}$$

$$\text{DAP} + \text{NADH} + \text{H}^{+} \xrightarrow{\text{GDH}} \text{L (-)-glycerol-3-P} + \text{NAD}^{+}$$

TIM: triosephosphate isomerase

GDH: glycerol-3-phosphate dehydrogenase

GAP: glyceraldehyde-3-phosphate

DAP: dihydroxyacetone phosphate

The reaction is carried out with a suitable excess of NADH and adequate enzyme concentrations. The decrease in absorbance at 340 nm associated with oxidation of NADH was used for the estimation of GAP concentration.

Reagents

The reaction mixture contained:

276 mM triethanolamine buffer, pH 7.6

0.2 mM NADH in a volume of 880 µl.

Assay enzymes

0.61 units GDH

$2.5 \times 10^{-3}$ units TIM

Procedure

A 100 µl sample was added to 880 µl reaction mixture and 10 µl glycerol-3-phosphate dehydrogenase. The mixture was incubated for 10 min at 30 °C in a 1 ml cuvette and reaction was started by the addition of 10 µl TIM. The decrease in absorbance was measured at 340 nm and the concentration of GAP calculated.

## 2.3 CHROMATOGRAPHIC METHODS

### 2.3.1 PAPER CHROMATOGRAPHY

#### 2.3.1.1 DESCENDING PAPER CHROMATOGRAPHY TECHNIQUE

Descending paper chromatography (Consden et al., 1944) was used for purification and separation of [$^{32}$P] G6P and [$^{32}$P] G1P6P from inorganic and other phosphates. All chromatography papers were used without prewashing with EDTA. A line of origin was drawn in pencil approximately 12 cm from the top end of the paper ('top': chromatography must be carried out down machine direction of paper). The $R_f$ values of phosphates are very low in the solvents used and separation is poor after a short run. The paper chromatograms were therefore overrun; the paper was serrated at its lower edge to ensure that the solvent flowed evenly off the end. The standard markers were applied 3 cm apart at the origin on one side of the chromatogram and the sample was applied as a 15 cm wide strip on the other side. The samples and markers were dried with an air jet; cold air was used to minimise chemical breakdown and irreversible adsorption of salts on the paper.

100 ml of the solvent to be used for separating sample components was placed in the bottom of the tank at least one hour before the run to saturate the tank with solvent vapour. At the end of the run any remaining solvent was removed from the trough and the paper was dried in a drying cabinet under a stream of cold air. The portion of the paper where standard markers had run was cut from the chromatogram and phosphates were developed by the technique of Burrows et al. (1952). The corresponding phosphates were identified on the chromatogram and eluted.

Since the chromatogram was overrun in order to separate radioactive compounds, $R_f$ values were replaced by 'position constant values' (Mortimer, 1952) defined as the position of the substances relative to that of inorganic phosphate ($R_{P_i}$) where,

$$R_{P_i} = \frac{\text{distance substance travels from origin} \times 100}{\text{distance } P_i \text{ travels from origin}}$$

Standard markers

The following amounts of standard markers were used:

0.3 µmol $KH_2PO_4$ (9.3 µg P)

0.15 µmol ATP (13.95 µg P)

0.4 µmol G1P (12.4 µg P)

0.4 µmol G6P (12.4 µg P)

0.2 µmol G1P6P (12.4 µg P)

### 2.3.1.2 CHROMATOGRAPHIC SYSTEMS

DEAE-CELLULOSE-AMMONIA SYSTEM

In this system DEAE-cellulose paper DE 81 with 0.12 M ammonia as solvent was used to separate G1P6P from G1P and G6P. Descending chromatography (2.3.1.1) was carried out for 6 h and G1P6P eluted from the dried paper with 5 M ammonia. The eluted fraction was freeze-dried, dissolved in a small volume of water and stored at -20 °C.

$R_{P_i}$ of compounds were:

$P_i$, 100; G1P, 119; G6P, 125; G1P6P, 19.

The distances travelled by compounds in 6 h:

$P_i$, 8 cm; G1P, 9.5 cm; G6P, 10 cm; G1P6P, 1.5 cm.

WHATMAN NO. 4, ETHYL ACETATE/ACETIC ACID/WATER SYSTEM

[$^{32}$P] G6P was purified from inorganic and other phosphates using Whatman no. 4 paper and ethyl acetate/acetic acid/distilled water (3:3:1 by vol) as solvent. The chromatogram was run for 14 h at 4 °C. After the run, the peak containing [$^{32}$P] G6P was eluted from the dried paper with a small volume of distilled water and stored at -20 °C.

$R_{P_i}$ values obtained on this system were as follows:
$P_i$, 100; ATP, 4; G6P, 30; G1P, 37.

The distances travelled by compounds in 14 h:
$P_i$, 28 cm; ATP, 1.12 cm; G6P, 8.4 cm; G1P, 10.4 cm.

WHATMAN NO. 4, 2-METHOXYETHANOL/ETHYL METHYL KETONE/AMMONIA SYSTEM

This system was used as a second purification step to purify [$^{32}$P] G6P from inorganic and other sugar phosphates, following the separation with the ethyl acetate/acetic acid/distilled water system. Chromatography was carried out for 24 h at room temperature using Whatman No. 4 paper and 2-methoxyethanol/ethyl methyl ketone/3M ammonia (7:2:3 by vol) as solvent. [$^{32}$P] G6P was eluted from the chromatogram with a small volume of water and stored at -20 °C.

The $R_{P_i}$ values of phosphates obtained were:
$P_i$, 100; ATP, 58; G1P, 215; G1P6P, 20.

The distance travelled by compounds in 24 h were:
$P_i$, 9.4 cm; ATP, 5.5 cm; G6P, 17 cm; G1P, 20.2 cm; G1P6P, 1.9 cm.

### 2.3.1.3 DETECTION OF PHOSPHATES ON PAPER

Phosphates on the paper chromatogram were detected using the colour reagent of Hanes and Isherwood (1949) as modified by Burrows et al. (1952).

Colour reagent

1 g ammonium molybdate was dissolved in 8 ml distilled water, 3 ml concentrated HCl and 3 ml 70% perchloric acid and the volume was made up to 100 ml with acetone.

Procedure

The paper was dipped in colour reagent and dried in a jet of air. Inorganic phosphate appeared as a yellow spot. All other unhydrolysed phosphates ( including G6P which hydrolyses with difficulty) appeared as blue spots when the chromatogram was irradiated at 254 nm under a Camag ultra-violet lamp. The intensity of the blue background was subsequently reduced by exposure of paper to ammonia vapour (Bandirski and Axelrod, 1951).

### 2.3.1.4 METHODS FOR LOCATING RADIOACTIVELY LABELLED COMPOUNDS ON PAPER

The following methods were used for locating the position of radioactive spots on paper chromatograms.

1. LOCATION BY REFERENCE

The standard markers were run on one side of the paper, and radioactive sample was applied on the other. The position of the radioactive substance was located by reference to the corresponding standards.

2. LOCATION BY GEIGER-MÜLLER COUNTER

The radioactive peak of $^{32}P$ was located using a Geiger-Müller counter, which was moved manually over the paper to detect the position of active spots. It was found to be difficult to locate small amounts of $^{14}C$ (low energy β emitter) by this method.

3. LOCATION BY RADIOACTIVE SCANNER

A 2cm wide strip was cut from the paper along the course taken by the radioactive sample. A Panax RCMS 2 chromatography scanner in conjunction with a Panax PX series modular control unit was used to scan the strips for radioactivity. Radioactivity was detected by an anthracene crystal in intimate contact with the window of a photomultiplier tube (EMI type 6097F). Radioactive counts in the form of pulses were integrated into a potential difference using the apparatus described by Dean (1982) and measured on a chart recorder.

## 2.3.2 THIN LAYER CHROMATOGRAPHY

Thin layer chromatography was used to check the purity of $[^{32}P]$ G6P, $[^{32}P]$ G1P6P and $[^{14}C]$ G6P.

0.1 mm cellulose MN polyethyleneimine impregnated Polygram cel 300 PEI supported on plastic of size 20 x 20 cm was prepared for used by drawing a line with lead pencil, 2 cm from the top edge of the plate to mark the extent of solvent migration. Small volumes (2 µl) of radioactive samples and standard markers were applied with a micropipette, 2 cm apart

and about 2-3 cm from the lower edge of the chromoplate. The spots were allowed to dry and the plate was placed in a chromatography tank containing solvent (0.25 M LiCl) to a depth of about 0.7-1.0 cm. The solvent was allowed to rise until it reached the marked line. The plate was removed from the tank and dried with a cold air jet. The part of the plate containing standard markers was cut off, dried and washed in methanol to remove LiCl. The phosphate colour reagent (see 2.3.1.3) was sprayed on the plate which was then irradiated under a Camag ultraviolet lamp. The phosphates appeared as blue spots on a light blue background.

To determine the position of radioactive compounds, 1 $cm^2$ areas of the plate were scraped along the course taken by radioactive samples and transferred directly to the scintillation vials containing 15 ml scintillant. The radioactivity was measured on the Packard liquid scintillation counter as described in 2.4.1.

The following $R_f$ values were obtained:
$P_i$, 0.18; ATP, 0.25; G1P, 0.65; G6P, 0.57; G1P6P, 0.07.

## 2.4 RADIOCHEMICAL METHODS

### 2.4.1 RADIOACTIVITY MEASUREMENT

Radioactivity assays were carried out by using a Model 3375 Packard Tri-carb liquid scintillation spectrometer. Samples were counted in the dark at 4 $^{o}$C.

#### 2.4.1.1 DETECTION OF $^{14}$C-RADIOACTIVITY: BY LIQUID SCINTILLATION COUNTING

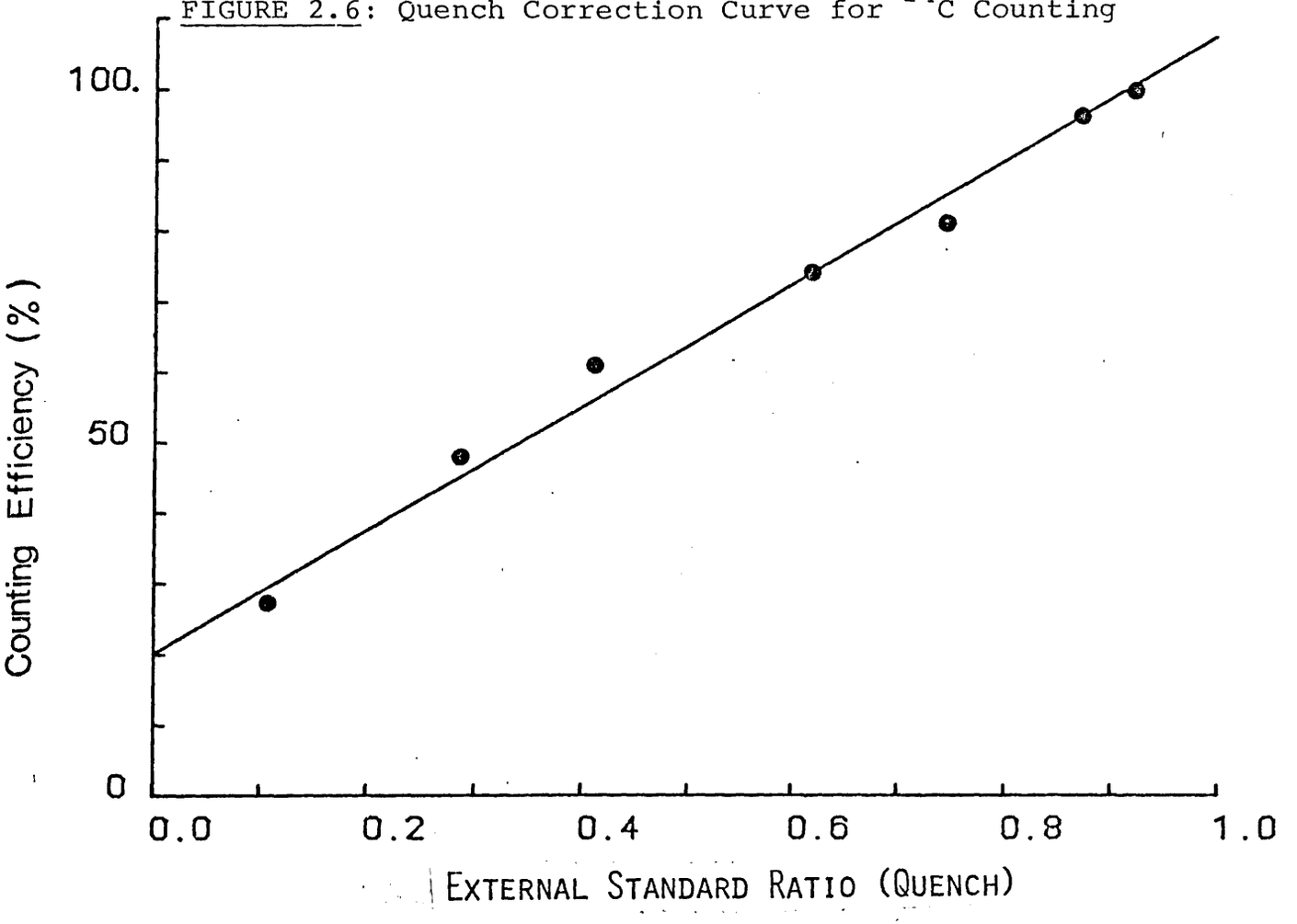
Scintillant for $^{14}$C counting was prepared by the method of Patterson and Green (1965). 1 vol Triton X-100 of scintillation grade was mixed with 2 vol toluene containing 0.4% PPO and 0.01% POPOP. The scintillant was stored in the dark for 24 h at room temperature before use.

For $^{14}$C radioactive assays, 10 ml of scintillant was placed in 20 ml screw cap glass vials and counted on the scintillation counter using a preset $^{14}$C energy window* at least three times (for 10 min/count) to give a reading for background activity. 0.01 to 1.0 ml of aqueous sample at neutral pH was then added to the vials, which were again counted three times.

The average counts obtained were corrected for quenching and efficiency of counting as described below.

* Energy window: Three identical and independent channels are available in scintillation spectrometer for pulse-height analysis. Each channel provides completely independent lower and upper level discriminators. Each channel can be set for fixed narrow energy window for β-spectral analysis.

FIGURE 2.6: Quench Correction Curve for $^{14}C$ Counting




The volumes of 1, 2, 4, 5, 6, 8 and 10 µl of nitromethane were added to scintillation vials containing 10 ml scintillant and a known amount of $^{14}C$. The graph shows percentage counting efficiencies at different levels of quenching.

The percentage of counting efficiency at different quench values was obtained as follows. 1-10µl of nitromethane (a known strong quencher) was added together with a known quantity of $^{14}C$ to the scintillation vials containing 10 ml scintillant, thereby giving a range of quenching. A graph at different levels of quenching was plotted, and this was used to determine the quench correction for $^{14}C$ radioactive sample (Fig. 2.6).

### 2.4.1.2 DECTECTION OF $^{32}P$-RADIOACTIVITY

#### 2.4.1.2.1 BY LIQUID SCINTILLATION COUNTING

The scintillant used for $^{32}P$ counting was the same as that described in 2.4.1.1 for $^{14}C$ counting but contained a 5% suspension of Cab-O-Sil (thixotropic gel powder). Cab-O-Sil prevents the decrease in counting efficiency due to adsorption of $^{32}P$ on the walls of glass vials.

0.01-1 ml of aqueous sample at neutral pH was added to 20 ml screw cap scintillation vials containing 10 ml scintillant (including 5% Cab-O-Sil), which had been counted for background activity. The radioactivity was determined on the Packard liquid scintillation spectrometer using a preset $^{32}P$ energy window, but no corrections were made for quenching. Counting efficiency was 99-100%.

#### 2.4.1.2.2 BY CHERENKOV RADIATION EMISSION

Principle

When a β-particle is emitted from the isotope at a velocity comparable to that of light in the medium through which it is travelling it interacts with the molecules of the medium,

and produces an electric shock wave. The resulting electromagnetic emmision is known as Cherenkov radiation and its light spectrum ranges from 300-700 nm. A particle must have energy greater than 0.263 MeV to produce a shock wave condition in water (in Fox, 1976). However, the production of shock wave also depends on the refractive index of the medium and this condition could also be produced by particles of lower energy (<0.263 MeV) in a medium of higher refractive index (Parker and Elrick, 1970).

The particle energy of $^{32}P$ is 1.71 MeV and its Cherenkov radiation emission could be easily detected in an aqueous medium on the Packard liquid scintillation counter and 40% efficiency is achieved using the preset tritium energy window. The Cherenkov radiation contributed by $^{14}C$ β-particles (energy 0.159 MeV) is negligible (theoretical counting efficiency 1.3%).

Procedure

Vials filled with 10 ml distilled water were counted using preset tritium energy window of scintillation counter for background counts. Samples (0-0.1 ml) were added in the vials and vials were counted again for radioactivity at the same channel setting. $^{32}P$-radioactivity was calculated on the basis of 40% counting efficiency.

### 2.4.1.3 *MULTIPLE ISOTOPE COUNTING*

A method has been devised for counting $^{32}P$ and $^{14}C$ when both are present in a sample. Experiments were conducted in which the concentration of either $^{32}P$ and $^{14}C$

was varied in the presence of a known concentration of the other. The amount of the variable was estimated by the method described in 2.4.1.1 and 2.4.1.2.1 and a good corelation between actual and calculated results was achieved. The actual counts of $^{32}P$ in a sample containing both $^{32}P$ and $^{14}C$ were determined by estimating the Cherenkov radiation emission (2.4.1.2.2).

Procedure

$^{32}P$ counts were determined in the presence of $^{14}C$ by the method of Cherenkov radiation emission (2.4.1.2.2). 0.1 ml samples were added to 10 ml distilled water in the scintillation vials. Scintillation vials were counted by using the preset tritium energy window of the scintillation counter. Background counts were obtained previously for each vial filled with only 10 ml distilled water.

For $^{14}C$ counting, 100 µl samples were added to 10 ml scintillant in the scintillation vials. The vials were counted using the preset $^{32}P$ channel with the window fully open to obtain the total counts of $^{32}P$ and $^{14}C$. To estimate the contribution of $^{14}C$ to this total, $^{32}P$ counts obtained by Cherenkov radiation emission were subtracted from the total counts and the remainder ($^{14}C$ counts) were corrected for quenching.

$^{32}P$ counts = 2.5 x counts obtained by Cherenkov radiation emission

$^{14}C$ counts = counts obtained on $^{32}P$ channel with the window full open - 2.5 x counts obtained by Cherenkov radiation emission

## 2.4.2 PREPARATION AND PURIFICATION OF LABELLED COMPOUNDS

### 2.4.2.1 PREPARATION OF [$^{32}$P] G6P

[$^{32}$P] G6P with high specific activity was prepared by a modification of the method of Schendel and Wells (1973) for the preparation of [$^{32}$P] ATP.

Principle

$^{32}P_i$ is incorporated into the substrate, D-glyceraldehye-3-phosphate, in the presence of glyceraldehyde phosphate dehydrogenase and $NAD^+$. At the same time lactate dehydrogenase in the presence of excess pyruvate ensures a high $NAD^+$/NADH ratio. In a coupled reaction ADP is phosphorylated by [$^{32}$P] 1,3-diphosphoglycerate in the presence of 3-phosphoglycerate kinase. The equilibrium is in favour of ATP formation. Finally, $^{32}$P is transferred from ATP to glucose by the action of hexokinase, giving $^{32}$P-labelled G6P.

Procedure

1 mCi of $^{32}$Pi carrier-free, supplied in HCl solution, pH 1-2, was evaporated to dryness in a vacuum desiccator for 24 h. Glyceraldehye - 3 - phosphate (GAP) was prepared from the commercially available diethylacetal derivative (Boehringer Mannheim, Germany) as follows. 100 mg GAP was dissolved in 5 ml water. The pH of the solution was adjusted to approximately 2.0 by adding Dowex resin in the $H^+$ form (50 x 8; 200-400 mesh). The Dowex resin was removed by filtration on a Buchner funnel and the filtrate

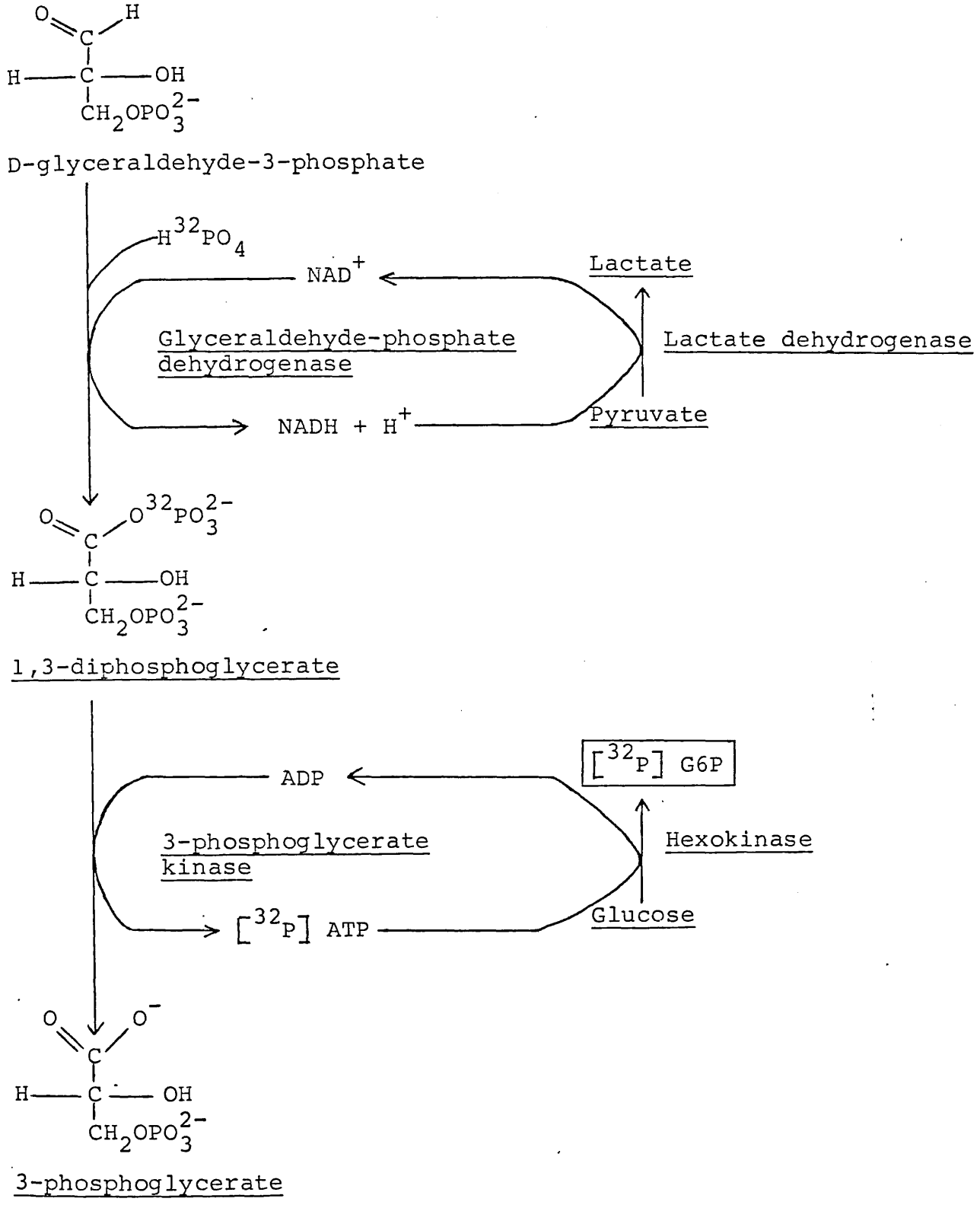



FIGURE 2.7: Schematic Diagram of the Preparation of $[^{32}P]$ G6P

made up to exactly 25 ml with distilled water. The resultant solution was incubated at 35 $^{o}$C for 15 h to complete the hydrolysis and assayed for concentration before use (see 2.2.7). This solution normally used at once, could be stored for 1 week at -20 $^{o}$C or for 2 days at 4 $^{o}$C.

The reaction mixture contained 15 nmol Tris-HCl, pH 8.3, 1 mCi $^{32}P_i$, 1.5 µmol $MgCl_2$, 40 nmol $K_2HPO_4$, 0.25 µmol ADP, 0.15 µmol sodium pyruvate, 0.134 µmol $NAD^+$, 0.15 µmol GAP, 6.0 µmol glucose together with 1.5 units of lactate dehydrogenase and 1.4 units of hexokinase in a final volume of 0.3 ml. The reaction was started by the addition of 13 units of 3-phosphoglycerate kinase and 13 units of glyceraldehyde-3-phosphate dehydrogenase. The mixture was incubated for 4 h at room tempeature and the reaction was stopped by the addition of 0.5 ml 5 mM EDTA. The incubation time for completion of the reaction in the above mixture had been determined previously (see 2.4.2.2).

### 2.4.2.2 DETERMINATION OF INCUBATION TIME FOR PREPARATION OF [$^{32}$P] G6P

The incubation time for completion of the reaction in the preparation of [$^{32}$P] G6P was determined as follows. 2 µl samples were withdrawn at timed intervals from the reaction mixture (2.4.2.1) and were applied to Whatman No. 4 paper. The samples and the standard markers of $P_i$, ATP and G6P were subjected to descending paper chromatography for 14 h at 4 $^{o}$C using ethyl acetate/acetic acid/distilled water as the solvent (2.3.1.2). The chromatogram was dried and the

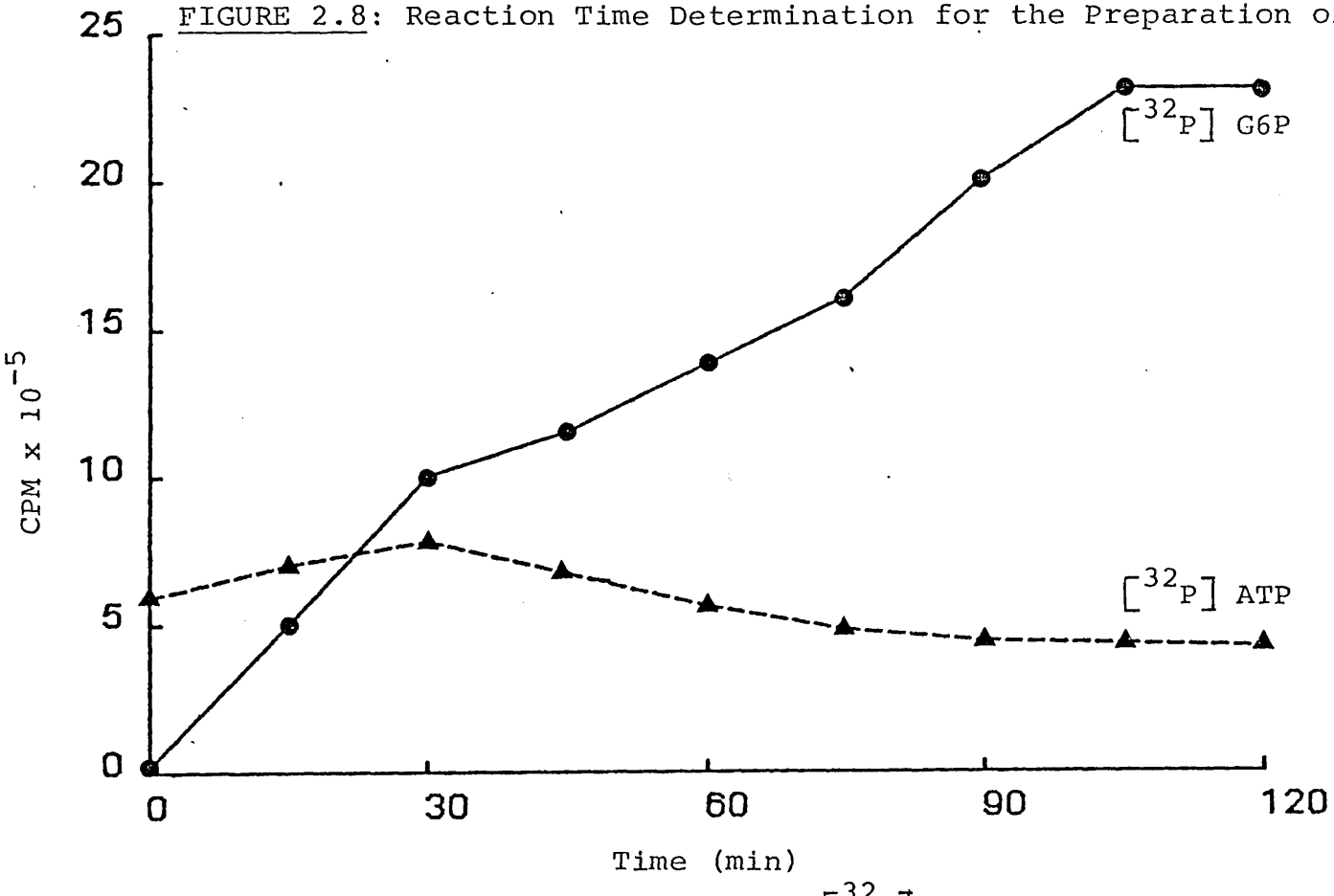



FIGURE 2.8: Reaction Time Determination for the Preparation of [$^{32}$P] G6P

Time for completion of reaction for [$^{32}$P] G6P preparation from $^{32}P_i$ was determined by incubating reagents for [$^{32}$P] G6P preparation and withdrawing 2 µl samples at timed intervals (see 2.4.2.2). The increase in amount of [$^{32}$P] G6P and decrease in [$^{32}$P] ATP were measured as functions of time as described in 2.4.2.2. 2 h was the minimum useful incubation time for preparative purposes.

peaks of [$^{32}$P] G6P and [$^{32}$P] ATP were identified with reference to the standard markers. 3 cm strips containing the peaks were placed in scintillation vials containing 10 ml scintillant and radioactivity counted on a Packard Tri-carb liquid scintillation counter (2.4.1.2.1).

A graph of [$^{32}$P] ATP and [$^{32}$P] G6P counts versus time was plotted (Fig. 2.8). 2 h was found to be the minimum incubation time. However, during preparative runs, the reaction mixture was incubated for 4 h in order to increase the yield of [$^{32}$P] G6P.

### 2.4.2.3 PURIFICATION OF [$^{32}$P] G6P

$^{32}$P[G6P] from 2.4.2.1 was separated by descending chromatography using ethyl acetate/glacial acetic acid/distilled water (3:3:1 by vol) as solvent (2.3.1.2). The mobility of G6P was less than that of $P_i$ in this solvent. In some experiments, [$^{32}$P] G6P was found to be contaminated with $^{32}P_i$. When this was the case, it was further purified by using a solvent system in which the mobility of G6P was greater than $P_i$. This second chromatography was performed on Whatman No. 4 paper at room temperature using 2-methoxyethanol/ethyl methyl ketone/3 M $NH_4OH$ (7:2:3 by vol) as solvent (2.3.1.2). The chromatogram was dried and the radioactive strip containing [$^{32}$P] G6P was eluted with a small volume of water. This solution containing [$^{32}$P] G6P was stored at -20 $^{o}$C.

A pure preparation of [$^{32}$P]G6P was obtained with a maximum specific radioactivity of 2.1 Ci mmol$^{-1}$ and less than 0.3%

*

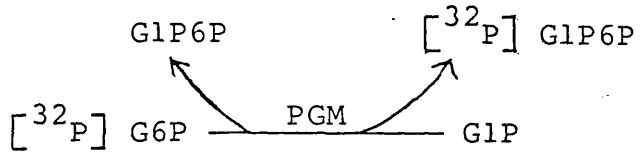
When $^{32}P$ G6P was incubated with PGM in the presence of $Mg^{++}$ and a catalytic quantity of G1P6P until equilibrium was reached, the relative quantities of $^{32}P$ G1P and $^{32}P$ G6P were found to be in the ratio 1:16.2 ($K_{eq}$ = 17.2). This indicates that the $^{32}P$ G6P must be virtually all enzymatically active.

contamination with $P_i$. The percentage yield was 46% based on $P_i$ incorporation.

*

2.4.2.4 PREPARATION AND PURIFICATION OF [$^{32}$P] G1P6P

[$^{32}$P] G1P6P was prepared from [$^{32}$P] G6P by the method used by Clarke et al. (1974).




50 µCi [$^{32}$P] G6P was incubated for 1 h at 30 °C with His-Tris-HCl buffer, pH 7.4 (40 mM histidine, 12 mM Tris) containing 1.1 mM $MgCl_2$, 0.25 µmol G1P6P and 10 µl rabbit muscle phosphoglucomutase in a final volume of 1 ml. [$^{32}$P] G1P6P was isolated by descending paper chromatography, using the DEAE-cellulose-ammonia system (2.3.1.2). The [$^{32}$P] G1P6P eluted from the paper with 5 M ammonia solution was freeze-dried, dissolved in 1 ml distilled water and stored at -20 °C. 9.85 µCi of [$^{32}$P] G1P6P was obtained with specific radioactivity of 34 Ci $mol^{-1}$.

2.4.2.5 PURIFICATION OF [$^{14}$C] G6P

D-[U-$^{14}$C] G6P was obtained from the Radiochemical Centre, Amersham as a sodium salt in aqueous solution containing 3% ethanol. It was found to be contaminated with 2.8% labelled glucose. The glucose was removed by purifying the [$^{14}$C] G6P on an ion-exchange column using the method described by Schendel and Wells (1973) for purification of [$^{32}$P] ATP.

Procedure

[$^{14}$C] G6P was freeze-dried, dissolved in 1 ml 10 mM $NH_4HCO_3$ buffer, pH 7.5 and applied to a DEAE-Sephadex A-50 column (1 x 5 cm) previously equilibrated with the same buffer. The column was washed with 15 ml of the buffer to remove labelled glucose and then with 10 ml 20 mM $NH_4HCO_3$ buffer, pH 7.5 to ensure that all traces of labelled glucose had been removed. Finally [$^{14}$C] G6P was eluted from the column with 10-15 ml 100 mM $NH_4HCO_3$ buffer, pH 7.5. The eluted fraction was freeze-dried. The residue, now free of $NH_4HCO_3$ was dissolved in 1 ml distilled water and stored at -20 °C.

## 2.4.3 RADIOCHEMICAL ASSAYS

### 2.4.3.1 DETERMINATION OF SPECIFIC RADIOACTIVITY OF [$^{32}$P] G6P and [$^{14}$C] G6P

The concentration of labelled G6P was determined spectrophotometrically by using the following reaction:

$$\text{G6P} + \text{NADP}^+ \underset{\leftarrow}{\xrightarrow{\text{G6PDH}}} \text{6-P gluconate} + \text{NADPH} + \text{H}^+$$

Procedure

Labelled G6P was incubated with 0.475 mM $NADP^+$ and Tris-HCl-$Mg^{++}$ buffer, pH 8.0 (100 mM Tris-HCl, 5 mm $Mg^{++}$, final concentration in cuvette) at 30 °C for 10 min in a final volume of 1 ml. Reaction was started by addition of 10 µl G6PDH and the G6P concentration was calculated from the change in absorbance at 340 nm.

The radioactivity of [$^{32}$P] G6P and [$^{14}$C] G6P was determined by the method described in 2.4.1.

### 2.4.3.2 DETERMINATION OF SPECIFIC RADIOACTIVITY OF [$^{32}$P] G1P6P

The specific radioactivity of [$^{32}$P] G1P6P was determined by hydrolysing G1P6P to G6P and then measuring the molar concentration of G6P as described in 2.4.3.1.

200 µl [$^{32}$P] G1P6P was added to 200 µl M HCl and was heated to 100 $^{o}$C in a boiling water bath for 20 min. After the hydrolysis of G1P6P to G6P, the solution was cooled and the acid neutralised with 200 µl M NaOH. The molar concentration of G6P in this solution was determined as described in 2.4.3.1.

The radioactivity of [$^{32}$P] G1P6P was determined in the scintillation counter after adding 10 µl of sample to 10 ml scintillant (2.4.1.2.1).

### 2.4.3.3 DETERMINATION OF [$^{32}$P] G1P IN THE PRESENCE OF [$^{32}$P] G6P FOR INDUCED-TRANSPORT TESTS

[$^{32}$P] G1P in the presence of [$^{32}$P] G6P was determined by a slight modification of a method of Ray *et al*. (1966), based upon the phosphate precipitation technique of Sugino and Miyoshi (1964). The hydrolysis and precipitation steps were carried out separately to protect the G6P from hydrolysis, because molybdate activates the hydrolysis of G6P (Weil-Malherbe *et al*., 1951).

G1P is acid and heat labile and hydrolyses completely when heated to 100 $^{o}$C for 10 min in acid solution. Leloir and Cardini (1957) have reported that the half-life of G1P

hydrolysis is 1.05-2.00 min in 1 M acid at 100 °C. The hydrolysis of G6P is very slight under these conditions. The inorganic phosphate from G1P hydrolysis can be precipitated with ammonium molybdate as phosphomolybdic acid in the presence of triethanolamine under acidic conditions. The unhydrolysed phosphate does not precipitate and remains in solution (Sugino and Miyoshi, 1964).

Procedure

For [$^{32}$P] G1P determination 0.1 ml samples from induced-transport tests were added to 0.14 ml 1.8 M $HClO_4$ in a 15 ml conical stoppered centrifuge tube. 0.5 ml solution containing 2 mM G1P and 2 mM G6P as carrier was then added. The mixture was brought to 100 °C in a boiling bath for 10 min and then immediately cooled to 4 °C in an ice water bath.

0.26 ml phosphate precipitating reagent, which had been prepared shortly before use by mixing 57 mM ammonium molybdate and 74 mM triethanolamine (1:1 by vol.), was then added to the centrifuge tube. The solutions were mixed within a few seconds to avoid chemical breakdown of [$^{32}$P] G6P. The tube was kept at 4 °C for 2 h to complete the precipitation of hydrolysed phosphate. After centrifugation at 2,000 g for 20 min, the supernatant containing [$^{32}$P] G6P was removed. The precipitate was washed three times with a solution containing 57 mM ammonium molybdate, 74 mM triethanolamine, 1.8 M $HClO_4$, distilled water (1:1:1:6 by vol.) and a trace of phosphate as carrier. The

precipitate was dissolved in 0.2 ml of M NaOH and transferred quantitatively with three washings of 0.2 ml distilled water to a scintillation vial containing 10 ml scintillant. 0.2 ml M HCl was also added to neutralize the alkali. The vials were counted in the Packard liquid scintillation spectrometer and no correction was made for quenching (2.4.1.2.1).

The same procedure can be used for determination of [$^{32}$P] G6P in the presence of [$^{32}$P] G1P. After precipitating the hydrolysed phosphate and centrifugation, 200 µl of supernatant containing [$^{32}$P] G6P was added to 10 ml scintillant in a scintillation vial. 0.1 ml M NaOH was added to discharge the yellow colour and neutralise the acid. The vials were counted for $^{32}$P radioactivity as described before (2.4.1.2.1).

### 2.4.3.4 DETERMINATION OF [$^{14}$C] G1P IN THE PRESENCE OF [$^{14}$C] G6P FOR INDUCED-TRANSPORT TEST

[$^{14}$C] G1P was estimated by the method of Britton and Clarke (1968) which was based on the inorganic phosphate and phosphate ester precipitation technique of Nelson (1944) and Somogyi (1945).

[$^{14}$C] G1P is hydrolysed completely to [$^{14}$C] glucose and $P_i$ at 100 $^{o}$C in a boiling water bath for 10 min. G1P6P is also hydrolysed to G1P and G6P, but the hydrolysis of [$^{14}$C] G6P is very slight under these conditions (Clarke, 1972). Hydrolysed $P_i$ and phosphate esters can be removed

from the solution by precipitation with $ZnSO_4$ and $Ba(OH)_2$, whilst [$^{14}C$] glucose remains in solution.

Procedure

0.1 ml samples from induced-transport tests were added to stoppered centrifuge tubes containing 0.1 ml M-$H_2SO_4$ to stop any enzyme reaction. The hydrolysis of [$^{14}C$] G1P was carried out by placing the tubes in a boiling water bath for 10 min. The tubes were cooled to room temperature and 0.5 ml glucose (1 mg $ml^{-1}$) was added as a carrier. 0.65 ml 0.2 M $Ba(OH)_2$ was then added to each tube to precipitate inorganic phosphate and G6P. The tubes were agitated thoroughly and followed by the addition of 0.2 ml 5% $ZnSO_4$ to each tube. The precipitate was compacted in the bottom of the tubes by centrifugation. 0.1 ml 5% $ZnSO_4$ was added again, followed by 0.1 ml 0.2 M $Ba(OH)_2$ to remove all traces of [$^{14}C$] G6P from the supernatant. After centrifugation 0.2 ml samples were added to 10 ml scintillant and radioactivity was counted as described in 2.4.1.1.

The amounts of $ZnSO_4$ and $Ba(OH)_2$ used in this experiment were suitable for determining G1P concentrations up to 5 mM (Clarke, 1972). When the concentration of G1P was higher than 5 mM, the amounts of $Ba(OH)_2$ and $ZnSO_4$ were increased appropriately.

### 2.4.3.5 DETERMINATION OF GLUCOSE-1,6-DIPHOSPHATASE ACTIVITY OF PURIFIED PHOSPHOGLUCOMUTASE BY USING LABELLED SUBSTRATE

Glucose-1,6-diphosphatase activity in purified preparations of rabbit liver phosphoglucomutase (100 units $mg^{-1}$ protein) was determined by using $^{32}P$-labelled G1P6P as substrate. The progress of the reaction can be determined by incubating the enzyme with [$^{32}P$] G1P6P and measuring the hydrolysed $^{32}P_i$ at various time intervals by the method of Ray et al. (1966) based upon the phosphate-precipitation technique of Sugino and Miyoshi (1964). In this method the $P_i$ is precipitated and the hydrolysis of G1P and G1P6P does not occur.

Procedure

0.486 μCi [$^{32}P$] G1P6P (21.75 nmol; specific radioactivity 22.34 Ci $mol^{-1}$) was warmed to 30 °C, with 9 nmol G1P6P (as carrier) in His-Tris-HCl buffer, pH 7.6 (40 mM Histidine, 12 mM Tris), containing 1.1 mM $MgCl_2$ in a total volume of 3.0 ml. 0.5 unit (23 μl) of phosphoglucomutase at 30 °C was added to the above mixture to start the reaction. 100 μl samples were withdrawn at various time intervals.

$^{32}P_i$ was determined in the samples as follows. 100 μl samples were added to 15 ml conical centrifuge tubes containing 600 μl 1.8 M $HClO_4$ to stop the enzyme reaction, followed by 20 μl 50 μM G6P, 10 μl 100 mM $KH_2PO_4$, pH 7.0 and 1 ml freshly prepared phosphate-precipitating reagent (1:1 mixture of 57 mM ammonium molybdate and 75 mM triethanolamine). The solutions were thoroughly mixed and left at 4 °C for 18 h.

The suspension was centrifuged at 2,000 g for 20 min and the supernatant, containing [$^{32}$P] G1P6P, [$^{32}$P] G1P and [$^{32}$P] G6P, was removed. The precipitate containing hydrolysed phosphate was washed three times with a solution containing 57 mM ammonium molybdate, 74 mM triethanolamine, 1.8 M $HClO_4$, distilled water (1:1:1:6 by vol.) and a trace of phosphate. The washed precipitate was dissolved in 200 µl M NaOH with gentle warming and was transferred quantitatively, with three washings of 200 µl distilled water, to vials containing 10 ml scintillant. The vials were counted for $^{32}$P-radioactivity as described in 2.4.1.2.1.

Addition of perchloric acid to the samples in $^{32}P_i$ determination also precipitated the phosphoglucomutase and $^{32}$P associated with the enzyme. However, the samples contained a very small amount of phosphoglucomutase, $5.04 \times 10^{-3}$ nmol, calculated by assuming the highest recorded specific activity of the enzyme, 484 units $mg^{-1}$ protein (3.1) and a molecular weight of 67,600 (3.2.3). If it is assumed that 1 mol enzyme binds 0.5 mol of P (see 3.6.1), the $^{32}$P-radioactivity associated with the enzyme would have increased by 8 cpm in the precipitate. This increase is insignificant and was ignored.

## 2.5 INDUCED-TRANSPORT TESTS

The mechanism of the rabbit liver phosphoglucomutase reaction was investigated using the induced-transport tests of Britton and Clarke (1968).

The flux-measurement method was introduced by Britton (1966) for investigating the mechanism of the enzyme reaction. Britton and Clarke (1968) carried out induced-transport tests with $^{32}P$- and $^{14}C$-labelled substrates in order to differentiate and characterise the mechanism of rabbit muscle PGM from three possible reaction mechanisms of the mutases (see 1.1.9).

The phosphate flux in the PGM reaction is defined as the actual number of phosphate groups transferred from the 1-position of G1P to the 6-position of G6P in unit time under particular conditions of concentration and temperature etc. The glucose flux is defined as the number of glucose molecules transferred from G1P to G6P in unit time (Britton and Clarke, 1968). Induced-transport experiments are performed by adding excess unlabelled G1P to an equilibrium mixture of labelled glucose monophosphates and measuring the total radioactivity in the G1P fraction during the progress of the reaction. Experiments are carried out with G1P containing $^{14}C$-labelled glucose or $^{32}P$-labelled phosphate separately. If the radioactivity remains the same in G1P this shows that the flux of G1P to G6P is independent of that from G6P to G1P (independence relationship obeyed). Transient decrease in radioactivity shows

co-transport. Lack of induced-transport occurs when both substrate and product compete for equivalent forms of the phosphoenzyme, and when label is transferred from substrate to product by a single catalytic cycle. Co-transport occurs when the label requires two or more catalytic cycles to move from substrate to product and increase in extent with the number of cycles required. Counter-transport occurs when substrate and product compete for different inter-convertible forms of the phosphoenzyme where this interconversion is partially rate limiting (Britton and Clarke, 1968).

Procedure

Induced-transport tests of rabbit liver PGM were carried out with [$^{32}$P] G6P at low substrate concentrations and with [$^{14}$C] G6P at both low and high substrate concentrations as described by Britton and Clarke (1968). The experimental details are given in 3.5.

## 2.6 EXPERIMENTS INVOLVING PREPARATION AND PROPERTIES OF LABELLED PHOSPHOGLUCOMUTASE

### 2.6.1 PREPARATION AND SEPARATION OF $^{32}$P-LABELLED PHOSPHOGLUCOMUTASE FROM RABBIT LIVER

$^{32}$P-labelled PGM from rabbit liver was prepared and separated on a Sephadex G-25 column by the method of Clarke et al. (1974).

10 units (0.306 nmol) rabbit liver PGM was incubated with 0.28 μCi [$^{32}$P] G6P (0.459 nmol, specific radioactivity 609 Ci mol$^{-1}$) and 0.28 μCi [$^{14}$C] G6P (1.97 nmol, specific radioactivity 142 Ci mol$^{-1}$) and 0.04 nmol G1P6P in His-Tris-HCl buffer, pH 7.6 (40 mM histidine, 12 mM Tris) containing 2 mM $MgCl_2$ at 30 °C for 5 min in a final volume of 0.25 ml. The incubation mixture was immediately cooled to 0 °C in an iced water bath and applied to a Sephadex G-25 column (20 x 1 cm) previously equilibrated with the same incubation buffer. The column was eluted with the same buffer and 0.3 ml fractions were collected. All fractions were assayed for enzyme activity and for $^{32}$P and $^{14}$C radioactivity. Enzyme activity was assayed spectrophotometrically (2.2.1.1.1). $^{32}$P and $^{14}$C radioactivity simultaneously present in fractions were counted by the method of multiple isotope counting (2.4.1.3).

The fractions containing enzyme activity were collected and used for studies of the lability of the phosphoenzyme in acid and alkali and for its stability at pH 7.6.

## 2.6.2 DETERMINATION OF EXCHANGEABILITY AND STABILITY OF PHOSPHOENZYME

The exchangeability of $^{32}P$-labelled phosphoenzyme with substrate and the half-life of labelled phosphoenzyme in His-Tris-HCl buffer, pH 7.6, containing 2 mM $MgCl_2$ at 30 °C were determined by the method of Clarke et al. (1974). $^{32}P$-labelled enzyme is incubated with substrate (G6P). The amount of $^{32}P$ subsequently found in isolated substrate is a measure of the exchangeability of enzyme-bound $^{32}P$ ('exchangeable $^{32}P$').

Approximately 10 units of $^{32}P$-labelled PGM, purified by gel filtration (2.6.1), in a volume of 1 to 2 ml, was warmed to 30 °C. 0.1 ml samples were withdrawn at timed intervals. Exchangeable $^{32}P$ in the substrate was estimated by adding the samples to a conical centrifuge tube containing 10 µl 0.1 M G6P and 10 µl 0.1 M $Na_2HPO_4$, pH 7.0 and incubating at 30 °C for 1 min. After cooling, the centrifuge tubes to 0 °C in a iced water bath, 1.7 ml 0.6 M $HClO_4$ was added, followed by 1 ml of a freshly prepared mixture of 57 mM ammonium molybdate and 74 mM triethanolamine (1:1 by vol.), all at 0 °C. Solutions were then left overnight at 0 °C. After centrifugation at 2,000 g for 20 min, 1 ml supernatant was added to 10 ml scintillant in a scintillation vial. 0.373 ml M NaOH was added to neutralize the acid. Total radioactivity in the GmP was then determined by scintillation counting (2.4.1.2.1).

## 2.6.3 DETERMINATION OF ACID STABILITY OF LABELLED PHOSPHOENZYME

The stability of labelled phosphoenzyme in acid was determined as follows. Approximately 1 unit of labelled enzyme in a volume of 0.1 ml, purified on Sephadex G-25 (2.6.1), was added to 0.85 ml 0.6 M $HClO_4$ pH 7.0 and 0.1 ml solution containing 1 mg Bovine serum albumin, all at 4 $^{o}C$. Perchloric acid was used to precipitate the enzyme. Serum albumin and $Na_2HPO_4$ were carriers for the enzyme and hydrolysed phosphate.

After centrifugation at 2,000 g for 10 min, the radioactivity associated with the protein in the precipitate and the $^{32}P$-labelled inorganic phosphate in the supernatant was estimated as follows. 0.2 ml of supernatant was added to 10 ml of scintillant in a scintillation vial. 0.2 ml of 0.5 M NaOH was further added to neutralise the acid and the liberated $^{32}P_i$ was estimated by scintillation counting as described previously (2.4.1.2.1). For estimation of radioactivity associated with the protein, the supernatant was removed and precipitate washed three times with a mixture of 0.85 ml 0.6 M $HClO_4$ and 10 µl 0.1 M $Na_2HPO_4$, pH 7.0. The precipitate was dissolved in 0.2 ml of 0.5 M NaOH and transferred to 10 ml scintillant in a scintillation vial. The centrifuge tube was washed with 0.2 ml 0.5 M HCl, which was added to the scintillant to ensure a quantitative transfer of radioactivity and to neutralize alkali in the scintillant. The radioactivity in the vials was then measured as described in 2.4.1.2.1.

## 2.6.4 ALKALINE HYDROLYSIS OF LABELLED PHOSPHOENZYME

The rate of hydrolysis of labelled PGM was measured in both 0.25 M NaOH and 0.33 M NaOH at 37 $^{o}$C.

Approximately 5 units of labelled enzyme in a volume of 1 ml, separated from substrates by gel filtration (2.6.1), was warmed to 37 $^{o}$C in a water bath. 1 M NaOH was added to the enzyme solution at 37 $^{o}$C to give a final concentration of 0.33 or 0.25 M NaOH and volume 1.5 ml. Samples were taken at timed intervals, cooled immediately to 4 $^{o}$C, and added to cold 0.1 ml 0.6 M $HClO_4$ in 15 ml conical centrifuge tubes, followed by 5 µl 0.1 M $Na_2HPO_4$, pH 7.0. After centrifugation at 2,000 g for 10 min, 0.1 ml supernatant was withdrawn and added to 10 ml scintillant in a scintillation vial. 0.07 ml 0.5 M NaOH was further added to the scintillant to neutralize the acid and the $^{32}P_i$ estimated by scintillation counting (2.4.1.2.1).

The labelled phosphate associated with protein in the precipitate was measured by the method described in the previous section (2.6.3).

## 2.6.5 QUANTITATIVE DETERMINATION OF PROTEIN-BOUND PHOSPHATE BY PHENOL EXTRACTION METHOD

PGM was phosphorylated using labelled substrate (2.6.1) and a quantitative determination of phosphate bound to enzyme was carried out by the phenol extraction method (Ramaley et al, 1967; Rose, 1970). In this method, the enzyme is selectively partitioned into the phenol fraction of a

phenol-water two phase system. All non-protein bound $^{32}P$ remains in the aqueous layer and can thus be removed.

Procedure

10 units of PGM was phosphorylated by incubation with 0.17 μCi[$^{32}P$]G6P (3.13 nmol; specific radioactivity 54.3 Ci $mol^{-1}$) and 0.12 nmol G1P6P in His-Tris-HCl buffer, pH 7.6 (40 mM histidine, 12 mM Tris), containing 2 mM $MgCl_2$ at 30 °C, for 30 min in a final volume of 0.2 ml. Reaction was terminated by the addition of 1 ml 50 mM Tris-HCl buffer, pH 8.0, 0.88 saturated with phenol and containing 1 mM sodium phosphate as carrier. The resulting solution was extracted with 2 ml phenol saturated with the same buffer mixture. The phenol layer containing the phospho-protein was clarified by centrifugation and the aqueous layer was carefully aspirated off. The phenol layer was then washed with 50 mM Tris-HCl buffer, pH 8.0. This washing procedure was repeated until the radioactivity in the aqueous layer approached background; usually 5 washes were found to be necessary to remove all traces of unbound phosphates from the protein. $^{32}P$-activity associated with protein was subsequently estimated by taking 30 ml of the phenol layer for scintillation counting (2.4.1.2.1).

## 2.7 POLYACRYLAMIDE GEL ELECTROPHORESIS FOR ISOENZYME SEPARATION

The PGM isoenzymes were separated and characterised by 'discontinuous polyacrylamide gel electrophoresis' (Ornstein, 1964; Davis,1964; Williams et al., 1964), using the Laemmli and Favre (1973) buffer system.

Acrylamide is completely soluble in aqueous solutions but can be polymerized to form a suitable supporting medium in the form of gels and slabs for electrophoresis (Raymond and Weintraub, 1959). Acrylamide will co-polymerize with bisacrylamide which acts as cross-linking agent. The polymerization reaction can be carried out either chemically or photochemically. The chemical polymerization of acrylamide requires an oxidising agent as catalyst (usually sodium or ammonium persulphate) with TEMED as a catalytic initiator. Photochemical polymerization is achieved by irradiating an acrylamide/TEMED/riboflavin solution with u.v. light. Polyacrylamide gel thus formed is thermostable, transparent, strong and chemically inert over a wide range of chemical and physical conditions. It is porous to proteins under certain conditions. Polyacrylamide gels and slabs can be prepared with a range of pore sizes by varying the relative properties of acrylamide and bisacrylamide. Proteins migrate faster through a gel of low concentration and large pore size than through one of high concentration and small pore size. The rate of movement of proteins through a particular gel depends on its molecular weight, shape and electric charge.

The disc electrophoresis method was developed by Ornstein (1964) and Davis (1964). The method employs gels of two porosities. The gels are set in a glass tube with a large pore gel (or stacking gel) on the top of a small pore gel (or separating gel). The method was named 'disc' because protein bands stack up in the separating gel in a series of concentrated discs after electrophoresis. In this method the proteins concentrate into the stacking gel according to a 'regulatory function' described by Kohlrausch (1897), and separate in the separating gel by the combined action of electrophoresis and molecular seiving. Because of discontinuity in porosities, pH and buffer anions the method was later called 'discontinuous electrophoresis'. This method separates proteins according to both charge and size and the extent of separation depends upon the concentration of polyacrylamide used in the polymerization of the separating gel.

Laemmli and Favre (1973) used Tris-HCl pH 6.8 for preparing the stacking gel and Tris-HCl pH 8.8 for the separating gel. The gels are set vertically in the electrophoresis apparatus and the compartment at each end of the tubes is filled with the same electrode buffer. There are three discontinuities in the whole system. (1) Pore size discontinuity at the interface of stacking and separating gels; (2) pH discontinuity at the interface of stacking and separating gels and at the interface of electrode buffer and stacking gel; (3) ion discontinuity because the stacking and separating gels contain chloride ions whilst the electrode buffer contains glycinate ions.

During the electrophoresis, boundaries form between leading ions (Chloride) and trailing ions (glycinate), bracketing the sample between them. At pH 6.8 in the stacking gel, glycinate ions have a lower mobility and chloride ions have a higher mobility than proteins. The sample therefore stays within the boundary of leading and trailing ions in the stacking gel. This process concentrates the proteins into discs. When the trailing ions of weak acid enter into the separating gel of higher pH, the mobility of the ions increases and overtake the slowly migrating protein sample. The proteins are left behind as thin discs and then separate by ordinary zone electrophoresis in the separating gel.

### 2.7.1 STOCK SOLUTIONS FOR ELECTROPHORESIS

Electrophoresis buffer (Laemmli and Favre, 1973):

0.05 M Tris

0.384 M Glycine pH 8.3 (4 $^{o}$C)

Separating gel buffer (Laemmli and Favre, 1973):

1.5 M Tris-HCl pH 8.8 (4 $^{o}$C)

Stacking gel buffer (Laemmli and Favre, 1973):

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

Stock acrylamide solution:

30% acrylamide

0.9% N,N'-methylbisacrylamide (bis) in water

Initiator:

N,N,N',N'-tetramethylethylenediamine (TEMED)

Catalyst:

18 mg sodium persulphate $ml^{-1}$ water

Photocatalyst:

4 mg riboflavin in 100 ml distilled water

Tracking Dye:

1% aqueous bromophenol blue

Sample preparation buffer (Scopes, 1968):

10 mM Tris-HCl pH 8.7 (4 $^{o}$C)

Sample dialysis buffer:

68 mM Tris-HCl pH 6.8

containing: 0.6 M sucrose

1.0 mM 2-mercaptoethanol

1.0 mM EDTA

## 2.7.2 SILICONISATION OF GLASS TUBES

Before gel electrophoresis, the glass tubes of 6 mm diameter and 10 cm long were siliconised to prevent gels permanently sticking to the tube walls. The glass tubes were first soaked overnight in 5% Decon-90 solution and washed with distilled water. After drying in an oven, the tubes were immersed in benzene containing 1% (v/v) dimethyldichlorosilane and brought to 60 $^{o}$C for 10 min. in a water bath. The siliconised tubes were then dried in an oven at 100 $^{o}$C, cooled and rinsed with methanol.

## 2.7.3 PREPARATION OF GELS

A 7.5% gel solution was prepared by mixing together 15 ml stock acrylamide, 15 ml separating gel buffer and 15 µl TEMED and making up to 59 ml with distilled water in a Buchner flask. The solution was degassed for 10 min with a water pump and then 1 ml sodium persulphate was added as

polymerization catalyst. The solution was transferred with a 20 ml syringe and needle to siliconised glass tubes (sealed on one end with parafilm) to give a column of gel about 7 cm long. Care was taken to avoid trapping air bubbles. 2 to 3 drops of water were placed immediately on top of the gel solution. Polymerization took about 20 min at room temperature.

A preliminary electrophoretic run was carried out at this stage to remove sodium persulphate from the separating gel. Persulphate is a strong oxidising agent and could produce artifacts in the electrophoretic pattern by oxidising free sulphydryl groups and causing a linkage to be formed between two adjacent free sulphydryl groups which could be in the same or in different proteins. Such artifacts in electrophoretic patterns have been observed by Brewer (1967), Fautes et al. (1967), Mitchell (1967) and Benwick (1968). It was thus important to remove persulphate ions prior to electrophoresis as it has been shown that rabbit muscle PGM contains six sulphydryl groups, two of which are easily accessible (Bocchini et al., 1967) and this type of heterogeneity has been shown to occur by Dawson and Mitchell (1973). The possibility of such artifacts in our experiment was removed to a low level by removal of persulphate from separating gels; separating gels were subjected to gel electrophoresis at 4 mA per gel for 1.5 min prior to setting the spacer gels (see Mitchell, 1967). To maintain the ionic and pH conditions of the separating gels after removal of persulphate, the buffer used for electrophoresis was the same as the separating gel buffer.

After preliminary electrophoresis, the stacking gels were photopolymerised on the top of separating gels as follows. The 3% stacking gel solution was prepared by mixing 3 ml stock acrylamide, 7.5 ml stacking buffer, 30 µl TEMED, 6 ml 50% sucrose solution and 3.5 ml riboflavin and made up to 30 ml with distilled water. The solution was degassed for 10 min in the dark. The buffer was removed from the surface of the separating gel to give a depth of 1.5 cm. The stacking gel set in 30 min under a 40 W fluorescent light at a distance of 10 cm. The stacking gel produces a discontinuity in the system and filters out any insoluble material from the sample.

The composition of the separating gel was T = 7.5%, C = 3% and that of the stacking gel was T = 3%, C = 3%, where T is the weight of acrylamide per 100 ml water and C is the amount of bisacrylamide expressed as a percentage of the total weight of acrylamide (Hjerten, 1962).

The separating gels could be prepared one day before the electrophoresis but the stacking gel must be prepared immediately before use.

### 2.7.4 PREPARATION OF SAMPLE FOR ELECTROPHORESIS

The preparation of the sample was carried out by a modified method of Scope (1968).

Tissues were homogenised with 2 vol 10 mM Tris-HCl buffer, pH 8.7, containing 2% (v/v) glycerol, 2 mM EDTA and 1 mM 2-mercaptoethanol. The homogenates were adjusted to

pH 7.0 ± 0.3 with M Tris and centrifuged at 25,000 g for 10 min. The supernatants were brought to pH 5.5 by the addition of M acetic acid and after 5 min the mixtures were centrifuged at 25,000 g for 10 min, to remove denatured proteins. The supernatants were finally adjusted to pH 7.0 with M Tris and dialysed overnight against 68 mM Tris-HCl buffer, pH 6.8 containing 0.6 M sucrose, 1 mM EDTA and 1 mM 2-mercaptoethanol. All steps were performed at 4 $^{o}$C.

A further step to exclude the possibility of artifacts in the electrophoresis pattern of phosphoglucomutase due to oxidation was taken by including 2-mercaptoethanol as sulphydryl group protecting agent (Spencer et al., 1964; Green and Dawson, 1973) in all stages of sample preparation. EDTA was included in the sample to protect the enzyme from heavy metals. 0.8 M sucrose was added to the dialysing buffer to increase the density of the sample and to concentrate the enzyme. High density of the sample minimises its mixing with electrode buffer.

25 µl of rabbit liver supernatant containing 0.94 unit of PGM activity and 2.9 mg protein and 80 µl of rabbit muscle supernatant containing 3.1 units of PGM activity and 0.81 mg protein were used for electrophoresis.

### 2.7.5 SAMPLE APPLICATION AND ELECTROPHORESIS

Electrophoresis was carried out in a Pharmacia GE-2/4 apparatus. The gels were inserted through the gasket in the upper chamber of the apparatus. Approximately 2 ℓ

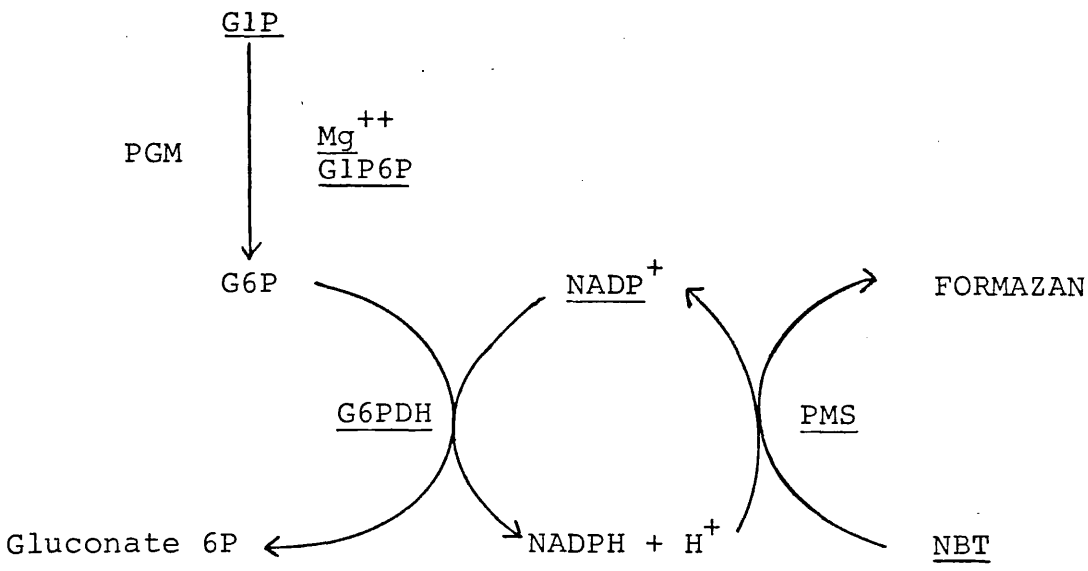



FIGURE 2.9: Sequence of reactions for detection of phosphoglucomutase activity on polyacrylamide gels.

The underlined components are used in the staining solution. The final compound formed is blue-purple insoluble Formazan, which is deposited at the site of PGM activity. For staining mixture see 2.7.6.

electrode buffer was poured into the lower chamber and the upper chamber was placed in position. Trapped air bubbles in the bottom end of the gel tubes were removed. The upper chamber was finally filled with the same buffer and trapped air in the top of each gel tube removed. 25 ml rabbit liver and 80 ml rabbit muscle supernatant were applied carefully to the tops of the gels.

A trial experiment showed that an electrophoretic run of 1.5 h at a current of 4 mA per gel brings the bromophenol blue band down to the end of the tube, leaving the isoenzyme bands poorly separated within 2 cm of the top of the gel. The electrophoresis of the samples was therefore carried out for 3 h at 4 mA per gel.

At the end of the run, gels were removed from the tubes by using a fine needle attached to a 20 ml syringe to inject a jet of water between the gel and the glass tube wall. The gels were stained selectively for PGM activity as described in 2.7.6.

### 2.7.6 STAINING

The gels were stained for PGM activity by the tetrazolium staining technique of Beitner et al. (1975) with slight changes in concentration of the chemicals. Similar staining procedures have also been used by Spencer et al. (1964), Scopes (1968), Turner and Lyerla (1980).

In the PGM coupled enzyme reaction (see Fig. 2.9), the G6PDH reduces $NADP^+$ to NADPH. This reaction can be coupled to the reduction of NBT by electron donor (NADPH) forming

the blue-purple insoluble compound formazan. This reaction proceeds rapidly in the presence of PMS which acts as an intermediary catalyst. The tetrazolium-PMS mixture is light sensitive, so that staining must be carried out in the dark.

A mixture containing the following was prepared just before use:

40 mM imidazole HCl buffer, pH 7.2 (30 °C)
2 mM G1P
2 µM G1P6P
3 mM $MgCl_2$
1 mM EDTA
0.5 mM $NADP^+$
0.4 mM NBT
0.1 mM PMS
100 units G6PDH per 100 ml stain.

The staining was carried out in the dark at 30 °C until the blue bands of PGM appeared (15-30 min)

### 2.7.7 STORAGE

Excess stain was removed by washing the gels with distilled water. The gels were fixed and stored in 5% acetic acid.

### 2.7.8 SCANNING OF GELS

The fixed gels were inserted in the silica trough of the densitometer filled with 5% acetic acid solution. All air bubbles were removed before the lid of the trough was

fitted. The trough was mounted on the carrier of the densitometer fitted in the Pye Unicam SP 8-100 spectrophotometer. PGM bands were scanned at 600 nm.

## 2.8 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Molecular weight determination of PGM was carried out on gels of constant polyacrylamide concentration (homogeneous gels; Weber and Osborn, 1969), using the Laemmli and Favre (1973) buffer system.

Shapiro, Vinuela and Maizel first reported in 1967 that the molecular sizes of polypeptides could be estimated from the relative electrophoretic mobilities of SDS-complexes on polyacrylamide gels. This technique was confirmed by Weber and Osborn (1969) using 40 well-characterised proteins of known molecular weights.

When an anionic detergent SDS is added to a protein solution, the protein denatures and dissociates into its subunits. SDS binds to each polypeptide forming a rod like molecule. SDS has ability to interact and denature a wide variety of proteins in a similar manner (Reynolds et al. 1970). Addition of DTT or 2-mercaptoethanol reduces all intra-chain and inter-chain disulphide bonds. Native proteins, having widely different charge, size and shape characteristics, can be separated into their constitutent polypeptide chains and converted into SDS-polypeptide complexes upon thiol reduction and SDS binding. SDS-polypeptide complexes have a constant charge per unit mass as each polypeptide binds approximately the same amount of negatively charged SDS (1.5 g $g^{-1}$ polypeptide) which swamps the native charge of the proteins. These complexes of the same charge per unit mass will separate solely on the basis of their size when subject to electrophoresis. Therefore the molecular weight

of particular polypeptides can be determined by comparing their mobility in SDS-polyacrylamide gels with those of well characterised proteins.

Polyacrylamide offers great possibilities for varying the experimental conditions. Molecular weight can be determined using a discontinuous system, as described by King and Laemmli (1971) and Neville (1971) with large and small pore gels and three buffers, or using a continuous system (Weber and Osborn, 1969; Shapiro et al., 1967) with homogeneous gels of one acrylamide concentration and one buffer. Since the resolving power is independent of the use of continuous and discontinuous buffer systems (Hjerten et al., 1965), the molecular weight determination is usually performed on homogeneous gels.

A coloured low molecular weight protein or tracking dye is generally used as a visible marker in electrophoresis and is mixed with the sample and the standard proteins. The position of this visible marker can be used as the reference point for the calculation of mobilities. Electrophoresis is usually continued until the visible marker is near the bottom of the gel. The mobilities can be calculated by taking measurements with a ruler on the stained gel, or more conveniently on a photograph of the gel or most accurately on a densitometer scan of the gel by using the following formula:

$$\text{Mobility} = \frac{\text{distance protein has migrated from origin}}{\text{distance from origin to the reference point}}$$

## 2.8.1 REAGENTS

10% sodium dodecyl sulphate - this solution was stored at room temperature to prevent precipitation. The preparations of all other reagents for gel preparation are described in 2.7.1.

Sample incubation buffer:

160 mM histidine

48 mM Tris

4.4 mM $MgCl_2$ adjusted to pH 7.6 with HCl

Electrophoresis buffer, pH 8.3 (Laemmli and Favre, 1973):

0.05 M Tris

0.384 M glycine

0.1% SDS

Reference protein:

1 mg cytochrome c from horse heart 1 mg $ml^{-1}$ containing 1% SDS, 40 mM DTT, 27% sucrose, prepared by the method used for sample preparation (2.8.3).

Protein stain:

A solution contained:

0.25% Coomassie blue

25.0 % methanol

4.6 % glacial acetic acid

15.0 % trichloroacetic acid

Insoluble material was removed by filtration.

Destainer:

7.5% glacial acetic acid

5.0% methanol

Storage solution:

7% glacial acetic acid

Molecular weight standard:

Pharmacia low molecular weight calibration kit contained: 64 μg rabbit muscle phosphorylase b (94,000), 83 μg bovine serum albumin (67,000), 147 μg hen's egg ovalbumin (43,000), 83μg bovine erythrocyte carbonic anhydrase (30,000), 80 μg Soybean trypsin inhibitor (20,100), 121 μg bovine α-lactalbumin (14,400) and 27 μg sucrose.

### 2.8.2 PREPARATION OF GELS

A gel solution was prepared by mixing together 15 ml stock acrylamide/bis (30/0.9), 15 ml 1.5 M Tris-HCl pH 8.8, 15 μl TEMED, 0.6 ml 10% SDS and making up to 59 ml with distilled water to give a final acrylamide concentration of 7.5% (after addition of 1 ml persulphate solution). The solution was deaerated and 1 ml sodium persulphate solution was added. The solution was deaerated again and transferred to siliconised glass tubes (6 mm diameter, 10 cm long) to give a column of gel about 8 cm long. 2-3 drops of 0.1% SDS were used to overlay the gel solution in the tubes. Gelling time was 20 min at room temperature. The composition of gel was T = 7.5%, C = 3% (see 2.7.2).

### 2.8.3 SAMPLE PREPARATION

In sample preparation, it is essential that the weight ratio of SDS to protein should be at least 3:1 and that all samples and standard molecular weight proteins should contain the same amount of SDS.

Two sets of experiments were run concurrently. In one set of experiments PGM was labelled with $^{32}P$ and then denatured

by treatment with SDS and DTT in order to prepare the sample for molecular weight determination of $^{32}P$-labelled phosphoenzyme. In the other, enzyme was treated directly with SDS and DTT. Radioactive labelled PGM was prepared by incubating 4 units (40 ml) of enzyme with 16 µl of sample incubating buffer, 5 µl [$^{32}P$] G6P (82 µCi, specific radioactivity 409 Ci $mol^{-1}$) and 3 µl G1P6P for 5 min at 30 °C. The final concentration of each was as follows: 40 µg enzyme; 40 mM histidine, 12 mM Tris, 1.1 mM $MgCl_2$ (buffer, pH 7.6); 1 nmol [$^{32}P$] G6P; 0.015 nmol G1P6P. To labelled enzyme was then added 8 µl 10% SDS and 8 µl 0.4 M DTT to give a final concentration of 1% SDS and 40 mM DTT. The enzyme was denatured by heating this solution to 60 °C for 15 min. After cooling to room temperature 10 µl EDTA, 30 µl 50% sucrose and 40 µl SDS/DTT-treated cytochrome c were added. The final volume of sample was 120 µl, 30 µl of which was used for one gel. For unlabelled enzyme 3 µl G1P6P and 5 µl [$^{32}P$] G6P were replaced by 8 µl distilled water.

### 2.8.4 PREPARATION OF STANDARD PROTEIN SAMPLE

The Pharmacia low molecular weight standard protein sample was dissolved in 60 µl distilled water and 20 µl sample incubation buffer and this was followed by the addition of 10 µl 10% SDS and 10 µl 0.4 M DTT. The final concentration of SDS and DTT were 1% and 40 mM respectively. Proteins were denatured by incubating the solution for 15 min at 60 °C. After cooling the solution to room temperature,

9 µl EDTA and 100 µl SDS/DTT-treated cytochrome c were added. 20-30 µl of this solution containing 10-15 µg of each protein was applied to one gel for electrophoresis.

### 2.8.5 ELECTROPHORESIS

Electrophoresis was carried out with Tris-glycine buffer, pH 8.3, containing 0.1% SDS. Before sample application, the gels were run for 4 h at 4 mA per gel in order to remove persulphate ions from the gels which would otherwise promote disulphide cross-linking (see 2.7). After this preliminary run, samples were applied to the top of the gels. Electrophoresis was performed at 4 mA per gel until the visible marker cytochrome c reached 1 cm from the base of the tubes (approximately 4 h).

### 2.8.6 STAINING AND DESTAINING

The gels were immediately removed from the tubes after electrophoresis and placed in narrow test tubes filled with protein staining solution. Trichloracetic acid was used in the stain in order to precipitate the proteins and thus prevent diffusion of protein bands in the gels. The gels were left in the staining solution for 1.5 h. After rinsing the excess stain from the gels, the gels were supported vertically in 12 cm perforated tubes and placed in 2 ℓ destaining solution. Destaining was carried out for 24 h with constant stirring during which time the destaining solution was changed once.

### 2.8.7 STORAGE

The destained gels were placed in narrow tubes which were filled with 7% glacial acetic acid and stored in the dark.

### 2.8.8 SCANNING OF PROTEIN BANDS

The gels were scanned for protein bands on the Pye Unicam SP8-100 spectrophotometer as described in 2.7.8.

### 2.8.9 DRYING OF GELS

Gels were dried with the aid of a Pharmacia gel slab drier modified to dry tube gels.

A wet Whatman No. 3 filter paper of size 15 cm was laid on the polythene support of the gel drier. The gels were sliced longitudinally in half using the gel slicer as described by Lyons (1978). The slices were arranged flat side down and side by side on the filter paper. The pack of gels was surrounded by blank gel slices to protect the outer ones from spreading during the drying process. The gels were then covered with thin plastic film. The rubber sheet of the gel drier was placed over the assembly and the gels were dried for 24 h at low pressure at room temperature.

### 2.8.10 SCANNING OF DRIED GELS FOR RADIOACTIVITY

When appropriate, dried gels were scanned for radioactivity in the Panax scanner as described in 2.3.1.4.

# CHAPTER THREE

# RESULTS

## 3.1 PURIFICATION PROCEDURE

Phosphoglucomutase (PGM) from rabbit liver was purified by a series of precipitations involving heat, pH and addition of ammonium sulphate, plus a modified method of ion-exchange chromatography as described by Joshi and Lane (1978). In the final stages of purification, affinity elution chromatography was performed on a phospho-cellulose column. The affinity elution procedure has been described by Scopes (1977a, 1977b) for isolation of rabbit muscle PGM. This procedure was developed specifically for the purification of rabbit liver enzyme.

Two well fed New Zealand white rabbits (28 days pregnant) were anaesthetised by intravenous injection of Saffan and then killed by injecting sodium pentabarbitone. This form of euthanasia was chosen as it should minimize any change in PGM activity due to distress. When the rabbits were dead, the livers were rapidly removed and washed three times with cold distilled water to remove as much blood as possible. The livers were cut into small pieces for homogenisation.

Unless otherwise specified, all the purification steps were performed at 0-4 $^{o}C$.

First Step: Homogenisation

The small pieces of liver were homogenised for 40 s in a chilled Waring blender with 3 vol 10 mM sodium phosphate buffer, pH 7.0, containing 1 mM $MgCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol and 50 mg $l^{-1}$ PMSF. $Mg^{++}$ ions activate

PGM and protect the enzyme during purification. EDTA was added in the buffer to protect the enzyme from heavy metals. 2-mercaptoethanol reduces all disulphide bonds and thereby protects against oxidation which could cause denaturation of the enzyme. 1 mM $MgCl_2$, 1 mM EDTA and 1 mM 2-mercaptoethanol were included in all purification steps, except in ion-exchange chromatography, where the concentrations were reduced to 0.1 mM in order to keep the ionic strength low. PMSF was included in the buffer to inactivate proteolytic enzymes.

All PGM activity was released from the liver cells during homogenisation. Preliminary experiments showed that homogenisation for longer than 40 s denatures a large percentage of PGM. The homogenate was centrifuged at 3,900 g for 30 min and filtered through glass wool to remove solid material.

The specific activity of PGM in the homogenate was only 0.12 unit per mg protein because of the presence of other proteins including myoglobin and haemoglobin. Liver also contains large amounts of nucleic acids which were removed in the subsequent steps.

Second Step: Heat Treatment

The turbid filtrate was brought to 59-60 $^{o}$C within 5 min by placing in a water bath at 70 $^{o}$C. The suspension was maintained at this temperature for 4 min, and cooled within 5 min to 4 $^{o}$C. The suspension was stirred constantly throughout the heating and cooling process. The PGM was found to be stable at 60 $^{o}$C for 4-5 min. The principle of heat treatment was to denature less stable proteins at a

temperature which did not significantly damage the PGM. Such treatment also denatures phosphoglucose isomerase which could not be removed by the subsequent procedures.

After heat treatment the solution was centrifuged at 3,900 g for 30 min and floating material was removed by filtration through glass wool. The specific activity was improved 3.6 fold with the loss of 21% of total enzyme activity.

Third Step: pH Treatment

The liver is a proliferating tissue and contains large amount of nucleic acids, much of it in the form of ribosomes (Scopes, 1978). Nucleic acids are strongly charged polymers and they co-precipitate with the enzyme in the ammonium sulphate fractionation step. Their presence changes the solubility and adsorption characteristics of the enzyme.

pH treatment was carried out to remove a large amount of nucleic acids and some other proteins which could be denatured and precipitated at pH 5.5. After heat treatment, the pH of the centrifugate obtained was lowered to 5.5 by the addition of M acetic acid. A weak acid was chosen to decrease the pH of the solution gradually and thereby to avoid denaturing the enzyme. The cloudy solution was left to stand at pH 5.5 for 5 min and then centrifuged at 3,900 g for 30 min. The residue was discarded and the supernatant was adjusted to pH 7.0 by the addition of 2 M Tris.

After this treatment, the solution was clear and approximately half of the proteins had been removed without a

significant loss of enzyme activity, thus raising the specific activity to 0.63 units $mg^{-1}$.

Fourth Step: First Ammonium Sulphate Fractionation

Most of the PGM activity (>90%) precipitates between ammonium sulphate saturation 0.3 and 0.7. When the enzyme solution was taken to 0.4 saturation, 30% of PGM activity was found in the precipitate. At 0.6 saturation, 10% PGM activity remained in the supernatant. The first ammonium sulphate fractionation was carried out as follows.

The supernatant fluid after pH treatment was brought gradually to 0.3 saturation by the addition of solid ammonium sulphate over a period of an hour with constant gentle stirring. After an equilibration period of 1 h, the precipitate was removed by centrifugation for 20 min at 23,000 g and discarded. No PGM activity was detected in the precipitate.

Step Five: Second Ammonium Sulphate Fractionation

Preliminary experiments showed that addition of solid ammonium sulphate at this stage resulted in the denaturation of large amounts of PGM and a decrease in pH of the solution of about 0.7 units. Therefore, the filtrate of 0.3 ammonium sulphate saturation was brought up gradually to 0.7 saturation over a period of 2-3 hours by the addition of a saturated solution of ammonium sulphate, adjusted to pH 7.0 with NaOH, containing 1 mM $MgCl_2$, 1 mM EDTA and 1 mM 2-mercaptoethanol. It was found that appreciable denaturation could be avoided by adding the saturated solution of ammonium sulphate drop wise from a burette whilst the solution was gently stirred.

A slight turbidity formed, followed by the formation of a precipitate. The solution was left overnight and centrifuged for 15 min at 23,000 g and the brown precipitate was collected. The precipitate was dissolved in a minimal volume of distilled water.

A small increase in specific activity with 27% loss in activity was observed (see Table 3.1).

Step Six: Dialysis

The resultant enzyme solution was dialysed for 24 h against eight successive changes (4 ℓ each) of cold distilled water to remove traces of salts. The removal of ammonium sulphate was followed after each change by adding a saturated solution of barium acetate to a small volume of the dialysis water and observing the appearance of $BaSO_4$ precipitate. Six successive changes of distilled water reduced the ammonium sulphate concentration to a very low level. Dialysis was continued for a further 24 h against 10 mM sodium phosphate buffer, pH 7.8, containing 0.1 mM $MgCl_2$, 0.1 mM EDTA and 0.1 mM 2-mercaptoethanol to prepare the enzyme solution for DEAE-cellulose chromatography. The concentration of enzyme protecting agents was reduced to 0.1 mM to maintain a low ionic strength for the adsorption of the enzyme on the ion-exchange column. Following dialysis the solution was centrifuged to remove any insoluble material. A small amount of PGM activity was lost with the precipitate with other proteins, but there was no change in the specific activity.

Step Seven: DEAE-Cellulose Chromatography

DEAE-cellulose was generated and equilibrated as follows.

Previously untreated DEAE-cellulose was suspended in 15 vol 0.1 M HCl. The mixture was stirred and allowed to stand at room temperature for 30 min. The resin was washed with distilled water in a Buchner funnel until the pH of the effluent reached 4. The resin was then suspended in 15 vol 0.5 M NaOH for 30 min. The resin was washed with water until the effluent pH was near neutral. For equilibration, the resin was suspended in 100 mM sodium phosphate buffer, pH 7.8. The mixture of resin and buffer was stirred and the resin allowed to settle for 20 min after which the supernatant and unsettled fines were removed. This settling procedure was repeated with 10 mM sodium phosphate buffer, pH 7.8, containing 0.1 mM $MgCl_2$, 0.1 mM EDTA and 0.1 mM 2-mercaptoethanol, until the supernatant was clear of fine particles. The resin was finally poured into the column and equilibration continued by passing the same buffer until the pH of eluent reached 7.8. Approximately 10 vol of buffer was found to be required.

The pH of the dialysate was checked after the dialysis step and equilibrating buffer was added until the concentration of protein reached 5 mg $ml^{-1}$. The enzyme at this protein concentration was found to be stable for only 2-3 h, even at 4 $^{o}C$, and the dilution of the entire enzyme sample would have resulted in a loss of enzyme activity. Accordingly, the enzyme solution was diluted in batches and applied to the bed resin of a 4 x 9 cm column. The diluted dialysate had a suitable ionic strength for the adsorption of the enzyme on the ion-exchanger. The enzyme was found to be stable when bound to the ion-exchanger column. The column was

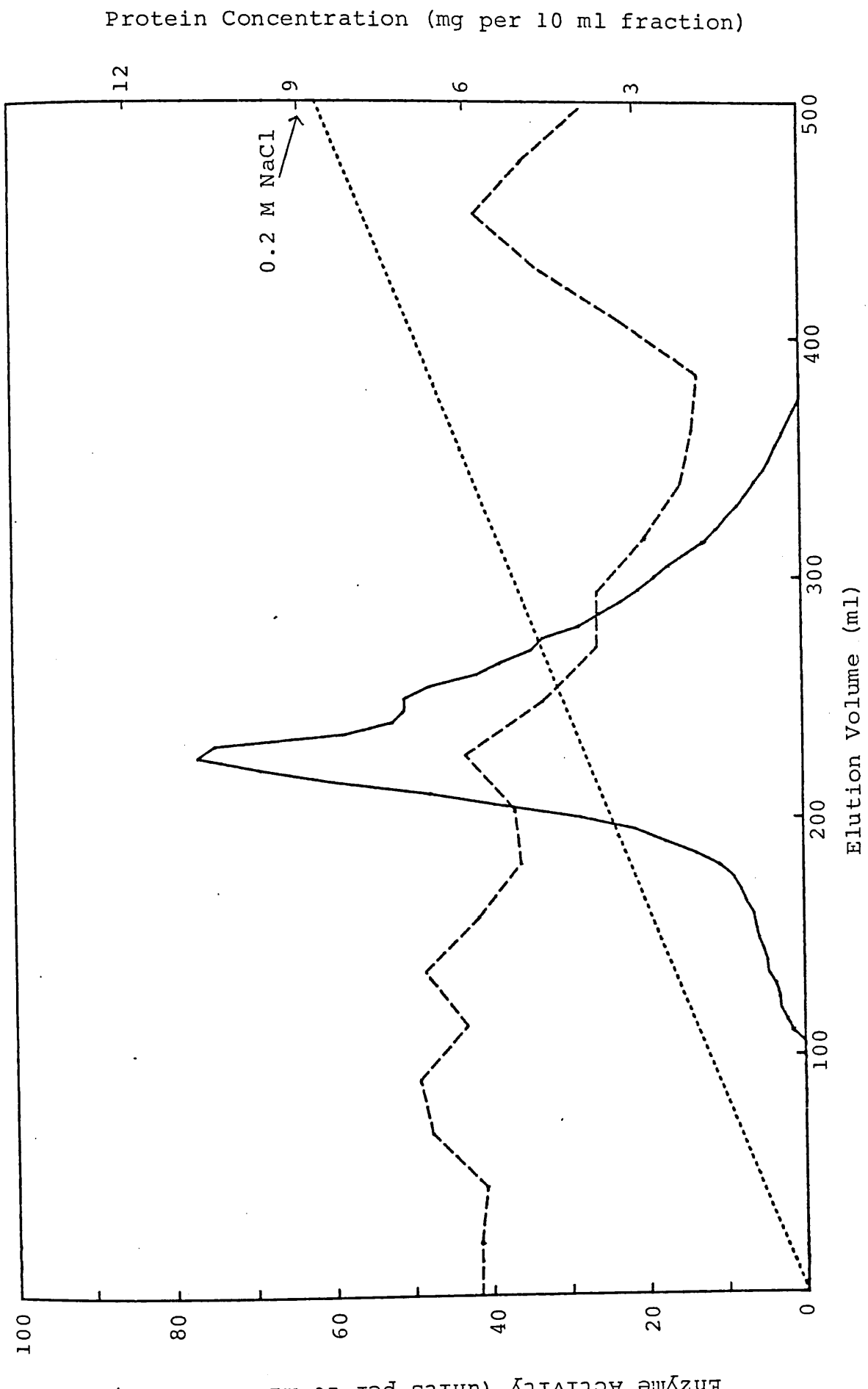
FIGURE 3.1: Elution profile in DEAE-cellulose chromatography of rabbit liver PGM

Dialysed enzyme solution was applied to a DEAE-cellulose column previously equilibrated with 10 mM sodium phosphate buffer, pH 7.8, containing 0.1 mM $MgCl_2$, 0.1 mM EDTA and 0.1 mM 2-mercaptoethanol. After washing the column with 500 ml of the same buffer the enzyme was eluted with a linear gradient of NaCl. Fractions of 10 ml were collected. (See step seven of purification procedure for experimental details).

———— PGM activity

---------- Protein concentration

..... NaCl gradient


Protein Concentration (mg per 10 ml fraction)
12
9
6
3
0.2 M NaCl
500
400
300
200
100
Elution Volume (ml)
100
80
60
40
20
0
Enzyme Activity (units per 10 ml fractions)

washed with 500 ml buffer. The adsorbed enzyme was eluted with the same buffer containing an increasing concentration of NaCl up to 0.2 M. 10 ml fractions were collected from the column and the protein concentration and enzyme activity were measured in each fraction. The elution profile is shown in Fig. 3.1. PGM was observed in two peaks; a first major peak and a second minor peak which can be seen as a shoulder of the major peak.

The fractions containing enzyme activity peaks were collected and combined. The enzyme was concentrated by the dropwise addition of saturated ammonium sulphate, pH 7.0 to give 0.75 saturation. The precipitated enzyme was collected by centrifugation and dissolved in a minimal volume of distilled water. The enzyme now had a specific activity of 12 units $mg^{-1}$ protein. More than 90% of protein was removed in this step without any loss of enzyme activity.

The enzyme solution was then prepared for the next step by dialysing against distilled water for 24 h to remove ammonium sulphate as described under Step Six. The solution was finally dialysed with 10 mM Tris-Mes buffer, pH 6.5, and diluted with the same buffer after centrifugation, so that the final solution contained 5 mg protein per ml.

Step Eight: Affinity Elution Chromatography

Affinity elution chromatography is defined as elution of the enzyme from an adsorbent by the use of a ligand that binds specifically to the enzyme in question thus displacing it from the adsorbent (Fifis and Scopes, 1978).

Scopes (1977) used this procedure to purify glycolytic enzymes. Most of the glycolytic enzymes have isoelectric points in the range of 6-8. At low pH, these enzymes can be adsorbed on a cation exchanger and can then be specifically eluted by a negatively charged substrate. It is necessary to use a pH just below the pI of the enzyme when it is weakly adsorbed. It can then be specifically eluted with substrate provided that binding of the ligand alters the interaction between enzyme and adsorbent, so that the partition coefficient between adsorbent and enzyme is decreased from nearly 1 to 0 (Scopes, 1978).

Scopes (1977a, 1977b) has purified rabbit muscle PGM by adsorbing the enzyme on phospho-cellulose at pH 6.0 and specifically eluting the phosphorylated and dephosphorylated forms of enzymes with 10 mM Tris-Mes buffer, pH 6.5, containing G1P and G1P6P substrates. However, the conditions for purification of rabbit liver PGM by affinity elution chromatography are somewhat different.

P-cellulose was generated and equilibrated by the method used for DEAE-cellulose (Step Seven), except that the resin was first treated with alkali then with acid and finally equilibrated with 10 mM Tris-Mes buffer, pH 6.5. The enzyme solution was then applied to the column of bed size 3.5 x 10 cm. PGM has a weak positive charge at pH 6.5 and was completely adsorbed at this pH. After adsorption of enzyme, the column was washed with 100 ml of adsorption buffer to remove unadsorbed proteins. Generally proteins adsorb on the top third of the column. Before eluting the

enzyme with substrate, the column was washed with 100 ml of 10 mM Tris-Mes buffer, pH 6.7, containing 0.5 mM EDTA, the ionic strength of which was the same as the eluting solution containing the substrates. This 'dummy substrate' wash eluted the non-specific proteins other than PGM which otherwise would elute with the enzyme under the same ionic conditions of eluting buffer. Finally the enzyme was specifically eluted with 200 ml of 10 mM Tris-Mes buffer, pH 6.7, containing 0.5 mM G1P and 0.1 mM G1P6P. The PGM exists in phosphorylated and dephosphorylated forms; the dephosphorylated enzyme only binds G1P6P whereas the phosphoenzyme binds monophosphates (G1P and G6P; Ray et al. 1973). Thus both forms of the enzymes were eluted with the mixture of G1P and G1P6P. The flow rate at the time of adsorption was 10 ml $h^{-1}$ and at the time of elution was 100 ml $h^{-1}$.

The fractions containing 95% of enzyme activity were collected and combined. The PGM solution was concentrated by adding saturated ammonium sulphate, pH 7.0 (containing 1 mM $MgCl_2$, 1 mM EDTA and 1 mM 2-mercaptoethanol) to give a final saturation of 0.75. After centrifugation, the precipitate was dissolved in 2 ml of 50 mM sodium phosphate buffer, pH 7.0 (containing 1 mM $MgCl_2$, 1 mM EDTA and 1 mM 2-mercaptoethanol). The enzyme solution was finally dialysed against the same buffer overnight in order to remove the ammonium sulphate.

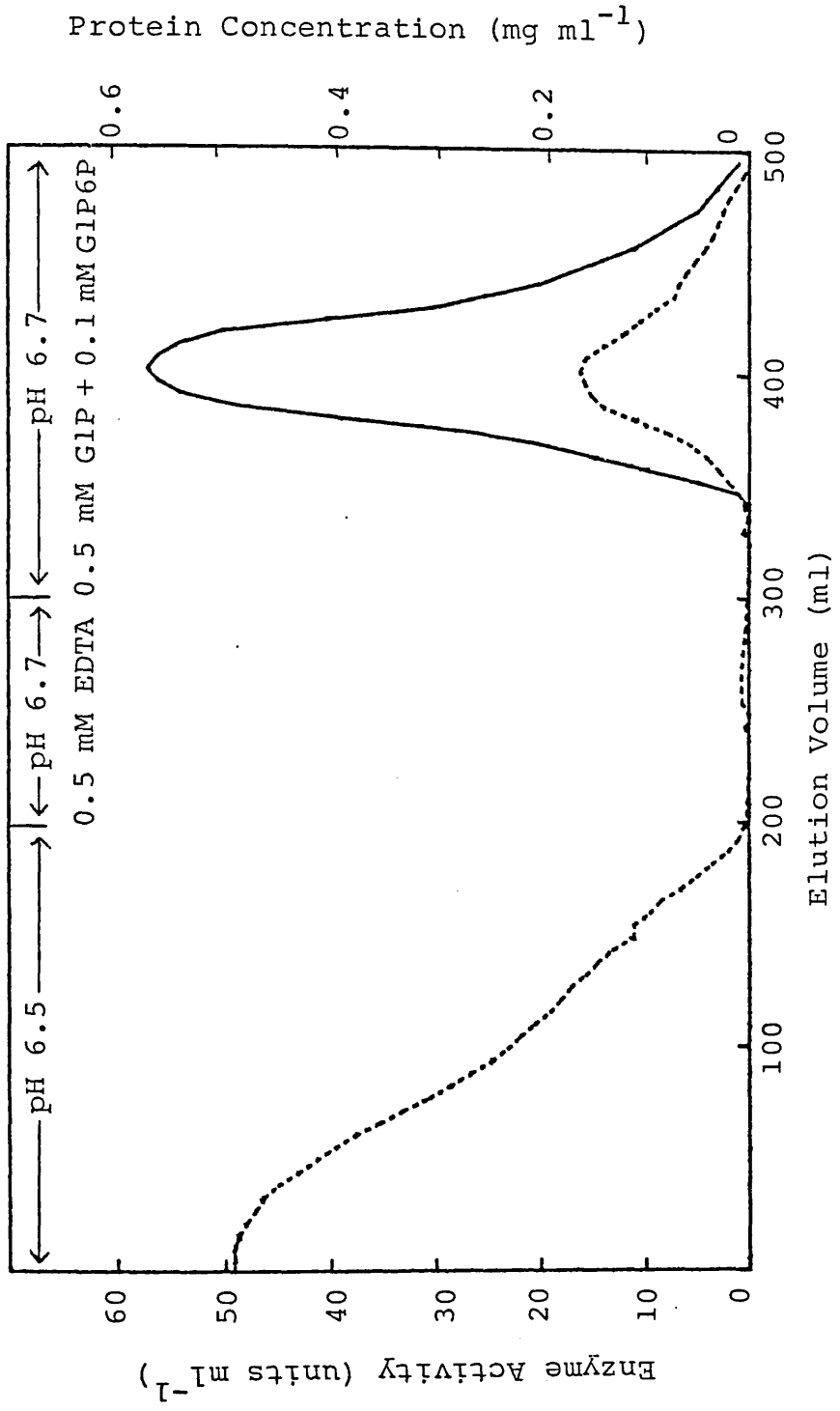
After affinity elution chromatography the enzyme solution had a specific activity of 160 units $mg^{-1}$ protein.

FIGURE 3.2: Elution profile for phosphocellulose chromatography of rabbit liver phosphoglucomutase

Enzyme was adsorbed on a P-cellulose column with 10 mM Tris-Mes buffer, pH 6.5. The column was eluted with 100 ml of the same buffer at pH 6.7 containing 0.5 mM EDTA to remove non specific proteins. PGM was specifically eluted with 200 ml of 10 mM Tris-Mes buffer, pH 6.7, containing 0.5 mM G1P and 0.1 mM G1P6P. Fraction size: 5 ml (see Step Eight of purification procedure for experimental details).

———— Enzyme activity

..... Protein concentration


Protein Concentration (mg ml$^{-1}$)
0.6
0.4
0.2
0
500
400
300
200
100
Elution Volume (ml)
Enzyme Activity (units ml$^{-1}$)
60
50
40
30
20
10
0
pH 6.5
pH 6.7
pH 6.7
pH 6.7
0.5 mM EDTA 0.5 mM G1P + 0.1 mM G1P6P

Step Nine: Gel Filtration

The enzyme solution after affinity elution chromatography was subjected to gel filtration to remove contaminating proteins. Sephadex G-100 of fractionation range 4000-150,000 molecular weight was used for this purpose.

The column was prepared by the method of Flodin (1962). Dry Sephadex powder was swollen for 5 h at 90 $^{o}$C in excess 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM $MgCl_2$, 1 mM EDTA and 1 mM 2-mercaptoethanol. The high temperature also deaerated the buffer. After cooling, the fines were removed by suspending the Sephadex in deaerated buffer at 4 $^{o}$C and decanting after 20 min several times until the supernatant was clear of fine particles. The Sephadex was then transferred to the column in slurry form. After packing, the homogeneity of the column was checked by running through a 1 ml sample (2 mg $ml^{-1}$) of blue dextran 2,000. The column was washed with 5 bed vols of the phosphate buffer before the application of the sample.

The dialysed enzyme solution in phosphate buffer was applied to the column and elution was accomplished with 500 ml buffer, flowing at about 60 ml $h^{-1}$. 5 ml fractions were collected and fractions containing in total 95% of the enzyme activity were combined. The enzyme was then concentrated by the addition of saturated ammonium sulphate to 0.75 saturation. After centrifugation the PGM precipitate was concentrated again by the addition of saturated

ammonium sulphate and stored as a suspension in 3.2 M ammonium sulphate at 4 $^{o}$C. The fraction eluted from Sephadex G-100 had a specific activity 484 units per mg protein at pH 7.6.

Comments

With this purification procedure an overall 4033-fold increase in specific activity, with a yield of 27% was attained. The final preparation had a specific activity higher than those of enzymes obtained from rabbit muscle (Najjar, 1948), human muscle (Joshi and Handler, 1969), yeast (Hirose et al., 1971), E.coli (Joshi and Handler, 1964) and Bacillus subtilis (Maino and Young, 1974a). Additional procedures failed to accomplish further increase in specific activity; among these were CM-cellulose chromatography, Sepharose 6B chromatography, chromatography at high ionic strength. A flow diagram of the steps in the preparation is shown in Fig 3.3 and the results of a typical purification procedure are summarised in Table 3.1.

The results obtained by this purification procedure were found to be reproducible. The entire procedure including three runs of column chromatography and three dialyses of the enzyme solution for each chromatographic step required about two weeks. However, the enzyme can be prepared with a specific activity of 290 in three days by using only one chromatogram step, that of affinity elution chromatography. A most crucial factor in the purification of this enzyme is

speed; long purification times affect the yield adversely. The recent introduction of HPLC techniques now could eliminate this problem.

In one purification the enzyme was prepared without using pH treatment and DEAE-cellulose chromatography. The enzyme obtained after affinity elution chromatography had a specific activity of 70. This low value was due to the presence of contaminating nucleic acids. Nucleic acids were found also to alter the binding characteristics of the enzyme towards the P-cellulose. The enzyme would not elute with substrates even when the pH of the eluent was increased to 7.0. In this preparation the enzyme had to be eluted with a gradient of increasing salt concentration.

TABLE 3.1: Isolation of phosphoglucomutase from rabbit liver.

For details of isolation steps and enzyme assay see 3.1 and 2.2.1.1.1.

| Step | Volume ml | Protein conc. mg $ml^{-1}$ | Total protein mg | Total PGM units | Specific activity units $mg^{-1}$ protein | Yield % |
|---|---|---|---|---|---|---|
| 1 | 560 | 156 | 87360 | 10534 | 0.12 | 100 |
| 2 | 337 | 574 | 19353 | 8322 | 0.43 | 79 |
| 3 | 340 | 34.9 | 11871 | 7479 | 0.63 | 71 |
| 4 | 370 | 293 | 10848 | 7268 | 0.67 | 69 |
| 5 | 41 | 148 | 6054 | 5267 | 0.87 | 50 |
| 6 | 270 | 1.48 | 398 | 4780 | 12 | 45 |
| 7 | 63 | 61.9 | 390 | 3260 | 8.4 | 31 |
| 8 | 45 | 0.42 | 18.89 | 3032 | 160 | 31 |
| 9 | 50 | 0.12 | 5.85 | 2832 | 484 | 27 |

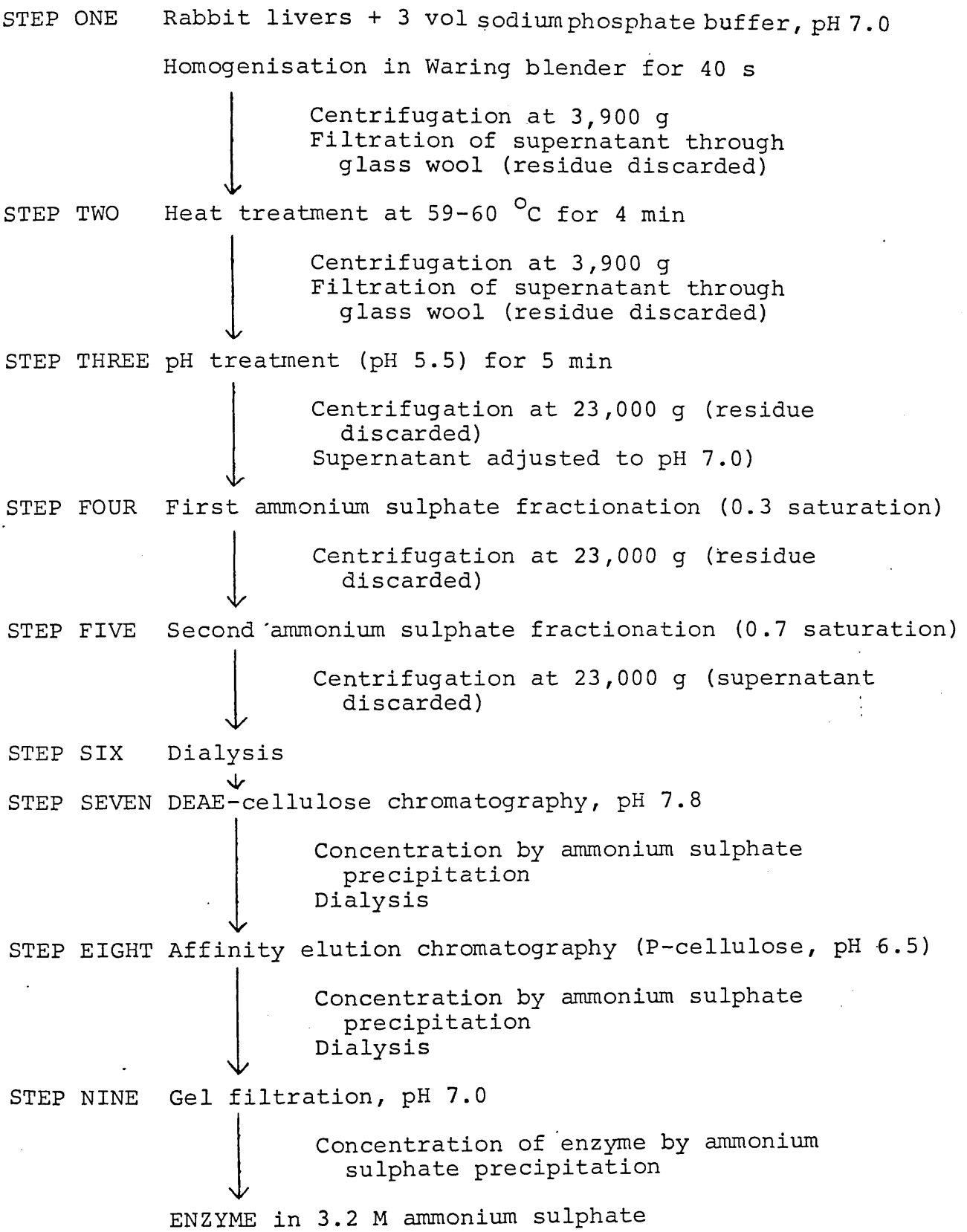



FIGURE 3.3: Isolation protocol of rabbit liver phosphoglucomutase

## 3.2 PROPERTIES OF RABBIT LIVER PHOSPHOGLUCOMUTASE

### 3.2.1 PURITY OF PURIFIED RABBIT LIVER PHOSPHOGLUCOMUTASE

Purity of PGM was investigated by measuring the activites of the other enzymes in the final preparations (see 2.2.1 for enzyme assays). Specific assays of individual enzymes, each at their optimum pH, indicated that the purified preparation was devoid of glucose-6-phosphate dehydrogenase and phosphoglycerate mutase activities. Hexokinase, glucose-6-phosphatase activities and phosphoglucose isomerase activities were 0.2%, 0.8% and 0.42% of the PGM activity respectively (see Table 3.2). However, at pH 7.6, the activities of contaminating enzymes were usually less than the values quoted in Table 3.2.

The purity of the enzyme was also examined on homogeneous polyacrylamide gels in a continuous buffer system. Electrophoresis was carried out on 7.5% polyacrylamide gels without SDS according to the method described in 2.8. 20 µg of enzyme per gel was used and the gels were stained for protein bands in Coomassie blue stain.

Gel electrophoresis showed the presence of only one type of protein containing more than 95% of total protein*. No bands of contaminating proteins were observed on the gel, showing that the purified enzyme was homogeneous.

### 3.2.2 STABILITY OF PHOSPHOGLUCOMUTASE

PGM was found to be extremely unstable when isolated under various conditions. The heat treatment and precipitation

* ignoring stained gel at the origin

TABLE 3.2: Percentage of activities of contaminating enzymes in purified PGM preparation (for assays see 2.2.1).

| Enzyme | pH of assay | Relative activity (units) |
|---|---|---|
| Phosphoglucomutase | 7.6 | 100 |
| Glucose-6-phosphatase | 8.5 | 0.8 |
| Phosphoglucose isomerase | 8.0 | 0.42 |
| Hexokinase | 8.0 | 0.2 |
| Glucose-6-phosphate dehydrogenase | 8.0 | 0 |
| Phosphoglycerate mutase | 7.4 | 0 |

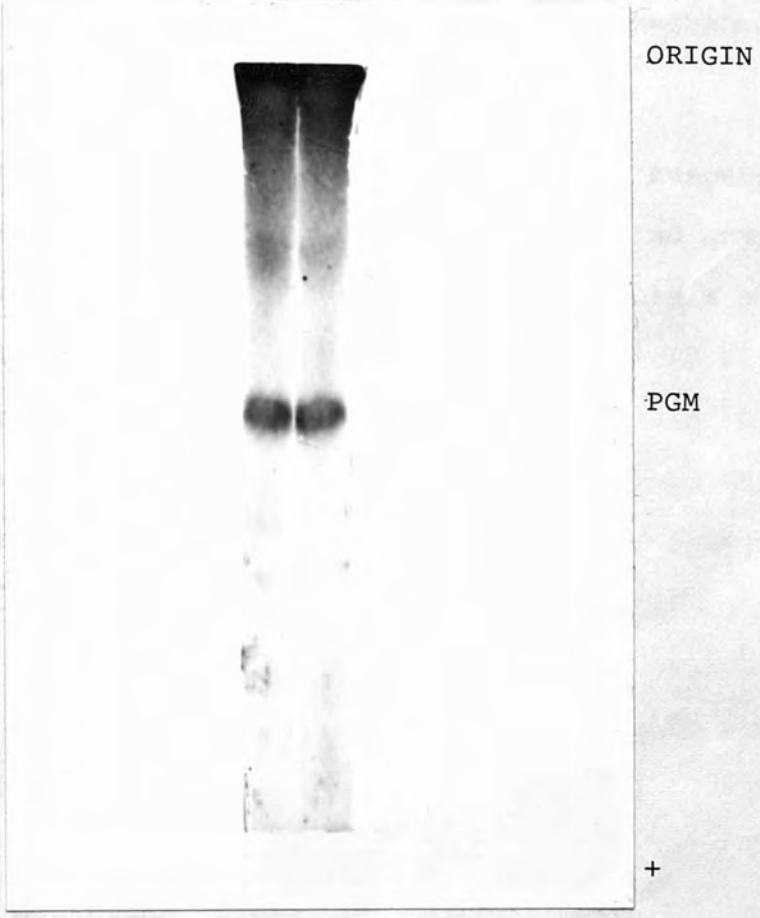



FIGURE 3.4: Polyacrylamide gel electrophoresis of purified phosphoglucomutase (in the absence of SDS)

The electrophoresis of purified PGM was carried out on 7.5% homogeneous polyacrylamide gels in a continuous buffer system for 2 h at 4 mA current per gel. The gels were stained for protein bands in Coomassie blue. (For experimental details see 2.8).

with ammonium sulphate resulted in substantial losses in enzyme activity. However, after treatment of liver extracts with PMSF, PGM was found to be significantly more stable. Stability of the enzyme was further enhanced when enzyme-protecting agents ($MgCl_2$, EDTA and 2-mercaptoethanol) were included in all preparative stages.

PGM was stable for 6 months when stored as a suspension in 3.2 M ammonium sulphate solution. The purified enzyme was found to be stable at low ionic strength. When a small amount of enzyme (0.2 mg) was diluted to 3 ml in His-Tris-$Mg^{++}$ buffer, pH 7.6 (40 mM Histidine, 12 mM Tris, 2 mM $MgCl_2$) and enzyme activity was tested, it retained full activity for at least 4 h at 30 $^{o}C$. During isolation, however, it was unstable at low ionic strength solution (see Step Six of 3.1). The protection offered PGM by PMSF suggests that proteases may be responsible for inactivation of the enzyme during isolation.

### 3.2.3 MOLECULAR WEIGHT

Molecular weight determination of PGM from rabbit liver was carried out on SDS-polyacrylamide gels as described in 2.8. A purified preparation of rabbit liver PGM of specific activity 150-200 units $mg^{-1}$ protein was used for molecular weight determination. In one set of experiments the enzyme was first labelled with $^{32}P$ (see 2.8.3) and then denatured with SDS and DTT prior to electrophoresis. The molecular weight of rabbit muscle PGM was also determined by the same method to confirm the results obtained with rabbit liver PGM.

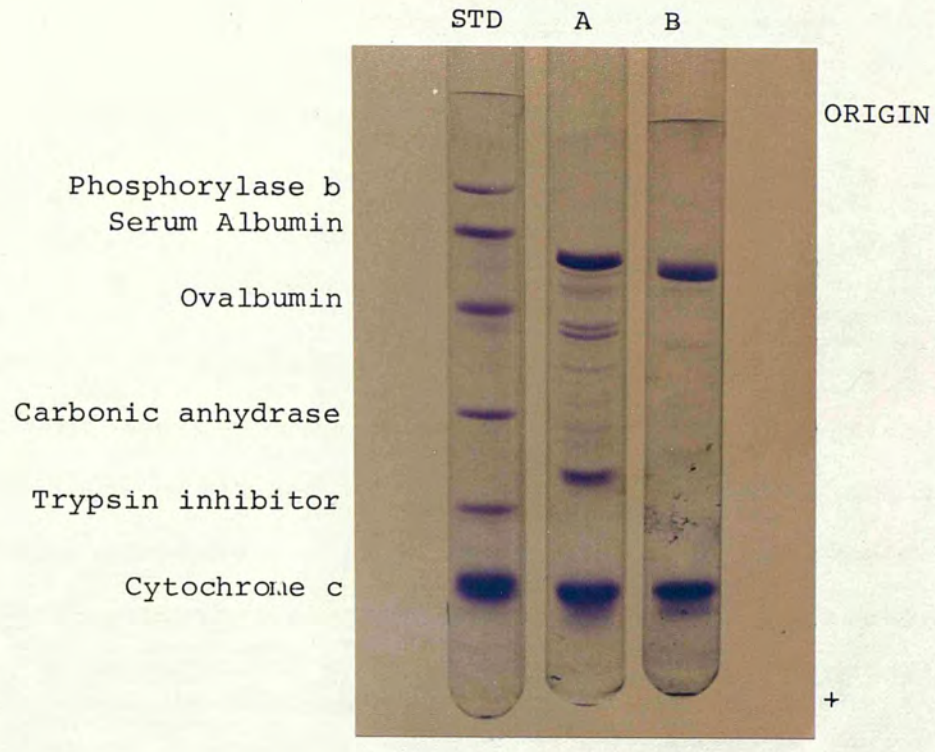



FIGURE 3.5: Molecular weight determination by SDS-polyacrylamide gel electrophoresis

Molecular weight of PGM from rabbit liver and rabbit muscle was determined as described in 2.8. Separation of SDS/DTT treated standard proteins sample, rabbit liver PGM and rabbit muscle PGM on SDS-polyacrylamide gels is shown.
STD - Mixture of standard proteins (Pharmacia): phosphorylase b from rabbit muscle (94,000), bovine serum albumin (67,000), ovalbumin from hen's egg (43,000), carbonic anhydrase from bovine erythrocytes (30,000), trypsin inhibitor from soy bean (20,100). Cytochrome c from horse heart (12,500) was used as coloured reference.
A - Rabbit liver PGM.
B - Rabbit muscle PGM.

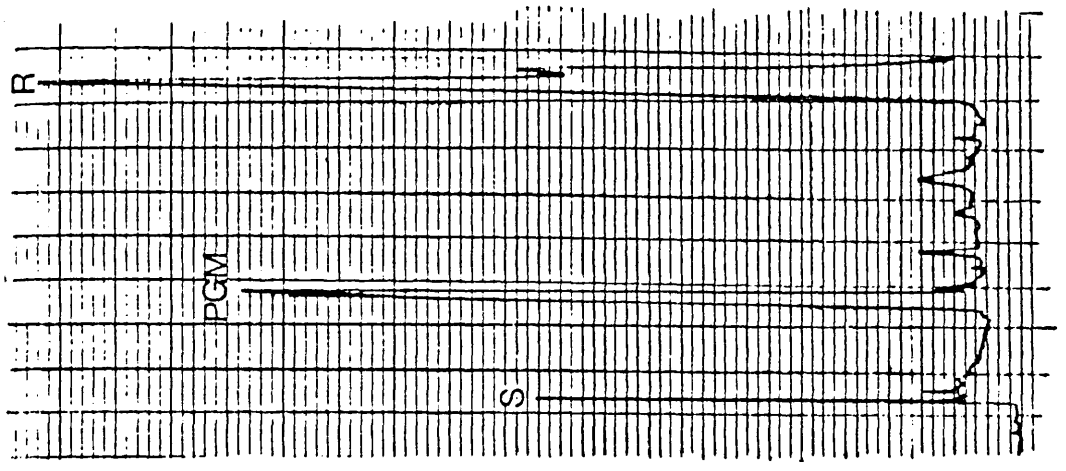
FIGURE 3.6: Scan of SDS-polyacrylamide gels for molecular weight determination

Molecular weight determination of PGM from rabbit liver and rabbit muscle was carried out on SDS-polyacrylamide gels as described in 2.8. Relative mobilities were determined from the absorbance scans of Coomassie blue stained gels. A photograph of gels is shown in Fig. 3.5.

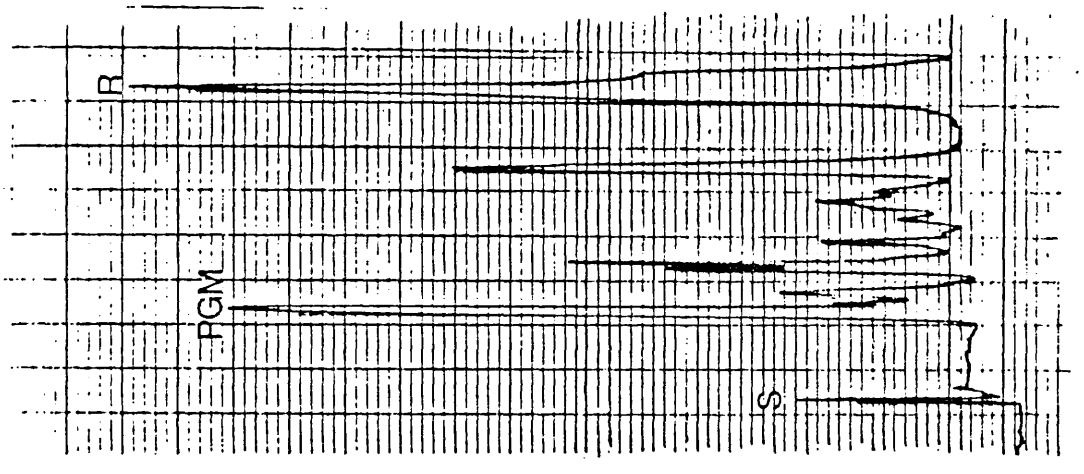
STD - Scan of gel containing standard proteins (Pharmacia): S - starting point; 1 - phosphorylase from rabbit muscle (94,000); 2 - bovine serum albumin (67,000); 3 - ovalbumin from hen's egg (43,000); 4 - carbonic anhydrase from bovine erythrocyte (30,000); 5 - trypsin inhibitor from soy bean (20,000); and R - cytochrome c (12,500) as coloured reference.

A - Scan of gel containing rabbit liver PGM.

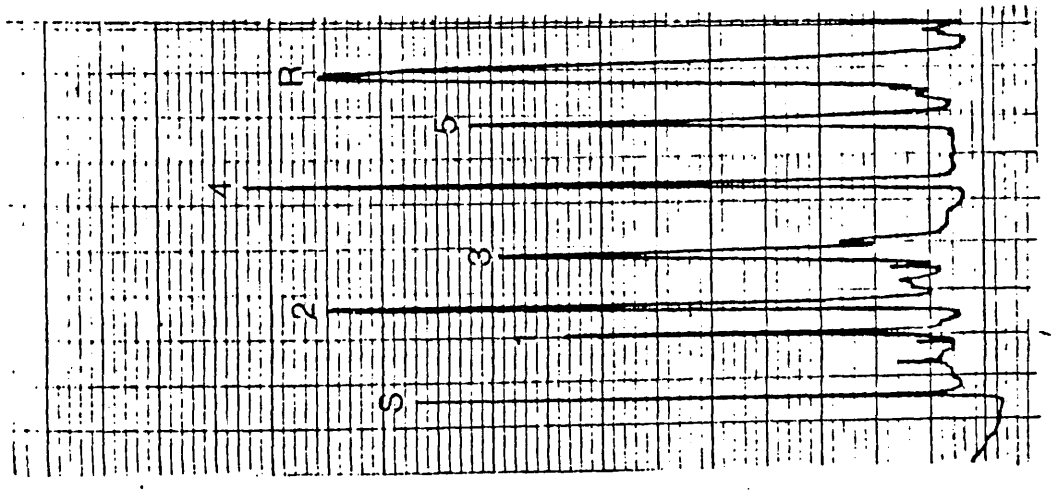
B - Scan of gel containing rabbit muscle PGM.




B



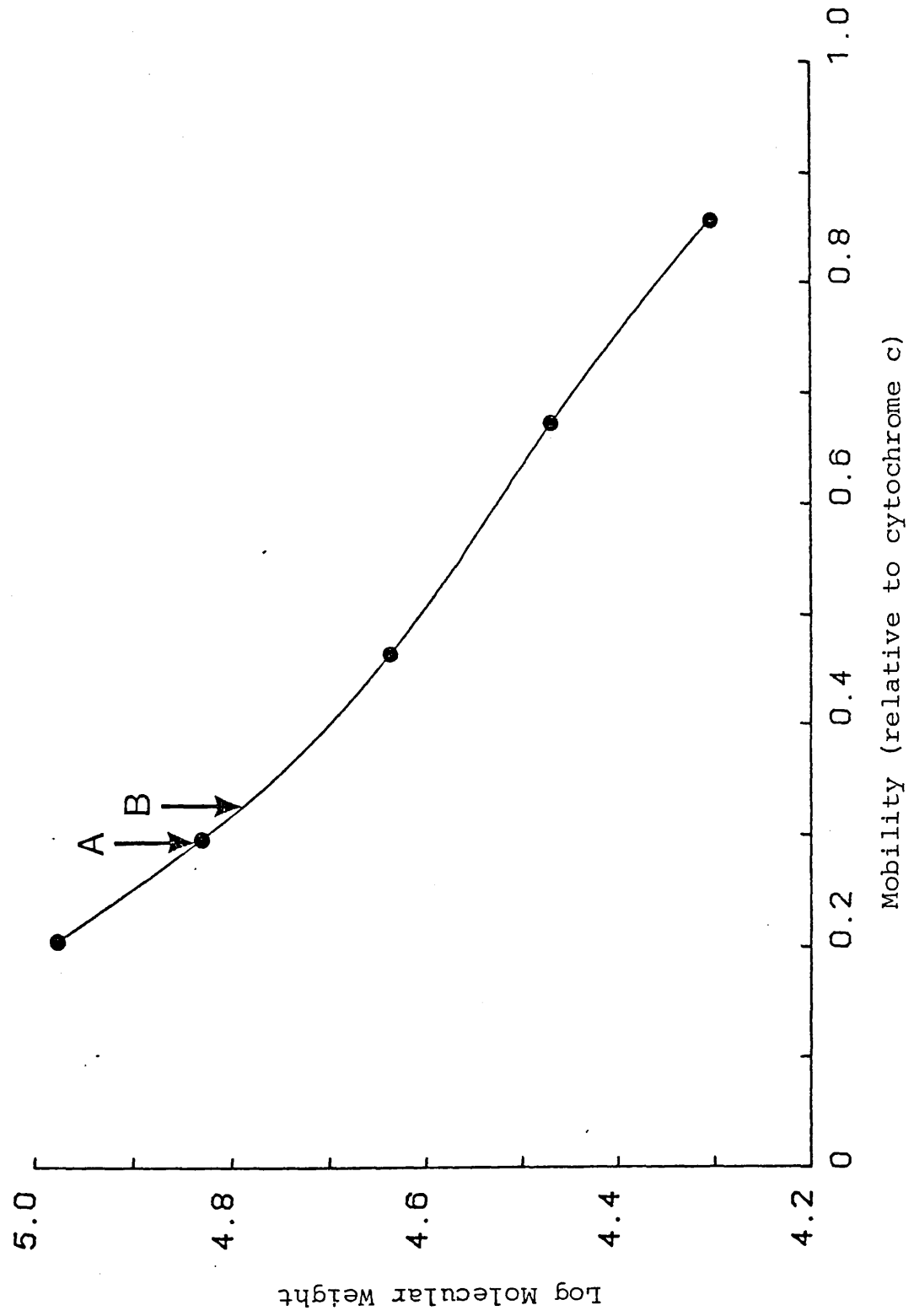

A




STD

FIGURE 3.7: Calibration curve of molecular weight determination by SDS-polyacrylamide gels

Molecular weight determination of PGM from rabbit liver and rabbit muscle was carried out by SDS-polyacrylamide gel electrophoresis as described in 2.8. Mobilities of proteins were determined from the absorbance scans (Fig. 3.6) of Coomassie blue stained gels. The graph shows log molecular weight versus mobility of standard proteins (see Fig. 3.6) in SDS-polyacrylamide gels. Arrows A and B show the positions of rabbit liver and rabbit muscle PGMs respectively.


Log Molecular Weight
5.0
4.8
4.6
4.4
4.2
0
0.2
0.4
0.6
0.8
1.0
Mobility (relative to cytochrome c)
A
B

Mobilities of enzymes and known molecular weight proteins in SDS-polyacrylamide gels were determined by optical density scans (Fig. 3.6) on a Pye-Unicam spectrophotometer (2.8.8). Mobility of $^{32}P$-labelled PGM was measured after drying the gels (see 2.8.9) on a Panax RCMS 2 chromatography scanner as described in 2.8.10. A graph of log molecular weight against the relative mobility of proteins with reference to cytochrome c was plotted (Fig. 3.7).

$^{32}P$-labelled enzyme appeared as a single radioactive peak and unlabelled enzyme as a major protein band (see Fig. 3.6A). The mobilities of labelled enzyme and major protein peak (PGM peak, Fig. 3.6A) were the same and the molecular weight of rabbit liver PGM was calculated to be 67,600 from the standard graph (Fig. 3.7). The molecular weight of rabbit muscle PGM was found to be 61,000, which is close to the frequently quoted value of 62,000 (see Filmer and Koshland, 1963; Yankeelov et al., 1967).

### 3.2.4 EFFECT OF pH ON RABBIT LIVER PHOSPHOGLUCOMUTASE

The activity of rabbit liver PGM was tested from pH 6.0 to 8.5 in His-Tris-HCl buffer (40 mM histidine, 12 mM Tris), containing 2.0 mM $MgCl_2$. Prior to assay maximal activity was ensured by incubation of enzyme in assay buffer for 10 min at 30 $^{o}C$. All the assays were subsequently carried out in the same buffer at 30 $^{o}C$, using the method described in 2.2.1.1.1.

As shown in Fig. 3.8 the optimal activity was observed at pH 7.6, similar to the optimum at 7.5 (Jagannathan and Luck

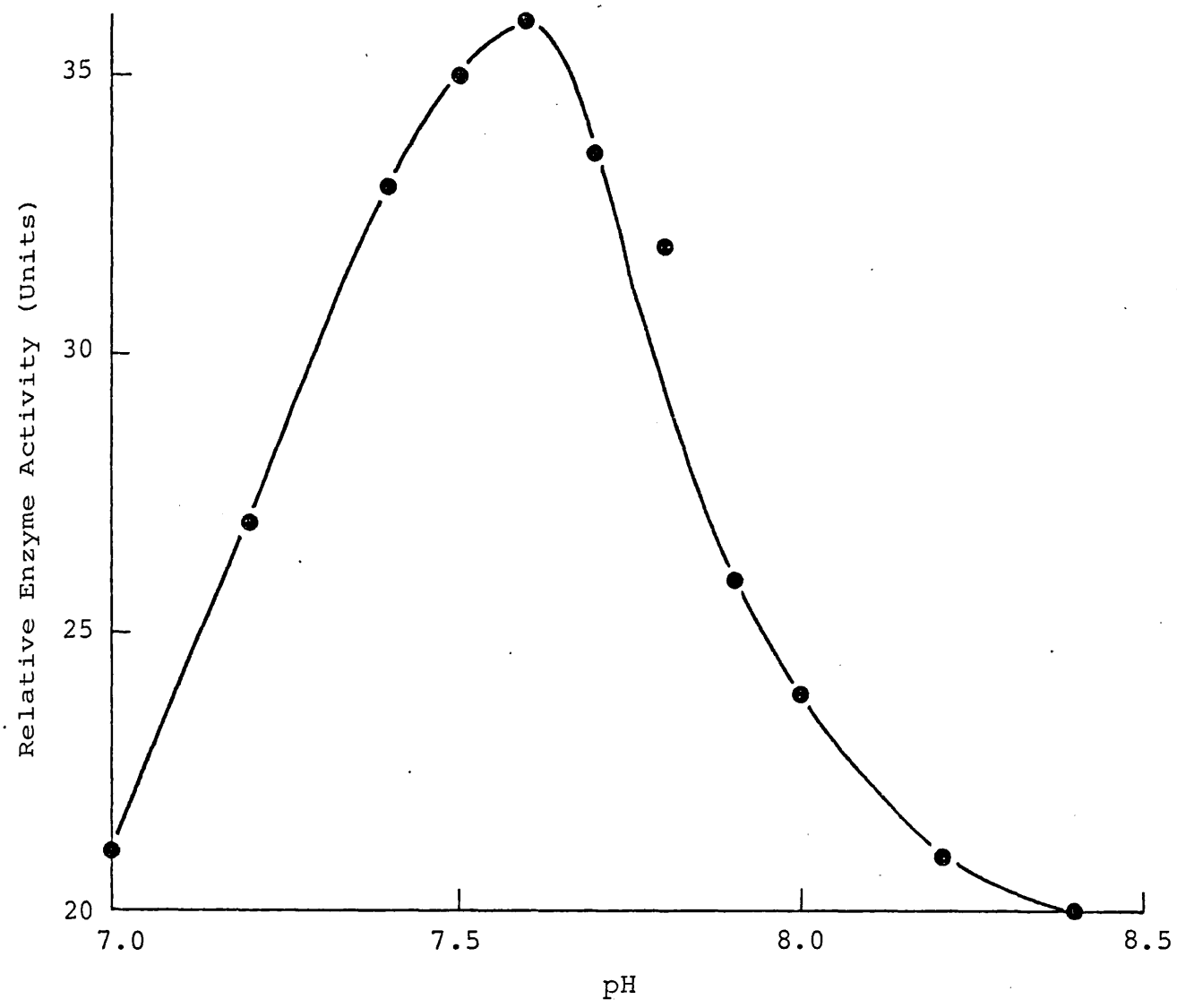



FIGURE 3.8: Variation of rabbit liver phosphoglucomutase activity with pH

PGM activity from rabbit liver was measured as a function of pH in a buffer containing 40 mM histidine, 12 mM Tris and 2.0 mM $MgCl_2$ with the assay system described in 2.2.1.1.1. The enzyme was preincubated in the buffer for 10 min prior to assay.

1949a) observed with the enzyme from rabbit muscle. At the optimum pH the enzyme activity was found to be the same before and after incubation with histidine and $Mg^{++}$ (see Table 3.4). Rabbit liver PGM was stable over the pH range used to measure pH optimum.

### 3.2.5 EFFECT OF PREINCUBATION WITH CHELATING AGENT AND MAGNESIUM

The effect of preincubation on the activity of rabbit liver PGM was observed by incubating the enzyme, prior to assay, in 40 mM histidine, 12 mM Tris-HCl buffer, pH 7.6, containing 2 mM $MgCl_2$, in absence of substrates for 10 min at 30 $^{o}C$. The activity was assayed as described in 2.2.1.1.1 and compared with that found without preincubation.

Preincubation of the enzyme with histidine and magnesium ion in the absence of substrate had little or no effect on enzyme activity. 97-98% of this activity was present in the original enzyme solution (see Table 3.4).

### 3.2.6 INFLUENCE OF METAL IONS AND EDTA ON ENZYME ACTIVITY

The influence of metal ions and EDTA on the activity of rabbit liver PGM was investigated using the spectrophotometric assay method (2.2.1.1.1) in the presence of 40 mM histidine, 12 mM Tris-HCl buffer, pH 7.6, 1 mM G1P and 2 μM G1P6P. The activity of rabbit muscle PGM was also measured under the same assay conditions. The results are summarised in Table 3.3.

Rabbit liver PGM showed a requirement for a divalent cation for enzyme activity, similar to the rabbit muscle enzyme

TABLE 3.3: Influence of metal ions and EDTA on the activity of phosphoglucomutase

| No. | Additions to buffer | RABBIT LIVER PGM | | RABBIT MUSCLE PGM | |
|---|---|---|---|---|---|
| | | Relative enzyme activity in units | Percentage of activity | Relative enzyme activity in units | Percentage of activity |
| 1 | None | 2.30 | 3.05 | 4.11 | 3.03 |
| 2 | 1 mM $Mg^{++}$ | 75.24 | 100 | 135.43 | 100 |
| 3 | 0.1 mM $Mg^{++}$ | 70.32 | 93.46 | 69.45 | 51.3 |
| 4 | 0.01 mM $Mg^{++}$ | 26.92 | 35.78 | 55.56 | 41 |
| 5 | 1 mM $Mg^{++}$, 5 µM EDTA | 85 | 113 | 135.02 | 99.7 |
| 6 | 0.1 mM $Mg^{++}$, 5 µM EDTA | 57.15 | 75.96 | 94.34 | 69.7 |
| 7 | 0.01 mM $Mg^{++}$, 5 µM EDTA | 32.56 | 43.27 | 55.9 | 41.3 |
| 8 | 1 mM $Mn^{++}$ | 10.56 | 14.04 | 31.19 | 23 |
| 9 | 0.1 mM $Mn^{++}$ | 10.71 | 14.23 | 37.8 | 27.9 |
| 10 | 0.01 mM $Mn^{++}$ | 10.48 | 13.93 | 34.72 | 25.6 |
| 11 | 1 mM $Zn^{++}$ | 0.46 | 0.6 | 2.0 | 1.5 |
| 12 | 0.1 mM $Zn^{++}$ | 0.87 | 1.2 | 3.07 | 2.3 |
| 13 | 0.01 mM $Zn^{++}$ | 2.03 | 2.7 | 4.03 | 3 |
| 14 | 100 mM EDTA | 0.08 | 0.1 | - | - |
| 15 | 10 mM EDTA | 0.10 | 0.13 | 0.13 | 0.1 |
| 16 | 5 mM EDTA | 0.83 | 1.1 | 1.65 | 1.2 |
| 17 | 1 mM EDTA | 1.88 | 2.5 | 4.2 | 1.4 |
| (S.A. of enzyme 100 U/mg) | | | | | |

All measurements of enzyme activity were carried out in 40 mM histidine, 12 mM Tris-HCl buffer, pH 7.6 at 30 $^{o}$C using the assay described in 2.2.1.1.1. The enzyme was not preincubated with buffer.

(cf. Milstein, 1961a). Both enzymes showed maximum activity with $Mg^{++}$ and 14-30% activity with $Mn^{++}$. $Zn^{++}$ inhibited the enzyme even at low concentrations. These results suggest that the active forms of both enzymes are magnesium-enzyme complexes. $Mg^{++}$ can be replaced by $Mn^{++}$ to some extent though replacement by $Zn^{++}$ leads to formation of an inactive complex. The established behaviour of the muscle enzyme (see Milstein, 1961a) is consistent with these observations.

When no metal ion was added to the reaction mixture, the activities of both enzymes were very low, ranging from 0 to 1%.This activity was completely eliminated with rabbit muscle enzyme by increasing the concentration of EDTA to 100 mM and removal of all metal ions. A small percentage (0.1%) of activity remained,however, with the rabbit liver enzyme. This may reflect the presence of phosphoribomutase which is present in liver but not in muscle and which appears not to require $Mg^{++}$ ions for activity (Kammen and Koo, 1969). However, as the level of this activity is less than that expected in the crude preparation (0.93% of the phosphoglucomutase activity), it is possible that a partial separation of the two enzymes occurred during affinity elution from phospho-cellulose (see 3.1, step 8).

The addition of 5 μM EDTA appeared to be beneficial with rabbit liver PGM, as would be expected if its activity was sensitive to the presence of heavy metal ions. This behaviour pattern is well established for the muscle enzyme (see Milstein, 1961a). However, rabbit muscle PGM did not

show any increase in activity with the addition of 5 μM EDTA (Table 3.3) probably because the assays were carried out without prior incubation with histidine and $Mg^{++}$ and this enzyme requires a preincubation step prior to assay for maximum activity (Milstein, 1961b).

### 3.2.7 EFFECT OF MAGNESIUM ION CONCENTRATION ON ENZYME ACTIVITY

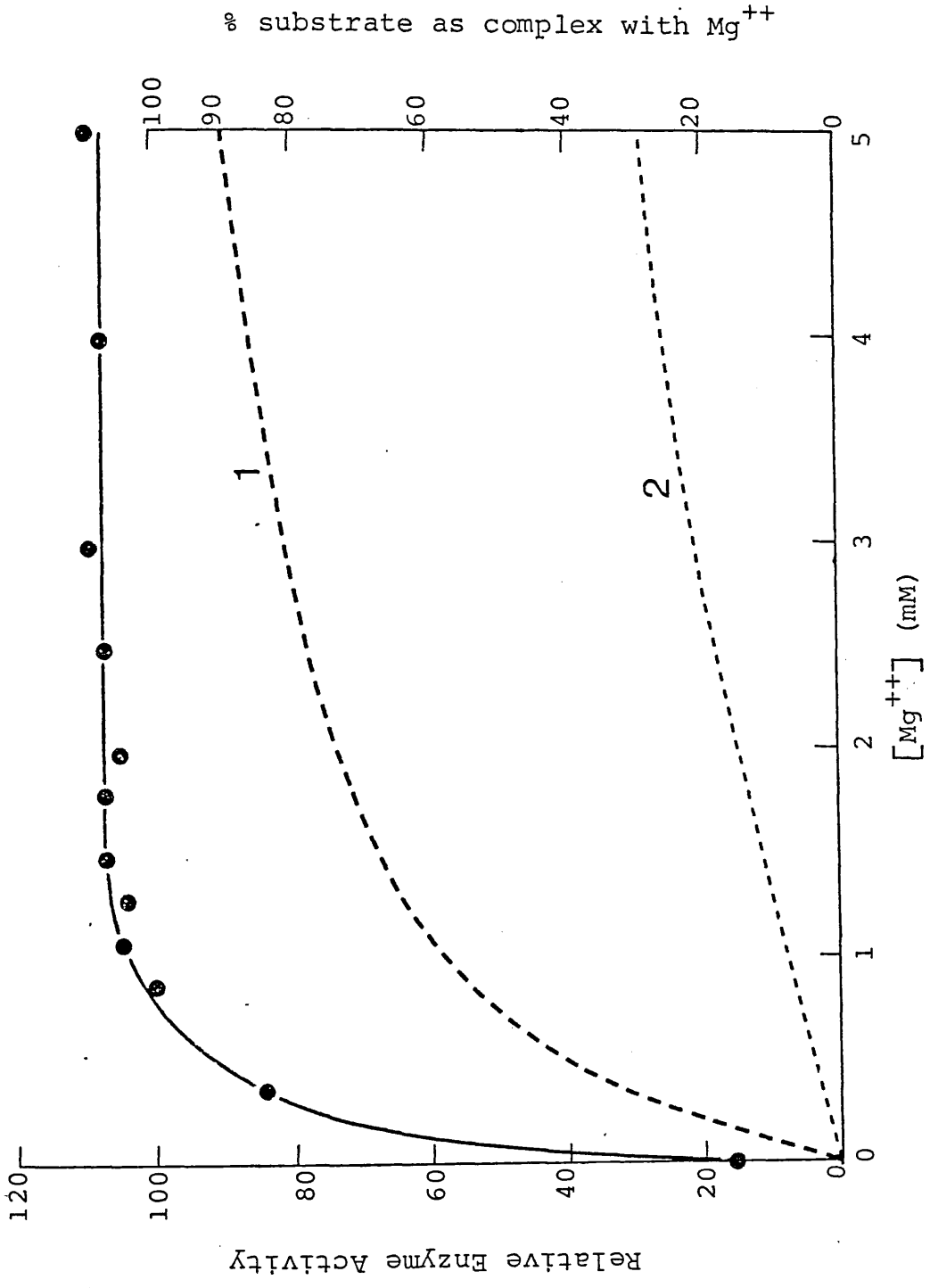
The activity of rabbit liver PGM was measured as a function of $Mg^{++}$ concentration in 40 mM histidine, 12 mM Tris-HCl buffer, pH 7.6, at 30 $^{o}C$ with the assay method described in 2.2.1.1.1, except that the magnesium ion concentration was varied.

Rabbit liver PGM activity was maximal at about 1.2 mM $Mg^{++}$ (Fig. 3 ) and remained constant up to 5 mM $Mg^{++}$. Similar results have been reported for the rabbit muscle enzyme where the maximum activity occurs at a magnesium ion concentration of between 2 and 4 mM. Above 8 mM, $Mg^{++}$ inhibition occurs owing to the accumulation of Mg-G1P and especially Mg-G1P6P complexes (Milstein, 1961; Robinson et al., 1965). The lack of inhibition found with liver phosphoglucomutase need not suggest that for this enzyme the magnesium complexes are the substrates. Although the Mg-G1P6P complex is formed extensively at 5 mM magnesium concentration (see Fig. 3.9), the free substrate is present at concentrations in excess of its $K_m$ under assay conditions.

FIGURE 3.9: Rabbit liver phosphoglucomutase activity as a function of magnesium ion concentration

PGM activity was measured in 40 mM histidine, 12 mM Tris-HCl buffer, at pH 7.6 and at 30 $^{o}$C, with the assay method described in 2.2.1.1.1, except that the magnesium ion concentration was varied as indicated. No preincubation of enzyme was carried out.

● indicates the phosphoglucomutase activity as a function of magnesium ion concentration. The dashed lines 1 and 2 indicate the expected binding of $G1P6P^{4-}$ and $G1P^{2-}$ by magnesium ions assuming dissociation constants of 0.67 mM (Ray and Roscelli, 1966) and 11 mM (Milstein, 1961) respectively for these complexes.


% substrate as complex with $Mg^{++}$
100
90
80
60
40
20
0
1
2
0
1
2
3
4
5
$[Mg^{++}]$ (mM)
Relative Enzyme Activity
120
100
80
60
40
20
0

### 3.2.8 INFLUENCE OF VARIOUS BUFFER SYSTEMS ON ENZYME ACTIVITY

The effect of various buffer systems on the activity of rabbit liver PGM was investigated in the presence of 2.0 mM $MgCl_2$, 1 mM G1P and 2 μM G1P6P at pH 7.6 and 30 °C, using the systems listed in Table 3.4. The concentrations of $NADP^+$ and G6PDH were the same as in 2.2.1.1.1. No preincubation of enzyme was carried out unless indicated.

As shown in Table 3.4, the activity of rabbit liver PGM was very low (2.8%) when no metal-chelating agent was present in the assay mixture. This phenomenon is well established for rabbit muscle enzyme (see Najjar, 1948). Maximum activity of the rabbit liver PGM enzyme was observed in the buffer system containing 40 mM histidine and 12 mM Tris-HCl. This buffer was used in all subsequent experiments. The enzyme is also active in the presence of cysteine (Najjar, 1948), imidazole (Harshman et al., 1965) and glutathione (Lehmann, 1939) as is the enzyme from rabbit muscle. However, there was a little activity when EDTA replaced histidine, imidazole or cysteine. The presence of 2-mercaptoethanol was also found to be beneficial for enzyme activity. These results appear to indicate that removal of metal ions other than magnesium is important if the enzyme is to have maximal activity.

### 3.2.9 INHIBITION BY ATP AND CITRATE

Preliminary studies of the effect of ATP and citrate on the activity of PGM from rabbit liver and rabbit muscle were

TABLE 3.4: Influence of buffer systems upon activity of rabbit liver phosphoglucomutase

The activity of the mutase was measured in the media listed in the presence of 2.0 mM magnesium chloride at pH 7.6 and at 30 °C. No preincubation of the enzyme was carried out unless indicated. The concentrations of G1P, G1P6P, $NADH^+$ and glucose-6-phosphate dehydrogenase are as shown for the assay system (see 2.2.1.1.1).

| Expt. No. | Reagent | Conc. mM | Relative activity in units | Percentage of activity |
|---|---|---|---|---|
| 1 | Histidine* | 40 | 33.5 | 100 |
| | Tris-HCl | 12 | | |
| 2 | Histidine | 40 | 32.5 | 97 |
| | Tris-HCl | 12 | | |
| 3 | Tris-HCl | 12 | 0.93 | 2.8 |
| 4 | Histidine† | 40 | 19.16 | 57.2 |
| 5 | Cysteine† | 20 | 33 | 98.5 |
| 6 | Histidine† | 40 | 27 | 81 |
| | Cysteine | 20 | | |
| 7 | Imidazole | 40 | 28.5 | 85 |
| 8 | Tris-HCl | 12 | 4.5 | 13.5 |
| | EDTA | 0.05 | | |
| 9 | Tris-HCl | 12 | 19.7 | 59 |
| | Mercaptoethanol | 20 | | |
| 10 | Glutathione | 20 | 27.3 | 81.5 |
| 11 | Sodium sulphite† | 20 | 0 | 0 |

* Enzyme preincubated with buffer for 10 mins prior to assay.
† No buffer was included, but the pH of these media was adjusted to pH 7.6.

carried out. The activities of the enzymes were measured at various concentrations of ATP or citrate under normal assay conditions and at constant concentration of ATP or citrate whilst varying the concentrations of magnesium and G1P6P. The assay conditions are described in Fig. 3.10 and Table 3.4 respectively. The enzyme was not preincubated before assay.

The results obtained for the effect of ATP or citrate at various concentrations are shown in Fig. 3.10. Activation by ATP of both rabbit liver and rabbit musle PGMs is observed at moderate G1P6P concentration (i.e. 7 times in excess of $K_m$) in the presence of low concentrations of ATP (0.5 mM). At higher concentrations, it is found that ATP has a strong inhibitory effect on both enzymes. This effect is slightly greater with rabbit liver PGM. At low concentrations of citrate (0-1 mM) the activity of neither enzyme was affected and at higher concentration a weak inhibition was observed.

Table 3.5 shows the effect of 3.5 mM ATP and 2.5 mM citrate upon enzyme activity at different concentrations of $Mg^{++}$ and G1P6P. PGM from rabbit liver and rabbit muscle showed a little activity (0-0.6% of the control) in the presence of 3.5 mM ATP or 2.5 mM citrate and in the absence of either $Mg^{++}$ or G1P6P.

Under normal assay conditions (2 μM G1P6P and 2 mM $Mg^{++}$) inhibition by ATP was greater than inhibition by citrate. Abolition of both ATP or citrate inhibitions occurred when the concentration of either $Mg^{++}$ of G1P6P was increased to

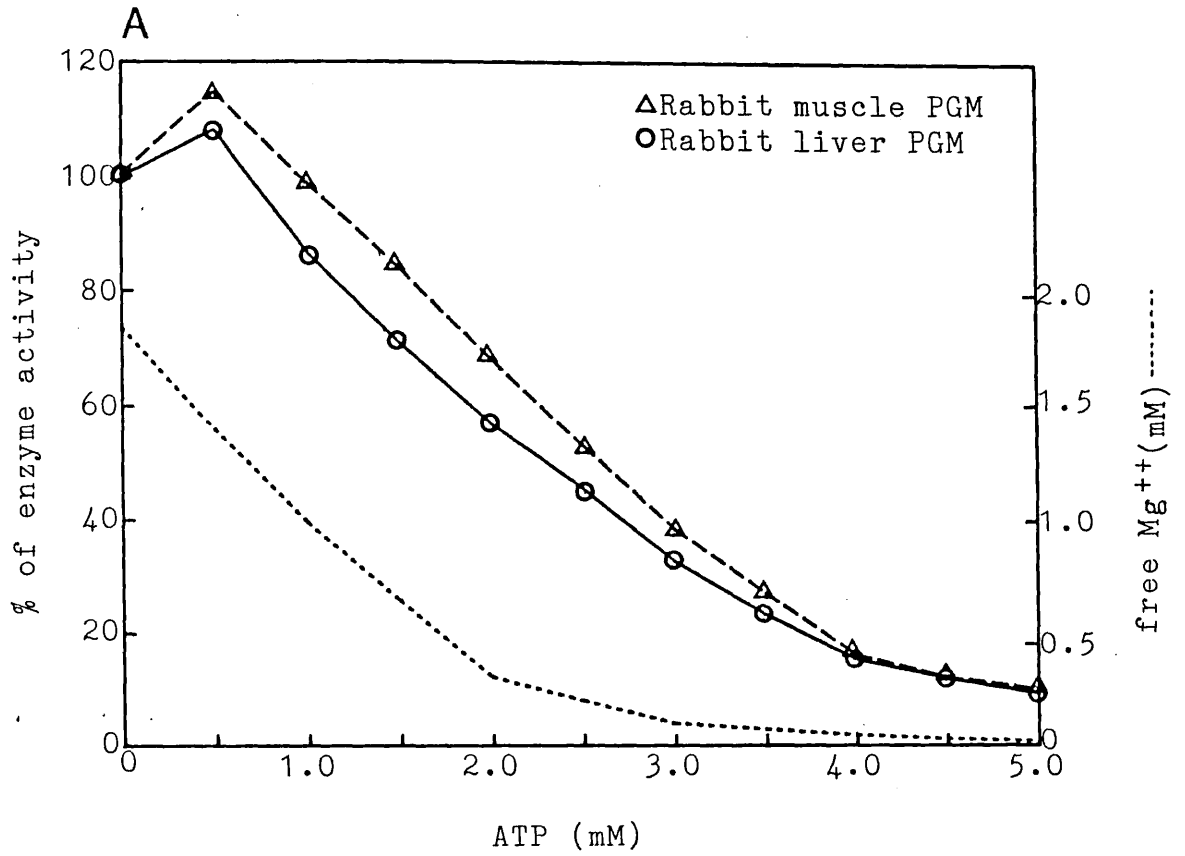
FIGURE 3.10: Effect of ATP and citrate concentration on enzyme activity

The rabbit liver and rabbit muscle PGMs were assayed in the presence of 0-5 mM ATP or citrate. The assay mixture contained 40 mM histidine, 12 mM Tris-HCl buffer, pH 7.6, 2.0 mM $MgCl_2$, 1 mM G1P, 2 µM G1P6P, 0.475 mM $NADP^+$, 0.7 unit of G6PDH and 0-5 mM ATP or citrate in a final volume of 2.995. The reaction was started by the addition of 5 µl PGM and the rate of reaction was determined as described in 2.2.1.1.1. The enzyme was not preincubated before assay.

Graphs A and B show the inhibition by ATP and citrate repectively.

- o Rabbit liver PGM activity.
- Δ Rabbit muscle PGM activity.

Indicate the concentration of free $Mg^{++}$ in mM, calculated by assuming dissociation constants of Mg-$G1P^{2-}$, $1.1 \times 10^{-2}$ M (Milstein, 1961a); Mg-$G1P6P^{4-}$, $6.7 \times 10^{-4}$ M (Ray and Roscelli, 1966a); Mg-$EDTA^{3-}$, $1.0 \times 10^{-6}$ M; Mg-$ATP^{2-}$, $5.0 \times 10^{-5}$ M; Mg-Citrate, $5.0 \times 10^{-4}$ M (Dawson et al., 1969).


A
△Rabbit muscle PGM
○Rabbit liver PGM
% of enzyme activity
free Mg++(mM)
ATP (mM)
0
20
40
60
80
100
120
0.5
1.0
1.5
2.0
3.0
4.0
5.0

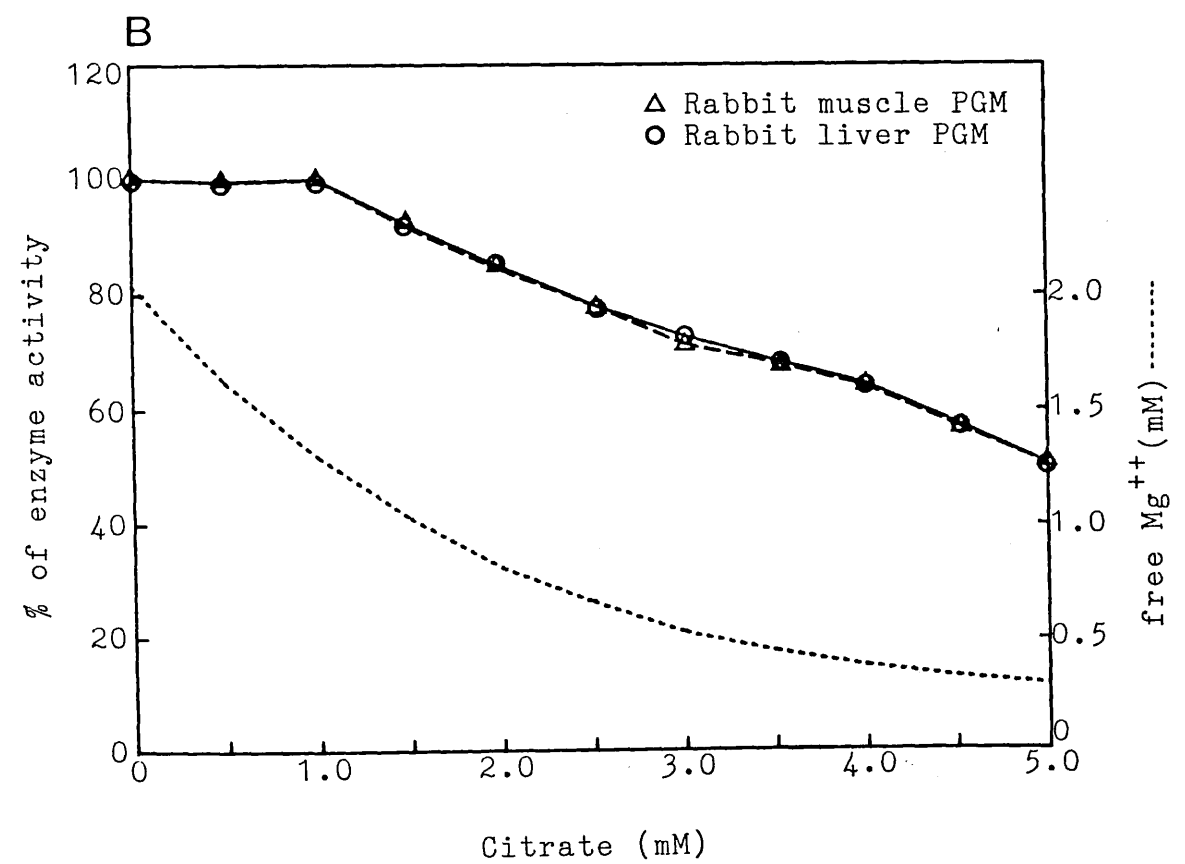

B
△ Rabbit muscle PGM
○ Rabbit liver PGM
% of enzyme activity
free Mg++(mM)
Citrate (mM)
0
20
40
60
80
100
120
0.5
1.0
1.5
2.0
3.0
4.0
5.0

TABLE 3.5: Inhibition by ATP and citrate at various concentrations of $Mg^{++}$ and G1P6P

PGM from rabbit liver and rabbit muscle were assayed under the conditions listed in the table, in the presence of 3.5 mM ATP or 2.5 mM citrate. The following reagents were also included in the assay mixture: 40 mM histidine, 12 mM Tris-HCl buffer, pH 7.6, 1 mM G1P, 0.475 mM $NADP^{+}$ and 0.7 unit G6PDH. The enzyme activity was determined spectrophotometrically as described in 2.2.1.1.1.

The concentration of free $Mg^{++}$ in each solution was calculated by assuming the following dissociation constants: Mg-G1P6P$^{4-}$, $6.7 \times 10^{-4}$ M (Ray and Roscelli, 1966a); Mg-G1P$^{2-}$, $1.1 \times 10^{-2}$ M (Milstein, 1961a); Mg-EDTA$^{3-}$, $1.0 \times 10^{-6}$ M; Mg-ATP$^{2-}$, $5.0 \times 10^{-5}$ M; Mg-citrate, $5.0 \times 10^{-4}$ M (in Dawson et al., 1969).

| Conditions | Free $Mg^{++}$ (mM) | Rabbit liver PGM, relative activity | Rabbit muscle PGM, relative activity |
|---|---|---|---|
| Optimum condition<br>2.0 µM G1P6P<br>2.0 mM $MgCl_2$ | 1.86 | 100 | 100 |
| 3.5 mM ATP<br>2.0 µM G1P6P<br>2.0 mM $MgCl_2$ | 0.064 | 24 | 27 |
| 3.5 mM ATP<br>- G1P6P<br>2.0 mM $MgCl_2$ | 0.064 | 0.4 | 0.3 |
| 3.5 mM ATP<br>2.0 µM G1P6P<br>- $MgCl_2$ | 0 | 0.2 | 0.4 |
| 3.5 mM ATP<br>2.0 µM G1P6P<br>5.0 mM $MgCl_2$ | 1.6 | 56 | 69 |
| 3.5 mM ATP<br>50.0 µM G1P6P<br>2.0 mM $MgCl_2$ | 0.064 | 51 | 67 |
| 2.5 mM citrate<br>2.0 µM G1P6P<br>2.0 mM $MgCl_2$ | 0.62 | 82 | 79 |
| 2.5 mM citrate<br>- G1P6P<br>2.0 mM $MgCl_2$ | 0.62 | 0.3 | 0.2 |
| 2.5 mM citrate<br>2.0 µM G1P6P<br>- $MgCl_2$ | 0 | 0.3 | 0.6 |
| 2.5 mM citrate<br>2.0 µM G1P6P<br>5.0 mM $MgCl_2$ | 2.87 | 91 | 89 |
| 2.5 mM citrate<br>50.0 µM G1P6P<br>2.0 mM $MgCl_2$ | 0.61 | 105 | 135 |
| 2.5 mM citrate<br>50.0 µM G1P6P<br>5.0 mM $MgCl_2$ | 2.28 | 128 | 142 |
| 3.5 mM ATP<br>50.0 µM G1P6P<br>5.0 mM $MgCl_2$ | 1.6 | 95 | 110 |

5.0 mM or 50 μM respectively. This is consistent with the findings of Beitner et al. (1975). The reversal of ATP inhibition by increasing the concentration of $Mg^{++}$ or G1P6P was greater with rabbit muscle PGM (67-69%) than with the rabbit liver enzyme (51-56%). The weak citrate inhibition was relieved from 80 to 90% of the original activity by increasing the concentration of $Mg^{++}$ to 5 mM. However, when an excess amount of G1P6P (50 μM) was added to abolish the citrate inhibition, the activities of liver and muscle PGMs were found to rise to 105% and 135% respectively of the control. The origin of this activation was not determined.

## 3.3 GLUCOSE 1,6-DIPHOSPHATASE ACTIVITY

Glucose 1,6-diphosphatase activity of rabbit liver homogenate was determined by using G1P6P as a substrate in 40 mM histidine, 12 mM Tris-HCl buffer, pH 7.6, containing 2 mM $MgCl_2$ and by measuring fluorimetrically G6P produced in a coupled dehydrogenase reaction as described in 2.2.2. Rabbit liver homogenate was centrifuged at 25,000 g for 10 min for this assay in order to clarify the solution. The diphosphatase activity in the crude extracts or rabbit liver could not be measured because of the presence of suspended material which made measurement of fluorescence difficult.

Purified preparations of rabbit liver PGM did not show any diphosphatase activity by the fluorimetic assay method. Therefore a radiochemical method (approximately 400 times more sensitive) was used in which hydrolysis of $[^{32}P]$ G1P6P was followed by measuring the rate of formation of $^{32}P_i$ per unit time at 30 $^{o}C$ as described in 2.4.3.5. A graph of log labelled substrate against time was plotted (Fig. 3.11). The diphosphatase activity was calculated from the rate constant ($k_1$) for the disappearance of substrate.

Glucose-1,6-diphosphatase activity of rabbit liver homogenate was found to be five times greater than that of rabbit muscle. However, rabbit liver phosphoglucomutase activity was approximately 1/3 that of rabbit muscle. The ratio of diphosphatase to mutase activity in rabbit liver homogenate should be 13 times higher than that of muscle.

FIGURE 3.11: Glucose-1,6-diphosphatase activity determination in purified rabbit liver phosphoglucomutase

Glucose-1,6-diphosphatase activity was determined by incubating 0.5 units of rabbit liver PGM with 0.486 μCi [$^{32}$P] G1P6P and 9 nmol carrier G1P6P in His-Tris-HCl buffer (40 mM histidine, 12 mM Tris), pH 7.6, containing 2.0 mM $MgCl_2$ at 30 $^{o}$C and measuring $^{32}P_i$ liberated per unit time as described in 2.4.3.5. A graph of log concentration of [$^{32}$P] G1P6P against time was plotted. The rate constant was calculated from the slope, the velocity of the reaction from the rate constant and the number of units of diphosphatase activity from the rate of the reaction by formulae given in appendix.

Slope = -0.00003547

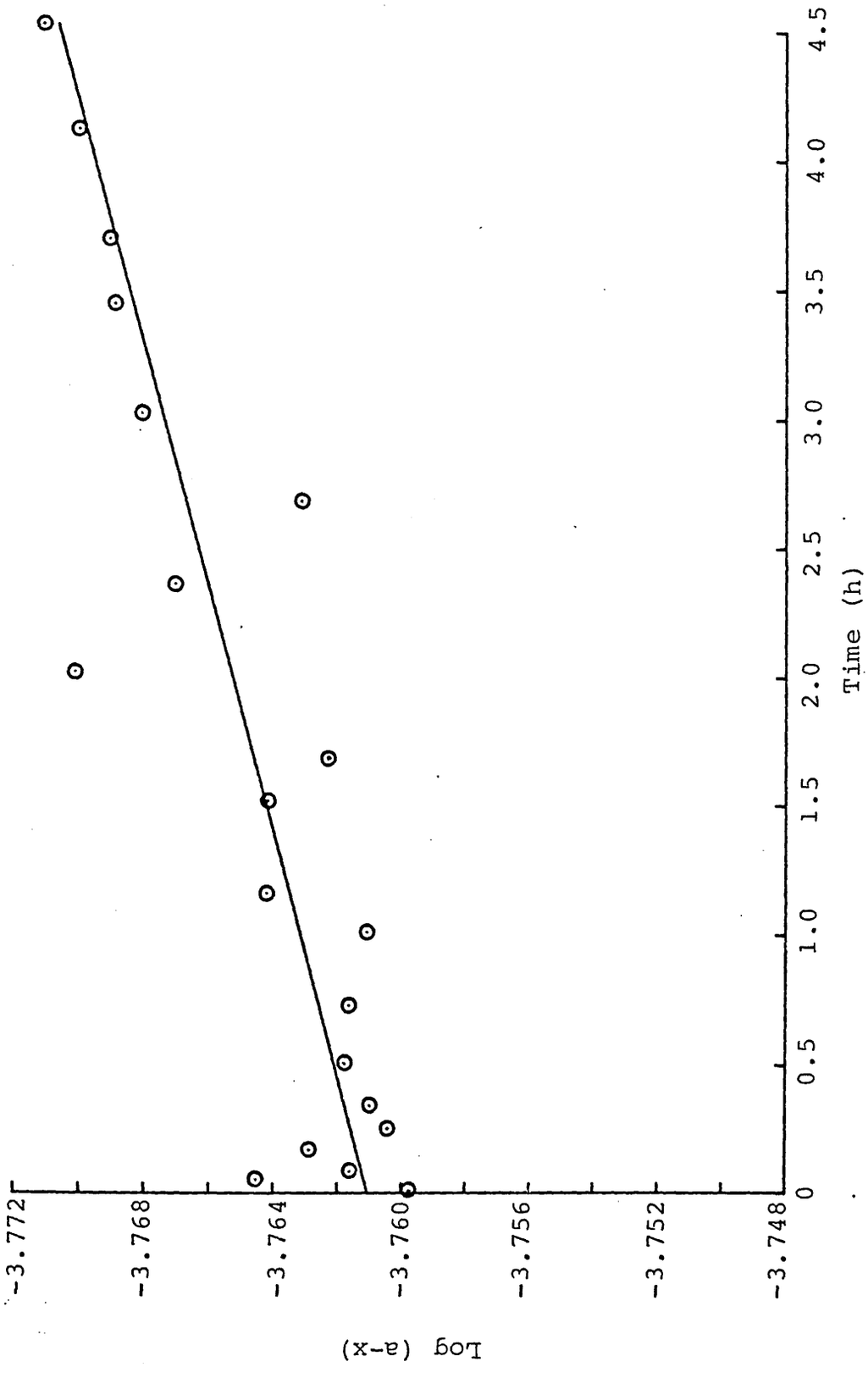
Intercept at y axis = -3.761

Rate constant $k_1$ = 0.00008168 $min^{-1}$

Velocity $v_i$ = 8.37 x $10^{-7}$ μmol $min^{-1}$

a = [$^{32}$P] G1P6P (initial)

x = $^{32}P_i$ (liberated)


Log (a-x)
-3.772
-3.768
-3.764
-3.760
-3.756
-3.752
-3.748
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
Time (h)

The values obtained for the ratio of diphosphatase to mutase activity were $3.46 \times 10^{-4}$ and $2.6 \times 10^{-5}$ for rabbit liver and rabbit muscle homogenates. The value obtained for diphosphatase to mutase activity in purified preparations of PGM (specific activity 150-200 units $mg^{-1}$) from rabbit liver was $1.67 \times 10^{-6}$. The value is much lower than that obtained with rabbit liver homogenate. This suggests that the removal of most of the diphosphatase activity occurred during the purification of the enzyme.

## 3.4 KINETIC STUDIES

The kinetic behaviour of rabbit liver PGM was investigated at low, non-inhibitory concentrations of G1P and G1P6P in 40 mM histidine, 12 mM Tris-HCl buffer at pH 7.6 and 30 $^{o}$C. A sensitive fluorimetric assay based upon the increase in fluorescence of NADPH was used (2.2.1.1.2) in order to permit measurements at sufficiently low concentrations of substrates.

Phosphoglucomutase, specific activity 290 units $mg^{-1}$ protein, was stored as a suspension in 3.2 M ammonium sulphate solution. The kinetic behaviour of phosphoglucomutase appears to be sensitive to the presence of anions, particularly sulphate (Ray and Roscelli, 1966a; 1966b). Before assay, the enzyme suspension in ammonium sulphate was centrifuged as described in 2.2.1.1.2 and the enzyme pellet dissolved in 40 mM histidine, 12 mM Tris-HCl buffer pH 7.6 containing 2 mM $Mg(NO_3)_2$. Prior to assay the enzyme was incubated for 10 min at 30 $^{o}$C in the same buffer. A volume of enzyme solution containing 0.44 µg of enzyme was then added to the assay mixture, at 30 $^{o}$C, so that the final assay mixture contained 40 mM histidine, 12 mM Tris-HCl buffer, pH 7.6, 2 mM $Mg(NO_3)_2$, 4-100 µM G1P, 0-0.05 µM G1P6P, 416 µM $NADP^{+}$ and 0.8 units of G6PDH (free from ammonium sulphate). The free $Mg^{++}$ concentration was kept constant at 2 mM so that the percentage of G1P and G1P6P complexed by magnesium would not vary. Consequently initial velocity plots would not be affected by changes in the extent of metal binding. Furthermore, the ionic strength of the assay

mixture was maintained at 0.05 mol $\ell^{-1}$ over the range of substrate concentrations by the addition of appropriate quantities of 200 mM $KNO_3$.

The rate of reaction after addition of phosphoglucomutase was followed at 30 °C by observing the increase in fluorescence at 455 nm (band width = 5 mm) with a fluorescence spectrometer. An excitation wave length of 240 nm was used (band width = 2.5 mm). Calibration of the fluorimeter was carried out as described in 2.2.1.1.2.

As shown in Fig. 3.12 and 3.13 plots of $1/v_i$ against $1/[G1P]$ at varying concentrations of G1P6P and of $1/v_i$ against $1/[G1P6P]$ at varying concentrations of G1P each gave a family of parallel lines, for which the following equation is appropriate initial velocity expression:

$$\frac{1}{v_i} = \frac{1}{V_m}\left(1 + \frac{K_m^A}{A} + \frac{K_m^B}{B}\right) \qquad \text{——— (1)}$$

A: concentration of G1P

B: concentration of G1P6P

The kinetic data were analysed using the above equation by linear regression analysis with simple errors (Cleland, 1963). A Sinclair ZX81 micro computer was used for calculations. A $V_{max}$ of 40.1 ± 0.44 x $10^{-3}$ µmol $ml^{-1}$ $min^{-1}$, (turnover No = 319 $s^{-1}$), a $K_m$ for G1P6P of 0.29 ± 0.019 µM and a $K_m$ for G1P of 30.8 ± 1.8 µM were obtained. These values do not take into account binding of the substrates by magnesium. If $Mg^{++}$ dissociation constants of 11 mM and 0.667 mM for G1P and G1P6P respectively (Milstein, 1961a; Ray and Roscelli, 1966a)

FIGURE 3.12: Lineweaver-Burk Plot for rabbit liver phosphoglucomutase at fixed concentrations of G1P6P

Lineweaver-Burk plots for rabbit liver PGM at low concentrations of substrates with G1P as the varied substrate and at fixed concentrations of G1P6P. Determinations were carried out at pH 7.6 in 40 mM histidine, 12 mM Tris-HCl buffer with a free magnesium concentration of 2.0 mm, at an ionic strength of 0.05 $mol^{-1}$ at 30 $^{o}C$. For further details see 3.4.

G1P6P concentrations: ● = 0.05 μM, O = 0.065 μM, ▲ = 0.085 μM, Δ = 0.12 μM, ■ = 0.25 μM, □ = 1.0 μM, ◆ = saturating concentration.

The last points with the dashed line are the intercepts on the $1/v_i$ axis of the Lineweaver-Burk plots shown in Fig. 3.13 and correspond to a saturating G1P6P concentration.

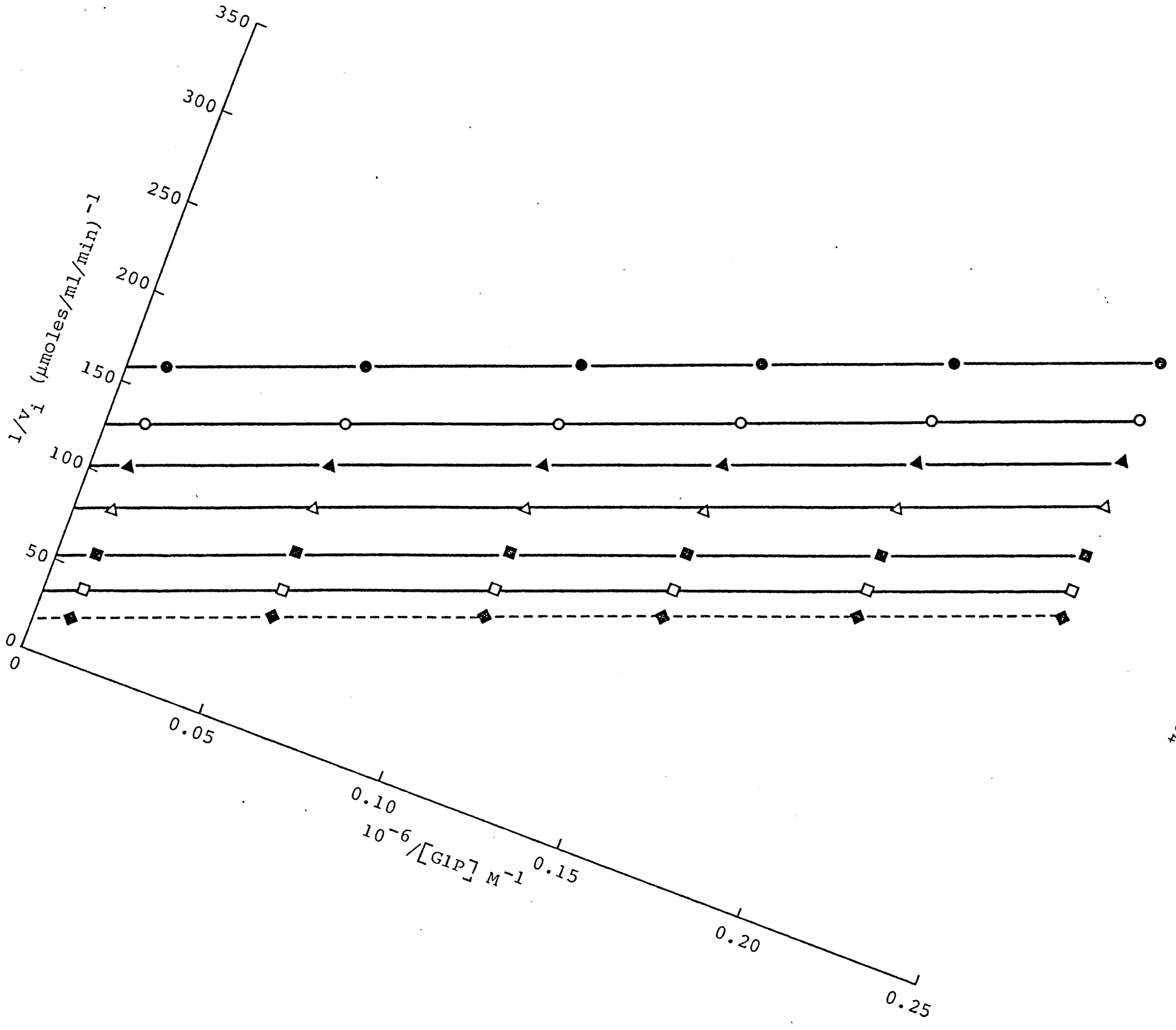
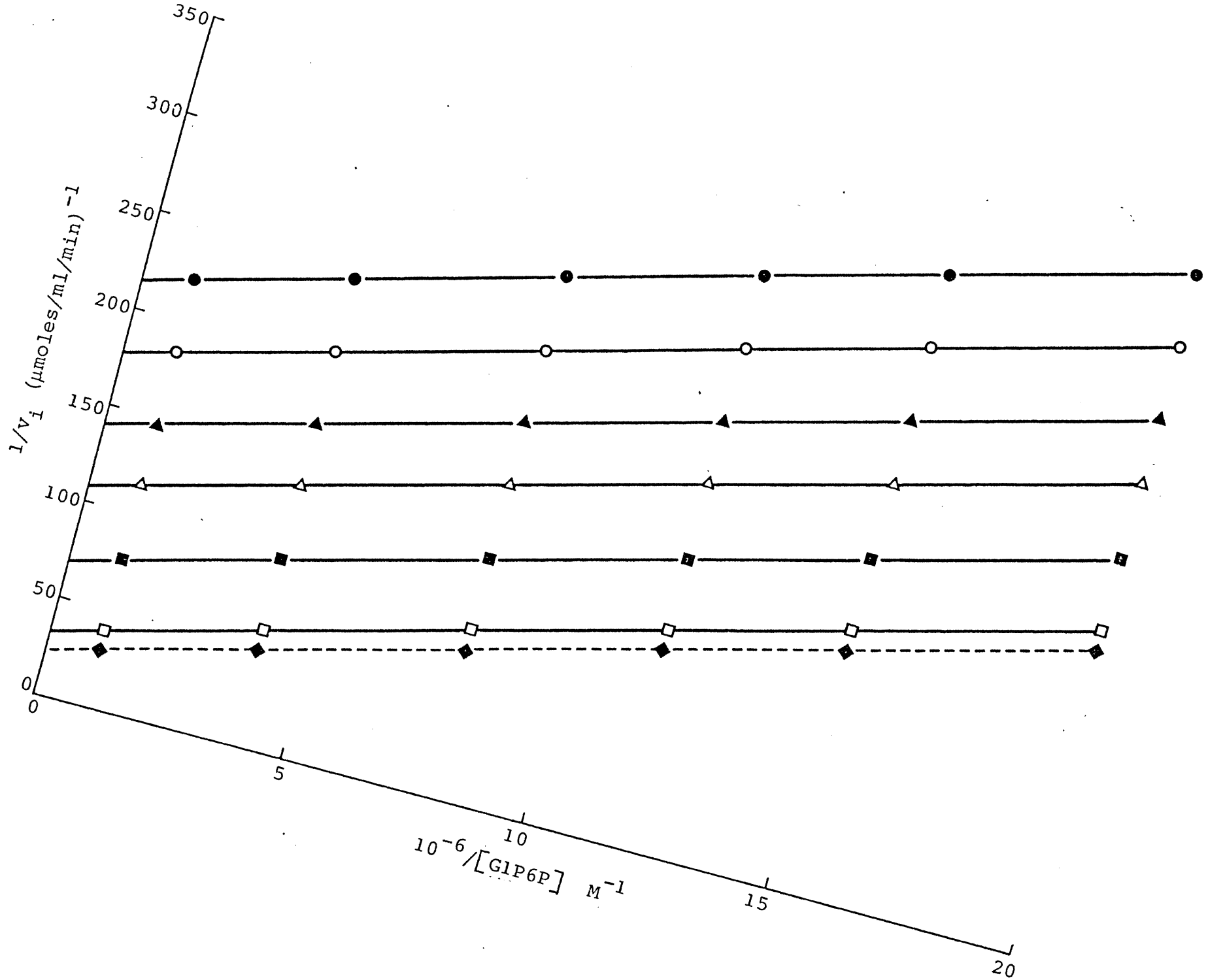

350
300
250
200
150
100
50
0
0
0.05
0.10
0.15
0.20
0.25
$1/v_i$ (μmoles/ml/min) $^{-1}$
$10^{-6}/[G1P]_M^{-1}$

FIGURE 3.13: Lineweaver-Burk Plots for rabbit liver phosphoglucomutase at fixed concentrations of G1P

Lineweaver-Burk plots for rabbit liver PGM at low concentrations of substrates with G1P6P as the varied substrate and at fixed concentrations of G1P. Determinations were carried out at pH 7.6 in 40 mM histidine, 12 mM Tris-HCl buffer with a free magnesium ion concentration of 2.0 mM, at an ionic strength of 0.05 mol $l^{-1}$ and 30 $^{o}$C. For further details see 3.4.

G1P concentrations: ● = 4.0 μM, ○ = 5.0 μM, ▲ = 6.5 μM, △ = 9.0 μM, ■ = 17.0 μM, □ = 100.0 μM, ◆ = saturating concentration.

The last points with the dashed line are the intercepts on the $1/v_i$ axis of the Lineweaver-Burk plots shown in Fig. 3.12 and correspond to a saturating G1P concentration.


350
300
250
200
150
100
50
0
0
5
10
15
20
$1/v_i$ (μmoles/ml/min) $^{-1}$
$10^{-6}/[G1P6P]$ $M^{-1}$

are assumed, then at the free $Mg^{++}$ ion concentration employed, 15.4% of G1P and 75.0% of G1P6P would be in the metal-complex form. The reported values may be appropriately adjusted if the Mg-substrate complexes do not react with the enzyme.

## 3.5 INDUCED-TRANSPORT TESTS

The mechanism of the rabbit liver PGM reaction was investigated using the induced-transport tests of Britton and Clarke (1968). A preliminary paper has been published on this work (Jamil and Clarke, 1981). Induced-transport tests were carried out with [$^{32}$P] G6P at low substrate concentrations and with [$^{14}$C] G6P at both low and high substrate concentrations. Preparation and purification of [$^{32}$P] G6P are described in 2.4.2.1 and 2.4.2.3 respectively. [$^{32}$P] G6P contained 0.2% of impurity as $^{32}P_i$ and it had specific radioactivity of 32 μCi μmol$^{-1}$. [$^{14}$C] G6P from the Radiochemical Centre, Amersham was purified on Sephadex A-50, as described in 2.4.2.5 to remove impurities. The specific radioactivity of [$^{14}$C] G6P was 34 μCi μmol$^{-1}$.

### Induced-Transport Experiment with [$^{32}$P] G6P or [$^{14}$C] G6P At Low Substrate Concentrations

[$^{32}$P] G6P or [$^{14}$C] G6P was incubated at 30 $^{o}$C with $2.2 \times 10^{-2}$ unit of rabbit liver phosphoglucomutase in 40 mM histidine, 12 mM Tris-HCl buffer, pH 7.4, containing 1.1 mM $MgCl_2$ in a total volume of 9.058 ml, until equilibrium was reached. The incubation mixture also contained a small amount of G1P6P (see below). Before the addition of non-radioactive G1P, 10 samples of 100 μl were withdrawn at timed intervals for determination of G1P concentration and radioactivity in G1P. G1P concentration and $^{14}$C or $^{32}$P-radioactivity in the samples were determined as described in 2.2.6, 2.4.3.3 and 2.4.3.4 respectively. At zero time

25 µl non-radioactive G1P was added which increased the volume of incubation mixture from 8.058 ml to 8.083 ml. 100 µl samples were withdrawn from the reaction mixture after zero time, and at timed intervals, to determine the concentration of G1P and radioactivity in G1P. Concentrations measured after the addition of non-radioactive G1P were corrected for dilution caused by this addition. Concentrations before the addition of non-radioactive G1P were: G1P, 7.0 µM; G6P, 40 µM; G1P6P, 0.5 µM. Concentrations immediately after the addition of non-radioactive G1P were: G1P, 112 µM; G6P, 39.8 µM; G1P6P, 0.5 µM.

Induced-Transport Experiment with [$^{14}$C] G6P at High Concentration of Substrate

[$^{14}$C] G6P was incubated at 30 $^{o}$C with 2.0 units of rabbit liver phosphoglucomutase in 40 mM histidine, 12 mM Tris-HCl buffer, pH 7.4, containing 1.1 mM $MgCl_2$ in a total volume of 5.0 ml until equilibrium was reached. The incubation mixture also contained G1P6P (see below). The samples were withdrawn before and after the addition of non-radioactive G1P (zero time) as described above; 6 samples of 100 µl were withdrawn before zero time and the volume of incubation mixture was increased from 4.4 ml to 4.6 ml after the addition of 0.2 ml of non-radioactive G1P. Concentrations measured after the addition were corrected for dilution. Concentrations before the addition of non-radioactive G1P were: G1P, 1.6 mM; G6P, 34.0 mM; G1P6P, 0.1 mM. Concentrations immediately after the addition were: G1P, 25.6 mM; G6P, 23.2 mM; G1P6P, 0.096 mM.

Three possible mechanisms have been proposed for phosphate transfer by phosphomutases (see Britton and Clarke, 1968 and introduction 1.1.9). These are the phosphoenzyme mechanism (Mechanism 1), the direct intramolecular transfer of phosphate (Mechanism 2) in which cofactor G1P6P may be involved indirectly, and the intermolecular transfer mechanism (Mechanism 3) in which the cofactor participates directly in the transfer of the phosphate (see 1.1.9). In the phosphoenzyme mechanism (Mechanism 1), the $^{14}C$ label obeys the independence relationship (transfer in a single catalytic cycle) but with $^{32}P$ co-transport occurs and two catalytic cycles are required. In direct transfer of phosphate (Mechanism 2) both $^{32}P$ and $^{14}C$ label of G1P will require one catalytic cycle for transfer. In the third mechanism where intramolecular transfer is involved, $^{14}C$ label requires two catalytic cycles whereas $^{32}P$ requires three catalytic cycles. In each mechanism, it is possible that the active form of the enzyme may exist in two isomeric forms, one which combines with G1P and one which combines with G6P. It is also conceivable that in Mechanism 1 (phosphoenzyme mechanism) that the intermediate (stable entity of enzyme created by reaction with a molecule of cofactor ) could be either a true phosphoenzyme or an enzyme-G1P6P complex which performs the role of a phosphoenzyme. This latter possibility was referred to as Mechanism $1^1$.

At low substrate concentrations with $^{32}P$-labelled substrate, co-transport of $[^{32}P]$ G1P to G6P was observed (Fig. 3.14). The level of $[^{32}P]$ G1P activity fell to 37% of its original

FIGURE 3.14: Induced-transport tests at low substrate concentrations

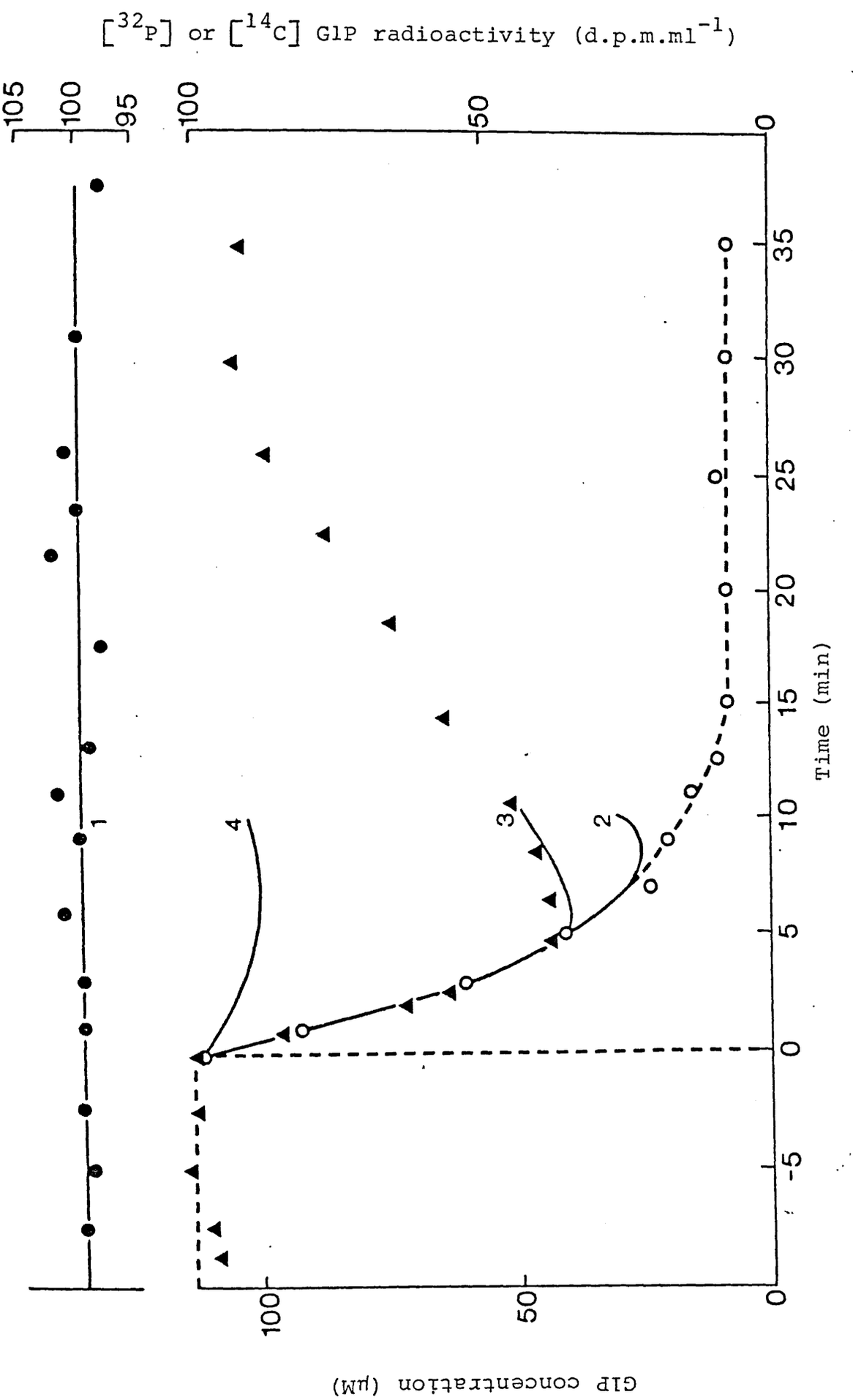
Induced-transport tests with $^{32}P$-labelled substrates showing co-transport, and $^{14}C$-labelled showing no induced transport. Labelled G1P was incubated at 30 $^{o}C$ with $2.2 \times 10^{-2}$ unit rabbit liver phosphoglucomutase in 40 mM histidine, 12 mM Tris-HCl buffer, pH 7.4, containing 1.1 mM $MgCl_2$, until equilibrium was reached. At zero time non-radioactive G1P was added in a small volume. Concentrations before additions: G1P, 7.0 μM; G6P, 40.0 μM; G1P6P, 0.5 μM. Concentrations after addition: G1P, 112.0 μM; G6P, 39.8 μM; G1P6P, 0.5 μM.

o concentration of G1P

▲ [$^{32}P$] G1P radioactivity (d.p.m./ml) corrected for dilution at zero time

● [$^{14}C$] G1P radioactivity (d.p.m./ml) corrected for dilution at zero time.

The continuous lines are theoretical curves for G1P radioactivity. For [$^{32}P$] G1P and [$^{14}C$] G1P radioactivity the expected curves for Mechanism 1 are 3 and 1 respectively, for Mechanism 2, 1 and 1 respectively and for 3, 2 and 3 respectively. Line 4 indicates the expected radioactivity changes for Mechanism 3 with [$^{14}C$] G1P if isomerisation of the E-G1P6P complex is slow with the rate constant 288 $S^{-1}$, or for Mechanism 1 with [$^{32}P$] G1P if isomerisation of the phosphoenzyme is slow, with the same rate of isomerisation. These mechanisms are described in the Introduction (1.1.9).


[32P] or [14C] GlP radioactivity (d.p.m.ml-1)
105
100
95
100
50
0
35
30
25
20
15
10
5
0
-5
Time (min)
1
2
3
4
100
50
0
GlP concentration (μM)

value then returned to that value as chemical equilibrium was reached. Any fall in $^{32}P$-activity in the 1-position of G1P6P would not affect this value significantly. The continuous lines in Fig. 3.14 are the theoretical curves calculated by the method of Britton and Clarke (1968) (see Appendix for flux equations). These curves were calculated on the assumption that any isomerization of free enzyme is rapid and they depend only on the concentrations of substrates and the equilibrium constant. Lines 1,2 and 3 are the expected curves for Mechanisms 2,3 and 1 respectively. The fall in $[^{32}P]$ G1P activity is similar to that expected for a phosphoenzyme mechanism (line 3, Fig. 3.14).

In similar experiments with $^{14}C$-labelled substrates no induced-transport was observed (Fig. 3.14). In Fig. 3.14 for $^{14}C$ radioactivity, line 1 is the theoretical curve for expected Mechanisms 1 and 2 and line 2 is for Mechanism 3. These data also support the phosphoenzyme mechanism.

Taken together these results support Mechanism 1 and exclude Mechanisms 2 and 3. However, Mechanism 3 could give rise to the observed induced-transport pattern if the isomerization of the enzyme-G1P6P complex is rate-limiting as the degree of co-transport for $^{14}C$- and $^{32}P$-labelled substrates is reduced towards that of Mechanism 1. This possibility has been excluded, for in order to reduce the $^{14}C$-co-transport to less than that indicated by line 4 in Fig. 3.14, the $K_m$ for G1P would need to be eighteen times less than the experimentally observed value of 31 μM (see 3.4). Similarly any isomerization of phosphoenzyme (Mechanism 1)

FIGURE 3.15: Induced-transport tests at high substrate concentrations

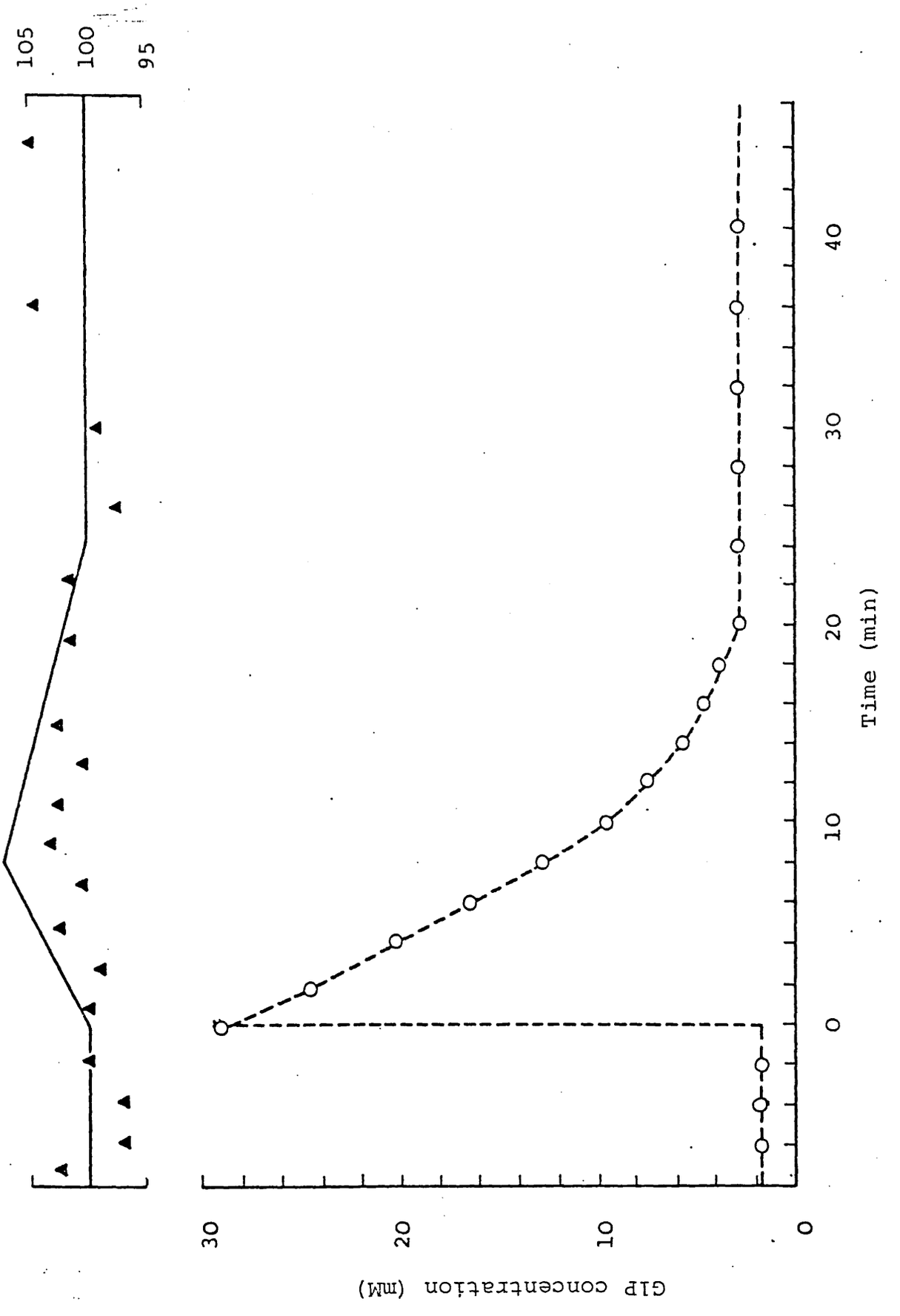
Induced-transport tests with $^{14}C$-labelled substrate at high substrate concentration showing absence of induced transport. [$^{14}C$] G6P was incubated with 2 units of rabbit liver phosphoglucomutase in 40 mM histidine, 12 mM Tris-HCl buffer, pH 7.4, containing 1.1 mM $MgCl_2$ until equilibrium was reached. At zero time 0.2 ml of non-radioactive G1P was added to 4.4 ml of this solution and the reaction allowed to proceed to equilibrium. Samples were taken from the reaction mixture before and after this addition and the concentration of G1P and radioactivity in G1P were corrected for dilution caused by the addition.

o concentration of G1P

▲ $^{14}C$ radioactivity in G1P (corrected for dilution at zero time).

Concentration before addition: G1P, 1.6 mM; G6P, 24.0 mM; G1P6P, 0.1 mM. Concentration after the addition: G1P, 25.6 mM; G6P, 23.2 mM; G1P6P, 0.96 mM.

The continuous line is the theoretical curve for Mechanism 1, assuming a rate for interconversion of the phosphoenzyme forms of $2.7 \times 10^7\ s^{-1}$.


[14C] GIP radioactivity (d.p.m.ml-1)
105
100
95
GIP concentration (mM)
30
20
10
0
Time (min)
0
10
20
30
40

should reduce the extent of $^{32}P$-co-transport and lead to $^{14}C$-counter-transport especially at high concentration. However, as shown in Fig. 3.15, no $^{14}C$-counter-transport was observed at high substrate concentrations. The continuous line in this figure is a theoretical curve for $^{14}C$ radioactivity for Mechanism 1, assuming a rate for interconversion of the phosphoenzyme forms of $2.7 \times 10^{7}$ $s^{-1}$. The finding that there was no induced transport indicated that the rate constant for any isomerization must be in excess of $2.7 \times 10^{7}$ $s^{-1}$. Thus these results are only compatible with Mechanism 1 or $1^{1}$.

## 3.6 $^{32}$P-LABELLED PHOSPHOENZYME

### 3.6.1 $^{32}$P-LABELLED PHOSPHOENZYME FORMATION

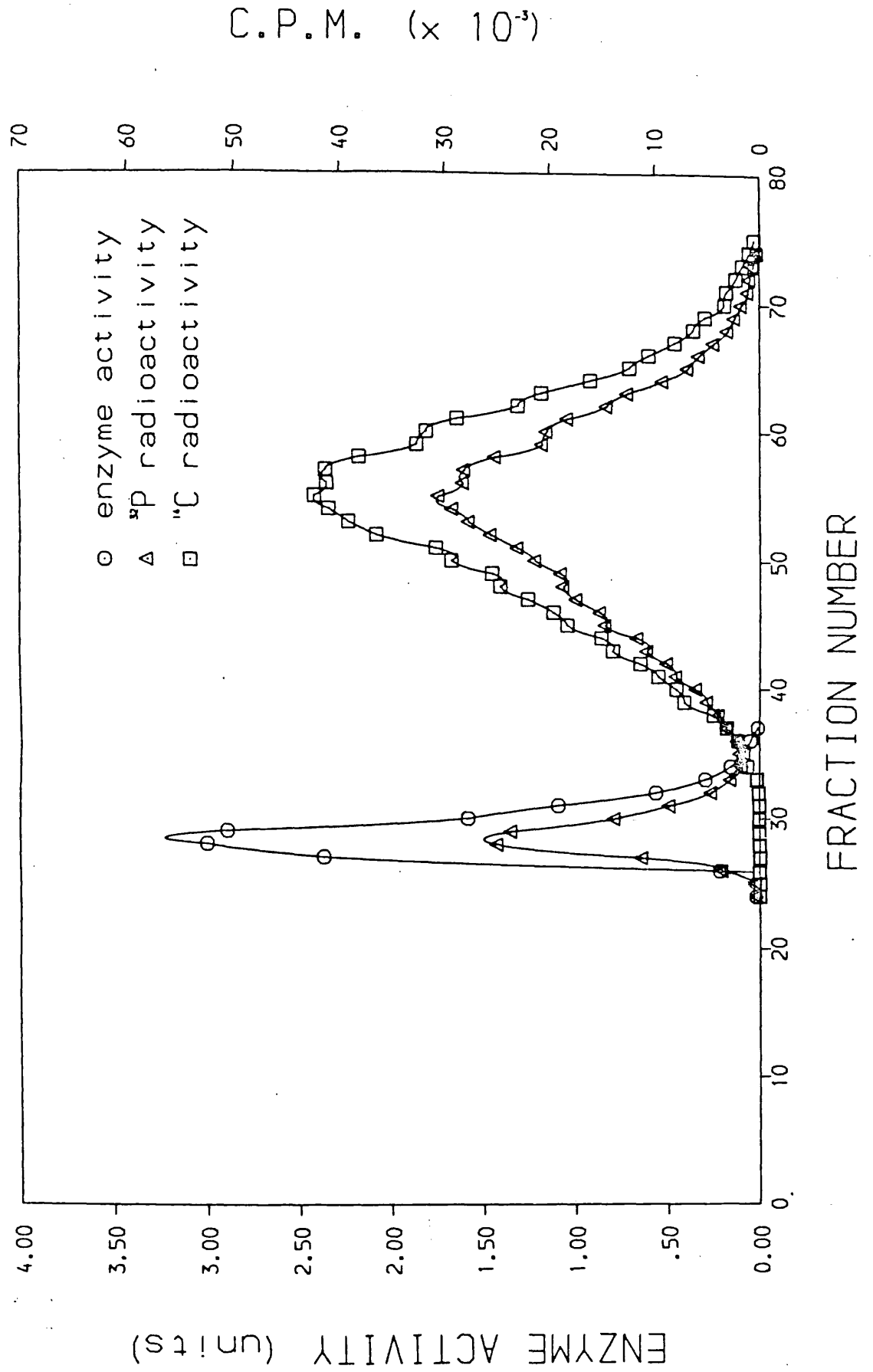
$^{32}$P-labelled rabbit liver PGM was prepared by incubating 10 units (0.306 nmol) of enzyme with 0.28 μCi [$^{32}$P] G6P (0.459 nmol, specific radioactivity 609 Ci mol$^{-1}$), 0.28 μCi [$^{14}$C] G6P (1.97 nmol, specific radioactivity 142 Ci mol$^{-1}$) and 0.04 nmol G1P6P in His-Tris-HCl buffer, pH 7.6 (40 mM histidine, 12 mM Tris), containing 2 mM $MgCl_2$ at 30 $^{o}$C for 5 min in a final volume of 0.25 ml. The mixture was immediately cooled and applied to a Sephadex G-25 column to separate labelled phosphoenzyme from labelled substrates. The detailed procedure for preparation and purification of $^{32}$P-labelled enzyme is given in 2.6.1.

The enzyme was eluted from the column in a single zone at the beginning of the elution profile whilst substrates were retarded in their migration through the bed. $^{32}$P radioactivity was eluted in two peaks, one associated with the enzyme peak and the other with the $^{14}$C radioactivity peak. $^{14}$C radioactivity was eluted in a single peak in the fractions where the substrates were expected. The elution profile is shown in Fig. 3.16. The association of $^{32}$P radioactivity, and not $^{14}$C, with the enzyme peak indicates the formation of $^{32}$P-labelled phosphoenzyme rather than an enzyme-G1P6P complex

Eluted fractions containing the enzyme activity with $^{32}$P radioactivity were collected and the incorporation of $^{32}$P into rabbit liver PGM was calculated. Evaluation of the

FIGURE 3.16: Purification of $^{32}P$-labelled phosphoglucomutase by gel filtration

The following were incubated at 30 °C for 5 min in a volume of 0.25 ml: His-Tris-HCl buffer, pH 7.6 (40 mM histidine, 12 mM Tris), 2 mM $MgCl_2$, 0.306 nmol (10 units) PGM, 0.459 nmol [$^{32}P$] G6P (0.28 μCi, specific radioactivity 609 Ci $mol^{-1}$), 1.97 nmol [$^{14}C$] G6P (0.28 μCi, specific radioactivity 142 Ci $mol^{-1}$) and 0.04 nmol G1P6P. The incubation mixture was immediately cooled to 0 °C in an ice bath and applied to a Sephadex G-25 column previously equilibrated with incubation buffer. The column was eluted with the same buffer and 0.4 ml fractions were collected. All fractions were assayed for $^{32}P$ and $^{14}C$ radioactivity, and for phosphoglucomutase activity. For detailed experimental procedure, see 2.6.1.


C.P.M. (x 10⁻³)
70
60
50
40
30
20
10
0
80
70
60
50
40
30
20
10
0
FRACTION NUMBER
enzyme activity
³²P radioactivity
¹⁴C radioactivity
4.00
3.50
3.00
2.50
2.00
1.50
1.00
.50
0.00
ENZYME ACTIVITY (units)

molar concentration of the enzyme was based on a molecular weight of 67,600 (see 3.2.3) and on the highest specific activity obtained, 484 units $mg^{-1}$ protein (see 3.1). The final specific radioactivity of P was calculated by assuming the following initial conditions of the enzyme: (1) the enzyme was in the non-phosphorylated form; (2) 1 mol enzyme bound 1 mol P ; (3) 1 mol enzyme bound 2 mol P. The molar concentration of rabbit muscle PGM was calculated by using the value 62,000 for the molecular weight (Yankeelov et al. 1964) and 1488 units $mg^{-1}$ protein for the highest specific activity obtained (Joshi and Lane, 1978).

The results are summarised in Table 3.6. If it is assumed that the PGMs were initially in the non-phosphorylated form before the label was added, 1 mol rabbit liver enzyme binds approximately 0.45 mol of P and 1 mol muscle enzyme binds approximately 0.85 mol P. The values obtained are comparable with the previously reported results that rabbit liver and rabbit muscle PGMs possess after isolation 0.5-0.7 mol and 1.0 mol P per mol of enzyme respectively (Najjar, 1962; Handler et al., 1965; Sidbury and Najjar, 1967).

The incorporation of $^{32}P$ into rabbit liver and rabbit muscle PGMs was also estimated by using the phenol extraction method as the rapid separation of labelled substrates and enzyme minimises phosphoenzyme degradation. 10 units of each enzyme were first labelled with $^{32}P$ by incubating with 0.17 $\mu Ci[^{32}P]$ G6P (3.13 nmol; specific radioactivity 54.3 Ci $mol^{-1}$) and 0.12 nmol G1P6P in His-Tris-HCl buffer

TABLE 3.6: Incorporation of $^{32}P$ into rabbit liver and rabbit muscle phosphoglucomutase determined by gel filtration

$^{32}P$-labelled PGM from rabbit liver and rabbit muscle were prepared and purified as described in 2.7.1. Evaluation of the molar concentration of the enzyme was based on the molecular weights 67,000 (see 3.2.3) and 62,000 (Yankeelov et al., 1964) and highest specific activities 484 units $mg^{-1}$ protein (see 3.1) and 1488 units $mg^{-1}$ protein (Joshi and Lane, 1978) for rabbit liver and rabbit muscle PGMs respectively. The number of mol of $^{32}P$ incorporated per mol of enzyme was calculated by assuming the following initial states of the enzyme: (1) non-phosphorylated form; (2) 1 mol P bound per mol enzyme; (3) 2 mol P bound per mol enzyme. PGM from rabbit muscle is generally purified as completely phosphorylated form and rabbit liver enzyme was purified incorporating affinity elution chromatography and would also be expected to be completely phosphorylated.

| Expt. No. | Conditions | mol P bound per mol enzyme | |
|---|---|---|---|
| | | rabbit liver PGM | rabbit muscle PGM |
| 1 | Non-phosphorylated form | 0.45 | 0.84 |
| | Phosphorylated form 1 mol P per mol enzyme | 0.63 | 1.19 |
| | Phosphorylated form 2 mol P per mol enzyme | 0.81 | 1.55 |
| 2 | Non-phosphorylated form | 0.43 | 0.82 |
| | Phosphorylated form 1 mol P per mol enzyme | 0.58 | 1.17 |
| | Phosphorylated form 2 mol P per mol enzyme | 0.73 | 1.56 |

TABLE 3.7: Incorporation of $^{32}P$ into rabbit liver and rabbit muscle phosphoglucomutases determined by the phenol extraction method

| Conditions | mol P bound per mol enzyme | |
|---|---|---|
| | rabbit liver PGM | rabbit muscle PGM |
| Non-phosphorylated form | 0.515 | 1.38 |
| Phosphorylated form 1 mol P per mol enzyme | 0.562 | 1.46 |
| Phosphorylated form 2 mol P per mol enzyme | 0.607 | 1.54 |

$^{32}P$-labelled phosphoenzyme samples from rabbit liver and rabbit muscle were prepared and freed from labelled substrates by the phenol extraction method. The details of the experimental procedure are described in 2.6.5 and Table 3.7.

(40 mM histidine, 12 mM Tris), pH 7.6, containing 2 mM $MgCl_2$ and total volume of 0.2 ml at 30 °C for 30 min. The enzyme incubation was terminated by the addition of 1 ml 0.88 phenol saturated with Tris-HCl pH 8.0, containing 1 mM sodium phosphate. $^{32}P$-labelled enzyme free from labelled substrate was extracted by using the phenol extraction method described in 2.6.5.

As shown in Table 3.7, if the liver enzyme was in the non-phosphorylated form at the beginning of the labelling reaction, 1 mol of enzyme would bind 0.515 mol P. The value obtained for this enzyme was close to that obtained by separating the labelled enzyme by gel filtration (see Table 3.6). However, with muscle enzyme, it was found that 1 mol enzyme binds 1.38 mol of P under similar conditions, a value higher than that obtained when labelled enzyme is isolated by gel filtration. This could be due to incomplete removal of $^{32}P_i$ from the phenol phase.

### 3.6.2 STABILITY OF LABELLED PHOSPHOENZYME AT 30 °C

The stability of purified $^{32}P$-labelled phosphoglucomutase was investigated in His-Tris-HCl buffer, pH 7.6 (40 mM histidine, 12 mM Tris), containing 2 mM $MgCl_2$ at 30 °C as described in 2.6.2.

A graph of log percentage phosphoenzyme versus time was plotted. The results (Fig. 3.17) show that decline in phosphoenzyme radioactivity is a first-order reaction. The half-life of phosphoenzyme was found to be 47.5 h. This is comparable with the half-life of phosphoenzyme from

FIGURE 3.17: Stability of labelled phosphoenzyme at pH 7.6 and 30 $^{o}C$

$^{32}P$-labelled PGM from rabbit liver was purified by the method described in 2.6.1. Approximately 10 units of labelled enzyme in His-Tris-HCl buffer, pH 7.6 (40 mM histidine, 12 mM Tris), containing 2 mM $MgCl_2$ in a volume of 1 to 2 ml was warmed to 30 $^{o}C$ in a water bath. 0.1 ml samples were withdrawn at timed intervals. $^{32}P$-radioactivity associated with enzyme was estimated by adding substrate to the samples, allowing label to exchange with substrate and measuring labelled substrate as described in 2.6.2.

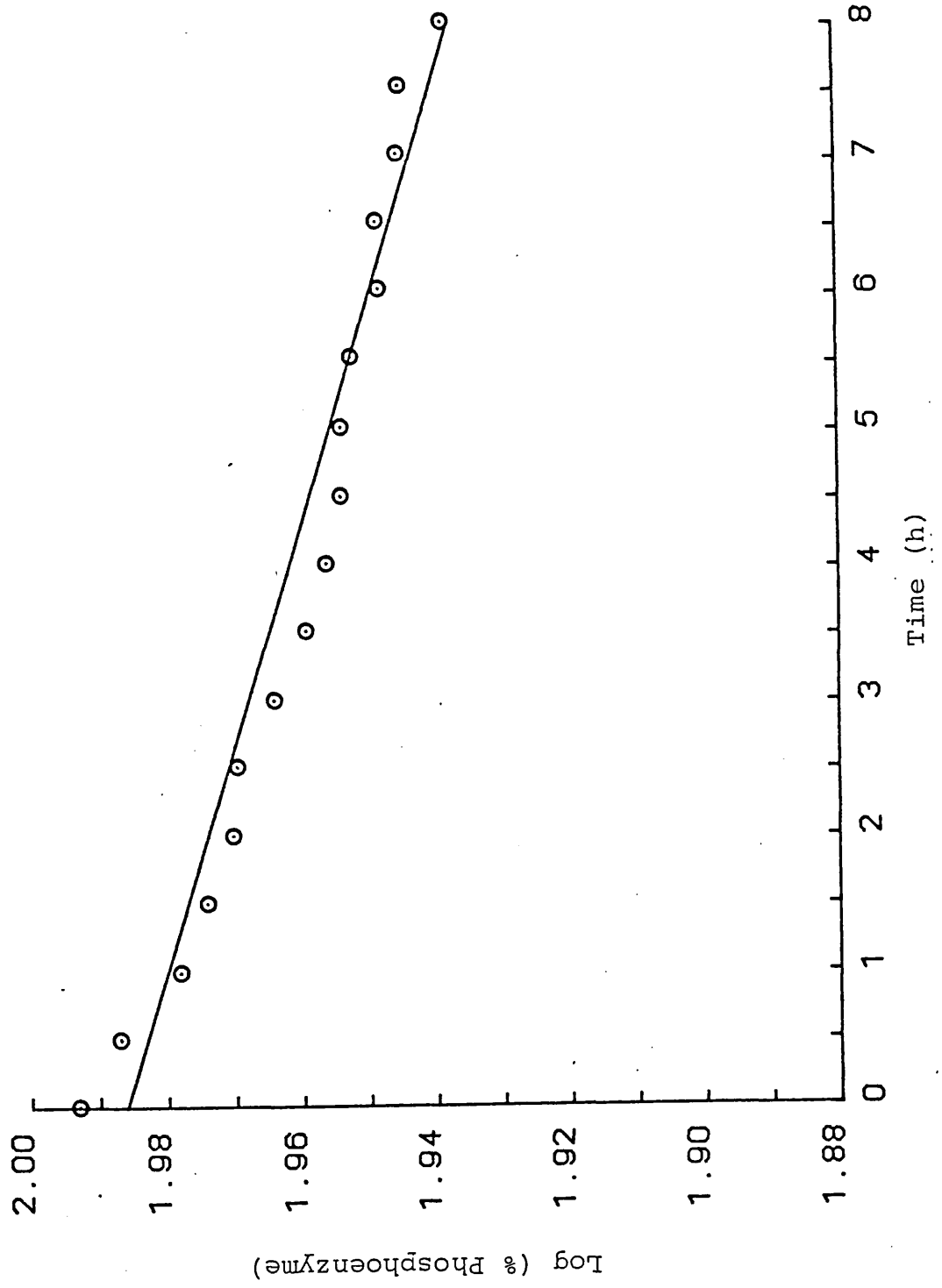
A graph of log percentage phosphoenzyme versus time was plotted. The half-life of phospho-enzyme was found to be 47.5 h.

Correlation coefficient = -0.975

Slope = 0.0001056

Intercept at y axis = 1.986

Rate constant $k_1 = 2.43 \times 10^{-4}$


Log (% Phosphoenzyme)
2.00
1.98
1.96
1.94
1.92
1.90
1.88
0
1
2
3
4
5
6
7
8
Time (h)

Micrococcus lysodeikticus, 50 h (Clarke et al., 1974).

The above results also show a rapid exchange of 96% of $^{32}P$ label from $^{32}P$-labelled phosphoenzyme to G6P (within a few seconds) at pH 7.6 and 30 °C. This suggests that phosphoenzyme is a true kinetic intermediate of the PGM reaction and is not an artifact of the isolation procedure.

### 3.6.3 NATURE OF THE PHOSPHATE BOND IN THE PHOSPHOENZYME

The nature of the phosphate bond in the phosphoenzyme of PGM from rabbit liver was investigated by treating purified $^{32}P$-labelled enzyme (3.6.1) with acid and alkali.

Stability of phosphoenzyme to acid

Stability of phosphoenzyme to 0.48 M perchloric acid was investigated as described in 2.6.3. 96-99% of the radioactivity was found to be associated with the enzyme, showing that the phosphate group is attached to the enzyme by an acid-stable linkage. This eliminated the possibility that the site of attachment of phosphate to the enzyme was a histidine (as with phosphoglycerate mutase; cf. Rose, 1970), aspartic acid or glutamic acid residues (cf. Kennedy and Koshland, 1957).

Stability of phosphoenzyme to alkali (Fig. 3.18)

Stability of phosphoenzyme to alkali was investigated in 0.25 M and 0.33 M NaOH at 37 °C as described in 2.6.4. The results show that the enzyme-phosphate bond is alkali-labile and that the hydrolysis of $^{32}P_i$ is a first-order reaction. Half-lives observed were 2.25 h in 0.25 M NaOH and 1.31 h

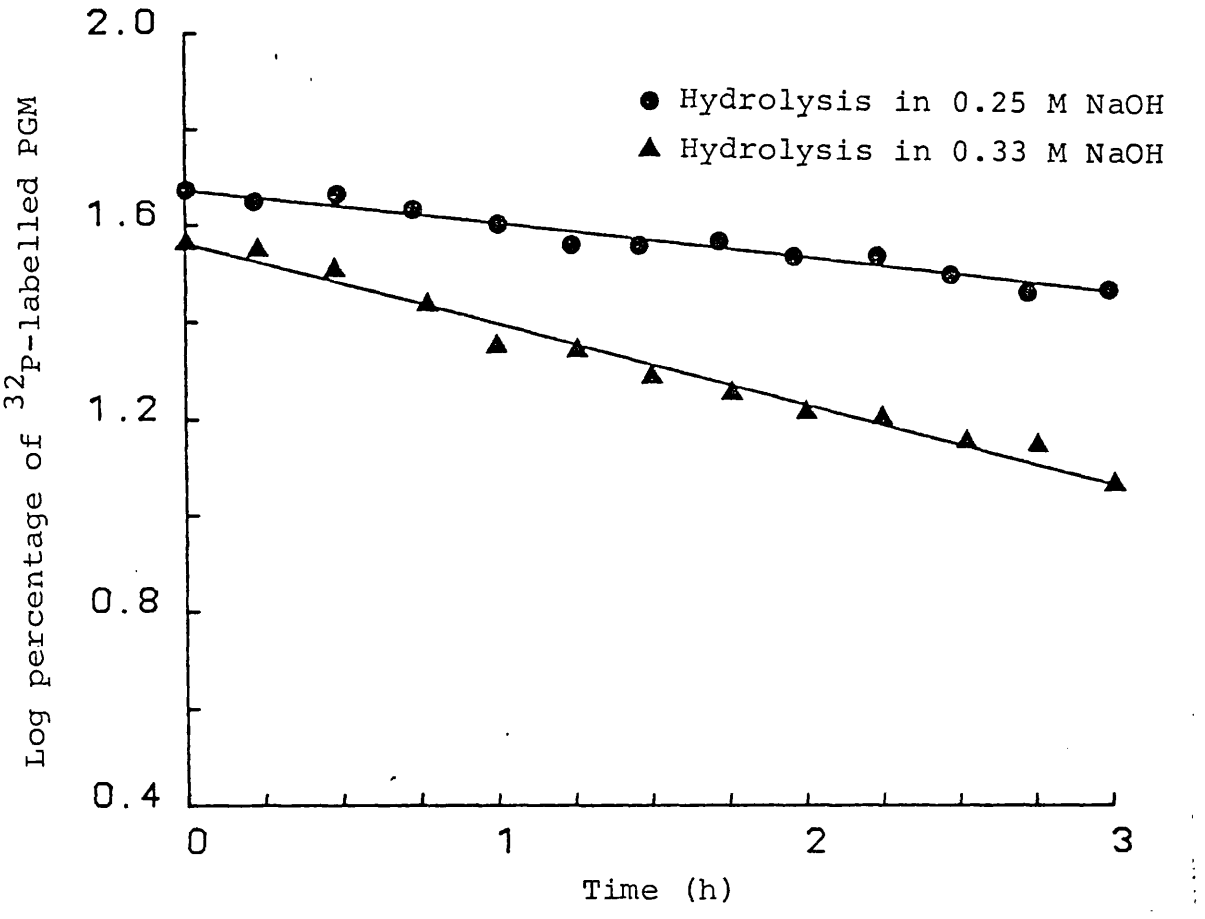



FIGURE 3.18: Hydrolysis of $^{32}$P-labelled phosphoenzyme in 0.25 M and 0.33 M NaOH

$^{32}$P-labelled PGM was prepared and purified by the method described in 2.6.1. Approximately 5 units of purified labelled enzyme in a volume of 1 ml was warmed to 37 $^{o}$C and added to an appropriate solution of NaOH, already at 37 $^{o}$C, to give a final concentration of NaOH 0.25 M or 0.33 M and volume 1.5 ml. The rate of hydrolysis of phosphoenzyme was estimated by withdrawing 0.1 ml samples from the above solution at timed intervals and measuring $^{32}P_i$ and radioactivity associated with the enzyme as described in 2.6.4.

The results show that hydrolysis of labelled phosphoenzyme in alkali is a first-order reaction. Half-lives were 2.25 h in 0.25 M NaOH and 1.31 h in 0.33 M NaOH at 37 $^{o}$C.

in 0.33 M NaOH at 37 °C. These values compare with the half-lives for the phosphoenzyme from M.lysodeikticus of 2.4 h and 1.7 h respectively under the same conditions (Clarke et al., 1974) and of 1.5 h for the rabbit muscle phosphoenzyme in 0.5 M NaOH (Kennedy and Koshland, 1957). The similarity of these values suggests that the phosphate group is attached to serine in the liver PGM.

## 3.7 ISOENZYME COMPOSITION OF PHOSPHOGLUCOMUTASE FROM RABBIT LIVER AND RABBIT MUSCLE HOMOGENATES

In an effort to separate PGM isoenzymes, a number of electrophoretic techniques have been tried including polyacrylamide gels using the discontinuous buffer system of Laemmli and Favre (1973) , cellulose acetate (Beitner et al., 1975) and starch gel (Spencer et al., 1964; Scopes, 1968; Dawson and Mitchell, 1969). The best and most reproducible separation of isoenzymes was achieved on polyacrylamide gels. Using this technique, the electrophoretic pattern of PGM from crude extract of rabbit liver was investigated and compared with that from rabbit muscle. The details of the experimental procedure are described in 2.7.

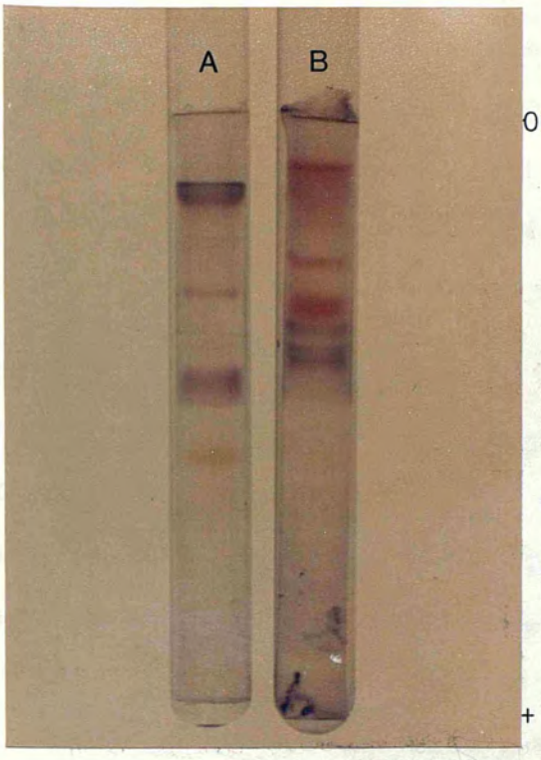
### 3.7.1 ELECTROPHORETIC PATTERN OF PHOSPHOGLUCOMUTASE FROM RABBIT LIVER AND RABBIT MUSCLE HOMOGENATES

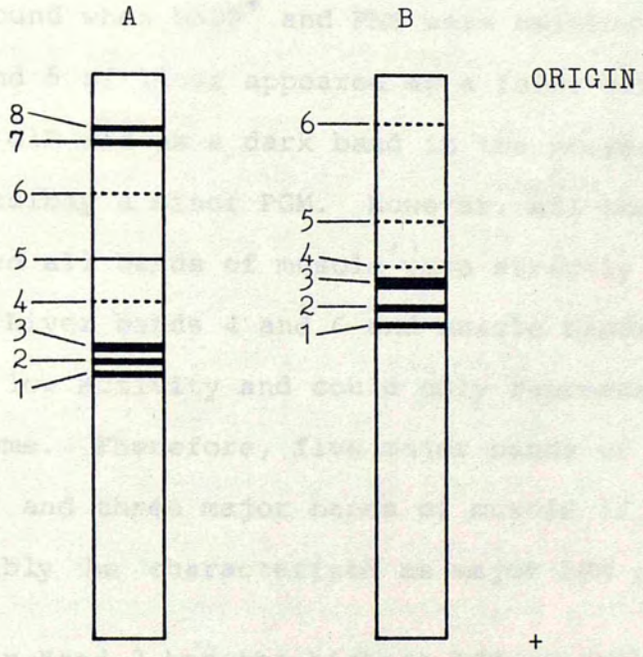
The electrophoretic pattern of the separation of PGM from rabbit liver crude extracts is shown in Fig. 3.19 in comparison with that obtained for the corresponding rabbit muscle enzyme. The line diagram below the photograph was drawn on the basis of the intensity of the blue-purple formazan formed by the PGM - specific reaction as described in 2.7.8.

Six major bands and two faint bands appeared with the rabbit liver PGM whereas three major bands and three faint bands appeared with muscle enzyme. The isoenzyme bands are numbered in decreasing order of negative charge (see Wilkinson, 1964; Webb, 1964).

FIGURE 3.19: Electrophoretic pattern of phosphoglucomutase from rabbit liver and rabbit muscle homogenates on polyacrylamide gels

A photograph of the gels and a schematic representation of the relative colour intensities of the bands obtained by scanning the gels on a densitometer are shown.
Gel A: rabbit liver PGM
Gel B: rabbit muscle PGM
Electrophoresis was carried out on polyacrylamide gels by the method of Ornstein (1964), Davis (1964) and Williams et al., (1964) using a discontinuous buffer system (Laemmli and Favre, 1973). Samples were prepared by a modified method of Scopes (1978). The gels were stained for PGM activity using the technique employed by Beitner et al. (1975). The details of the experimental procedure are described in 2.7. The staining solution contained 40 mM imidazole-HCl buffer, pH 7.2, 2 mM G1P, 2 μM G1P6P, 3 mM $MgCl_2$, 1 mM EDTA, 0.5 mM $NADP^+$, 0.4 mM NBT, 0.1 mM PMS and 2 units G6PDH per ml stain. Gels were stained for 15-20 min in the dark.


A
B
ORIGIN
+


A
B
ORIGIN
8
7
6
5
4
3
2
1
6
5
4
3
2
1
+

The greatest activity of the PGM bands was observed when imidazole was used as the staining buffer. In other buffers e.g. His-Tris-HCl, all isoenzyme bands exhibited about 30% less activity. With the Tris-HCl staining buffer, as used by Scopes (1968) and Turner and Lyerla (1980), the activity of isoenzyme bands was 10-20% of that in imidazole buffer.

To confirm that the development of bands was due to the action of PGM activity, the bands were developed in the absence of G1P, G6PDH, PMS or $NADP^+$ from the staining mixture (see 2.7.6 for staining mixture). When G1P was omitted from the staining solution, none of the bands developed with muscle and only a faint band (No. 5) appeared with liver. The same results were obtained when G6PDH was omitted from the staining mixture. There was no development of bands found when $NADP^+$ and PMS were omitted from the stain. Band 5 of liver appeared as a faint band in the absence of G1P and as a dark band in the presence of G1P. This is possibly a minor PGM. However, all the other bands of liver and all bands of muscle were strictly due to PGM activity. Liver bands 4 and 6 and muscle bands 4, 5 and 6 had very low activity and could only represent a minor PGM isoenzyme. Therefore, five major bands of liver (1, 2, 3, 7 and 8) and three major bands of muscle (1, 2 and 3) could possibly be characterised as major PGM isoenzymes.

Rabbit liver band 3 has the highest PGM activity and band 7 has the lowest among the major five bands. The activities decrease in the order 3, 2, 1, 8 and 7. Muscle band 3 similarly has the highest PGM activity and the activities

decrease in the order 3, 2, 1. The pattern of liver bands 1 and 3 was observed to be similar with the muscle bands pattern 1 to 3, but there were differences in their electrophoretic mobilities. These three bands close to each other could represent a zone of isoenzyme bands (see Spencer et al., 1964) and in the case of liver PGM a second zone exists containing isoenzyme bands 7 and 8. This was the main difference observed in the electrophoretic patterns of liver and muscle PGM at optimal staining conditions.

To detect the presence of phosphoenzyme bands, cofactor was omitted from the stain and to determine whether any isoenzyme bands were due to phosphoribomutase, some gels were stained in the absence of $Mg^{++}$ (Fig. 3.20). These gels were compared to others run concurrently and stained in the presence of both $Mg^{++}$ and G1P6P.

When G1P6P was excluded from the staining solution, G1P, free from G1P6P was used (see 2.2.1.1.2 for purification). As shown in Fig. 3.20 (Gels C and F), exclusion of cofactor and increasing the period of staining from 15 min to 45 min, resulted in appearence of liver PGM band 3 as a dark band and bands 1 and 2 as faint bands, and muscle bands 3 and 2 as dark and 1 as a faint band. Liver band 5 also appeared in the absence of cofactor (see later). The intensities of bands 1, 2 and 3 of liver and muscle PGM were lower than for the bands of those gels stained in the presence of G1P6P, even after staining for a longer time. However, decrease in activities of these bands were on the same order as those in the controls (Gels A and D). The 2nd zone bands 7 and 8 of

FIGURE 3.20: Electrophoretic pattern of phosphoglucomutases from rabbit liver and rabbit muscle homogenates in the presence and in the absence of $Mg^{++}$ or G1P6P

A photograph of the gels and a schematic representation of the relative colour intensities of the bands obtained by scanning the gels on a densitometer are shown. The experimental procedure is given in Fig. 3.19. The gels were stained under the following conditions:

Gel A: rabbit liver PGM control (3 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P). Staining time: 15-20 min.

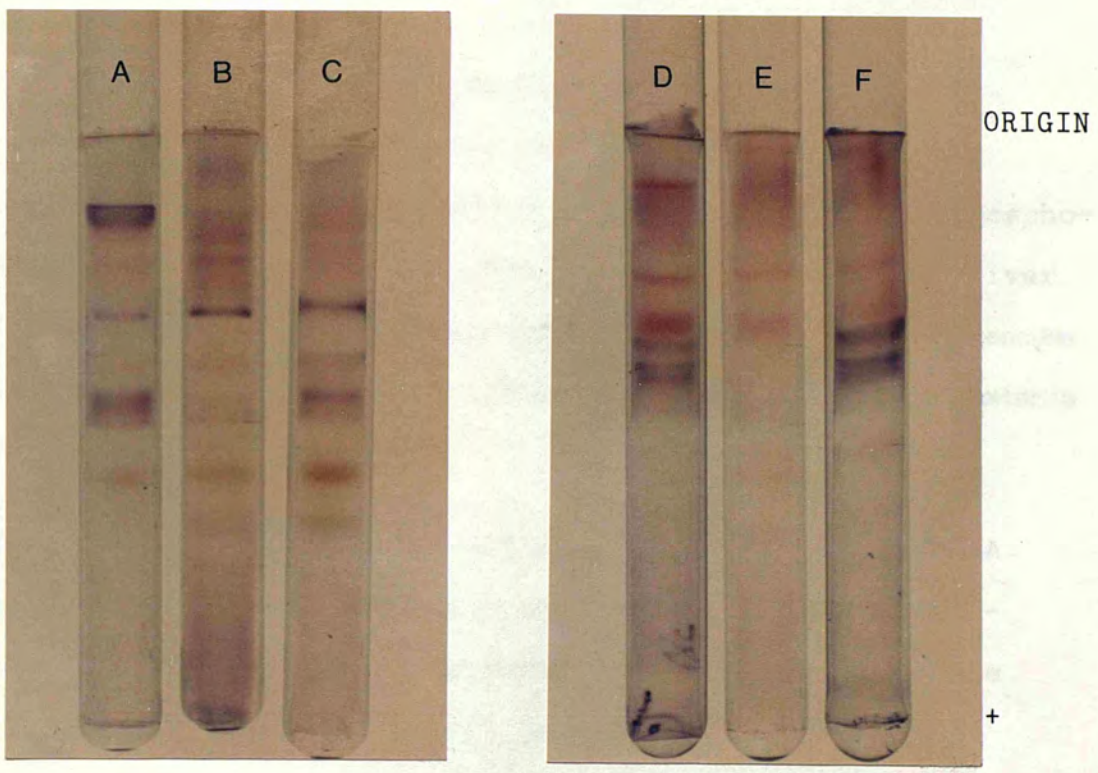
Gel B: rabbit liver PGM in the absence of $Mg^{++}$ ( 3.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P). Staining time: 45-50 min.

Gel C: rabbit liver PGM in the absence of G1P6P ( 3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P). Staining time: 45-50 min.

Gel D: rabbit muscle PGM control (3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P). Staining time: 15-20 min.

Gel E: rabbit muscle PGM in the absence of $MgCl_2$ (3.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P). Staining time: 45-50 min.

Gel F: rabbit muscle PGM in the absence of G1P6P (3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P). Staining time: 45-50 min.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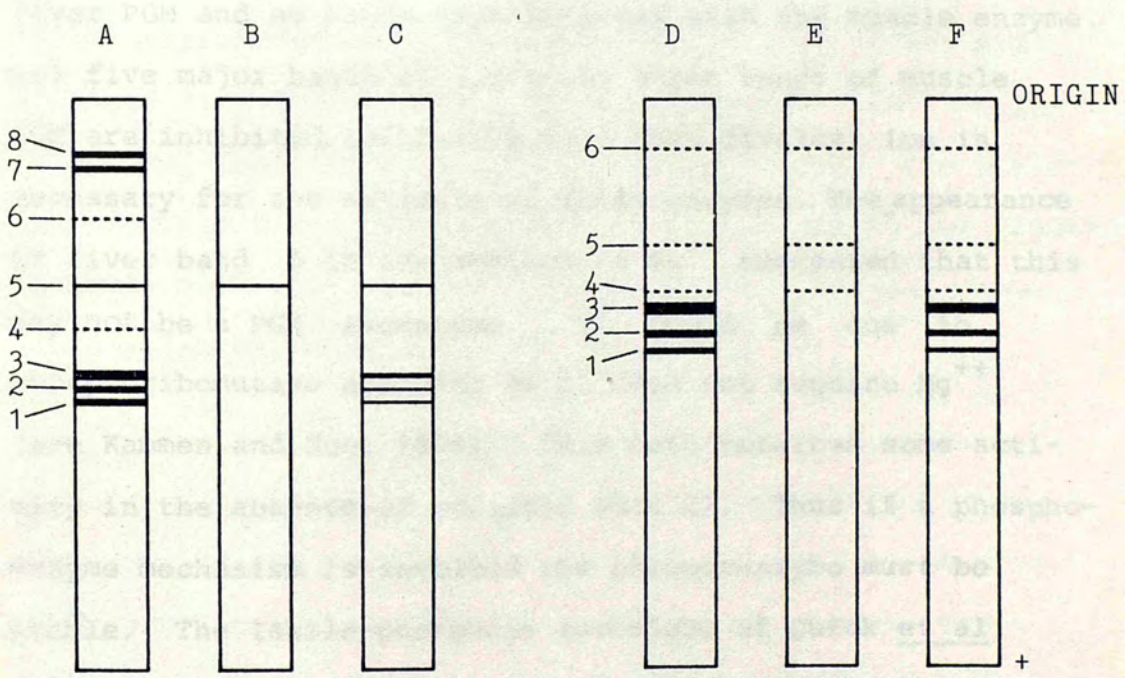
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liver PGM did not appear in the absence of G1P6P even when the gels were stained for 1.5 h. This suggests that liver PGM bands 1, 2 and 3 and similarly muscle PGM bands 1, 2 and 3 are phosphoenzyme and that the liver PGM 2nd zone bands 7 and 8 may be dephosphoenzymes. Their position is in accord with their expected relative electrophoretic mobilities. Appearance of all three bands as phosphoenzyme with rabbit muscle, but only three of the five liver PGM bands as phosphoenzyme, suggests that liver PGM phosphoenzyme is unstable or that other factors influence the relative extents of phosphorylation.

When gels were stained in the absence of $Mg^{++}$, 3 mM EDTA was included in the staining solution in order to eliminate the effect of contaminating divalent cations on the enzyme activity. As shown in Fig. 3.20 (Gels B and E), with the omission of $Mg^{++}$ only band No. 5 appeared with liver PGM and no bands were detected with the muscle enzyme. All five major bands of liver and three bands of muscle PGM are inhibited, indicating that this divalent ion is necessary for the activity of these enzymes. The appearance of liver band 5 in the absence of $Mg^{++}$ suggested that this may not be a PGM isoenzyme. It could be due to phosphoribomutase activity as it does not require $Mg^{++}$ (see Kammen and Koo, 1969). This band retained some activity in the absence of cofactor (Gel C). Thus if a phosphoenzyme mechanism is involved the phosphoenzyme must be stable. The labile-phosphate technique of Quick et al. (1972) was also tried in order to detect phosphoribomutase activity in band No. 5. This

technique failed, however, to produce any colourless phosphoribomutase bands on blue backgrounds; possibly inactivation of the enzyme occurred during prolonged staining (16 h). Other coupled enzymic methods (Quick et al., 1972) for staining phosphoribomutase isoenzymes could not be used because of the requirement of expensive enzymes.

### 3.7.2 EFFECT OF ATP AND CITRATE ON PHOSPHOGLUCOMUTASE ACTIVITIES IN RABBIT LIVER AND RABBIT MUSCLE HOMOGENATES

ATP and citrate are inhibitors of PGM (Beitner et al., 1975; Beck, 1979). The effect of these inhibitors was observed on PGM isoenzymes taken from rabbit liver and muscle homogenates and separated on polyacrylamide gels as described in 2.7 (see also 3.7.1). The gels were stained in the presence of either 3.5 mM ATP or 2.5 mM citrate at different concentrations of $Mg^{++}$ and G1P6P (Figs 3.21 to 3.24) and compared with the gels run concurrently and stained in the absence of inhibitors under optimal conditions. The staining conditions and staining times are mentioned in the figures. Normally gels were stained for a longer time in the presence of inhibitors.

When a strong $Mg^{++}$ chelator, ATP, was present in excess relative to $Mg^{++}$ (Fig. 3.21, Gel B), it strongly inhibited all forms of PGM from rabbit liver, though band 3 which is the major isoenzyme band and is probably a phosphoenzyme (see 3.7.1) retained some activity. Bands 2 and 3 also appeared faintly but ATP completely inhibited the bands of

FIGURE 3.21: Effect of ATP on phosphoglucomutase from rabbit liver homogenate

A photograph of the gels and a schematic representation of relative colour intensities of the bands obtained by scanning the gels on a densitometer are shown. The experimental procedure is given in Fig. 3.19. The gels were stained under the following conditions:

Gel A: control without ATP (3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P). Staining time: 15-20 min.

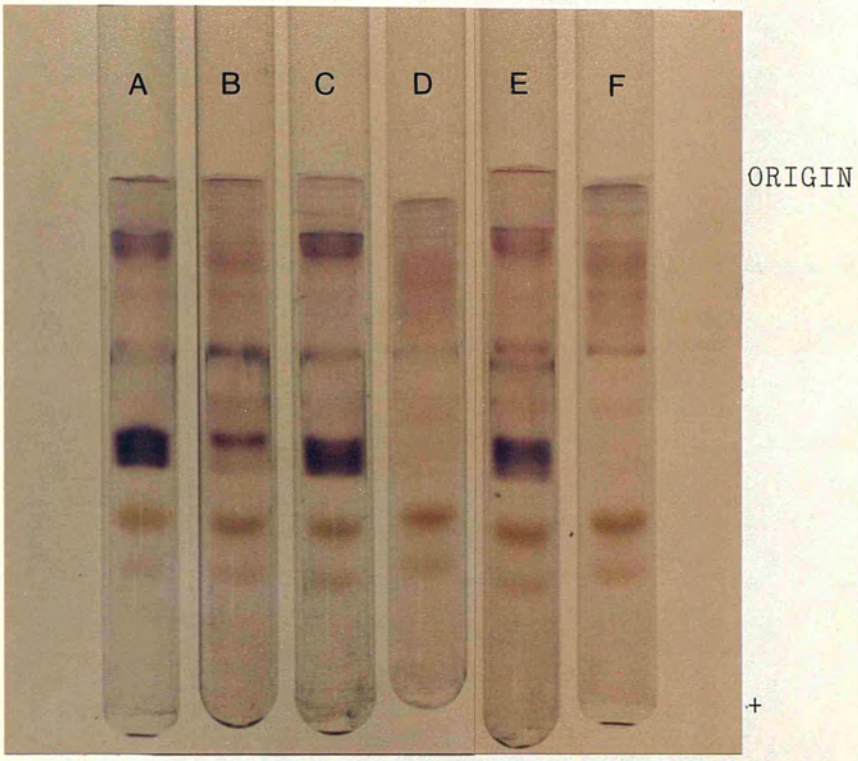
Gel B: 3.5 mM ATP, 3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P. Staining time: 45 min.

Gel C: 3.5 mM ATP, 3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 50.0 μM G1P6P (in presence of excess G1P6P). Staining time: 30 min.

Gel D: 3.5 mM ATP, 3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P (in absence of G1P6P). Staining time: 45 min.

Gel E: 3.5 mM ATP, 5.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P (in presence of excess $MgCl_2$). Staining time: 30 min.

Gel F: 3.5 mM ATP, 3.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P (in absence of $MgCl_2$). Staining time: 45 min.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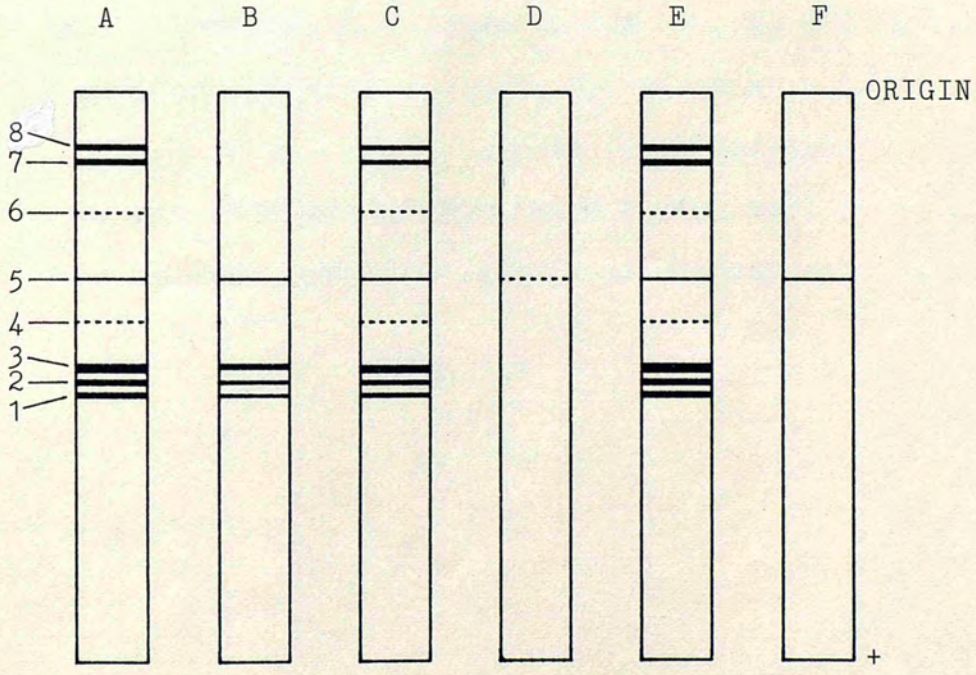
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FIGURE 3.22: Effect of ATP on phosphoglucomutase from rabbit muscle homogenate

A photograph of the gels and a schematic representation of relative colour intensities of the bands obtained by scanning the gels on a densitometer are shown. The experimental procedure is given in Fig. 3.10. The gels were stained under the following conditions:

Gel A: control without ATP (3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P). Staining time: 15-20 min.

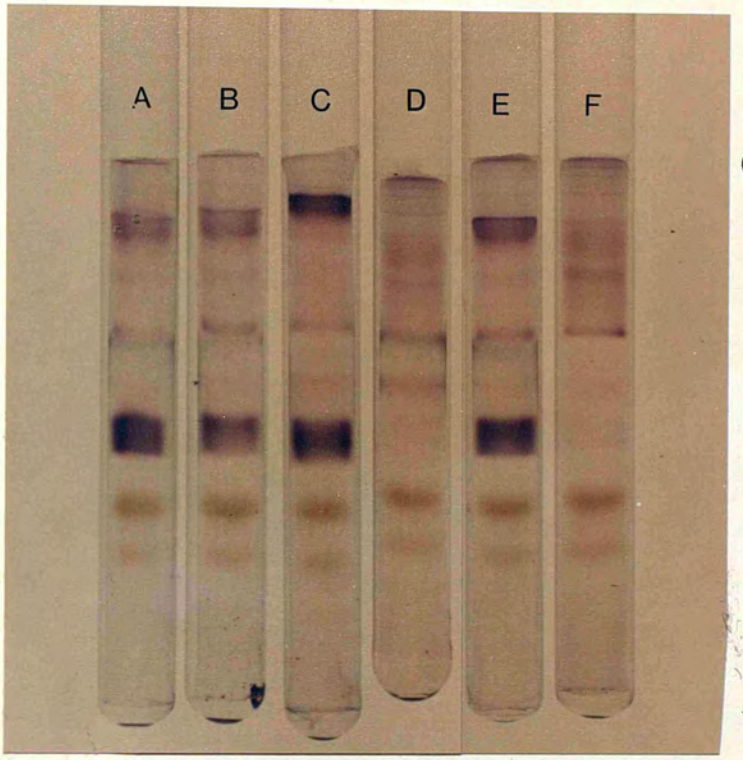
Gel B: 3.5 mM ATP, 3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P. Staining time: 45 min.

Gel C: 3.5 mM ATP, 3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 50.0 μM G1P6P (in presence of excess G1P6P). Staining time: 30 min.

Gel D: 3.5 mM ATP, 3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, (in absence of G1P6P). Staining time: 45 min.

Gel E: 3.5 mM ATP, 5.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P (in presence of excess $MgCl_2$). Staining time: 30 min.

Gel F: 3.5 mM ATP, 3.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P (in absence of $MgCl_2$). Staining time: 45 min.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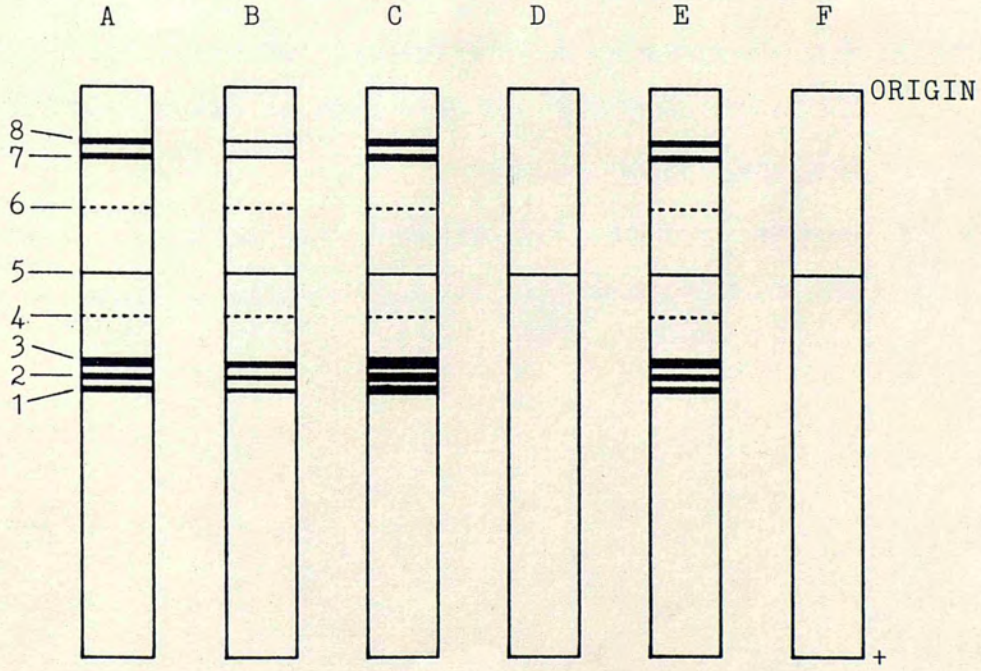
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FIGURE 3.23: Effect of citrate on phosphoglucomutase from rabbit liver homogenate

A photograph of the gels and a schematic representation of relative colour intensities of the bands obtained by scanning the gels on a densitometer are shown. The experimental procedure is given in Fig. 3.19. The gels were stained under the following conditions:

Gel A: control without citrate (3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P). Staining time: 15-20 min.

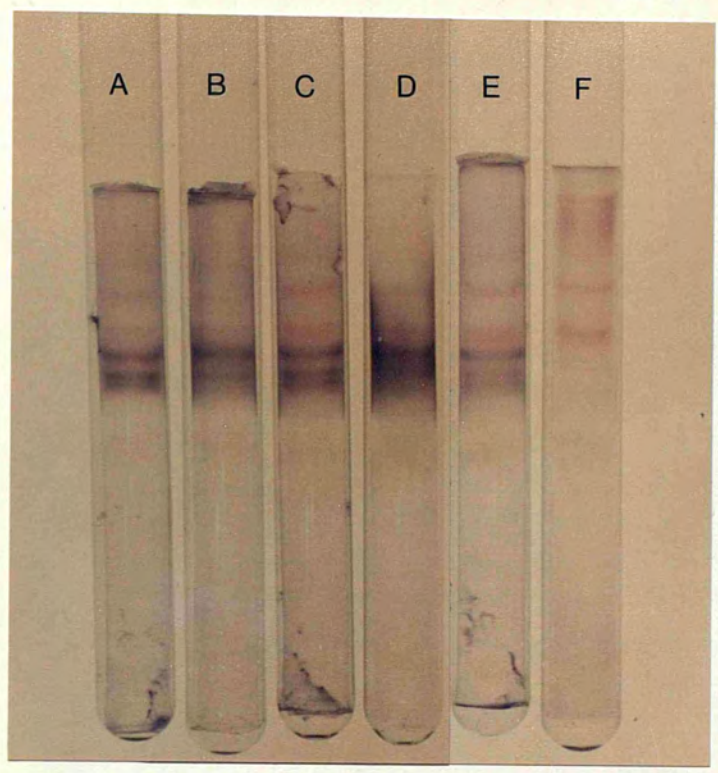
Gel B: 2.5 mM citrate, 3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P. Staining time: 25 min.

Gel C: 2.5 mM citrate, 3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 50.0 μM G1P6P (in presence of excess G1P6P). Staining time: 15 min.

Gel D: 2.5 mM citrate, 3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mm G1P (in absence of G1P6P). Staining time: 45 min.

Gel E: 2.5 mM citrate, 5.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P (in presence of excess $MgCl_2$). Staining time: 20 min.

Gel F: 2.5 mM citrate, 3.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P (in absence of $MgCl_2$). Staining time: 45 min.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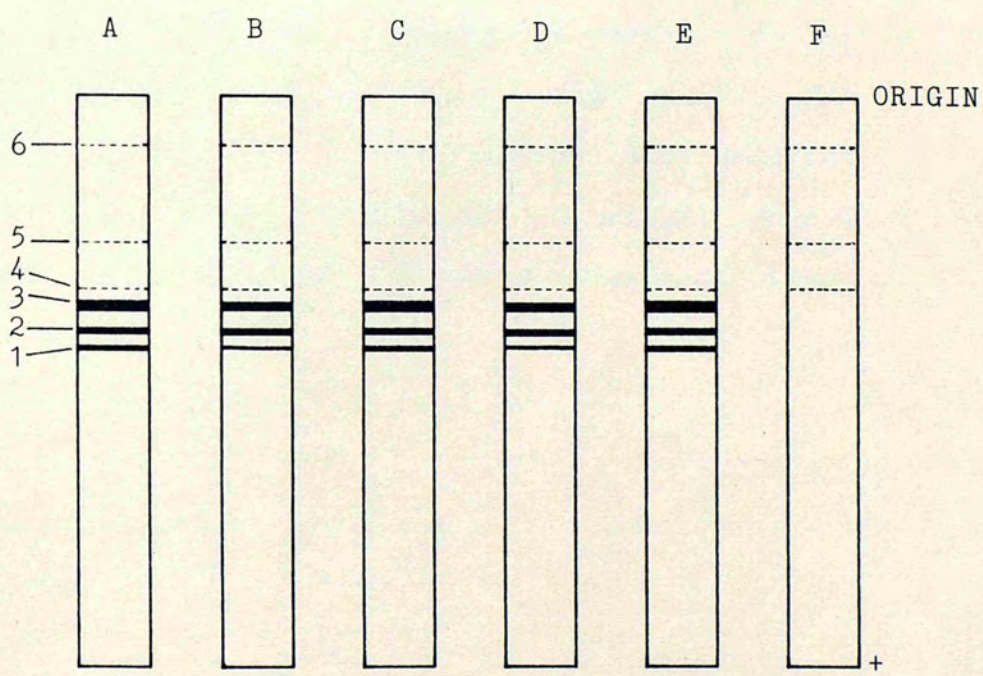
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FIGURE 3.24: Effect of citrate on phosphoglucomutase from rabbit muscle homogenate

A photograph of the gels and a schematic representation of relative colour intensities of the bands obtained by scanning the gels on a densitometer are shown. The experimental procedure is given in Fig. 3.19. The gels were stained under the following conditions:

Gel A: control without citrate (3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P). Staining time: 15-20 min.

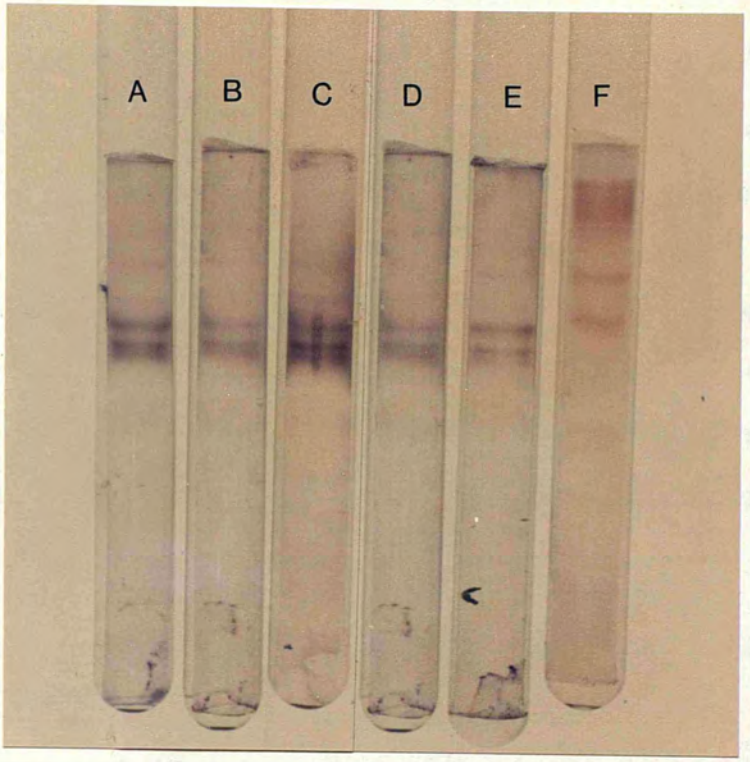
Gel B: 2.5 mM citrate, 3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P. Staining time: 25 min.

Gel C: 2.5 mM citrate, 3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 50,0 μM G1P6P (in presence of excess G1P6P). Staining time: 15 min.

Gel D: 2.5 mM citrate, 3.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P (in absence of G1P6P). Staining time: 45 min.

Gel E: 2.5 mM citrate, 5.0 mM $MgCl_2$, 1.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P (in presence of excess $MgCl_2$). Staining time: 20 min.

Gel F: 2.5 mM citrate, 3.0 mM EDTA, 2.0 mM G1P, 2.0 μM G1P6P (in absence of $MgCl_2$). Staining time: 45 min.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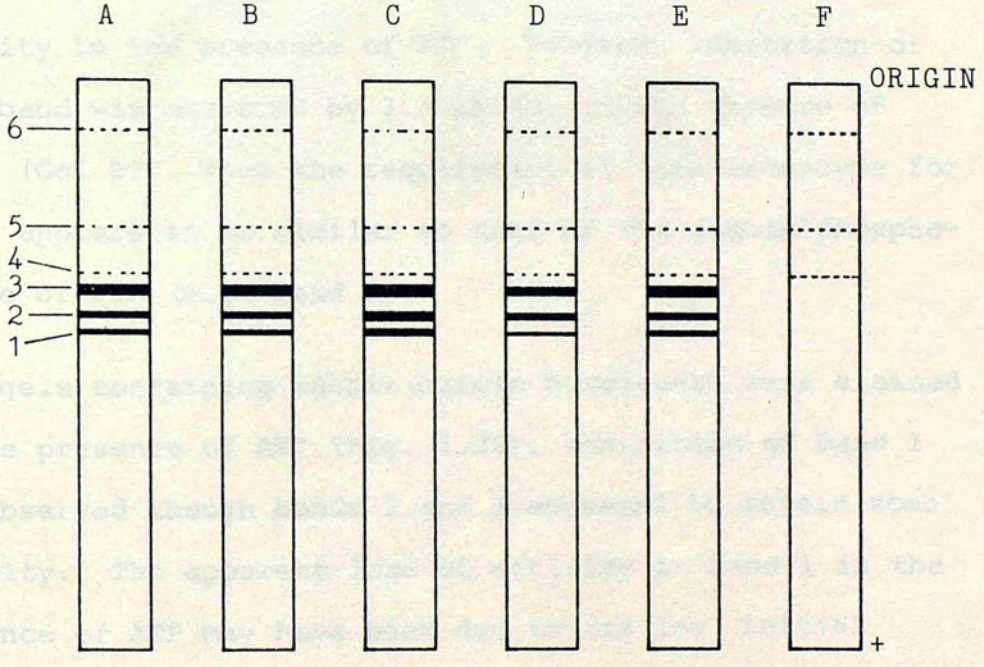
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the 2nd zone (bands 7 and 8) which are probably dephosphoenzyme. This suggested that ATP predominantly binds the dephosphoenzyme.

Reversal of ATP inhibition was observed with 5 mM $Mg^{++}$ (Gel E) when $Mg^{++}$ was present in excess of the ATP concentration. Similarly the inhibition was relieved in the presence of excess G1P6P (Gel C), indicating that ATP acts competitively with cofactor. However, some inhibition was still observed in the presence of excess $Mg^{++}$ or G1P6P and ATP, since the bands required 30 min to reach a given intensity whereas the controls only required 15-20 min.

Liver band 5, which is probably a phosphoribomutase (see 3.7.1), retained activity in the presence of ATP (Gel B) and also retained activity when $Mg^{++}$ was completely removed (Gel F). The lack of the requirement for $Mg^{++}$ by this enzyme might contribute to retention of activity in the presence of ATP. However, inhibition of this band was observed by 3.5 mM ATP in the absence of G1P6P (Gel D). Thus the requirement of this isoenzyme for G1P6P appears to be similar to that of the stable phosphoenzyme of PGM (e.g. band 3).

When gels containing rabbit muscle homogenate were stained in the presence of ATP (Fig. 3.22), inhibition of band 1 was observed though bands 2 and 3 appeared to retain some activity. The apparent loss of activity in band 1 in the presence of ATP may have been due to its low initial activity. As before with rabbit liver, removal of

this inhibition was observed when the G1P6P concentration was increased to 50 μM (Gel C) or the $MgCl_2$ concentration to 5 mM (Gel E).

When gels containing samples from liver (Fig. 3.23) or muscle (Fig. 3.24) homogenate were stained in the presence of 2.5 mM citrate under the same conditions, weak inhibition was observed with all forms of liver and muscle PGM. It was difficult to differentiate the extents of inhibition for individual bands as the inhibition was weak in comparison to that found with ATP. Citrate inhibition was completely removed in the presence of excess $Mg^{++}$ concentration (Gel E of Figs. 3.23 and 3.24) with bands of both liver and muscle PGM. However, when the gels were stained with an excess amount of G1P6P (50 μM), more prominent bands appeared than in the absence of citrate under optimal conditions (Gel C, Figs 3.23 and 3.24). A similar activation of the purified rabbit liver and muscle enzymes was observed in the presence of 2.5 mM citrate and 50 μM G1P6P (see 3.2.9). The origin of this activation was not determined.

# CHAPTER FOUR

# DISCUSSION

## 4.1 PURIFICATION OF RABBIT LIVER PHOSPHOGLUCOMUTASE

During isolation of PGM from rabbit liver, care was taken to prevent modification of the enzyme *in vivo*. Hashimoto *et al*. (1967) showed that PGM from adrenalin-injected rats has a greater requirement for preincubation with chelating agent and $Mg^{++}$ for maximum activity than has PGM from normal rats, but this requirement is lower in insulin-treated rats. These effects are possibly due to fluctuation in the amount of active enzyme (4.3). Similar results have also been reported for the effect of insulin on the enzyme of rabbit skeletal muscle (Peck and Ray, 1971). The physiological conditions of the animal may thus have an influence on the enzyme *in vivo*. Hence, before removal of livers, rabbits were injected with Saffan to minimise changes in the levels of hormones.

Steps to prevent changes in the enzyme *in vivo* during isolation were taken as follows. Proteolytic enzymes were inactivated by the addition of PMSF during the first step of purification to avoid degradation of the enzyme. $Mg^{++}$ and EDTA at concentrations of 1 mM were included, whenever possible, to remove heavy metal ions from the enzyme and replace them with $Mg^{++}$, thereby preventing inactivation. 1 mM 2-mercaptoethanol was added to prevent oxidation of sulphydryl groups and formation of conformational isomers.

Joshi and Lane (1978) using mild conditions, isolated a comparatively undegraded form of rabbit muscle PGM with a

molecular weight slightly greater (64,500) than that usually recorded (1.1.2). In the preparation of rabbit liver PGM, steps taken to protect the enzyme from inactivation and degradation appeared to be successful, since the value obtained for the molecular weight of the purified enzyme (67,600; see 3.2.3) was slightly greater than those reported for other PGMs, except for that from B.subtilis which is a dimer (1.1.2). This indicated that the purified rabbit liver enzyme was in a comparatively undegraded form. The value for the molecular weight of rabbit liver PGM was thought to be unbiassed as the molecular weight of rabbit muscle enzyme (obtained from Boehringer, Mannheim, West Germany) was found to be 61,000 (see 3.2.3), a value close to the frequently quoted figure of 62,000 (see Filmer and Koshland, 1963; Yankeelov et al., 1964; Harshman and Six, 1969). Purified liver PGM appeared as a single band on polyacrylamide gels (3.2.1), suggesting that the enzyme was not substantially degraded.

## 4.2 ISOENZYME COMPOSITION AND HETEROGENEITY OF PHOSPHOGLUCOMUTASE

PGMs from rabbit liver and muscle homogenates were separated on polyacrylamide gels (see 3.7). Rabbit liver PGM was separated into five major bands and the muscle enzyme into three major bands (see 3.7.1). It was difficult to reconcile these results with those already published owing to differences in separation techniques and conditions. Dawson and Mitchell (1969) have observed that different techniques for the separation of isoenzymes give rise to dissimilar isoenzyme composition; the use of the same technique under different conditions alters the electrophoretic patterns (see Scopes, 1968; Dawson and Mitchell, 1969). Electrophoresis of rabbit muscle PGM on starch gel has variously been shown to separate the enzyme into two zones, each containing 4 to 6 bands (Spencer et al., 1964); into three forms due to progressive oxidation of sulphydryl groups during separation (Green and Dawson, 1973); into three conformational isomers (Dawson and Mitchell, 1969); and into three major and three minor bands (Scopes, 1968). Rabbit muscle PGM also separates as two major forms on CM-cellulose (Joshi et al., 1967) and into four phosphorylated forms by isoelectric focussing (Harshman and Six, 1969). In general the observed number of isoenzymes of muscle PGM is 2, 3 or 4. The appearance of three major bands of rabbit muscle PGM on polycarylamide gels, as described in 3.7.1, is consistent with most results hitherto reported. The five major bands of rabbit liver PGM observed under similar conditions suggest that the isoenzyme compositions of the liver and muscle enzymes are dissimilar.

The major difference in the electrophoretic patterns of PGM from rabbit liver and rabbit muscle homogenates on polyacrylamide is that liver PGM appears as two zones containing 3 and 2 bands and muscle PGM as one zone containing 3 bands, similar in pattern to one of the zones of the liver enzyme. The slowest migrating anodal bands 7 and 8 of the 2nd zone of the liver PGM were not observed with muscle enzyme. Although PGM bands designated as 1, 2 and 3 were found with both the liver and the muscle homogenates, there were differences in the electrophoretic mobilities. There must hence be a small charge or size difference between the proteins in liver and muscle bands 1, 2 and 3. Three sets of human PGM isoenzymes with different mobilities on starch gel have been shown to be different in their properties (see 1.1.7). The difference in the mobilities of liver and muscle bands 1, 2 and 3 and that in the overall patterns of the liver and muscle isoenzyme is further evidence for a difference in the properties of PGMs from the two tissues. Environmental and metabolic conditions could be responsible for this difference as suggested by Wilkinson (1969).

The heterogeneity of PGMs from rabbit liver and muscle separated on polyacrylamide gels cannot be explained in terms of progressive oxidation of sulphydryl groups of PGM (cf. Green and Dawson, 1973), as the experiments described here involved removal of the oxidising agent (persulphate) from the polyacrylamide (2.7.3) and the inclusion of a sulphydryl protective agent (2-mercaptoethanol) in the sample (2.7.4). This minimised the possibility that the

isoenzyme pattern on the gels was an artifact. Similarly, the isoenzyme pattern cannot be explained on the basis of "genetic frequencies" (Tsoi and Douglas, 1965; Hopkinson and Harris, 1966, 1968). Although three gene loci may give rise to isoenzyme zones with mammalian PGMs, it appears that it is usual for locus 1 ($PGM_1$) to predominate (1.1.7). The single zone (of three bands) found with the rabbit muscle PGM is consistent with this pattern. Though a second zone of lower mobility is present with the rabbit liver enzyme, it is not likely thatthis zone is due to loci 2 or 3 as these would be expected to be more negatively charged than locus 1 isoenzymes and therefore have higher mobilities. It was concluded that the second, slower isoenzyme bands are dephospho forms of the isoenzymes present in the zone of higher mobility. This conclusion was tested by observing the enzyme activity present in individual bands in the presence and absence of cofactor and inhibitors (see below). Moreover, the slower dephosphoenzyme bands of the rabbit liver PGM show a staining pattern similar to that of the faster phosphoenzyme bands.

Rabbit liver 1st zone isoenzyme bands and rabbit muscle PGM bands 1, 2 and 3 showed activity in the absence of G1P6P (3.7.1) suggesting that they are phosphoenzymes, In contrast, the rabbit liver 2nd zone bands (7 and 8) did not show activity in the absence of G1P6P and are probably dephosphoenzymes. The electrophoretic mobilities of these two zones of isoenzymes on polyacrylamide gels are compatible with their being phospho and dephospho forms. Thus the 1st

zone bands showed higher mobilities than the 2nd zone bands, as would be expected, since the phosphcenzyme at alkaline pH would have a higher negative charge (cf Duckworth and Sanwal, 1972). ATP inhibition should also be more pronounced with the dephosphoenzyme than the phosphoenzyme (see 4.4). When the effect of ATP was investigated it was found that ATP had a greater inhibitory effect on the bands of the 2nd zone than on those of the 1st zone, as expected. Rabbit muscle PGM bands 1, 2 and 3 were similar to liver 1st zone bands (3.7.2). This further suggests that these are due to phosphoenzymes, as no dephosphoenzymes would be expected with rabbit muscle PGM.

The above results indicate that a large amount of liver enzyme (25-30% of total) may exist in the unphosphorylated form in contrast to muscle enzyme which is probably completely phosphorylated. This finding is consistent with the results of Joshi et al. (1967) and Handler et al. (1965) who reported, respectively, that 60% of rabbit liver PGM was separated as phosphoenzyme (1.1.7) and that this enzyme contained 0.5-0.7 mol phosphate per mol enzyme (1.1.8).

## 4.3 METAL IONS AND ENZYME ACTIVITY

Rabbit liver PGM requires $Mg^{++}$ for activity, maximum activation being achieved at a concentration of about 1.2 mM (3.2.6). This behaviour is very similar to that of other PGMs including rabbit muscle and beef liver enzymes (Milstein, 1961a; Chiba et al., 1976). Rabbit liver enzyme showed a small activity (3.05%) in the absence of $Mg^{++}$, like the enzymes of rabbit muscle and beef liver, and this activity decreased to a very low level in the presence of 100 mM EDTA (see 3.2.6). This indicates that, in the absence of added $Mg^{++}$, the activity was probably due to traces of metal ions bound to the enzyme (cf. Milstein, 1961a).

In the absence of $Mg^{++}$, $Mn^{++}$ also activates rabbit liver enzyme to some extent, though $Zn^{++}$ ions are inhibitory. This is comparable to the behaviour of all other known PGMs except the yeast enzyme, which retains activity in the presence of $Zn^{++}$ (McCoy and Najjar, 1959).

Rabbit liver PGM requires the presence of a chelating agent in the assay mixture for maximum activity, like all other known animal PGMs (see 1.1.5). Liver PGM shows very low activity (2.8%) in the absence of a chelating agent (Table 3.4). Maximum activity of liver enzyme was observed in the presence of histidine, together with a low concentration of EDTA. The enzyme was also found to be activated by cysteine, imidazole and glutathione. Milstein (1961a) suggested, for the muscle enzyme, that the chelating agent

binds inhibitory metal ions (e.g. $Cu^{++}$ and $Zn^{++}$), found in distilled water and towards which the enzyme is very sensitive. He showed that removal of such ions from enzyme solution and assay reagents eliminated the requirement for chelating agent. Histidine, cystein and imidazole increase the rate of release of inhibitory ions from the enzyme far more effectively than does EDTA, but are less effective than the latter in binding these ions (Ray and Peck, 1972). The highest activity is consequently observed when both EDTA and histidine (or cystein or imidazole) are present in suitable concentrations (see 1.1.5).

In contrast to the rabbit muscle enzyme, rabbit liver PGM showed a lack of stimulation when incubated with histidine and $Mg^{++}$ prior to assay (3.2.5) though Milstein (1961a) and Harshman et al. (1965) had demonstrated that PGM is normally contaminated with inhibitory metal ions. Similarly Peck and Ray (1971) reported that approximately 65% of the total enzyme binds $Zn^{++}$ in rabbit skeletal muscle under normal conditions, in vivo. Ray and Roscelli (1966b) interpreted the requirement for incubation of the enzyme with a chelating agent and $Mg^{++}$ prior to assay in terms of the accelerated removal of inhibitory metal ions and their replacement with $Mg^{++}$. This process occurs efficiently in the absence or in the presence of low concentration of substrate (1.1.5).

Removal of inhibitory ions is believed to be slower from the phosphorylated than the dephosphorylated form of the enzyme, because of the higher affinity of the former for

the metals (1.1.5). As the enzyme is normally isolated in a phosphorylated form, removal of inhibitory metal ions by a chelating agent alone would be slow. In these circumstances it is necessary to preincubate the enzyme with both chelating agent and $Mg^{++}$ if it is to be fully activated before assay. Such a step would still be necessary if the enzyme were to be isolated in a partially phosphorylated form.

It has been reported for enzymes which appear to be only partially phosphorylated in vivo (flounder and shark muscle PGMs; Hashimoto and Handler, 1966) that incomplete phosphorylation is associated with a lower extent of activation during preincubation. The mechanism underlying this effect is unclear and it seems probable that the extent of phosphorylation and the extent of activation are not correlated. It is more likely that the latter depends principally on the relative levels of free $Zn^{++}$, $Mn^{++}$ and $Mg^{++}$ in the tissue concerned. In this context it should be noted that no activation was found with pure rabbit liver PGM though it was isolated as partially phosphorylated enzyme. The lack of activation was simply due to replacement of inhibitory metal ions by $Mg^{++}$ during the isolation procedure (all solutions contained some $Mg^{++}$; see 3.1). The same effect was noted during purification of the human muscle enzyme (Joshi and Handler, 1969).

The requirement for activation of the enzyme prior to assay may also depend on the levels of hormones at the time of the animal's death. Raising the level of adrenalin increases this requirement for the PGMs from rat muscle and liver

whereas raising the level of insulin has the opposite effect (Hashimoto et al., 1967). Peck and Ray (1971) observed that 35% of total rabbit muscle PGM, in vivo, under normal conditions, exists as a $Mg^{++}$ complex (active form), while all the enzyme was metal-complexed. Since the concentration of free $Mn^{++}$ and $Ca^{++}$ in muscle is very low, they suggested that the greater part of the PGM binds $Zn^{++}$. Insulin shock in both fasted and fed rabbits increases the fraction of enzyme bound to $Mg^{++}$ owing to an increase in the $Mg^{++}/Zn^{++}$ ratio (Peck and Ray, 1971). Comparison with the results of Hashimoto et al. (1967) suggests that increase in the level of insulin decreases the requirement for preincubation. Adrenalin possibly increases the free $Zn^{++}/Mg^{++}$ ratio and the requirement for preincubation increases. For rabbit liver homogenate it was calcualted from the rate increase during the lag period of the assay (no preincubation) that 55% of the total enzyme was in the active form. The remainder probably binds $Zn^{++}$ and $Mn^{++}$, and will be effectively inactive as the $Zn^{++}$-enzyme complex is inactive and the $Mn^{++}$ complex has only 15% activity of the $Mg^{++}$ complex (Ray, 1969). In liver some $Mn^{++}$-enzyme complex would be expected as the concentration of $Mn^{++}$ in that tissue is 20 times that in muscle (Underwood, 1977) and $Mn^{++}$ binds PGM tenaciously. It is possible that the extent of binding to $Mn^{++}$ is variable, as the level of free $Mn^{++}$ appears to fulfil a regulatory function in liver (Schramm, 1982). Thus hormones may, to some extent, regulate the activity of liver PGM through alteration of both the $Zn^{++}/Mg^{++}$ and $Mn^{++}/Mg^{++}$ ratios. This effect should be independent of regulation via the extent of phosphorylation of the enzyme.

## 4.4 ATP AND CITRATE INHIBITION

Inhibition by the chelators ATP and citrate was investigated on PGMs from rabbit liver and rabbit muscle in order to compare their effects on enzymes from these two sources (3.2.9). ATP and citrate bind to $Mg^{++}$ and their binding constants are $5 \times 10^5$ $M^{-1}$ and $5 \times 10^4$ $M^{-1}$ respectively (in Dawson et al., 1969). When PGM is assayed in the presence of ATP or citrate, these anions occur as complexes with $Mg^{++}$. The inhibition which results has been thought to occur by chelation of $Mg^{++}$ and competition of the $Mg^{++}$-inhibitor complexes with G1P6P for a binding site (see 1.1.6). This was suggested by Kovács and Bot (1965) for ATP when they found that its partial inhibition could be relieved by the addition of excess $Mg^{++}$ or G1P6P, but the complete removal of ATP inhibition only occurred when both $Mg^{++}$ and G1P6P were added in excess. Similar inhibitory effects of citrate have also been observed (Beitner et al., 1975). However, when the inhibition by ATP and citrate of PGMs from rabbit muscle and liver was investigated, it was found that citrate was less effective than ATP for both enzymes (3.2.9). This is consistent with the results of Beitner et al. (1975). The lower inhibitory effect of citrate in comparison to that of ATP is due to its lower binding affinity for $Mg^{++}$ and possibly to the lower charge of its complex.

Both liver and muscle PGM activities decreased to 10% of their optimum values in the presence of 5 mM ATP. The free $Mg^{++}$ concentration was calculated to be 0.03 mM (Fig. 3.10).

At this $Mg^{++}$ concentration, liver PGM should have shown approximately 30% of its optimum activity (3.2.7) if ATP inhibition were simply due to the lowering of the free $Mg^{++}$ concentration. Similarly, at 5 mM citrate the free $Mg^{++}$ concentration would be 0.27 mM and the rabbit liver enzyme should have shown more than 70% activity (3.2.7). Instead the observed activity was 50% (Fig. 3.10). These results suggest that the activities of both enzymes in the presence of ATP or citrate were not only affected by the removal of $Mg^{++}$ but also by competition of $Mg\text{-}ATP^{--}$ or $Mg\text{-}citrate^{-}$ with G1P6P. This suggestion was supported by the observation that inhibition by 3.5 mM ATP or 2.5 mM citrate was not completely relieved by increasing the free $Mg^{++}$ concentration to an adequate level (Table 3.5).

When inhibitions by ATP and citrate were compared for the two enzymes, it was observed that the degree of ATP inhibition was slightly greater with the liver enzyme (Fig. 3.10). Similarly, in the presence of 3.5 mM ATP, the removal of ATP inhibition on increasing the concentration of $Mg^{++}$ or G1P6P was found to be greater with the muscle PGM than with the liver enzyme (Table 3.5). Inhibition by anions competitive with G1P6P is thought to be caused by binding to the dephosphorylated form of the enzyme (Ray and Peck, 1972). Thus Duckworth *et al.* (1973) observed that *E.coli* PGM isolated in the dephosphorylated form is somewhat more sensitive to nucleotide inhibitors than is the rabbit muscle enzyme which is usually isolated in the completely phosphorylated form. Furthermore, after electrophoretic separation of the enzymes into phospho- and dephosphoenzyme zones, ATP appears

to inhibit the activity of isoenzymes in the latter zone to a greater extent (3.7.2). It is hence possible that the slightly greater inhibition by ATP observed with the liver enzyme was due to a high $K_m$ for G1P6P. Citrate is a weaker inhibitor than ATP (see above) and its effects on the enzymes from the two sources could not be differentiated.

At low concentrations of ATP (0-0.5 mM), activation of both rabbit liver and rabbit muscle enzymes up to 110% and 115% respectively of their optimum activities was observed (Fig. 3.10). The $K_i$ of ATP is approximately 0.5 mM (see 4.7) and at the concentration mentioned above, the ATP would have little inhibitory effect due to the presence of adequate levels of $Mg^{++}$ and G1P6P. ATP has been shown to be contaminated with approximately 1% $Al^{+++}$ in commercial preparations (Schloss et al., 1982). Activation observed at low concentration of ATP could therefore have been caused either by the presence of ATP as a chelator or by the action of $Al^{+++}$. The enzyme-$Al^{+++}$ complex is active and as $Al^{+++}$ binds the enzyme powerfully it may displace other inhibitory metal ions (Milstein, 1961a). This explanation of activation requires that the enzyme is at the outset partially complexed with inhibitory metal ions and that it competes effectively with ATP for $Al^{+++}$. Though activation of the rabbit muscle enzyme might be explained in this way, no such effect would be expected with the rabbit liver enzyme as this is initially present as the enzyme-magnesium complex (4.3). Similarly in the presence of 2.5 mM citrate and 50 µM G1P6P, the activities of the

liver and the muscle enzymes increased by up to 105% and 135% respectively. This could also have been explained on the basis of removal of inhibitory metal ions if the muscle PGM alone had shown activation. Further information is required to explain the activation of the PGMs under these conditions.

TABLE 4.1: Ratio of glucose 1,6-diphosphatase activity and phosphoglucomutase activity

| Source of enzyme | PGM activity units $g^{-1}$ wet tissue | Diphosphatase activity (units x $10^3$ $g^{-1}$ wet tissue) | Ratio of diphosphatase/mutase activity x $10^5$ | Reference |
|---|---|---|---|---|
| Rabbit liver | | | | |
| Homogenate | 28.5 | 9.85 | 34.56 | This thesis |
| Purified PGM | | | 0.167 | This thesis |
| Rabbit muscle | | | | |
| Homogenate | 74.9 | 1.96 | 2.6 | This thesis |
| Purified PGM | | | 0.0072 | Hashimoto & Handler (1966) |
| Beef liver | | | | |
| Homogenate | 22.8 | 4.68 | 20.53 | Ueda et al. (1976) |
| Purified PGM | | | 0.649* | Hirose et al. (1976) |
| Flounder muscle | | | | |
| Purified PGM | | | 0.209 | Hashimoto & Handler (1966) |
| Shark muscle | | | | |
| Purified PGM | | | 0.0839 | Hashimoto & Handler (1966) |

* glucose 1,6-diphosphatase activity in the presence of xylose-1-phosphate
Units of rabbit, flounder and shark muscle PGMs were calculated from the enzyme concentrations (Hashimoto and Handler, 1966) assuming values of 62,000, 63,000 and 63,000 for molecular weights and 1488 (Joshi and Lane, 1978), 606 and 630 (Hashimoto and Handler, 1966) for the highest specific activites respectively.

## 4.5 DIPHOSPHATASE ACTIVITY

The ratio of diphosphatase to mutase activity in a purified preparation of PGM from rabbit liver was found to be $1.67 \times 10^{-6}$ (3.3). This ratio is higher than that recorded for PGMs from shark and rabbit muscle (Hashimoto and Handler, 1966) and beef liver (in the absence of xylose-1-P) (Hirose et al., 1976) but comparable with the value for flounder muscle enzyme (Hashimoto and Handler, 1966). A comparison of the diphosphatase and mutase activities of rabbit liver PGM and those of other PGMs is presented in Table 4.1.

Hydrolytic activity of G1P6P may arise in the liver as a result of thermodynamic reversal of routes for G1P6P synthesis, the presence of specific diphosphatase enzymes, or a hydrolytic activity associated with the PGM. In liver, G1P6P synthesis appears to involve several routes (see 1.1.10): (1) synthesis by a side reaction of phosphofructokinase from G1P and ATP, (2) synthesis by PGM from G1P and F1P6P which solely depends on the PGM activity, and (3) synthesis by a specific synthetase utilizing G1P and glycerate-1,3-diphosphate, which may be a particular PGM isoenzyme. Thus PGM could be a multi-functional enzyme like phosphoglycerate mutase from yeast, human erythocytes and pig erythrocytes which also have diphosphoglyceromutase activity (synthesis of 2,3-diphosphoglycerate) and 2,3-diphosphoglycerate phosphatase activity (degradation of 2,3-diphosphoglycerate) (Ueda et al., 1976). However, the reversal of G1P6P synthesis is unlikely to account for significant G1P6P hydrolysis under experimental conditions as, of the relevant reactants, only G1P6P was present in the assay medium.

Specific diphosphatases do appear to be present in mitochondrial and microsomal fractions of beef liver cells and these seem to be responsible for more than 80% of the diphosphatase activity in liver (Ueda et al., 1976). However, 17% of the diphosphatase activity was found in the cytosol and this could be due to the hydrolytic activity of PGM arising from instability of the phosphoenzyme. During purification of rabbit liver PGM the ratio of diphosphatase to mutase activity decreased from $3.46 \times 10^{-4}$ to $1.67 \times 10^{-6}$ (Table 4.1). Furthermore, the yield of PGM was observed after purification to be 27% of the original present in crude extracts (Table 3.1), whereas the diphosphatase activity was reduced to 0.4% of that in the centrifuged homogenate. (The activity in the crude liver extract before centrifugation must have been higher, but this could not be measured; see 3.3).

These results show that there was a progressive fall in the diphosphatase activity of the PGM fraction during purification and the final diphosphatase activity indicates that the phosphoenzyme is stable over the time scale of the experiments. The diphosphatase activity in the cytosol was probably due to contaminating enzymes from disrupted microsomes and mitochondria. The same explanation must apply for the beef liver cytoplasmic diphosphatase activity, since the beef liver phosphoenzyme is stable.

The ratio of diphosphatase to mutase activity of beef liver PGM even in the presence of an activator ($6.49 \times 10^{-6}$), was lower than the ratio of G1P6P synthesis activity to mutase ($1 \times 10^{-4}$) (Ueda et al., 1976). This showed that

beef liver PGM may be partly responsible for synthesis of G1P6P but not for its breakdown. If the G1P6P synthesis activities of beef liver and rabbit liver PGMs are similar, then PGM only will be responsible for G1P6P synthesis in both tissues.

## 4.6 KINETICS AND REACTION MECHANISM

Lineweaver-Burk plots of $1/v_i$ against 1/G1P at varying concentrations of G1P6P and $1/v_i$ against 1/G1P6P at varying concentrations of G1P for rabbit liver PGM gave a family of parallel lines (Figs. 3.12 and 3.13). This kinetic behaviour of the enzyme is a characteristic of a phosphoenzyme mechanism (ping-pong mechanism). In this sense rabbit liver PGM behaves like the other major forms from rabbit muscle (Ray and Roscelli, 1964a, 1964b), flounder and shark muscle (Hashimoto and Handler, 1966) and beef liver (Chiba et al., 1976).

The $K_m$ values of rabbit liver PGM obtained were 30.8 ± 1.8 μM and 0.29 ± 0.019 μM for G1P and G1P6P respectively (3.4). For G1P, enzymes from different sources exhibit a range of $K_m$ values from 2.2 μM with the yeast enzyme (Hirose et al., 1970) to 400 μM with the flounder muscle enzyme (Hashimoto and Handler, 1966). The rabbit liver PGM value, 31 μM, falls within this range. However, for G1P6P the $K_m$ values of enzymes from different sources are very similar, with values ranging from 0.03 μM for the flounder muscle enzyme (Hashimoto and Hanler, 1966) to 0.065 μM for beef liver enzyme (Chiba et al., 1976), although the yeast enzyme has a somewhat larger $K_m$ value of 0.14 μM (Hirose et al., 1970). It might thus be expected that rabbit liver enzyme would have a $K_m$ value within, or close to, this range. The finding of a higher $K_m$ value (0.29 μM), when great care was taken to ensure that the enzyme was isolated in its native form (4.1) and

and when only pure G1P was employed as substrate (2.2.1.1.2) and both the free magnesium ion concentration and ionic strength were kept constant (2.2.1.1.2), could suggest that other reported values are low estimates or that the higher $K_m$ is the consequence of a regulatory function. In determinations for the beef liver enzyme, neither free $Mg^{++}$ nor ionic strength was kept constant (Chiba et al., 1976).

Failure to prevent enzyme modification during isolation or to eliminate G1P6P completely from the G1P solution could also lead to differences of this sort. Underestimation of the $K_m$ for G1P6P may arise if G1P acts as an inhibitor during the estimation of the apparent $K_m$ for G1P6P. In this context it should be noted that use of a sensitive fluorimetric assay in the current investigation permitted measurements of initial velocity at slightly lower concentrations of G1P than were used by other authors. Thus the higher $K_m$ of the rabbit liver enzyme is not an artifact and may reflect the metabolic role of the enzyme (see 4.7).

The parallel-line double reciprocal plots obtained with rabbit liver PGM indicate that the low diphosphatase activity associated with this enzyme is not sufficient to influence significantly its kinetic behaviour. On the other hand the possibility that the enzyme may follow a sequential addition mechanism cannot be unequivocally excluded by kinetic evidence alone, as discussed earlier (1.1.9). Furthermore, kinetic data do not elimate the possibility that 2nd zone PGM (3.7.1), the minor PGM component present in liver, may follow a different reaction

mechanism. Induced-transport tests were therefore carried out to make an unambiguous assignment of the mechanism and to detect the presence of minor pathways.

Induced-transport tests (3.5) with [$^{32}$P] G6P at low substrate concentration and with [$^{14}$C] G6P at both low and high substrate concentrations showed that the rabbit liver enzyme has a phosphoenzyme mechanism in which the phosphoenzyme intermediate could be either a true phosphoenzyme (Mechanism 1) or the enzyme-G1P6P complex (Mechanism 1$^{1}$) (for mechanisms see 1.1.9). However, the demonstration that $^{32}$P-label only was associated with the enzyme after incubation with $^{14}$C- and $^{32}$P-labelled G6P (3.6.1) indicated that liver PGM follows Mechanism 1 and not Mechanism 1$^{1}$. Thus the active form of the enzyme is a true phosphoenzyme and not an enzyme-G1P6P complex. Incubation of $^{32}$P-labelled enzyme with excess G6P for a few seconds resulted in the transfer of 96% of the $^{32}$P activity to G6P (3.6.2). This showed that phosphoenzyme was capable of behaving as a true kinetic intermediate as the transfer of label occurred at a rate in excess of the expected rate of conversion of G1P to G6P.

$^{32}$P-labelled phosphoenzyme was found to be stable in acid and the rate of hydrolysis in alkali of $^{32}$P-labelled phosphoenzyme was comparable with that of the phosphoenzymes from rabbit muscle and M.lysodeikticus (see 3.6.3). This suggests that phosphate in rabbit liver PGM is attached to a serine residue and possibly the sequence around the serine residue may be similar to those found with rabbit muscle and M.lysodeikticus enzymes, Thr-Ala-Ser(P)-His-Asp.

The half-life of the phospho-enzyme at 30 $^{o}$C in His-Tris-HCl buffer, pH 7.6, containing 2 mM $MgCl_2$, was found to be 47.5 h and the rate constant for hydrolysis was $2.4 \times 10^{-4}$ $min^{-1}$ (3.6.2). This compares with the value of $8.2 \times 10^{-5}$ $min^{-1}$ obtained from measurement of diphosphatase activity (Fig. 3.11). A precise agreement between these values may not be possible as the latter was measured in the presence of G1P6P (3.3) and this might have altered the enzyme reactivity. The half-life of the phosphoenzyme is comparable to that from M.lysodeikticus, 50 h (Clarke et al., 1974) and represents only $1.67 \times 10^{-4}$% of the mutase activity. Thus under most experimental conditions the phosphoenzyme may be considered to be stable. For the liver enzyme, the reaction probably follows the exchange mechanism for phosphate transfer proposed for the rabbit muscle enzyme (Ma and Ray, 1980).

In the preparation of $^{32}$P-labelled enzyme, it was observed that only 0.45-0.63 mol phosphate was bound per mol of PGM from rabbit liver, in comparison with 0.84-1.19 mol phosphate per mol of enzyme for PGM from rabbit muscle, though the G1P6P concentration was well in excess of its $K_m$ in each case (3.6.1). Attempts to improve the incorporation of $^{32}$P-label into the phosphoenzyme by using the phenol extraction method were not successful. 0.52-0.56 mol phosphate per mol enzyme was incorporated when this method was used (Table 3.7). Several explanations are possible for this low level of phosphorylation. The enzyme may be partially bound to $Zn^{++}$, it may not be pure, or it may show a significant diphosphatase activity. However, in the

TABLE 4.2: Tentative scheme for low incorporation of $^{32}P$-label in liver PGM

$$E + G1P6P \rightleftharpoons E.G1P6P \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EP.GmP$$

| Source of PGM | $k_1$ | $k_2$ | Favoured form | Relative rate of incorporation of label from GmP into G1P6P | Form isolated | Expected relative $\frac{V_{max}}{E}$ |
|---|---|---|---|---|---|---|
| Rabbit liver | slow | fast | E.G1P6P | fast ($k_1 < k_2$) | EP + E | < 1 |
| Rabbit muscle | fast | slow | EP.GmP | slow ($K_2 < k_1$) | EP | 1 |

E.G1P6P: Dephosphoenzyme-G1P6P complex

EP.GmP: Phosphoenzyme - glucose monophosphate complex

experiments with liver PGM, the enzyme was purified by a process incorporating affinity elution chromatography, which should separate active from inactive enzyme (3.1), the diphosphatase activity was very low (4.5) and the enzyme isolated was completely $Mg^{++}$-bound (4.3). Thus other factors must be involved. Using the scheme given in Table 4.2, one possible explanation is that the central complex in the catalytic reaction is distributed between two forms E.G1P6P and EP.GmP and that the relative stabilities of these two species depends upon which enzyme is involved. Thus, if the rate of interconversion of these forms is sufficiently slow, the extent of enzyme phosphorylation will depend upon the relative values of $k_1$ and $k_2$. Such an explanation is compatible with the known rate of incorporation of radioactivity from monophosphate into cofactor for the rabbit muscle enzyme (Ray and Roscelli, 1964a). Unfortunately no similar data are available for the rabbit liver enzyme, so that this suggestion must remain tentative. It is attractive, however, in that it would explain much of the variability of enzyme phosphorylation found with PGMs. No other simple explanation for this phenomenon seems to be possible.

TABLE 4.3: Concentration of metabolites in animal tissues

| Metabolite | Source | Condition | Concentration | Reference |
|---|---|---|---|---|
| G1P6P | Mouse liver | Normal | 14 μM | Bergmeyer,1974 |
| | Mouse muscle | Normal | 45 μM | Bergmeyer,1974 |
| ATP | Rat liver | Normal | 1.6 mM | Baguer et al., 1976 |
| | | Normal | 3.5 mM | Faupel et al., 1972 |
| | | Stress | 2.5 mM | Faupel et al., 1972 |
| | | Normal | 2.94 mM | Bergmeyer,1974 |
| | Rat muscle | Normal | 5.8 mM | Bergmeyer,1974 |
| Citrate | Rat liver | Normal | 0.21 mM | Bergmeyer,1974 |
| | | Starved (24h) | 0.18 mM | Bergmeyer,1974 |
| | | Starved (27h) | 0.15 mM | Bergmeyer,1974 |
| | | Starved (48h) | 0.18 mM | Bergmeyer,1974 |
| | | Normal | 0.298 mM | Baguer et al., 1976 |
| | | Normal | 0.278 mM | Faupel et al., 1972 |
| F1P6P | Rat liver | Normal | 25 μM | Bergmeyer,1974 |
| | | Starved (28h) | 26 μM | Bergmeyer,1974 |
| | | Normal | 27 μM | Faupel et al., 1972 |
| | | Normal | 11.3 μM | Baguer et al., 1976 |
| | Mouse liver | Normal | 25 μM | Bergmeyer,1974 |
| | Cow liver | Normal | 62 μM | Bergmeyer,1974 |
| | Rat muscle | Normal | 36 μM | Bergmeyer,1974 |
| 3-PGA | Rat liver | Normal | 0.089 mM | Baguer et al., 1976 |
| | | Normal | 0.285 mM | Faupel et al., 1972 |
| | | Normal | 0.32 mM | Bergmeyer,1974 |
| | Rat muscle | Normal | 0.03 mM | Bergmeyer,1974 |
| | Mouse liver | Normal | 0.22 mM | Bergmeyer,1974 |
| PEP | Rat liver | Normal | 0.10 mM | Bergmeyer,1974 |
| | Rat muscle | Normal | 6.5 mM | Bergmeyer,1974 |

## 4.7 PHOSPHOGLUCOMUTASE: REGULATORY ASPECTS

It has been suggested by Johnson (1974) that PGM is a regulatory enzyme. Since PGM catalyses an important reaction step linking glycogen metabolism and glycolysis and the intracellular concentrations of G1P and G6P in liver and muscle are not those of the PGM reaction in equilibrium (Hashimoto et al., 1966), this enzyme may have a minor regulatory function.

A variety of metabolites, such as ATP, citrate, F1P6P, 1,3-diPGA, PEP and several others have been shown to inhibit PGM. The normal levels of some of those metabolites is given in Table 4.3. The concentrations do not seem to vary dramatically under different physiological conditions. The inhibition produced by them is principally competitive with G1P6P (1.1.6). The normal concentrations of G1P6P have been reported to be 14 μM in mouse liver and 45 μM in muscle (in Bergmeyer, 1974). The concentration of G1P6P in muscle may vary from 50% to 300% of the original level under various conditions (1.1.10). If it be assumed that the level of G1P6P in rabbit liver and muscle is similar to that in mouse and the concentration of G1P6P in liver fluctuates in a similar way then the concentration of G1P6P will be about 24 to 145 times higher in liver, and 363 to 2177 times higher in muscle than the appropriate $K_m$'s for PGM (see 3.4 and Ray and Roscelli, 1964a, from rabbit liver and rabbit muscle respectively). In the case of ATP we calculated that $K_i$ for the competitive element versus G1P6P must be about 0.5 mM (from Duckworth et al., 1983). Although the

concentration of ATP in liver and muscle is higher than its $K_i$ value, in the presence of physiological levels of G1P6P, inhibition by ATP should be small. The $K_i$ values of other metabolites (see 1.1.6) are of the same order or higher than their physiological concentrations. Thus it does not seem possible that the activity of PGM is significantly affected by metabolites in vivo. Similarly, although 1,3-diPGA has a low $K_i$ value for PGM from rabbit muscle (5 μM) and beef liver (10 μM) (1.1.6), its concentration in the tissues is probably less than this and the extent of inhibition should also be small (Hirose et al., 1976).

As the level of G1P6P is greatly in excess of the $K_m$ for this substance, it follows that the activity of PGM should be relatively independent of the G1P6P concentration unless high concentrations produce enzyme activation or inhibition of G1P by G1P6P occurs. Such an activation of phosphoglycerate mutase by 2,3-diPGA has been proposed by Mantle and Garfinkel (1969). No G1P6P activation has been reported at high concentrations of G1P6P, nor would any inhibition of G1P binding be expected (see below). It should be noted, however, that at high G1P6P concentrations (50 μM) citrate did produce an activation of 105% activity in the rabbit liver enzyme and 135% activity in the rabbit muscle enzyme which could not be accounted for by the increase in G1P6P concentrations. Similar studies, in the presence of 2.0 mM G1P6P, provide evidence that ATP at low concentrations (0-0.5 mM) can activate the enzyme by up to 115% (Fig. 3.10). The activation is not very high and it seems unlikely that this could be an

effect of great metabolic significance. Some inhibition of G1P binding by the elevated level of G1P6P could occur if the $K_i$ for G1P6P were less than about 3 μM in liver or 9 μM in muscle. For rabbit muscle PGM this $K_i$ value is 0.7 mM (Ray and Roscelli, 1964b) and so no inhibition would be expected.

The activity of PGM *in vivo* may depend upon the concentrations of specific metal ions as these can activate and inhibit PGM to various extents (1.1.4). Thus as rabbit muscle PGM binds $Zn^{++}$ $10^6$ more strongly than $Mg^{++}$ and is strongly inhibited by it, extensive inhibition of the enzyme would be expected *in vivo*, since the quantity of $Zn^{++}$ in muscle is comparable with that of $Mg^{++}$ (Harrison and Hoare, 1980). However, in skeletal muscle the concentration of free $Mg^{++}$ is only 1% and that of $Zn^{++}$ less than $10^{-4}$% of the total concentrations, while 35% of the PGM is found complexed with $Mg^{++}$ and the rest with $Zn^{++}$ (Peck and Ray, 1971). Most of the $Mg^{++}$ is bound to ribosomes, nucleoside phosphates and other organic phosphates. $Zn^{++}$ is similarly bound to various phosphates (Harrison and Hoare, 1980). Peck and Ray (1971) found that the activity of PGM was regulated by insulin in a manner which suggested that it exerted the effect through an increase in free cytoplasmic $Mg^{++}$, possibly by release from the inner cell membrane, since insulin is known to affect membrane permeability and affinity for solutes. Similarly Hashimoto *et al.* (1967) reported that insulin decreases the extent of activation of PGM by $Mg^{++}$ and chelating agent. Thus the effect

of insulin appears to be due to an increase in the $Mg^{++}/Zn^{++}$ ratio, which could control the PGM activity *in vivo*. In liver the concentration of $Mn^{++}$ is appreciable (see 4.4) and may also affect enzyme reactivity. As mentioned elsewhere (4.2), the free $Mn^{++}/Mg^{++}$ ratio also appears to be dependent on the hormonal status of the liver, since the free $Mn^{++}$ level is enhanced three-fold in the fed as compared with the fasting state (Ash and Schramm, 1982). However, it should be noted that this leads to an effect upon PGM activity opposite to that produced by $Zn^{++}/Mg^{++}$ levels. In the fed state the insulin level should be elevated and this will decrease the $Zn^{++}/Mg^{++}$ ratio. Thus if the concentration of metal ions influences the activity of PGM in a metabolically significant way, the effect must be complex.

A newly discovered compound, F2P6P, may also regulate PGM activity. This compound is not an intermediary metabolite of glycolysis, but is a potent regulator (review by Hers and Van Schaflinger, 1982). The normal concentration of F2P6P is 3.5 μM in rat liver and changes from about 1 μM to 10 μM when glucagon and glucose respectively are administered (Hue *et al.*, 1981). F2P6P is a positive effector of phosphofructokinase and a negative effector of fructose-1,6-diphosphatase. Its concentration increases when F6P is plentiful. Its presence may be regarded as a signal of glucose abundance. Although the data are not available for PGM, it appears that F2P6P could regulate PGM activity if this enzyme had a greater affinity for F2P6P than for

G1P6P. It is also possible that the failure to isolate a completely phosphorylated liver PGM could be explained by the presence of tightly bound F2P6P, especially as this enzyme has a relatively high $K_m$ for G1P6P. There is a delay before F2P6P formation in liver, after administration of glucose, for initially glycogen synthetase is stimulated and G6P kept low. Only after the glycogen stores are filled do G6P and F6P increase in concentration and initiate F2P6P synthesis. Thus F2P6P is apparently concerned more with glycolysis and lipogenesis than with glycogen metabolism. However, elevated levels of F2P6P may inhibit PGM (by competing with G1P6P) and this could allow F2P6P to switch off glycogen breakdown when glucose is readily available. This inhibition would act in a manner which was complementary to the principal methods of regulating carbohydrate metabolism.

## 4.8 CONCLUSIONS

From the results obtained, the following conclusions can be drawn.

1. Rabbit liver PGM appears to have properties very similar to those of rabbit muscle PGM in most major respects. The two enzymes are similar in their pH optima (3.2.4), requirements for $Mg^{++}$ (4.3), sensitivity to the inhibitory metal ions (4.3) and the requirement for a chelating agent in the assay mixture for maximum activity.

2. In contrast to rabbit muscle PGM, the liver enzyme did not require preincubation with $Mg^{++}$ and chelating agent for maximum acitvity (4.4). This is because the removal of inhibitory metal ions from the enzyme occurred during isolation of this enzyme (4.3).

3. The molecular weight of rabbit liver PGM is found to be higher than that of the rabbit muscle enzyme (3.2.3). It is uncertain whether liver PGM has, in fact, a higher molecular weight, or whether it was isolated in a comparatively undegraded form. However, the difference in mobilities on polyacrylamide (3.7.1) of bands of phosphoenzyme zones from rabbit liver and rabbit muscle showed that a small difference in molecular weight is possible.

4. PGM from rabbit liver appeared to be only partly phosphorylated, as isoenzymes appeared in electrophoresis as phospho- and dephosphoenzyme zones (3.7.1). However, from the levels of G1P6P in liver, it might be expected that

PGM would occur in the completely phosphorylated form (4.7). The presence of F2P6P may be responsible for the incompleteness of phosphorylation, if the affinity of rabbit liver PGM for F2P6P is higher than that for G1P6P.

5. Only 0.45 mol $^{32}P$ $mol^{-1}$ could be incorporated into rabbit liver PGM in comparison with 0.84-1.19 mol $^{32}P$ $mol^{-1}$ for rabbit muscle enzyme (3.6.1). This low incorporation of $^{32}P$-label into rabbit liver cannot be explained by the presence of impurities or that of enzyme binding $Zn^{++}$ instead of $Mg^{++}$ (4.6). Moreover, the stability of the rabbit liver phosphoenzyme and evidence from other PGMs indicates that the diphosphatase activity of PGM is insufficient to account for this phenomenon (4.5). It is possible that the distribution of the enzyme between the EP.GmP and E.G1P6P forms could account for this behaviour if the formation of EP.GmP from E.G1P6P were relatively slow (4.6).

6. The kinetic properties of rabbit liver PGM were found to be similar to those of the rabbit muscle enzyme (3.4), although the rabbit liver PGM showed a small diphosphatase activity. In contrast to all other PGMs investigated, rabbit liver enzyme showed a high $K_m$ for G1P6P. This may have a metabolic importance, for although inhibition by metabolic intermediates can scarcely be significant, inhibition by F2P6P may be more so (4.7).

7. A phosphoenzyme mechanism has been unequivocally established for rabbit liver PGM, using isotopic evidence (4.6). All PGMs may have the same mechanism of action. This similarity may have an evolutionary importance.

8. The level of G1P6P in tissues appears to be a regulatory factor for several key metabolic enzymes (1.1.10). The low diphosphatase activity associated with PGM in liver is inadequate to regulate the G1P6P concentration (4.5). The enzyme may, however, contribute to G1P6P synthesis.

9. PGM activity in rabbit liver and rabbit muscle should be independent of the concentration of G1P6P <u>in vivo</u>, since this substance is present in excess (4.7). From the data available, free $Mg^{++}$ and $Zn^{++}$ concentrations in muscle and free $Mg^{++}$, $Mn^{++}$ and $Zn^{++}$ concentrations in liver could be responsible for the regulation of PGM activity <u>in vivo</u>.

## 4.10 SUGGESTIONS FOR FUTURE WORK

The following points can be considered for future investigations.

(1) In the present study, the activation of PGM has been observed in the presence of low concentrations of ATP (0-0.5 mM) and 2 μM G1P6P and in the presence of citrate (2.5 mM) and high concentrations of G1P6P (50 μM). The possibility that these chelators could under some conditions activate the enzyme by chelating inhibitory metal ions has been excluded (4.3). This activation could be further investigated by determining the extent of activation produced by ATP at high G1P6P concentrations and citrate at low G1P6P concentrations. For all experiments the concentrations of free $Mg^{++}$ should be carefully controlled. It may then be possible to show that there is a binding site for ATP and citrate which is associated with the activation process.

(2) A small diphosphatase activity has been observed to be associated with rabbit liver PGM. However, this activity was measured in the presence of $Mg^{++}$ ions. In vivo PGM activity appears to be regulated by the ratio of the free metal ion concentrations, $Mg^{++}$ and $Zn^{++}$ in muscle and $Mg^{++}$, $Mn^{++}$ and $Zn^{++}$ in liver (4.3). The possibility that binding of the enzyme by $Zn^{++}$ (or $Mn^{++}$) enhances the diphosphatase activity should be investigated.

(3) It has been shown that most of the G1P6P in liver is synthesized bythe action of PGM upon G1P and F1P6P and by

the action of G1P6P synthetase upon G1P and 1,3-diPGA. The latter enzyme is possibly an isoenzyme of the PGM (1.1.10). Since the presence of $Mg^{++}$, $Mn^{++}$ and $Zn^{++}$ affects the PGM activity, it would be interesting to investigate the effects of individual metal ions on the rate of synthesis of G1P6P.

(4) In the preparation of labelled phosphoenzyme, a lower incorporation of $^{32}P$-label was observed with rabbit liver PGM in comparison to rabbit muscle enzyme (4.6). In order to explain this lower incorporation, a tentative scheme has been presented. This scheme shows that the low incorporation may be due to an equilibrium between the two possible forms of the central complex, E.G1P6P and EP.GmP. As a consequence of this, the ratio of rate with which $^{32}P$- or $^{14}C$-label from G1P accumulates in G1P6P compared to G6P should be higher than in the case of the rabbit muscle enzyme. This prediction could be easily tested.

(5) A phosphoenzyme mechanism has been unequivacally established for rabbit liver PGM in the present study, and PGMs from rabbit muscle and M.lysodeikticus, using isotopic evidence (1.1.9). It is possible that all PGMs have the same phosphoenzyme mechanism. To test this hypothesis further, induced-transport tests should be carried out with PGMs from B.cereus, B.subtilis and yeast as the evidence for a phosphoenzyme mechanism with these enzymes is not conclusive.

(6) PGM may be a regulatory enzyme. From the data available it is unlikely that the G1P6P concentration could regulate PGM activity in vivo, because of the presence of excess G1P6P in comparison to its $K_m$ (4.7). Similarly other anionic metabolites could not be responsible for the regulation of PGM activity at physiological concentrations of G1P6P (4.7). However, the free $Mg^{++}$ and $Zn^{++}$ concentrations do seem to have an effect on PGM activity in rabbit muscle (Peck and Ray, 1971). Similarly in liver, in addition to free concentrations of $Mg^{++}$ and $Zn^{++}$, $Mn^{++}$ could also possibly be responsible for the regulation of PGM activity because the free $Mn^{++}$ concentration fluctuates under different physiological conditions (Ash and Schramm, 1982). Furthermore, although no data are available, F2P6P may also have an effect on PGM activity. This compound is a potent regulator of phosphofructokinase and its concentration fluctuates under different physiological and hormonal conditions (Hers and Schaftingen, 1982). Thus the $K_i$ value for F2P6P with PGM should be determined.

It may also be of interest to see whether F2P6P can act as a cofactor for the enzyme, as this would implicate PGM in the control of the level of F2P6P in the cell.

(7) The presence of a small amount of phosphoribomutase in comparison to PGM has been reported in rabbit liver and the amount of this enzyme is higher in rabbit liver in comparison to muscle (Kammen and Koo, 1969). Phosphoribomutase requires G1P6P but does not require $Mg^{++}$ for its activity. In recent studies in this laboratory, rabbit liver

phosphoribomutase has been separated from PGM on a chromatofocussing column. The isoelectric points of PGM and phosphoribomutase have been found to be 6.8 and 5.8 respectively. It would be interesting to investigate the properties and the mechanism of action of this enzyme, so that the mode of phosphate transfer could be compared with that of other phosphomutases.

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APPENDIX

A. Transfer of labelled glucose and labelled phosphate from G1P to G6P by three possible mechanisms of PGM (Britton and Clarke, 1968)

1. Phosphoenzyme mechanism (Mechanism 1)

$$G^*1P^* + EP_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP_2^* + G^*6P$$

$$EP_2^* \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP_1^*$$

$$G1P + EP_1^* \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP_2 + G6P^*$$

$$EP_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP_1$$

$$ES \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + G1P6P$$

2. Intramolecular transfer mechanism (Mechanism 2)

$$G^*1P^* + EP_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP_2 + G^*6P^*$$

$$EP_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP_1$$

$$ES \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + G1P6P$$

3. Intermolecular transfer mechanism (Mechanism 3)

$$G^*1P^* + [E.G1P6P]_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} [E.G^*1P^*6P]_2 + G6P$$

$$[E.G^*1P^*6P]_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} [E.G^*1P^*6P]_1$$

$$G1P + [E.G^*1P^*6P]_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} [E.G1P6P^*]_2 + G^*6P$$

$$[E.G1P6P^*]_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} [E.G1P6P^*]_1$$

$$G1P + [E.G1P6P^*]_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} [E.G1P6P]_2 + G6P^*$$

$$[E.G1P6P]_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} [E.G1P6P]_1$$

G* and P* represent labelled molecules. ES represents the enzyme substrate complex; $EP_1$ and $EP_2$ represent different forms of phosphoenzyme, $[E.G1P6P]_1$ and $[E.G1P6P]_2$ represent different forms of the dephospho-enzyme-G1P6P complex, and E dephosphoenzyme. $k_1$ and $k_2$ etc are rate constants. For details of mechanisms see 1.1.9.

B. Flux Equations (see Britton and Clarke, 1968)

The following equation was used to calculate the change in label of G1P ($^{14}C$ or $^{32}P$) during the progress of the reaction for various possible mechanisms of PGM

$$\frac{d[G1P^*]}{d[G1P]} = \frac{[G1P^*]r}{[G1P](r-1)} - \frac{[G6P^*]}{[G6P](r-1)} \qquad \text{——— ①}$$

where

$[G1P^*]$ = concentration of $[^{32}P]$ G1P or $[^{14}C]$ G1P

$[G6P^*]$ = concentration of $[^{32}P]$ G6P or $[^{14}C]$ G6P

r = flux ratio

Change in radioactivity depends on the flux ratio (r) in equation 1, which is related with various possible mechanisms:

1. For direct transfer of label ($^{32}P$ or $^{14}C$)

$$r = \frac{[G1P]\ (1 + \alpha\ K\ [G6P])}{K[G6P]\ (1 + \alpha\ \ [G1P])} \qquad \text{——— ②}$$

2. For indirect transfer of label ($^{32}P$ or $^{14}C$) requiring two catalytic cycles

$$r = \frac{[G1P]^2\ (1 + \alpha\ K\ [G6P])}{K^2[G6P]^2\ (1 + \alpha\ \ [G1P])} \qquad \text{——— ③}$$

3. For indirect transfer of label ($^{32}P$ or $^{14}C$) requiring three catalytic cycles

$$r = \frac{[G1P]^3\ (1 + \alpha\ K\ [G6P])}{K^3\ [G6P]^3\ (1 + \alpha\ \ [G1P])} \qquad \text{——— ④}$$

In equations 2, 3 and 4

K = equilibrium constant define as $\frac{[G1P]}{[G6P]}$

$\alpha = k_{+1}\ k_{+2}\ /k_{-3}\ (k_{-1} + k_{+2})$

$\alpha$ is a measure of the rate at which the isomerisation of the two isomeric forms of the enzyme occurs. $\alpha$ is zero when the isomerisation is rapid. Its upper limit for slow isomerization was evaluated using the following equation:

$$\alpha < \frac{1}{K_{m(G1P)}} \left(1 + \frac{V^f_{max}}{V^r_{max}}\right) \qquad \text{——— ⑤}$$

$V^f_{max}$ = $V_{max}$ of forward direction (G1P to G6P)
$V^r_{max}$ = $V_{max}$ of reverse direction (G6P to G1P)

Since $V^r_{max}$ for rabbit liver PGM was not determined, the ratio of $\frac{V^f_{max}}{V^r_{max}}$ of rabbit muscle PGM (in Ray and Roscelli, 1964b) was used to evaluate $\alpha$. It was assumed that this ratio would not differ significantly for enzymes from two sources.

The following equation was used to evaluate the rate of isomerisation of two isomeric forms of the enzyme at high substrate concentrations.

$$\frac{\alpha K_{m(G1P)}}{V^f_{max}} = \frac{1}{E_{k_3}} + \frac{1}{E_{k_{-3}}} \qquad \text{——— ⑥}$$

mechanism (e.g. Ricard *et al.*, 1972; Colowick, 1973). Although evidence for the formation of binary complexes between glucose and hexokinase II has been provided by the protection of the enzyme by glucose against proteolytic digestion, no similar effect could be demonstrated with ATP (Grossbard & Schimke, 1966).

To resolve the question of the mechanism of substrate addition, rat skeletal-muscle hexokinase II, purified to homogeneity by the method of Holroyde & Trayer (1976) was studied by the flux-ratio method introduced by Britton (1966). The ratio of the flux of [$^{14}$C]glucose 6-phosphate to glucose over the flux of glucose 6-[$^{32}$P]phosphate to ATP measured at constant [ADP] and [glucose 6-phosphate] concentrations in the absence of ATP as [glucose] was increased was found to be independent of [glucose] and equal to unity. This provided evidence for an obligatory order of addition of glucose and ATP, with glucose binding to the free enzyme. The dependence of the ratio flux of glucose 6-phosphate to ATP/flux of glucose 6-phosphate to glucose on [ATP] was consistent with an ordered mechanism in which ATP was the second substrate. Furthermore, the slope of the plot of this ratio against [ATP] increased with [ATP] and with [glucose 6-phosphate]. This finding was consistent with the binding of glucose 6-phosphate to an allosteric site on hexokinase II.

There is some controversy in the literature concerning the mode of feedback inhibition of the hexokinases by glucose 6-phosphate. Kinetic evidence obtained by Ning *et al.* (1969) and Purich & Fromm (1971) supports action of glucose 6-phosphate at the catalytic site and of ADP at a regulatory site, whereas Kosow & Rose (1970) argue that glucose 6-phosphate acts at an allosteric site and ADP at the catalytic site. Binding of glucose 6-phosphate to hexokinase I has been shown to cause a conformational change reflected in the protection of 8 out of 12 thiol groups that react with 5,5′-dithiobis-(2-nitrobenzoate) in the absence of glucose 6-phosphate and protects completely against inactivation by the reagent (Redkar & Kenkare, 1972). Glucose 6-phosphate has also been shown to release mitochondrially bound hexokinases in a specific manner, presumably through conformational changes. However, there is little evidence that an allosteric site is involved, even though this seems to be the most popular view. The unusual dependence of the ratio flux of glucose 6-phosphate to ATP/flux of glucose 6-phosphate to glucose on both [ATP] and [glucose 6-phosphate] provides kinetic evidence for such an allosteric site.

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## Mechanism of action of rabbit liver phosphoglucomutase

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Phosphoglucomutases that need magnesium ions for activity also require glucose 1,6-bisphosphate as cofactor. Transfer of phosphate from the 1- to the 6-position of glucose may occur by a phosphoenzyme intermediate (mechanism 1), an intramolecular transfer of phosphate (mechanism 2) or by an intermolecular transfer of phosphate from glucose 1,6-biphosphate to the monophosphates (mechanism 3) (Britton & Clarke, 1968). In mechanism 1 the intermediate may be either a true phosphoenzyme or an enzyme–glucose 1,6-biphosphate complex, which performs the role of a phosphoenzyme (mechanism 1′). The appropriate mechanism for rabbit liver phosphoglucomutase was determined by induced-transport experiments as described below.

Livers were removed from freshly killed rabbits and phosphoglucomutase isolated by a modification of the method of Scopes (1977). A partially purified preparation (sp.activity 100 units/mg) was used in all experiments. Phosphoglyceromutase, hexokinase, glucose 6-phosphate dehydrogenase and glucose 6-phosphatase activities were less than 0.1%, and phosphoglucose isomerase activity less than 1% of the phosphoglucomutase activity.

Induced-transport tests were carried out using [$^{14}$C]glucose 6-phosphate (Sp.radioactivity 34 Ci/mol) purified chromatographically from [$^{14}$C]glucose and [$^{14}$C]glucose 1,6-bisphosphate, and pure glucose 6-[$^{32}$P]phosphate (Sp.activity 32 Ci/mol) prepared by a modification of the method of Schendel & Wells (1973). Labelled substrate was incubated with the enzyme until chemical equilibrium was obtained. Then excess

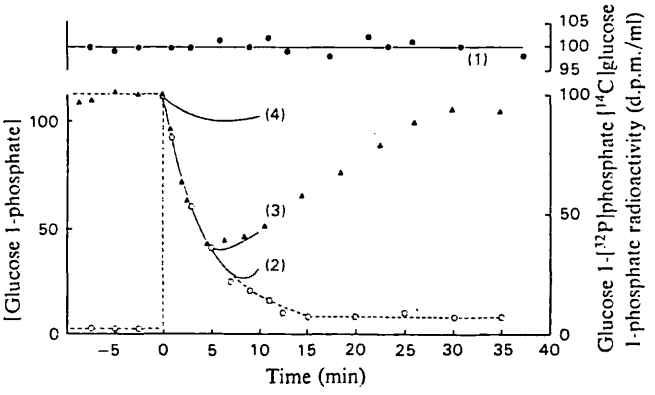


Fig. 1. *Induced-transport tests with $^{32}$P- and $^{14}$C-labelled substrates for rabbit liver phosphoglucomutase*

▲ Glucose 1-[$^{32}$P]phosphate radioactivity; ●, [$^{14}$C]glucose 1-phosphate radioactivity (both in d.p.m./ml and corrected for dilution at zero time); ○, glucose 1-phosphate. The continuous lines are theoretical curves for glucose 1-phosphate radioactivity. For glucose 1-[$^{32}$P]phosphate and [$^{14}$C]glucose 1-phosphate radioactivity the expected curves for mechanism 1 are lines 3 and 1 respectively, for mechanism 2, lines 1 and 1 respectively and for mechanism 3, lines 2 and 3 respectively. Line 4 indicates the expected radioactivity changes for mechanism 3 with [$^{14}$C]glucose 1-phosphate if isomerization of the enzyme–glucose 1,6-biphosphate complex is slow with a rate constant of 288 $s^{-1}$, or for mechanism 1 with glucose 1-[$^{32}$P]phosphate if isomerization of the phosphoenzyme is equally slow.

unlabelled glucose 1-phosphate was added and changes in the concentration of glucose 1-phosphate and glucose 1-[$^{32}$P]-phosphate activity were followed as the system relaxed back towards equilibrium. The results from a number of such experiments at low concentrations of glucose monophosphates are shown in Fig. 1. With $^{32}$P-labelled substrates, co-transport of glucose 1-[$^{32}$P]phosphate into glucose 6-phosphate was observed. The activity of glucose 1-[$^{32}$P]phosphate fell to 37% of its original value and then returned to that value as chemical equilibrium was reached. Any fall in $^{32}$P radioactivity in the 1-position of glucose 1,6-biphosphate would not affect this value significantly. The fall in glucose 1-[$^{32}$P]phosphate radioactivity is very similar to that expected for a phosphoenzyme mechanism (see line 3, Fig. 1).

In similar experiments with $^{14}$C-labelled substrates no induced transport was observed (line 1, Fig. 1). Taken together these results support mechanism 1 and exclude mechanisms 2 and 3. However, mechanism 3 could give rise to the observed induced-transport pattern if the isomerization of the enzyme–glucose 1,6-biphosphate complex is rate-limiting as the degree of co-transport for $^{14}$C- and $^{32}$P-labelled substrates is decreased towards that of mechanism 1. This possibility has been excluded, for to decrease the $^{14}$C-co-transport to less than that indicated by line 4 in Fig. 1, the $K_m$ for glucose 1-phosphate would need to be five times less than the experimental value (see Britton & Clarke, 1968).

Similarly any isomerization of the phosphoenzyme (mechanism 1) should decrease the extent of $^{32}$P-co-transport and lead to $^{14}$C-counter-transport especially at high substrate concentrations. No $^{14}$C-counter-transport was observed, and under these conditions, experiments indicated that the rate constants for any isomerization must be in excess of $2.7 \times 10^7 s^{-1}$. Thus these results are only compatible with mechanism 1 or 1′.

To determine the nature of the phosphoenzyme, 10 units of enzyme were incubated for 1 min at 30°C with 0.25 $\mu$Ci each of $^{32}$P- and $^{14}$C-labelled glucose 1,6-biphosphate in 160 mM-histidine/40 mM-tris/5 mM-$MgCl_2$, pH 7.4. In the same buffer at 4°C, the mixture was passed down a Sephadex G-25 column. No radioactivity was associated with the enzyme fraction, which suggests that the phosphoenzyme is unstable (probable half-life <6 min at 4°C). In contrast the phosphoenzyme from muscle is stable. Gel electrophoresis by the method of Laemmli & Favre (1973) showed that the isoenzyme patterns of the rabbit muscle and liver enzymes are different. It is therefore possible that the apparent bisphosphatase activity associated with the liver enzyme is of metabolic importance.

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# Hexose monophosphate shunt and labelling of amino acids in rat brain after injection of [U-$^{14}$C]glucose

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Glucose is metabolized in the adult brain mainly by glycolysis; only a small amount, up to 1.3% in rat brain and 5.8% in monkey brain, is oxidized directly by the hexose monophosphate shunt pathway (Hostetler *et al.*, 1970; Gaitonde & Arnfred, 1971; Coleman & Allen, 1978). Since [U-$^{14}$C]glucose administered to rats and cats (see Gaitonde *et al.*, 1965) is rapidly converted into $^{14}$C-labelled glutamate and associated amino acids, it was of interest to investigate whether a part of the labelled amino acids was formed subsequent to the direct oxidation of [U-$^{14}$C]glucose by the hexose monophosphate pathway. It was decided, therefore, to block the hexose monophosphate pathway by inhibiting the activity of 6-phosphogluconate dehydrogenase by injecting 6-aminonicotinamide to rats. 6-Aminonicotinamide is incorporated into NADP and NAD giving 6-aminonicotinamide analogues of these cofactors, i.e. 6-aminonicotinamide–adenine dinucleotide phosphate and 6-aminonicotinamide–adenine dinucleotide respectively, which are strong competitive inhibitors of the enzymes utilizing the natural cofactors. In the brain the main biochemical observation is a severalfold increase of 6-phosphogluconate, indicating almost complete inhibition of 6-phosphogluconate dehydrogenase (Herken & Lange, 1969). Animals treated with 6-aminonicotinamide show a variety of neurological abnormalities, e.g. irreversible paralysis, impaired righting responses, unsteadiness of gait, anorexia and blindness. In 2 days the animals show lesions in the central nervous system (spinal cord and brain) confined to the grey matter, sparing the white matter and peripheral nerves (Wolf *et al.*, 1959).

At 30 min after administration of [U-$^{14}$C]glucose to rats paralysed in 4 h after an injection of 6-aminonicotinamide the $^{14}$C content of brain amino acids decreased by 16% ($P < 0.001$). This decrease was reflected in glutamate and associated amino acids. The pool sizes of glutamate and 4-aminobutyrate decreased ($P = 0.01$), glucose increased ($P < 0.01$), lactate slightly decreased ($P < 0.05$) in the brain of the treated rats. The specific radioactivities of brain glucose and lactate in the treated group were similar to those in the control group (five animals in each group). The $^{14}$C content of brain 6-phosphogluconate was significantly higher in the treated group; this indicated that the hexose monophosphate shunt pathway was blocked or strongly inhibited. The results are interpreted as evidence that the triose phosphate formed in the adult brain by the hexose monophosphate pathway and subsequently glycolysed contributes to a measurable extent to the labelling (or formation) of glutamate and 4-aminobutyrate. Thus the inhibition of the hexose monophosphate shunt pathway in certain (neuronal) brain cells might be responsible for the disturbance in the normal functioning of the brain.

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