A thesis entitled

# "THE MECHANISMS OF SOME PHOSPHOMUTASE REACTIONS"

by

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## ABSTRACT

The mechanism of reaction of rabbit-muscle phosphoglucomutase and phosphoglycerate-mutase have been examined using the flux approach of Britton. By induced-transport tests and by the measurement of the ratio of the fluxes of $^{14}C$- and $^{32}P$-labelled substrates from reactant to product at chemical equilibrium it has been possible to distinguish between the three mechanisms proposed for these enzymes on the basis of evidence from conventional techniques. The rate constant for isomerization of the phosphoenzyme of phosphoglucomutase has been measured and a lower limit determined for the isomerization of the phosphoenzyme moiety of Phosphoglycerate-mutase. Unlike kinetic methods the results obtained by this technique are unequivocal, and do not rely on either a detailed knowledge of the reaction mechanism or upon the assumption that the rate constants are invarient. The influence of isotope effects and the non-ideal behaviour of solutions upon the interpretation of results is also considered and the theoretical background for the induced transport test is presented.

For the phosphoglycerate-mutase system a series of preliminary experiments were carried out to determine the

magnesium binding constants with 2PGA and 3PGA, and a redetermination of the equilibrium constant was carried out under standard conditions. A kinetic investigation using an $NAD^+$/NADH coupled assay at low substrate concentrations showed the basic Lineweaver-Burke kinetic pattern to be one of parallel-line plots. The basis of this pattern as a kinetic test for a phosphoenzyme pathway is criticised and it is shown that a random sequential pathway can produce such a pattern without any assumptions about the nature of the enzyme intermediate. The $K_m$'s, $K_i$'s, $V^f_{max}$, $V^r_{max}$, of reaction were determined at constant $\mu$ and $[Mg^{2+}]_{free}$.

On the basis of the available evidence a new reaction scheme for phosphomutases is proposed and further applications of the flux technique suggested.

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CONTENTS

Page

Abstract ... ... ... ... ... ... ... i

Acknowledgements ... ... ... ... ... iii

Contents ... ... ... ... ... ... ... iv

Chapter I: Historical Survey

Introduction ... ... ... ... ... 1

Section H.1. Phosphoglucomutases ... ... 2

Section H.2. Phosphoglycerate-mutases ... 20

Chapter II: Materials and Methods

Section M.1. Phosphoglucomutase system ... 32

Section M.2. Phosphoglycerate-mutase system 51

Chapter III: Results

Section R.1. Phosphoglucomutase system:- Preliminary studies; Validation of techniques 78

Section R.2. Phosphoglucomutase system:- Equilibrium constant; Effect of monophosphate concentration ... ... ... ... ... 100

Section R.3. Phosphoglycerate-mutase system:- Preliminary studies; Validation of techniques 109

Section R.4. Binding constants of 2PGA, 3PGA and 8-hydroxyquinoline with $Mg^{2+}$ ... ... ... 129

Page

Section R.5. Studies of the equilibrium constant of the phosphoglycerate-mutase reaction 149

Section R.6. Measurement of $K_m$'s of 3PGA and 23PGA and $V^f_{max}$ and $V^r_{max}$ for phosphoglycerate-mutase ... ... ... ... ... ... 158

Section R.7. Phosphoglucomutase system:- Induced transport experiments ... ... 194

Section R.8. Phosphoglycerate-mutase system:- Induced transport experiments ... ... 223

Chapter IV: Theory

Section T.1. Theory of induced transport tests 257

Section T.2. Kinetics of reaction ... 312

Section T.3. Calculations of $Mg^{2+}$ binding constants with 8-hydroxyquinoline, 2PGA and 3PGA; Equilibrium constant of the phosphoglycerate-mutase reaction ... ... 335

Chapter V: Discussion

Introduction ... ... ... ... ... 342

Section 1. Preliminary investigations of the phosphoglycerate-mutase system: Binding constants of $Mg^{2+}$ with 2PGA and 3PGA ... 344

Section 2. Induced transport tests ... 359

References ... ... ... ... ... 393

# CHAPTER 1

# HISTORICAL SURVEY

## INTRODUCTION

The purpose of this survey is to outline the present state of knowledge of the mechanism of reaction of phosphoglycerate mutase and phosphoglucomutase and to compare and contrast what is known about the nature of action of each of these enzymes when obtained from different sources. Furthermore the survey is designed to show in what way this knowledge is ambiguous and to suggest means by which these ambiguities can be removed and understanding of the mode of action of these enzymes extended.

The important features of these enzyme reactions revolve around the necessity for a cofactor and its role in catalysis. In addition the phosphoglucomutases require magnesium for activity and they are activated when incubated with a suitable buffer such as histidine or cysteine. Broadly speaking the enzyme reactions have been studied by three separate experimental approaches which are: kinetic investigations, isotopic labelling experiments, and isolation of enzyme intermediates. The results of these investigations are reported in the subsequent sections.

SECTION H1. PHOSPHOGLUCOMUTASES

The phosphoglucomutases appear to have very similar molecular weights (62,000 for the rabbit-muscle enzyme[130], 63,000 for the flounder muscle enzyme[56] and the shark muscle enzyme, 58,000 for that from Micrococcus lysodeikicus[80]). The molecular weights for the enzymes from Bacillus cereus and Escherichia coli are less certain but lie in the ranges 58,000-63,000 and 62,000-65,000 respectively[80, 117]. The rabbit muscle phosphoglucomutase exists in multiple forms[82], four active peaks of the non-aggregated enzyme being obtained by isoelectric focussing.

Most of the enzymes contain SH groups. The rabbit muscle enzyme has 2 though they have no effect on the activity of the enzyme[56]. Flounder muscle enzyme has four SH groups. Removal of one by C.M.B. titration stimulates the enzyme activity but after the second has reacted the enzyme activity returns to its original level. Titration of the remaining groups has no effect[56]. The enzyme from shark-muscle has two SH groups but they exert little or no effect upon the enzyme reactivity whereas the E. coli enzyme has only one SH group which is essential for enzyme activity[117]. M. lysodeikticus phosphoglucomutase has 4 SH groups. Titration of these with C.M.B. only partially suppresses enzyme activity. The pH optimum for the muscle enzymes is

7.4 whereas for the bacterial enzymes it is higher; 8.3 for B. cereus, 8.5 for M. lysodeikticus and 8.5-9.0 for E. coli

All the enzymes require G16P and $Mg^{2+}$ for maximum activity, and all, apart from the enzymes from M. lysodeikticus and B. cereus have been isolated as phosphoenzymes[80, 56]. It is interesting that these latter two enzymes are said to show no diphosphatase activity whereas the rabbit muscle enzyme shows a small, and the flounder and shark muscle enzymes show a moderate diphosphatase activity[56, 80].

Phosphoglucomutase from rabbit muscle normally requires in addition to magnesium ions[35, 76] a complexing agent such as imidazole, histidine, or EDTA for optimal activity[76, 77]. Milstein[77] suggested that the role of the complexing agent was to bind heavy metal ions such as $Zn^{2+}$ or $Cu^{2+}$ to which the enzyme was very sensitive. Inhibition is caused by competition between $Mg^{2+}$ and $Zn^{2+}$ or $Cu^{2+}$ for the enzyme (rather than by mercaptide formation with the reactive SH groups)[77, 78] with inhibition constants of $4 \times 10^{-14}$M for $Zn^{2+}$ and $2 \times 10^{-17}$M for $Cu^{2+}$. Complete removal of the inhibiting metal ions from both the enzyme and the reagents eliminates the assay requirements for the complexing reagent[77, 6] but it is still necessary to

pre-incubate the enzyme prior to assay with a complexing agent (E.D.T.A., histidine, etc.) and magnesium[77, 79] to obtain maximum activity. Histidine was a particularly effective activator for the rabbit-muscle enzyme. Harsham et al.[79] postulated that the activated state of the enzyme was maintained by virtue of a magnesium:ligand:enzyme complex, though Milstein[77] favoured activation by a slow removal of inhibiting metal ions by the complexing agents followed by their replacement by magnesium ions. Ray and Roscelli[6] in a later investigation, preferred Milstein's interpretation.

Though rabbit muscle phosphoglucomutase shows a 4-5 fold increase in activity when the untreated enzyme is incubated with histidine or imidazole and magnesium at pH 7.4[33], the phosphoglucomutases from flounder or shark muscle only showed a 1.5-2 fold stimulation in activity under the same conditions[56]. Maximal stimulation was obtained with cysteine rather than histidine (1.7-2.2 fold stimulation)[56]. Preincubation of phosphoglucomutase from bacterial sources (Micrococcus lysodeikticus, Bacillus cereus, and Escherichia coli) with histidine or imidazole and magnesium at pH 8.3 showed no stimulation of activity[80] and the requirement of the M. lysodeiktius enzyme for cysteine was not reduced by this treatment.

The early literature concerning the rabbit muscle phosphoglucomutase reaction, defining the properties of the enzyme, the thermodynamics and kinetics of reaction can be found in reviews by Najjar[33], and Hummel and Kalnitsky[81]. Leloir et al.[101] showed that the enzyme required G16P cofactor for activity. Najjar[86] isolated a phosphoenzyme and proposed a mechanism in which the enzyme alternates between a phospho- and dephospho form (i.e. a ping-pong mechanism), see Scheme H.1.1. Najjar also proposed that the EP which combines with G1P may be different from that which combines with G6P.

Scheme H.1.1.

$$\text{G1P} + \text{P-enzyme} \underset{}{\overset{K_1}{\rightleftharpoons}} \text{G16P} + \text{enzyme} \quad \ldots \quad \text{H.1.1.}$$

$$\text{G16P} + \text{enzyme} \underset{}{\overset{K_2}{\rightleftharpoons}} \text{G6P} + \text{P-enzyme} \quad \ldots \quad \text{H.1.2.}$$

An alternative mechanism was proposed by Cleland[53, 128] on the basis of Bodansky's substrate-velocity measurements[129]. In this Scheme (M.1.2 below) the diphosphoenzyme facilitates the direct transfer of phosphate

between G1P (or G6P) and G16P. This is essentially a sequential addition mechanism and is very similar to mechanism 3 in Fig. T.1.3 of the Theory section.

Scheme H.1.2.

$$E + G1P + G16P \rightleftharpoons E + G16P + G6P \quad \ldots \quad H.1.3.$$

The kinetics of rabbit-muscle phosphoglucomutase have been extensively investigated by Ray and Roscelli[1, 6, 17, 32, 83, 84] in an attempt to discriminate between these two alternative pathways. This work has been reviewed by Cleland[85]. A series of careful experiments[1] showed that varying the cofactor G16P, without which the reaction could not be sustained (apart from a small fraction, representing 0.7-2% of the total enzyme activity which appeared to be G16P independent) and G1P gave parallel initial velocity patterns, apparently confirming the ping-pong mechanism proposed by Najjar and Pullman[86] in which the enzyme alternates between the phospho- and dephospho-forms as shown in the Scheme H.1.1. The phosphate is covalently bound to the enzyme by a seryl hydroxyl group[91, 100].

However, when $^{32}P$-labelled phosphoenzyme was incubated with G1P and G16P cofactor for short periods (less than 1 min), at intermediate ionic strength $\mu \simeq 0.1$ most of the label was found in G6P rather than the diphosphate. This observation shows that G16P cofactor need not be an obligatory intermediate in the reaction as would be required by a ping-pong mechanism. Thus G16P does not have to dissociate from the enzyme and reassociate as required by scheme H.1.1 and the EP-G1P complex must directly isomerize into the EP-G6P complex. The requirement of the reaction for cofactor would be met by the two EP-monophosphate complexes being in equilibrium with a dephosphoenzyme-G16P complex which releases G16P slowly at about 5% of the rate of release of G6P.

Such a mechanism, Scheme H.1.3, would also have a parallel line initial velocity pattern typical of a ping-pong mechanism, and is identical with the phosphoenzyme mechanism shown in Fig. T.1.1 of the Theory section.

Scheme H.1.3.

$$G1P + EP \rightleftharpoons ES \rightleftharpoons G6P + EP$$

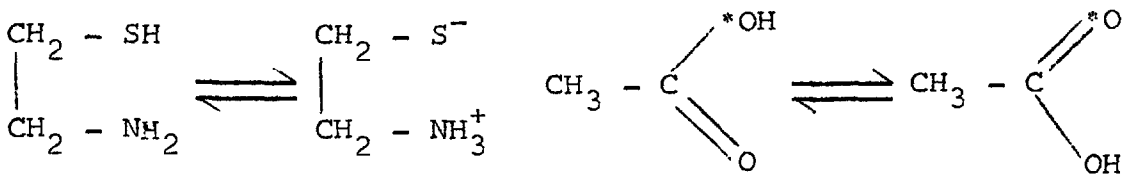
$$ES \rightleftharpoons E + G16P$$

In order that the kinetic scheme should not be complicated by the addition and release of magnesium (which may also be regarded as substrate) to the various intermediate enzyme complexes these reactions were carried out at saturating levels of magnesium (see later).

Hashimoto and Handler also conducted experiments in which the fate of $^{32}P$ labelling of G1P during the course of reaction was investigated. They confirmed Ray and Roscelli's[1] observations that the rate of incorporation of $^{32}P$ into G16P was extremely slow. Indeed their results suggested that the rate of dissociation of G16P from the *ES* complex was even slower. However they found that when they attempted to prepare the phosphoenzyme under conditions which preserved the integrity of the enzyme, almost one mole of G16P remained bound[56]. They considered this difficult to reconcile with the phosphoenzyme mechanism and suggested that reaction might after all occur via the mechanism shown in Fig. T.1.3 of the Theory section as suggested by Cleland. If this is so the phosphoenzyme represents an artifact of the separation procedure.

Ray and Roscelli also looked for evidence for isomerization of the phosphoenzyme by trying to detect an uncompetitive component in product inhibition[32]. The

inhibition constant for iso-pathways[53] due to G6P was measured by comparing the mean velocity of reaction for 80% conversion of G1P to G6P with the integrated rate equation. This procedure could not, however, be used to measure the corresponding inhibition constant of G1P due to the unfavourable equilibrium constant so that it was necessary to use the appropriate Haldane relationship. From these inhibition constants it was deduced that in order to explain the strictly competitive inhibition observed at product concentrations up to 0.1 M that the rate constant for interconversion of the two forms of the phosphoenzyme must be greater than $10^7$ $sec^{-1}$. This rate constant is considerably larger than the maximum velocities for any known group transfer in an enzyme reaction[87] and is of the same order of magnitude as rate constants for simple hydrogen transfer in aqueous solution of the types shown below[87, 88].



Consequently Ray and Roscelli concluded that there was no evidence of a phosphoenzyme isomerization in the magnesium-phosphoenzyme complex.

Phosphomutases from other sources have not been so intensively investigated. However, the flounder and shark muscle enzymes[56] as well as the bacterial enzymes have an obligate requirement for cofactor G16P and magnesium in order to remain active. Kinetic studies of the flounder and shark muscle enzymes showed that the double reciprocal Lineweaver-Burke plots had a parallel line pattern characteristic of the ping-pong type mechanism. However, the $K_m$'s for G1P and G16P were between one and two orders of magnitude greater than for the rabbit-muscle enzyme. Moreover, the phosphorylated forms of the enzymes were comparatively unstable, spontaneously hydrolysing to dephosphoenzyme and inorganic phosphate at a significant rate, and this may affect the initial velocity pattern (see Theory section).

Joshi and Handler[117] prepared the bacterial phosphoglucomutases from *E. coli* and Hanabusa *et al.*[80] isolated the phosphoglucomutases from *M. lysodeikticus* and *B. cereus*. The pH optima of these enzymes is approximately pH 8.4, which is different from the pH optima of the muscle enzymes, pH 7.4. Further as mentioned previously they require cysteine for activity. The enzyme from *E. coli* was

shown to form a phosphoenzyme but no phosphoenzymes were isolated from B. cereus or M. lysodeikticus nor did these enzymes exhibit phosphatase activity. The initial velocity pattern for the mutases from B. cereus and M. lysodeikticus showed intersecting straight lines whereas the mutase from E. coli gave parallel lines. However, the patterns of the latter two enzymes were distorted by a marked competitive inhibition of G1P by G16P. The $K_m$'s for G16P and G1P for the bacterial enzymes were roughly one to two orders of magnitude larger than the $K_m$'s for the rabbit muscle. Though the failure to isolate phosphoenzymes from M. lysodeikticus and B. cereus might have been due to this, the intersecting initial velocity pattern suggested to the authors of these papers that the true mechanism of reaction is shown in Scheme H.1.2 (i.e. mechanism 3 in Fig. T.1.3). The distribution of $^{32}P$ labelling into the substrates from $[^{32}P]$ G1P during the course of reaction with the M. lysodeikticus enzyme showed that unlike the reaction with the rabbit muscle enzyme isotopic equilibrium between the monophosphate and diphosphate species was achieved very rapidly even in the presence of excess G16P[80]. They considered that this also favoured the proposed scheme.

It is rather surprising that phosphoglucomutase from

different sources should apparently have such different mechanisms. In this connection it is of interest that Brody and Tatum[90] found that phosphoglucomutase from the wild forms of Neurospora crassa gave kinetics resembling those of M. lysodeikticus (i.e. intersecting initial velocity pattern) whereas the enzyme obtained from a mutated strain gave kinetics resembling the rabbit muscle enzyme (i.e. parallel line pattern). Unless there are two distinct enzymes involved it is unlikely that a mutation would alter the reaction mechanism.

Thus phosphoglucomutases show a wide range of behaviour, and two mechanisms (Schemes H.1.2 and H.1.3) have been proposed to account for this behaviour. However, a third mechanism should be considered in which there is a direct intramolecular transfer of phosphate. Although transfer of $^{32}P$-labelled phosphate to G1P and G6P rather than G16P supports the first two mechanisms reaction times of the order of seconds were used which is a relatively long period compared with the turnover number, 750 $sec^{-1}$ (for the rabbit enzyme). It is conceivable, therefore, that phosphate may have been transferred by side reactions and the normal enzyme reaction might be the direct transfer of phosphate from the 1-position to the 6-position of glucose with the phosphate from

the phospho-enzyme (or a phosphate from the EG16P complex) performing some indirect role such as the formation of a pyrophosphate bond. This mechanism is shown in Fig. T.1.2 of the Theory section. Such a direct transfer of phosphate might well explain the residual activity of rabbit-muscle phosphoglucomutase in the absence of co-factor.

It may be shown (see Theory section) that the kinetic evidence just described may not distinguish unequivocally between the proposed mechanisms. Thus the phosphoenzyme mechanism need not necessarily give rise to a parallel-line Lineweaver-Burke pattern. If some phosphatase activity is associated with the mutase activity, as is the case with the flounder and shark muscle phosphoglucomutases, either a parallel or intersecting line reciprocal initial velocity pattern can be obtained depending upon the relative rates of dissociation of G16P from the enzyme, $k_{-4}$ (see Theory section T.2, Scheme 1), and the rate of hydrolysis of the phosphoenzyme, $k_5$. If $k_{-4} > k_5$ then a parallel line plot is obtained; if $k_5 = k_{-4}$ an intersecting line pattern is observed. Thus as the diphosphatase activity of the enzymes have rate constants of about 1 mole/mole enzyme/hr. the rate constant for EG16P dissociation ($k_{-4}$) must be greater than this value. Such a value would not be inconsistent with the rate at which radioactivity accumulates in G16P from G1P.

For the flounder muscle the rate is much greater than for the rabbit-muscle enzyme, though not as great as the *M. lysodeikticus* enzyme[56]. This latter enzyme, however, has been reported to possess no diphosphatase activity and if this is true the intersecting line initial velocity pattern found for the enzyme cannot be explained in terms of a modified phosphoenzyme mechanism.

Similarly, from a consideration of the generalized mechanism for sequential addition of substrates (in which the order of addition of substrates may be random, see Theory section), it can be shown that under certain conditions this mechanism can give a parallel line initial velocity pattern as well as one in which the lines are intersecting. In particular, for example, if the affinity of the free enzyme for G16P is much greater than that of the enzyme combined with substrate (i.e. in Scheme T.2.5, if $h \gg g$, for d, c small) then the parallel line pattern is obtained. Consequently, this test cannot be used to distinguish between an EP and an EG16P sequential mechanism.

The situation is even more complicated for although the catalytic enzyme complex may be EG16P only one of the phosphates may take part in the reaction. The actual mechanism may therefore be essentially that of a phosphoenzyme. Consequently isolation of enzyme intermediates

may be no guide to mechanism.

Thus it seems that the conventional kinetic approach even aided by information regarding the enzyme intermediates is unable to distinguish between the possible mechanisms for these enzymes. Hence there remain three possible mechanisms of action for the phosphoglucomutases, which are shown in Figs. T.1.1-3 of the Theory section. Consequently it was decided to investigate the rabbit-muscle enzyme reaction using the flux approach of Britton[59-63]. In Figs. T.1.1-3 of the Theory section are shown the fates of $^{14}C$-labelling of the glucose chain and $^{32}P$-labelling of the phosphate group of G1P during reaction by each mechanism. As can be seen by inspection, of the figs. the minimum number of sequential reactions of enzyme with G1P required to transfer $^{14}C$- and $^{32}P$-label from G1P to G6P is different for each. Consequently this approach in which "Induced transport tests" are carried out can be used to distinguish between them (see Theory section). The method also provides a very sensitive test for isomerization of the phosphoenzyme. Further details of these tests are reported in the theory section and the interpretation of the results is considered in the Discussion section. No attempt has been made in the present investigation to conduct studies upon a single isoenzyme.

The enzyme has been used as supplied by the manufacturer (C.F. Boehringer and Soehne).

Role of Magnesium in rabbit-muscle phosphoglucomutase reaction

As discussed earlier magnesium is an obligate activator for rabbit-muscle phosphoglucomutase. It is initially required in conjunction with a complexing agent such as E.D.T.A. or histidine to remove heavy metals from the enzyme which render it inactive. This process usually takes place during preincubation before the fully activated enzyme is used in kinetic studies. The subsequent role of magnesium during reaction is complex as it behaves not only as an activator which may dissociate from enzyme intermediates but also as an inhibitor. Ray and Roscelli[1] showed that inhibition was competitive with G1P and G16P and that the increase in $K^{app}_{G16P}$ with increasing magnesium concentrations was comparable with the expected decrease in the free tri- or tetraanion of G16P due to complex formation with magnesium. Similarly the increase in $K^{app}_{G1P}$ could be shown to parallel the formation of the G1P magnesium complex. Ray and Roscelli therefore concluded that the magnesium inhibition observed at high magnesium ion concentrations was a result of magnesium forming complexes with the substrates which did not react

with the enzyme.

If $Mg^{2+}$ is also a substrate for the enzyme the kinetic pathways become very complex. The initial velocity plots obtained by varying magnesium at different G1P levels (G16P saturating) and at different G16P levels (G1P saturating) yielded an intersecting line pattern in contrast to the parallel line patterns obtained at saturating magnesium for the two phosphates[17]. The initial velocity of reaction obeyed the equation H.1.4 shown below:

$$\frac{V_{max}}{V_i} = 1 + \frac{K_{Mg}}{[Mg^{2+}]} + \frac{K_{G1P}}{[G1P]}\left(1 + \frac{{}^{1}K_i[Mg]}{[Mg^{2+}]}\right) + \frac{K_{G16P}}{[G16P]}\left(1 + \frac{{}^{1,6}K_i[Mg]}{[Mg^{2+}]}\right)$$

..... H.1.4.

which reduces to the simple equation for a ping-pong mechanism at saturating levels of magnesium. The symbols have their usual interpretations. Linear reciprocal plots were obtained when the initial rate at which $[^{32}P]$ G6P appears after the addition of a trace of $^{32}P$-labelled G1P was added to an equilibrium mixture of the phosphates was plotted against G1P concentration (with G16P and magnesium saturating) and when plotted against free magnesium

concentration (G1P and G16P saturating)[17]. Raising the magnesium level in the first case and the G1P level in the second did not measurably affect the gradient of the reciprocal plots, thus showing that the concentration of G1P producing half maximal exchange rate is independent of magnesium concentration, and that the magnesium ion concentration producing half maximal exchange rate is independent of G1P concentration. Ray, Roscelli and Kirkpatrick assumed a phosphoenzyme scheme of reaction and compared the expected initial velocities and isotope exchange rates at different substrate concentrations for each of the possible ordered or random pathways with the predicted values from equation H.1.4. They concluded from this analysis that the addition and release of magnesium and reactants was random during at least some of the stages in the reaction.

When enzyme solutions containing known levels of magnesium were added to a solution containing substrate, cofactor and $Zn^{2+}$ ions, the enzyme was slowly inactivated. This was interpreted to mean that the magnesium had dissociated from the enzyme and had been replaced by $Zn^{2+}$ ions[83]. At sufficiently high levels of zinc, every enzyme molecule that releases magnesium should immediately react with zinc, and the rate of inactivation should be

determined by the rate of dissociation of $Mg^{2+}$. This "all or none" assay was used to determine the dissociation constants of the magnesium-phosphoenzyme complex and to determine the rates of dissociation of magnesium from the dephosphoenzyme, the phosphoenzyme, and the central enzyme complex MgEG16P by appropriate choice of experimental concentrations[83, 84]. In this way rate constants for the release of magnesium from the $Mg^{2+}$-phosphoenzyme, $Mg^{2+}$-central complex and $Mg^{2+}$-dephosphoenzyme of 0.07, 0.03 and 0.22 $sec^{-1}$ respectively were obtained, and the dissociation constant of the $Mg^{2+}$-phosphoenzyme was found to be 29 μM. The dissociation rates can be compared with the rate constants for release of G16P, G6P and G1P from their respective enzyme complexes of 38, 750 and 250 $sec^{-1}$. Thus though the mechanism is effectively random the disparity in the rate constants implies that it is effectively kinetically ordered. Magnesium thus seems to dissociate so rarely from any of the complexes that alternative pathways of formation and breakdown of the active enzyme complexes do not contribute to the observed kinetics of reaction. The Lineweaver-Burke plots remain linear despite the random order of addition of reactants as the rate of release of magnesium from the various enzyme intermediate forms was very slow compared with the rate of reaction.

SECTION H.2. PHOSPHOGLYCERATE MUTASES

The phosphoglycerate-mutases derived from muscle tissue, yeast and plants have been investigated and the preparations and properties of the enzymes described by various authors prior to 1962 summarized in reviews by Pizer[48] and Grisolia[99]. Molecular weights of 57,000[92] and 64,000[103] have been reported for the rabbit-muscle enzyme and there is evidence that the molecule is made up of two subunits with molecular weights of about 27,000[123]. The chicken breast enzyme has a molecular weight of 65,690[70] and the yeast enzyme one of 112,000[92]. The enzymes have SH groups which are necessary for activity[48] and most show a small extent of diphosphatase activity (see later). Unlike phosphoglucomutase attempts to detect metal ion activation failed even when steps were taken to reduce the level of metal ions bound by protein though zinc, silver, and cupric ions were shown to be inhibitory[44, 93]. In the case of zinc the inhibition has been shown to be due to the formation of $Zn^{2+}$-2PGA and $Zn^{2+}$-23PGA complexes which are unable to react with the enzyme[93, 94]. However, the specific nature of the inhibition of other metals is unknown.

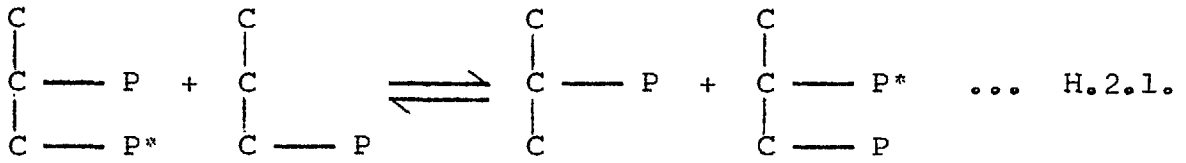
The muscle enzyme has been shown to require 23PGA as cofactor for activity[95] as has the enzyme isolated from

yeast[92], though in this case about 10% of the activity was retained in the absence of 23PGA[125]. On the other hand the enzymes purified from plant sources do not require 23PGA for activity[45, 96]. It is of interest that arsenate has been claimed to partially activate the muscle and yeast enzymes[97].

Boundary electrophoresis of the purified rabbit-muscle enzyme has shown that two isoenzymes exist, though the enzyme was predominantly in one form[98]. The same procedure applied to purified preparations of the yeast enzyme showed the presence of six components[43] five of which possessed phosphoglycerate mutase activity. No data, however, is available for the presence of isoenzymes in preparations of phosphoglycerate mutase from wheat or rice germ.

The mechanism of action of rabbit and chicken breast muscle phosphoglycerate-mutase has been investigated by several authors. Sutherland et al.[95] showed that 23PGA was necessary as cofactor for the enzyme and showed that $^{32}P$-labelled 23PGA phosphoryl groups could interchange with 3PGA phosphoryl groups. The stimulation of the mutase by 23PGA was interpreted by analogy with the phosphoglucomutase system[101, 126] to indicate that enzymatic conversion of 3PGA to 2PGA occurred via an intermolecular transfer of a phosphoryl group as shown in equation H.2.1 below (this is the

equivalent of Scheme T.1.3).




... H.2.1.

It has also been shown that the enzyme will transfer $^{32}P$ from 23PGA to 3PGA analogues[102]. Unlike many of the phosphoglucomutases (see earlier), the phosphoglyceratemutases have not been isolated as stable phosphoproteins[44, 75]. An early claim was made that a protein with $^{32}P$ bound to serine could be obtained from the rabbit muscle enzyme but the $^{32}P$ could not be exchanged with the substrates[103, 48]. A later investigation by Zwaig and Milstein[22, 104] showed that these findings were due to contamination of the enzyme with phosphoglucomutase and to an impure preparation of $^{32}P$ 23PGA which contained G16P. Using mild conditions for isolating the active enzyme intermediate Grisolia et al.[105] found, after incubation of the rabbit muscle with [$^{32}P$] 23PGA two moles of phosphate bound per mole of enzyme. Almost all of the phosphate of this intermediate exchanged with 3PGA but was acid labile (and alkali labile) and consequently could not be bound to a serine group. Subsequently Jacobs and Grisolia[75] chromatographed this

complex and found approximately equal quantities of $P_i$ and 3PGA. They therefore suggested that it was an enzyme 23PGA complex rather than a phosphoenzyme. (Binding of 23PGA has also been demonstrated for the chicken-breast enzyme[70].) The release of $P_i$ from this enzyme 23PGA complex by acid and alkali contrasts markedly with the extreme stability of the phosphate ester bonds of the uncomplexed 23PGA[106]. Consequently the enzyme bound cofactor must be so distorted that it is no longer a diphosphoglycerate ester in the chemical sense[22, 104]. It has since been shown that the phosphate-protein bond in the complex becomes stable to alkali after treatment with sodium dodecyl sulphate (S.D.S.) although its lability to acid is retained[123]. The stability to different pH's at 46°C is very similar to that of 3-phospho-histidine and after alkaline hydrolysis [$^{32}$P] phosphohistidine was isolated. Thus it seems that in the case of rabbit-muscle phosphoglycerate-mutase the phosphate may be bound to histidine groups rather than serines as in the case of rabbit-muscle phosphoglucomutase. Electrophoresis of the complex after S.D.S. treatment on polyacrylamide gels containing S.D.S. indicated two equal subunits of molecular weight about 27,000, each containing one $^{32}$P. The author considered it to be more likely that the complex was a true phosphoenzyme (as

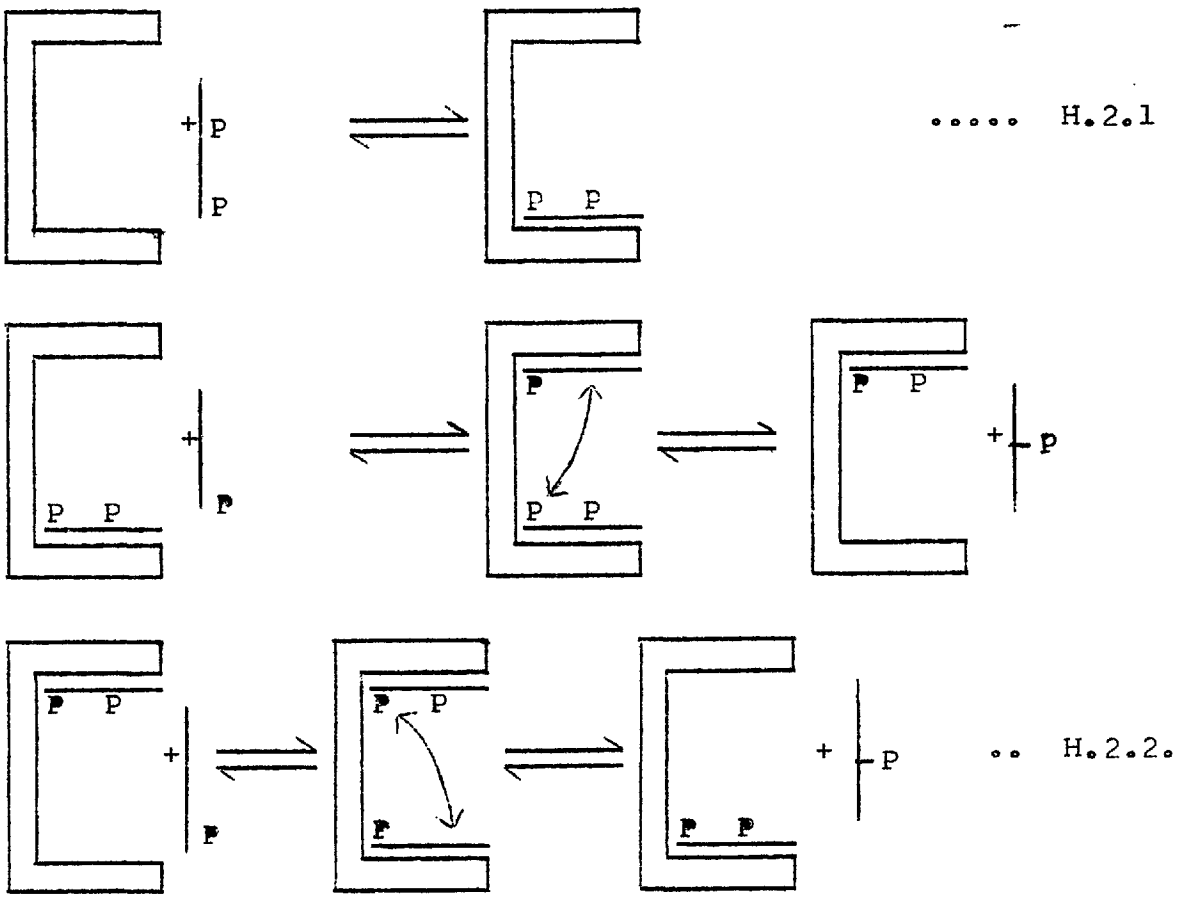
it was readily hydrolysed) rather than as enzyme-23PGA complex, although the absence of the glyceric acid moiety was not confirmed by $^{14}C$-labelling experiments. The author also concluded that the phosphoglycerate-mutase has two active centres. Jacobs and Grisolia[75], in contrast had ascribed the cause of hydrolysis to distortion of the 23PGA and had concluded that there is only one active centre. It does not seem to be possible to distinguish between these possibilities on the data at present available.

Jacobs and Grisolia[75] showed that free 23PGA need not be an obligatory intermediate in conversion of 3PGA to 2PGA or vice versa. Cascales and Grisolia[57] confirmed this observation using $^{32}P$-labelled substrates for both the yeast and the chicken breast muscle enzymes. At low ionic strength $^{32}P$-label from 3PGA appears almost exclusively in 2PGA with virtually none in 23PGA provided that 2PGA was rapidly removed with enolase, pyruvate kinase, and lactic dehydrogenase. As the ionic strength is raised 23PGA becomes more extensively labelled and in 0.2-0.4 M KCl the specific activity begins to exceed that of 2PGA. In solutions containing 1 M or more KCl then incorporation of $^{32}P$-label into 23PGA is almost complete whereas incorporation into 2PGA is negligible. At low ionic strength 23PGA may dissociate from the enzyme only

once in every 100 catalytic cycles whereas at high ionic strength the dissociation of 23PGA is faster than the turnover rate of the enzyme. The maximum rate of exchange between E23PGA and 23PGA induced by salt occurs between pH 7 and 8 and has a pH profile unlike that of the catalytic reaction[44] where the pH optimum is from 1 to 3 pH units lower (muscle and yeast enzymes, respectively); however, it resembles the pH profile of the non 23PGA dependent mutases[99]. These investigations were carried out at concentrations of substrates which were several orders of magnitude greater than their $K_m$'s. The initial velocities of reaction under these conditions showed no inhibition of mutase activity by salt.

These findings together with the evidence for an enzyme-3PGA complex led Grisolia and Cascales[75] to suggest that the reaction proceeds by a sequential addition of substrates to the enzyme according to the mechanism set out in Scheme H.2.1 below:-

Scheme H.2.1.



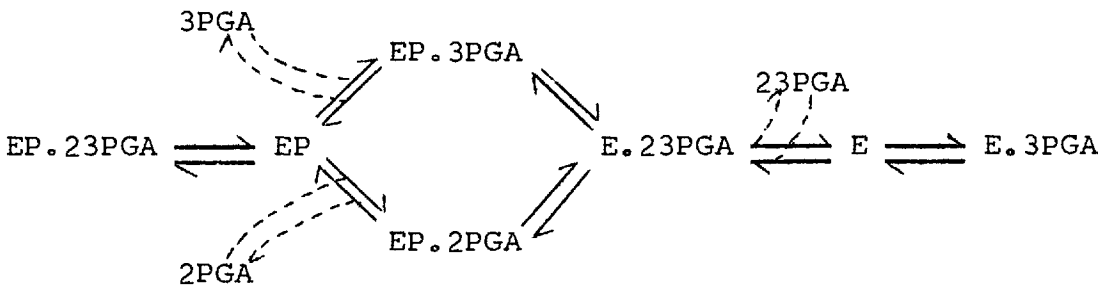

..... H.2.1

.. H.2.2.

The differences in mechanism observed at low and high ionic strength can be explained if salt is considered to labilize the E23PGA complex as shown in equation H.2.1. This scheme is a variant of that proposed by Sutherland et al.[95] (see also Theory section).

Grisolia and Cleland[54], however, subsequently examined the initial velocities at different concentrations of 3PGA and 23PGA. Their data was complicated by substrate inhibition but they concluded that the Lineweaver-Burke plots were basically of parallel line pattern at both low and high ionic strength. They therefore suggested in contradiction to Grisolia's earlier findings that the enzyme has a phosphoenzyme mechanism, as shown diagrammatically in Fig. H.2.2 even though no phosphoenzyme intermediate has been demonstrated. However, according to Koshland[107] such an intermediate is not necessarily isolable, nor demonstrable other than through kinetic data.

Scheme H.2.2.




The change in the labelling of 2PGA and 23PGA from [$^{32}$P] 3PGA with ionic strength was attributed to changes in the rate constant for dissociation of 23PGA from E.23PGA with

ionic strength.

In their study Grisolia and Cleland took no account of the variation in $Mg^{2+}$-binding by the substrates (see Theory section) which may alter the initial velocity pattern. Moreover, as discussed for phosphoglucomutase a parallel line initial velocity pattern does not necessarily signify a phosphoenzyme mechanism. In this context it should be noted that all phosphoglycerate mutases show some phosphatase activity, which expressed as a fraction of the mutase activity is 1/40,000 for the chicken breast muscle enzyme[57]; 1/100,000 for the yeast enzyme[57] and 1/152,000 for the wheat germ enzyme[105]. The reverse reaction, incorporation of inorganic phosphate into 3PGA or 23PGA does not happen, at least with the rabbit muscle enzyme[22]. Mantle and Garfinkel[51] have criticised Grisolia and Cleland's interpretation on the basis of computer stimulation studies as the experiments were carried out under the conditions such that $[23PGA] \ll K_{i23PGA}$ and $[3PGA] \gg K_{i3PGA}$. Simulation showed that the basic kinetic pattern is so overlaid by competitive inhibition under these conditions that this underlying pattern cannot be reliably discerned. This complication can be minimized by carrying out measurements of phosphoglycerate mutase activity with $[3PGA] < \frac{1}{2} K_{i3PGA}$ and $[23PGA] < \frac{1}{2} K_{i23PGA}$. (This requirement could not be met by their enolase coupled assay method, but the

$NAD^+$/NADH coupled method described in this thesis is capable of measuring initial velocities of reaction in the required concentration range.) It should be mentioned that determinations of $K_m$'s, $K_i$'s and $K_{eq}$ carried out in this thesis under conditions in which both the ionic strength of solution, and the free magnesium ion concentration are buffered yielded values differing significantly from those employed in the computer simulation study. However, the conclusions of Mantle and Garfinkel still probably apply.

In view of the contradictory nature of the observations and the difficulties of interpretation it seems that there are still at least three mechanisms of reaction still open to the muscle enzyme. These are shown for the phosphoglucomutase system in Figs. T.1.1-3 (Theory section). To obtain the corresponding mechanisms for phosphoglycerate mutase put G1P = 2PGA; G6P = 3PGA, and G16P = 23PGA. First of all there is the mechanism in which 23PGA plays the role of an allosteric activator. In this mechanism labelling of 23PGA would be achieved by side reactions. Such a mechanism is attractive as a common mechanism for phosphoglycerate mutase reaction as it could easily explain the ability of arsenate ions to activate the muscle enzyme, whereas if the cofactor 23PGA were more intimately involved

in phosphate and glycerate transfer the reason why arsenate can replace 23PGA as cofactor remains unclear. Mechanism 2 is the phosphoenzyme mechanism, or the alternative in which only one phosphate from the 23PGA takes part in the reaction and in which the remainder of the cofactor molecule may not dissociate from the surface of the enzyme. Finally mechanism 3 is the sequential mechanism referred to in Scheme H.2.1. These mechanisms are essentially identical to those proposed for the phosphoglucomutase.

No comparable kinetic studies have been carried out with the yeast enzyme. However, it would seem that as the yeast and muscle enzymes show many similarities their mechanisms of reaction are probably very similar.

As already discussed for phosphoglucomutase (see also Figs. T.1.1-3), the number of sequential reactions required to transfer $^{14}C$- and $^{32}P$-label from G1P to G6P is different for each mechanism. Consequently the flux approach of Britton[59, 60, 61, 62, 63] can be used to distinguish between them as described in the Theory section. Evidence was also sought for an isomerization of the "phosphoenzyme intermediate".

In the present work no attempt has been made to isolate a single isoenzyme for study. The rabbit-muscle

phosphoglycerate mutase was used as supplied by the manufacturer (C.F. Boehringer und Soehne). A series of preliminary investigations were also necessary to establish the values of the $K_m$'s, $K_i$'s and $K_{eq}$ for the rabbit muscle enzyme reaction and to establish the extent of magnesium binding of substrates under the experimental conditions employed.

# CHAPTER 2

# MATERIALS AND METHODS

## SECTION 1. PHOSPHOGLUCOMUTASE SYSTEM

### 1. Materials

G1P, G6P, and G16P, the monophosphates as the sodium salts and the diphosphate as the cyclohexylamine salt, rabbit skeletal muscle phosphoglucomutase as a crystalline suspension in 2.5M $(NH_4)_2SO_4$ and tris(trihydroxymethylaminoethane) were obtained from C.F. Boehringer & Soehne G.m.b.H.(Mannheim, Germany). L-histidine was obtained from Koch-Light Labs. Ltd., Colnebrook, Bucks., England. All glassware was cleaned with Decon 75 (A. Gallenkamp & Co. Ltd., London E.C.2) before use.

### Purification of Substrates

Both G1P and G6P were found to contain G16P as an impurity (see enzyme assay section). The $K_m$ of the enzyme for G16P is very low ($K_m = 6.2 \times 10^{-8}M$)[1] and it was therefore important that all traces of this impurity should be removed from the substrates used for the enzyme assay studies.

The purification of the sugar phosphates by column chromatography was described by Bartlett[2] with Dowex-1-Cl column and HCl as eluate, and by Ray and Roscelli[3] with

Dowex-1-formate and gradient elution with a pyridine-formic-acid buffer. The latter method was adopted since extremes of pH are not involved and the products are saline free. A Dowex-1-formate column 1.8 x 25 cm. was prepared from Dowex-1-Cl (8% cross linked 20-50 mesh). The column was first converted from the chloride to the hydroxide form with 2N NaOH. Complete conversion was confirmed by the absence of chloride in the eluate when tested with acidified $AgNO_3$ solution. It was then converted to the formate form with 2M formic acid. Pyridine-formic acid buffer was made up by the addition of formic acid, of known concentration, approximately 5M, to 10-20 ml. of pyridine until the pH reached a value of 3.0 (indicator paper). This solution was diluted until the concentration of formic acid was 0.75M. 500 mg. of G6P or G1P dissolved in about 10 ml. of water were applied to the column which was eluted with 1.2 l. of a linear gradient of water and the pyridine-formic acid buffer, at about 1 ml./min. Samples of 20 ml. were collected and G6P or G1P detected by Anthrone[4]. Recovery was greater than 400 mg. in 120 ml. of eluate. The fractions containing G1P or G6P were placed in a 1 litre flask and the solvent removed completely by freeze drying. The sugar phosphates were reconverted to the sodium salts by the method of McCready & Hassid[5] as follows. The solid was dissolved in a small volume of water (0.5-2 mls.) and 1 ml. of

a 1% solution of NaOH and 3 vols. methanol were added. The crystals of the $Na^+$ salt were collected by filtration. These were washed with alcohol and ether and then dried.

Assays carried out on both purified G6P and G1P showed that G16P had been completely removed by chromatography (see enzyme assay section).

Desalting of Phosphoglucomutase solution

The solution of phosphoglucomutase contained 2.5 M ammonium sulphate to stabilize the enzyme. Sulphate is an inhibitor of PGM[6] and therefore it had to be removed when concentrated solutions of enzyme were required. This was achieved with a Sephadex G-25 column. Sephadex G-25 was soaked in 9.6 mM tris chloride buffer pH 7.4 for about 1 hour, a column 1 x 15 cm. was prepared and was washed with more buffer. A sample of 0.025 ml. of the enzyme solution was applied and washed through the column with tris chloride solution taking care not to mix the enzyme solution with the buffer. The elution rate was restricted to less than one drop per second. Either 10 or 20 drops of the eluate were collected at a time and each successive sample was analysed for enzyme and sulphate. Sulphate ion was detected by adding a few drops of each sample to a drop of barium chloride solution and allowing the mixture to stand. Phosphoglucomutase was detected by observing the conversion of

G1P to G6P. Two drops of each elution sample were added to 0.2 ml. 50 mM G1P, 0.01 ml. 1 mg./ml. G16P and 0.5 ml. tris buffer pH 7.4 in a total volume of 1.0 ml. The mixture was incubated for 2 min. at room temperature (or 25°C) and the reaction stopped by the addition of 4.4 ml. of ammonium molybdate in 1.0 N $H_2SO_4$.

Freshly prepared Fiske-Subbarow reagent[7] (0.2 ml.) was added and the mixture was heated in boiling water for ten minutes. A blue colour developed in proportion to the quantity of G1P remaining (see Methods Section); samples containing the enzyme gave only a slight blue colouration. A typical elution pattern is shown in the Table below. The column was thoroughly washed with 9.6 mM tris chloride before re-use.

Table 1

| Drops | 100-120 | 130 | 140 | 150 | 160 | 170 | 180 | 190 | 200 |
|---|---|---|---|---|---|---|---|---|---|
| Enzyme | - | - | + | ++ | ++ | ++ | ++ | ++ | ++ |
| Sulphate | - | - | - | - | - | - | - | - | + |

| Drops | 210 | 220 | 230 | 240 |
|---|---|---|---|---|
| Enzyme | + | - | - | - |
| Sulphate | + | + | + | + |

Drops 130-200 were collected for use in kinetic and other experiments. The total volume of these drops was very consistent at 3.37 ml. provided that the drop rate did not exceed 1 drop per second.

Preparation of [$^{32}$P] G1P

[$^{32}$P] G1P was prepared by the following procedure. A 1 mC sample of sodium [$^{32}$P] phosphate (specific activity 36.5 curies/mg.$P_i$ phosphorus) supplied as a sterilized solution, pH3, by the Radiochemical Centre, Amersham, was evaporated to dryness in vacuum desiccator containing NaOH. Then 0.2 ml. of a 10 mM sodium phosphate buffer, pH7 was added followed by 0.1 ml. of a 4% (w/v) aqueous solution of glycogen which had been standing for a few hours to ensure complete solution of the glycogen, and finally 0.1 ml. of rabbit muscle α phosphorylase solution (supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A., as a 2 mg./ml. suspension in 1.5 mM E.D.T.A., 1.92 mM sodium glycerophosphate adjusted to pH 6.6 with acetic acid and 0.1 M NaF; the activity was 1434 units/mg.[8]). After incubation at 30°C for 90 min. the mixture was evaporated to dryness in a vacuum desiccator. The [$^{32}$P] G1P was separated from [$^{32}$P] $P_i$ by the following chromatographic procedures.

The radioactive mixture dissolved in a small quantity of water was dried onto Whatman No. 4 paper as a 2" wide strip with

a cold air jet. After chromatography with Butan-1-ol, acetic acid, water (12:3:5 v/v) for approximately 22 hrs. the chromatogram was dried by a cold air draft. The [$^{32}$P] G1P was eluted with a small volume of water after the area containing the [$^{32}$P] G1P was identified from the position of the marker spots and with a G.M. monitor. The eluate was dried onto Whatman No. 4 paper as a 2" wide strip as before. Chromatography was carried out with 2-methoxyethanol, ethylmethylketone, 3N $NH_4OH$ (7:2:3 v/v) for 26 hrs. and after drying the [$^{32}$P] G1P area was identified and eluted in a small volume of water. It was rechromatographed on the latter system and finally eluted with water to give a total volume of solution of approx. 1 ml. The $R_f$ of G1P was less than that for $P_i$ in the first system and greater in the second (see later). The two systems together gave a very good separation of [$^{32}$P] G1P from $P_i$. The yield of pure [$^{32}$P] G1P (approx. 50-100 $\mu$C), was measured by taking 0.01 mls. for estimation by standard scintillation techniques (see later). The final product was stored as a frozen solution at approximately -20°C.

Purification of [$^{14}$C] G6P

Chromatography of [$^{14}$C] on Whatman No. 4 paper using 2-methoxyethanol, ethylmethylketone, 3N $NH_4OH$ (7:2:3 v/v)

showed that [$^{14}$C] G16P was present as an impurity. Although present as less than a few per cent of the total [$^{14}$C] G6P for some experiments it was necessary to remove all traces of G16P. This was achieved by chromatography with the same system. 0.1 ml. of [$^{14}$C] G6P solution concentration 16 mM, Specific Activity 3.1 mC/mM (supplied by the Radiochemical Centre, Amersham, Bucks.) was dried as a 2" wide strip on to Whatman No. 4 chromatography paper and chromatographed for 24 hrs. The radioactive strip containing [$^{14}$C] G6P was eluted with distilled water in small volume (> 1 ml.) and the eluate was reduced to about 0.2 ml. in a vacuum desiccator. [$^{14}$C] G6P was assayed by taking 0.01 ml. for scintillation (see Radioactivity Assay section). It was stored as a frozen solution at approximately -20°.

Chromatographic Methods

The descending method of chromatography has been used unless otherwise stated. Chromatography paper was used as supplied since in preliminary experiments pre-washing with E.D.T.A. to remove divalent metal ions was found to give no substantial improvement. The length of the chromatogram was usually about 40 cm. and the end was serrated to promote even elution when the solvent overran. Samples were dried on to strips 1"-2" wide at the origin of the chromatogram, with

a jet of cold rather than hot air to minimize irreversible adsorption of salts on to the chromatogram paper and to minimize chemical breakdown. Marker spots of the appropriate phosphates were run on either side of the test strip. Chromatography was carried out in glass tanks which had previously been saturated with solvent vapour for at least one hour. The chromatograms were dried with a stream of cold air in a drying cabinet. The marker strips were cut off from the main chromatogram and developed by the technique of Burrows[9]. They were dipped in a reagent solution freshly made up of 1 gm. A.R. Ammonium molybdate dissolved in 8 ml. water, 3 ml. conc. HCl, 3 ml. 70% $HClO_4$ and diluted to 100 ml. with acetone and after drying they were irradiated with a UV lamp. After a few minutes the phosphates appeared as blue spots. The corresponding phosphates were found in the same positions on the main chromatogram and no difference in position (i.e. $R_F$) which could have been attributed to salt effects was noted.

DEAE - Cellulose, Ammonium formate system

This system was used to separate G16P from G1P and G6P, and to measure the radioactive labelling in G16P during the course of the enzymic conversion of labelled G1P to G6P. Samples (0.1 ml.) from the reaction mixture were heated

briefly to 100°C to stop the enzyme reaction and then they were applied as 1" wide strips on DEAE cellulose chromatography paper (Whatman DE 81) as described previously. Development was carried out for six hrs. with 0.18M Ammonium formate ($R_{FG1P} = R_{FG6P} \simeq 0.5$, $R_{FG16P} \simeq 0.16$).

Whatman No. 4 Butan-1-ol, Acetic acid, Water system

This system was used to separate radioactive G1P from radioactive phosphate ($R_{FG1P} \simeq 0.14$, $R_F\ PO_4^{3-} \simeq 0.33$[9]). For consistent results the chromatograms were run in the same direction as the paper (Whatman No. 4) was machined and freshly made up solvent Butan-1-ol, Acetic acid, water (12:3:5 v/v) was used as esterification of the butanol occurs slowly and leads to smeared spots if the solvent is not fresh. Typically chromatography was carried out for 20-22 hr.

Whatman No. 4 2-methoxyethanol, methyl ethyl ketone, 3N $NH_4OH$ system

This system was used to purify G1P from inorganic phosphate during the preparation of [$^{32}$P] G1P in conjunction with the preceding system. ($R_{FG1P} = 0.36$, $R_F\ PO_4 = 0.21$.) For consistent results chromatography was carried out in the machine direction of the paper and the solvent; 2-methoxyethanol, methyl ethyl ketone, 3 N $NH_4OH$ (7:2:3 v/v) was freshly prepared. Typical chromatography times were 25-27 hr.[121]

## Liquid Scintillation Counting of [$^{32}$P] and [$^{14}$C]

The measurement of [$^{14}$C] activity was carried out as follows. Aliquots of 0.1 to 1.0 ml. of solution at neutral pH (to reduce quenching of counts) were pipetted into 15 ml. of scintillation mixture (60 gm. naphthalene, 4 gm. of 2.5 diphenyloxazole, 0.2 g. of 1-4 bis(5-phenyloxazol-2-yl) benzene, 100 ml. methanol, 20 ml. of ethylene glycol made up with dioxan to 1 litre) in a scintillation vial. The vials were counted in a Packard Tri-carb liquid-scintillation counter (Packard Instruments Co.) and corrected for quenching by the internal channels-ratio method[10].

For the measurement of [$^{32}$P] activity 0.2 to 1.0 ml. of solution at neutral pH were pipetted into 15 ml. of the dioxan scintillation medium described above in a scintillation vial to which Cab-o-Sil (Packard Instrument Co., Wembley, Middlesex) had been added as a 5% suspension. This was necessary to prevent loss of radioactivity as a result of adsorption on to the walls of the glass vial. The vials were counted in a Packard Tri-carb liquid scintillation counter as for [$^{14}$C] but no correction was applied for quenching.

## Enzyme Protein determination

The total enzyme protein was determined from the

extinction at 278 mμ using the extinction coefficient $E^{1\%}_{1cm} = 7.70$[11]. 0.1 ml. of the enzyme solution supplied by Boehringer und Soehne was added to 1.14 ml. of distilled water in a ½ cm. quartz cuvette and the optical density measured at 278 mμ on a Hilger Spectrophotometer against a water blank.

G1P and G6P estimations by the Anthrone Method

The hexose phosphates when eluted from the chromatographic columns were determined by the Anthrone Method[12] according to the following procedure. 2.0 ml. of 0.2% anthrone in 95% sulphuric acid were added to 1.0 ml. of sample with cooling in a bath of cold water. The mixture was heated for 10 min. at 100°C then cooled and the optical density of the solution measured at 630 mμ against a suitable sulphuric acid/anthrone blank. For a 1 cm. cuvette the optical density for 0.2 μmoles of either G1P or G6P was 0.275. The colour was stable.

Chemical Estimation of G1P

G1P was measured by a slight modification of Bartlett's phosphate method[13] as described by Ray and Roscelli[14]. Samples of 0.1 ml. were taken from the reaction mixture and added to 4.40 ml. of 0.18% (w/v) ammonium molybdate in N $H_2SO_4$ in stoppered test tubes. Then 0.2 ml. of Fisk-Subbarow reagent[7] (2.4 g. of anhydrous sodium sulphite,

12 g. of sodium metabisulphite and 0.2 g. of 1-amino-2-naphthol-4-sulphonic acid in 100 ml. of water) was added and the test tubes heated to 100°C in boiling water for 10 min. G1P is hydrolysed and the blue colour due to $P_i$ is fully developed. During this period very little G6P is hydrolysed. After cooling the solutions were transferred to 1 cm. cuvettes and the extinctions measured at 830 mμ against a water blank. The method was standardized by plotting a graph of optical density at 830 mμ against samples of known G1P concentration. G1P concentrations of 0.2-5.0 mM could be assayed without dilution.

Enzyme Activity Assay

The enzyme activity was assayed as described by Ray and Roscelli[14]. A sample (0.01 ml.) of stock enzyme solution (2 mg/ml.) was diluted with 4.0 ml. distilled water and 1.0 ml. of histidine/tris buffer pH 7.4 (histidine 40 mM, tris 12 mM, $MgCl_2$ 1.1 mM and gelatin 50 μg/ml.). 0.05 ml. of this diluted enzyme solution was added to a further 0.2 ml. histidine/tris buffer and incubated at 30°C for 10 min. to activate the enzyme. Then 0.2 ml. of a solution containing 2.0 μ moles G1P and 0.01 μ moles G16P was added with thorough mixing. After five minutes the reaction was stopped by the addition of 1.0 ml. N $H_2SO_4$. Three samples of 0.1 ml. were

taken and analysed for G1P by the method of Bartlett[13]. From the quantity of G1P converted to G6P (which should not be more than 30%) and the concentration of enzyme the specific activity of the enzyme can be calculated (see Results section).

Determination of [$^{14}$C] G1P

[$^{14}$C] G1P is hydrolysed to inorganic phosphate and $^{14}$C glucose by dilute acid. The hydrolysis of [$^{14}$C] G6P is very slight under these conditions and both G16P (hydrolysed to G6P) and [$^{14}$C] G6P can be removed from solution by precipitation with $ZnSO_4$ and $Ba(OH)_2$[15, 16]. Thus [$^{14}$C] G1P may be separated from [$^{14}$C] G1P and G16P. In experiments at low concentrations of substrate 0.1 ml. samples from the reaction mixture were each added to 0.1 ml. of N $H_2SO_4$ in glass stoppered test tubes, and were well shaken to stop the enzyme reaction. The G1P was hydrolysed by heating the tubes in boiling water for ten minutes. After cooling, 0.5 ml. of a solution containing glucose (0.1 mg./ml.) as carrier was added to prevent loss of radioactivity by adsorption in the subsequent steps. This was followed by 0.65 ml. of $Ba(OH)_2$ solution (approx. 0.15 M). The tubes were agitated thoroughly to complete the $BaSO_4$ precipitation before 0.2 ml. of $ZnSO_4$ solution

(approx. 5% w/v) was added to each. Again the tubes were thoroughly agitated before the precipitate was compacted by centrifugation. The remaining few per cent of [$^{14}$C] G6P was taken down with a further precipitation step of 0.1 ml. of $ZnSO_4$ solution followed by 0.1 ml. $Ba(OH)_2$ solution. After thorough agitation the precipitate was again compacted by centrifugation. Samples 0.1-0.2 ml. (0.7-1.0 ml. in equilibrium flux experiments) of each supernatant was pipetted into 15 ml. dioxan scintillator and the radioactivity assayed as described previously. The $Ba(OH)_2$ solution was made up by dissolving solid AR. $Ba(OH)_2$ in distilled water from which $CO_2$ had been removed by boiling. The concentrations of $Ba(OH)_2$ and $ZnSO_4$ solutions were adjusted until 9.8-9.9 ml. of $Ba(OH)_2$ added to 10.0 ml. of $ZnSO_4$ solution produced a faint pink colour with phenolphthalein. It was found that the addition of 2 ml. $ZnSO_4$ solution plus 1 ml. of N $H_2SO_4$ and 6.4 ml. $Ba(OH)_2$ solution gave a faint pink colour with phenolphthalein. Thus there was a slight excess of $Ba^{2+}$ ions at the end of each precipitation step.

The procedure outlined above was suitable for experiments in which the total substrate concentration did not exceed about 5 mM. For higher concentrations the volumes of reagents were increased. At 30.4 mM total concentration the volumes of the reagents were doubled and the sample volume reduced to

0.05 ml. At 86.4 mM total concentration the reagent volumes were increased twentyfold apart from the final addition of $ZnSO_4$ and $Ba(OH)_2$ where 1 ml. of each solution was added. 2 ml. of supernatant were taken for counting.

Determination of [$^{14}$C] G16P

[$^{14}$C] G16P was separated from [$^{14}$C] G1P and G6P by chromatography on DEAE cellulose paper as described previously with 0.18M ammonium formate. The chromatogram was scanned for radioactive peaks due to G16P (and G1P + G6P) by a Packard radio-chromatogram scanner. The area on the scan trace corresponding to the G16P was measured using a fixed arm planimeter (Allbrit Ltd., England), and the radioactivity in G16P determined from a calibration curve of radioactivity against area on the scan trace.

Determination of [$^{32}$P] G1P and [$^{32}$P] G6P

[$^{32}$P] G1P was determined by a variation of the method of Ray et al.[17] based upon the phosphate precipitation technique of Sugino and Miyoshi[18]. Inorganic phosphate from the hydrolysis of G1P was selectively precipitated in the presence of the G6P. A mixture of 57 mM ammonium molybdate, 74 mM triethylamine and 0.9 N $HClO_4$ (1:1:2 v/v) was prepared shortly before use and 0.4 ml. portions were placed in 15 ml.

conical centrifuge tubes. Samples (0.1 ml.) taken from the enzyme reaction mixture were added to the centrifuge tubes followed by 0.5 ml. of a solution containing carrier G1P (0.4 mM) and G6P (0.4 mM). The centrifuge tubes were placed in a boiling water bath for 10 min, cooled thoroughly in cold running water and then kept for at least 1 hr. at 4°C. After centrifugation at 2000 g for 20 min. the supernatant containing [$^{32}$P] G6P was removed and the precipitates washed three times with a mixture consisting of 57 mM ammonium molybdate, 74 mM triethylamine, 0.9 N $HClO_4$ and water (1:1:2:6 v/v) to which a trace of phosphate had been added. After each washing centrifugation was repeated for 5 minutes before the supernatant was removed. The precipitates were then dissolved with gentle warming in 0.2 ml. N. NaOH and each solution was transferred quantitatively with three washings of 0.2 ml. of water to a scintillation vial containing 15 ml. dioxan scintillator with a 5% suspension of Cab-O-Sil. 0.1 ml. N HCl was added to each vial to obtain a neutral pH and the vials were counted as described previously.

[$^{32}$P] G6P was counted using the phosphate precipitation technique described for [$^{32}$P] G1P. After centrifugation, portions (0.4 ml.) of the supernatants were added to scintillation vials containing scintillation mixture and Cab-O-Sil as in the determination of [$^{32}$P] G1P.

Approximately 0.15 ml. of N NaOH was added to discharge the yellow colour and the vials were counted as described previously.

Depression of freezing point measurements

The depression of freezing point of 0.4 ml. of solutions containing G1P or G6P at various concentrations in histidine/tris buffer pH 7.4 at the concentration employed in the induced transport tests was measured with an Advanced Osmometer (Advanced Instruments Inc., Newton Highlands, Mass., U.S.A.).

Induced transport tests

The enzyme was activated in histidine-tris buffer, pH 7.4, containing: histidine 40 mM, tris 12 mM, $MgCl_2$ 1.1 mM and gelatine 50 µg/ml., by incubation for 10 mins. at $30^{o}C$ (1). A sample from the diluted and activated enzyme solution was added to the G1P and [$^{32}$P] G1P, or G6P and [$^{14}$C] G6P, with G16P and the system incubated for 60-90 mins. to allow it to reach equilibrium. Non-radioactive G1P was then added in small volume to the reaction mixture. Samples (0.1 mls.) were withdrawn for chemical and radioactivity determinations both before and after the addition of non-radioactive G1P. In one series of experiments (1A, 1C, 4, 5, see results section R.7), 4.0 mls. of the enzyme solution

were taken and 1.0 ml. of a solution containing G1P or G6P, and G16P was added; the non-radioactive G1P was added in a volume of 0.4 mls. to 4.6 mls. of this solution. Immediately after the addition of non-radioactive G1P the concentrations of histidine, tris and $MgCl_2$ were: 29.4 mM, 8.8 mM, and 0.8 mM respectively. In the other series of experiments (1B, 2, 3, see results section R.7), 1.35 mls. of a solution containing G1P, G6P, and G16P were added to 4.0 mls. of the enzyme solution; the non-radioactive G1P was added in a volume of 0.4 mls. to 4.95 mls. of this solution. In this case the final concentrations of histidine, tris, and $MgCl_2$ were 27.7 mM, 8.3 mM, 0.76 mM respectively. $[^{32}P]$ G1P and $[^{14}C]$ G1P were estimated as described earlier. For experiments 2 and 3, in which high concentrations of hexose phosphates were used it was found to be necessary to adjust the pH of the stock G1P and G6P solutions to pH 7.4 before addition to the reaction solution in order to keep the pH of the latter solution at pH 7.40.

Comparison of glucose and phosphate fluxes at equilibrium

A solution of enzyme was activated by incubation in histidine-tris buffer pH 7.4 at 30°C for 10 mins. Then 0.42 mls. of G1P and G6P were added to 7.78 mls. of the enzyme buffer solution and the system allowed to come to

equilibrium. The concentration of the buffer solution was then: histidine, 37.5 mM; tris, 11.2 mM; $MgCl_2$, 1.03 mM; and gelatine 47 µg/ml. The solution was divided into two equal parts of 4.00 mls. and [$^{32}$P] G1P was added to one and [$^{14}$C] G6P to the other in small volume (0.01-0.025 mls.). Samples (0.1 mls.) were taken at intervals for radioactive assay while the radioactivity equilibrated between the two substrates to determine the final distribution.

## SECTION 2. PHOSPHOGLYCERATE MUTASE SYSTEM

### Reagents and Materials

2PGA and 3PGA as the sodium salts and 23PGA as the cyclohexylamine salt, ADP as the free acid, ATP and NADH as the sodium salts, and the following enzymes; phosphoglycerate mutase, specific activity (S.A.) 30 U/mg. as a crystalline suspension 2 mg./ml. in 2.6 M $(NH_4)_2SO_4$ solution, pH $\simeq$6; enolase S.A. 27 U/mg. as a crystalline suspension 2 mg./ml. in 2.8 M $(NH_4)_2SO_4$ solution, pH $\simeq$6; pyruvate kinase S.A. 150 U/mg. as a crystalline suspension 2 mg./ml. in 2.1 M $(NH_4)_2SO_4$ solution, pH $\simeq$6 and lactic dehydrogenase S.A. 360 U/mg as a suspension concentration 5 mg./ml. in 2.2 M $(NH_4)_2SO_4$ solution pH $\simeq$7, were obtained from C.F. Boehringer und Soehne, G.m.b.H. (Mannheim, Germany). All glassware was cleaned with Decon 75. (A.Gallenkamp and Co. Ltd., London E.C.2).

### Purification of Substrates

3PGA (and 2PGA) as supplied were found to contain sufficient 23diPGA impurity to catalyse the phosphoglycerate mutase reaction in the absence of added co-factor. The substrates were therefore purified since in the kinetic investigations the concentration of 23diPGA had to be known accurately, especially at low co-factor concentrations. The

usual purification of 3PGA by precipitation of the barium salt, followed by conversion to the sodium salt by agitation of solid precipitate with Amberlite 1R120 (14-52 mesh in the $Na^+$ form) ion exchange resin[19] does not separate 3PGA from 23diPGA[20] and the method of Bartlett[4] using Dowex 1-Cl ion exchange resin was used.

A column of Dowex 1-Cl, 8% cross linked 20-50 mesh, 1 x 30 cm. was prepared and washed with 1N HCl followed by distilled water. 0.4 gm. 3PGA was applied to the column dissolved in water to give a dilute solution (approx. 3 mM) whose ionic strength was less than that of the ionic strength of the HCl (0.02 M) used as eluant. Elution was carried out with 0.02 M HCl solution which does not elute 23PGA. 10 ml. fractions were collected with a fraction collector, and each fraction was neutralised by the addition of NaOH. A sample of 0.2 mls. was taken from every second fraction for assay of 3PGA. The sample was added to 1.0 ml. Tris/$Mg^{2+}$ buffer (Tris = 25.0 mM, $MgCl_2$ = 5.0 mM), pH 7.4, 0.1 ml. 10 mM ADP solution, 0.1 ml. of 3 mg./ml. NADH solution and 0.01 ml. of a mixture of equal parts of the stock solutions of pyruvate kinase, lactic dehydrogenase and enolase were added. The solution was made up to a volume of 3.0 ml. in a 1 cm. cuvette and the change in optical density at 340 mμ which was proportional to the concentration of 3PGA was measured

upon the addition of 0.003 ml. of phosphoglycerate mutase solution. The combined fractions containing 3PGA of volume of 700 ml. was reduced to 25 ml. by freeze drying in a Mudd-Flosdorf apparatus. Separation of 3PGA from NaCl was achieved by conversion to the barium salt. A 4 x excess of $BaCl_2$ was added followed by 2 volumes of ethanol. After standing overnight at -20°C the precipitate was filtered off and dried with alcohol and ether. Recovery was 72% (by weight).

To convert the barium salt to the sodium salt 10 ml. of distilled water was added to the precipitate and it was agitated in a conical flask with excess Amberlite 1R120 (14-52 mesh) resin in the $Na^+$ form. After the precipitate had dissolved the suspension was filtered and the resin washed with a small volume of water. The combined filtrate was passed through a 1 μ millipore filter to remove traces of resin which rendered the solution cloudy and diluted to 20 ml. to give a solution of concentration approx. 55 mM from recovery weight. The concentration of 3PGA determined subsequently by enzymatic assay (see substrate assay section) was found to be 49.29 mM. The concentration of 23diPGA was assayed by measuring the reaction rate for the conversion of 3PGA to 2PGA in the absence of added 23diPGA. The extremely slow rate of reaction showed that 23PGA had been

virtually completely removed (see kinetic assay section).

2PGA was purified from 23diPGA in the same way but only 0.04 gm. was taken and a proportionately smaller Dowex 1-Cl column used. The separation of 2PGA from NaCl was omitted. The concentration of the final solution was found to be 25.93 mM giving a recovery of 2PGA of 99%. It contained 1.22 M NaCl.

## Preparation of [$^{32}$P] labelled substrates

### 1. Equilibrium mixture of 2PGA and 3PGA

A mixture of [$^{32}$P] 2PGA and 3PGA was prepared by the following procedure based on the method of Grisolia *et al.* (1968)[21].

AlmC sample of sodium [$^{32}$P] phosphate, supplied by the Radiochemical Centre, Amersham, Bucks. as a sterilised solution, pH 3, was evaporated to dryness overnight in a vacuum desiccator containing caustic soda. Fresh human blood (1.0 ml.) containing 0.5 mg. dry heparin (anticoagulant) was added and the solution agitated for a period of 6 hr. at 37°C in an atmosphere whose composition was roughly that of alveolar air (approx. 20% $O_2$; 5% $CO_2$). 0.2 ml. 10% trichloracetic acid (T.C.A.) was then stirred in thoroughly with a glass rod. After standing overnight at room temperature the mixture was centrifuged and the

supernatant removed. The precipitate was washed with an equal volume of water and after recentrifugation the supernatants were united. The majority of T.C.A. in the supernatant was extracted by agitation with twice its volume of ether which had been previously saturated with water. This procedure was repeated twice. The solution was then neutralised with 2-3 drops of NaOH (conc. approx. 0.3N). 0.1 ml. of a solution of 3PGA in tris buffer pH 7.4 (containing 3 mg./ml. 3PGA, 50 mM tris) and 0.3 units of phosphoglycero-mutase (0.002 ml. stock solution) were added. After incubation at 37°C for 2 hrs. the solution was allowed to stand overnight. The equilibrium mixture of [$^{32}$P] 2PGA and [$^{32}$P] 3PGA which resulted was separated from [$^{32}$P] 23diPGA and inorganic phosphate by paper chromatography on Whatman No. 4 paper, once with di-iso-propyl ether - 90% formic acid (3:2 by volume), and once with butan-1-ol, acetic acid, water (12:3:5 by volume). For details see section on chromatography. The mixture of 2- and 3PGA was eluted from the final chromatogram with distilled water in small volume (0.4 ml.) and assayed by scintillation counting of 0.01 ml. Total yield was approximately 100 μC.

Purity was assayed by chromatography on Whatman No. 4 paper with Di-iso-propyl ether, 90% formic acid (3:2 v/v) and scanning for radioactivity (Packard Instruments Co.).

Marker spots of 2PGA, 3PGA and Pi were developed with the molybdeum blue[9]. A good separation was obtained between these materials. The majority of the radioactivity was found at the pcsition of 3PGA and less than 5% of the $^{32}P$-activity was associated with inorganic phosphate or 23PGA. The solution was stored at -20° to reduce the rate of substrate decomposition due to radiochemical change.

2. Preparation of $^{32}P$ 3PGA

The preparation of [$^{32}P$] 3PGA free of 2PGA was carried out by an extension of the above method. After [$^{32}P$] 23diPGA had been incubated with 3PGA and phosphoglycerate mutase in tris buffer pH 7.4, 0.1 ml. of 0.25 M $HClO_4$ was added to the solution to destroy the enzyme. The solution was evaporated to dryness in a desiccator containing caustic soda and the residue was dissolved in 0.2 ml. of triethanolamine buffer containing ADP/$Mg^{2+}$, pH 7.6 (9.3 gm. triethanolamine, 5.48 gm. KCl, 0.611 gm. $MgSO_4.7H_2O$ and 0.128 gm. ADP made up to 250 ml.). 0.05 ml. (3 mg./ml.) NADH solution and 0.01 ml. of a solution containing equal parts by volume of the stock solutions of enolase, pyruvate kinase and lactic dehydrogenase were added and the solution then incubated at 30°C for 30 minutes. 2PGA was thus converted to lactate and ATP. The solution was left

overnight at 4°C to complete the reaction and the [$^{32}$P] 3PGA was then separated from [$^{32}$P] inorganic $PO_4$, ATP and 23diPGA by chromatography on Whatman's No. 4 paper, using first di-iso-propyl ether, 90% formic acid (3:2 by vol.), and secondly butan-1-ol, acetic acid, water (12:3:5 by vol.). The purity of [$^{32}$P] 3PGA was assessed by chromatography on Whatman No. 4 paper using the di-iso-propyl ether, 90% formic acid (3:2 by volume) system as described in the previous section. Less than 5% of the [$^{32}$P] activity was associated with 23diPGA or inorganic phosphate. The [$^{32}$P] 3PGA was stored as a frozen solution at -20°C.

An enzymic procedure was used to separate 3PGA from 2PGA since no convenient and reliable chromatographic system for separation of 2PGA and 3PGA was found.

Purification of [$^{14}$C] 3PGA

3PGA generally labelled with [$^{14}$C] of specific activity 28 mC/mM (Calbiochem. Ltd., Los Angeles, California 90054) was usually used without further purification. However, for the equilibrium flux ratio experiments it was necessary to remove [$^{14}$C] 2PGA which was also present. 0.16 µmoles [$^{14}$C] 3PGA in 0.1 mls. was incubated at 30°C for 20 min. with 0.1 ml. triethanolamine/ADP buffer pH 7.6 (see previous section), 0.025 ml. of 3 mg./ml. NADH solution, and 0.003 ml.

each of enolase, pyruvate kinase and lactic dehydrogenase, to convert [$^{14}$C] 2PGA to [$^{14}$C] lactate. The solution was applied to a Whatman No. 4 paper and chromatographed with butan-1-ol, acetic acid, water (12:3:5 v/v). The [$^{14}$C] 3PGA was eluted with water and stored at -20°C.

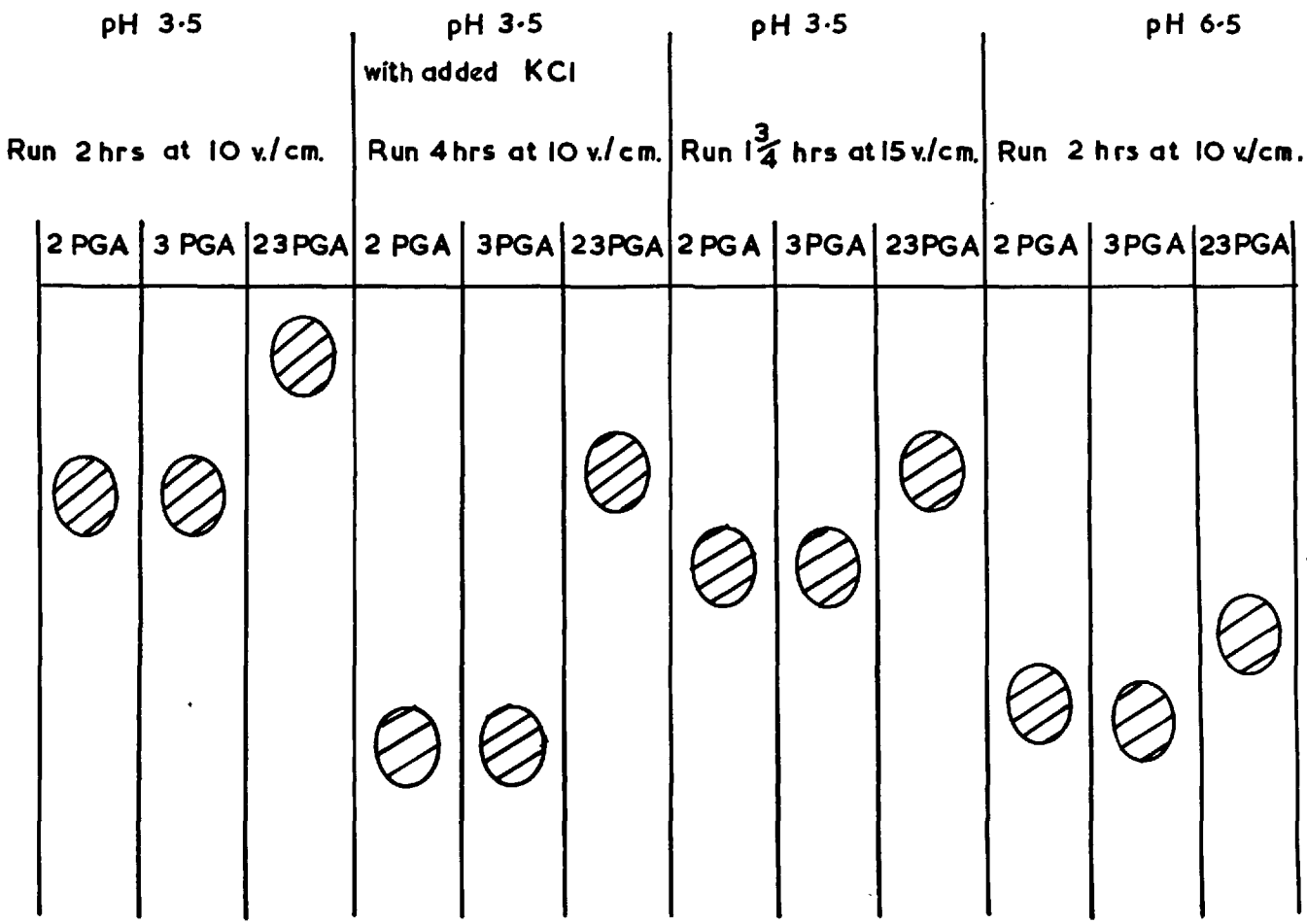
## Chromatography and Electrophoresis Techniques

### 1. Electrophoresis

The separation of 2- and 3PGA from 23diPGA during the preparation of the [$^{32}$P] labelled substrates had to be carried out in the presence of salts. Consequently it was decided to investigate electrophoretic separation based on the technique of Zwaig and Milstein[22] as it seemed probable that it would be less susceptible to the effects of salts than paper chromatography. Test electrophoretograms were carried out at pH 3.5 since at this pH 2- and 3PGA are well separated from 23PGA. Whatman No. 1 chromatography paper rather than Whatman 3 MM paper (Zwaig and Milstein) was used, of length approx. 20 cms. Approximately 0.5 μmoles of 2PGA, 3PGA and 23diPGA markers were dried on to the origin with a stream of cold air. These quantities were just sufficient to develop colour with the molybdenum blue reagent. The paper was then saturated with buffer pH 3.5 (pyridine:acetic acid: water, 1:10:189 by vol.) without moving the position of the

FIG. M.2.1.

High voltage electrophoresis on Whatmans No. 1 paper.



phosphates. A second electrophoresis strip was set up, identical with the first, except that approx. 40 µmoles of KCl were added to each marker spot. A potential difference of 200 volts was applied across the chromatogram strips for 2-4 hr. and after drying they were developed with molybdenum blue reagent[9]. The results are illustrated in Fig. M.2.1. below. It will be seen that the addition of the KCl did not affect the separation of 2- and 3PGA from 23diPGA. Increasing the potential difference across the chromatogram strip to 300 volts decreased the resolution of the mono- and di-phosphates. Electrophoresis at pH 6.5 with a pyridine, acetic acid, water buffer (10:0.4:90 by vol.) gave a very poor separation (Fig. M.2.1).

Despite these satisfactory results paper chromatography proved to be a more effective method of separation. In part at least this was due to the limited width and length of the strips that could be accommodated on the electrophoresis apparatus.

2. Paper Chromatography

The chromatogram systems and the separations obtained are described below. The salts in the incubation mixture had an insignificant effect on the $R_F$'s and did not cause appreciable streaking.

2-methoxyethanol, methyl ethyl ketone, 3N $NH_4OH$. Whatman No. 4 system

This system was used for the separation of [$^{14}$C] 23diPGA from [$^{14}$C] 2PGA and [$^{14}$C] 3PGA, and in an unsuccessful attempt to separate 2PGA from 3PGA.

The solvent was freshly prepared 2 methoxyethanol, methyl ethyl ketone and 3N $NH_4OH$ (7:2:3 by volume). Chromatography was continued for 25-30 hr. and a typical chromatogram for various phosphates has been shown in Fig. M.2.2.

Butan-1-ol, Acetic Acid, Water. Whatman No. 4 system

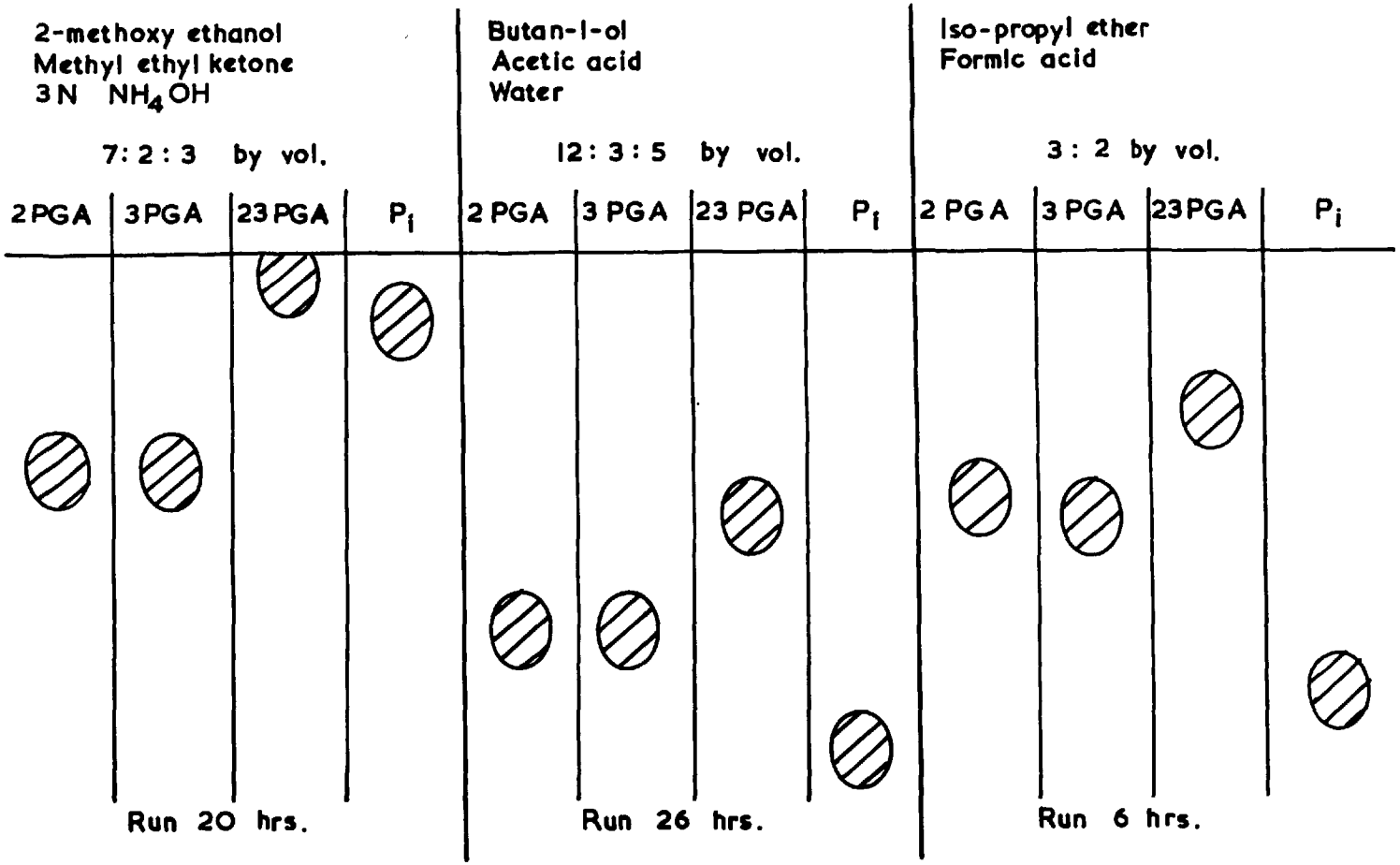
This system was used as the second purification step of [$^{32}$P] 2PGA and 3PGA from 23diPGA (and the same as that used in the phosphoglucomutase system). During chromatography the direction of chromatography was arranged to be the same as the machine direction of the chromatography paper. Freshly made up solvent, butan-1-ol, acetic acid, water (12:3:5 by volume) was used and the distribution of phosphate after chromatography for 16-24 hr. is illustrated in Fig. M.2.2.

Iso-propylether, formic acid. Whatman No. 4 system

Isopropylether, 90% formic acid (3:2 by vol.) is the system of Hanes and Isherwood[23] and was the primary separation step of [$^{32}$P] 2- and 3PGA from 23diPGA and

FIG. M. 2.2.

Descending chromatography on Whatmans No. 4 paper.



inorganic phosphate. Descending chromatography was carried out for 6 hr. on a 40 cm. (approx.) strip of Whatman No. 4 paper. The distribution of the various phosphates after this period of time is illustrated in Fig. M.2.2.

Enzyme Protein Determination

An aliquot (0.05 mls.) of the enzyme solution was diluted to 2.55 mls. in a 1 cm. quartz cuvette. The optical density at 280 nm was measured and the total enzyme protein calculated assuming an extinction coefficient of 12.5.

Determination of 2PGA

2PGA was determined by a modification of the method described by Czok and Eckert in "Methods of Enzymatic Analysis"[24]. Samples (0.10 ml.) were taken from the reaction mixture and added to 0.10 ml. 0.25 N $HClO_4$ in stoppered test tubes to denature the enzyme. The solutions were neutralised by the addition of 0.02 ml. N.NaOH to each tube, then 0.50 ml. of triethanolamine buffer pH 7.6 containing 1.0 mM ADP (9.3 g triethanolamine hydrochloride, 5.48 g. KCl, 0.611 g. $MgSO_4.7H_2O$ and 0.128 g. ADP made up to 250 ml.), was added followed by 0.10 ml. freshly prepared NADH (3 mg/ml.). The volume of the solution was made up to 2.27 ml. with distilled water, and 0.01 ml. LDH (18 units)

and 0.01 ml. PK (3 Units) were added. The solutions were each thoroughly mixed in a 1 cm. cuvette and the optical density of NADH measured at 340 nm. Enolase (0.01 ml. 0.5 Units) was then added to enable 2PGA to be converted to ATP and lactate. The conversion was completed in a few minutes under these conditions and the final optical density was measured at 13 minutes. After adjusting for dilution by enolase the change in optical density was proportional to the concentration of 2PGA present since the conversion of 2PGA to lactate thermodynamically goes to completion. The apparent equilibrium constant of pyruvate kinase is 6290 at pH 7.4 and 30°C when measured by conversion of PEP to pyruvate[25], and the mean equilibrium constant of lactic dehydrogenase from pH 7 to 10 is $3.03 \times 10^{11}$ in favour of lactate at low enzyme concentration[26].

In the method of Czok and Eckert[24] the triethanolamine buffer, contained 40 mM EDTA but with this buffer there was no reaction. This appeared to be a result of the EDTA extensively complexing magnesium ($Mg^{2+}$ : EDTA = 1:4), and reducing the concentration of free magnesium to a sufficiently low level to inactivate enolase and pyruvate kinase, each of which has an obligate requirement for the metal ion.

Measurement of Initial Velocities of reaction

The initial rates of conversion of 3PGA to 2PGA, and of

2PGA to 3PGA were measured for a variety of substrate and cofactor concentrations, at 30°C, and at pH 7.4 and at constant ionic strength (I = 0.08). $MgCl_2$ was added to maintain constant the free $Mg^{2+}$ ion concentration at 2.25 mM.

Conversion of 3PGA to 2PGA

The initial velocity of conversion to 3PGA to 2PGA by phosphoglyceratemutase was followed by an NADH coupled assay in 1 cm. quartz cuvettes with a total solution volume of 3.00 mls. The cuvettes contained 1.0 ml. of 50 mM tris buffer, pH 7.40, 0.10 ml. 3 mg./ml. NADH solution, 0.10 ml. 10 mM ADP solution and 0.01 ml. of a mixture of enolase, pyruvate kinase, and lactic dehydrogenase (0.2, 1.5, and 9.0 units respectively, made up from equal volumes of the stock enzyme solutions. Various quantities of 3PGA, 23diPGA, KCl, and $MgCl_2$ were then added according to Tables M.2.1., M.2.2. and M.2.3. $MgCl_2$ was standardized by titration with $AgNO_3$ before use and the 3PGA had been chromatographed to remove 23diPGA. The quantities of $MgCl_2$ and KCl were calculated to keep $[Mg^{2+}]_{free}$ constant at 2.250 mM approx. for experiments 1A to 8H, and to keep the ionic strength constant at I = 0.08 for all experiments (for details in calculations see Tables M.2.1.-M.2.3.). The volume of each solution was made up to 2.99 mls. by the addition of distilled water and mixed well. The

Table M.2.1. Table of total $[Mg^{2+}]$ concentration required to keep $[Mg^{2+}]_{free}$ a constant at pH 7.4 at given concentrations of 3PGA and 23PGA. $[Mg^{2+}]_{total}$ was calculated using binding constants of 302 $M^{-1}$ for $3PGA^{3-}$ and 1111 $M^{-1}$ for 23PGA.

All values in (mM)

| Runs | 23PGA (μM) / (mM) 3PGA | A | B | C | D | E | F | G | H | * |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 50.0 | 25.0 | 10.0 | 5.0 | 2.0 | 1.0 | 0.5 | 0.33 | $[Mg^{2+}]_{free}$ (mM) |
| 1 | 5.00 | 4.183 | 4.166 | 4.155 | 4.152 | 4.149 | 4.149 | 4.148 | 4.148 | 2.380 |
| 2 | 2.00 | 2.966 | 2.949 | 2.938 | 2.935 | 2.932 | 2.932 | 2.931 | 2.931 | 2.247 |
| 3 | 1.00 | 2.560 | 2.543 | 2.532 | 2.529 | 2.526 | 2.526 | 2.525 | 2.525 | 2.188 |
| 4 | 0.50 | 2.357 | 2.340 | 2.329 | 2.326 | 2.323 | 2.323 | 2.322 | 2.322 | 2.155 |
| 5 | 0.25 | 2.255 | 2.238 | 2.227 | 2.224 | 2.221 | 2.221 | 2.220 | 2.220 | 2.137 |
| 6 | 0.10 | 2.195 | 2.178 | 2.167 | 2.164 | 2.160 | 2.160 | 2.160 | 2.160 | 2.127 |
| 7 | 0.05 | 2.174 | 2.157 | 2.146 | 2.143 | 2.140 | 2.140 | 2.139 | 2.139 | 2.123 |
| 8 | 0.02 | 2.162 | 2.145 | 2.134 | 2.131 | 2.128 | 2.128 | 2.127 | 2.127 | 2.121 |

Volume of 19.845 mM $MgCl_2$ solution required to make up 3.0 mls of reactant solution to total magnesium ion concentrations given in Table M.2.1. above.

Table M.2.1a.

In mls $MgCl_2$ solution

| 23PGA (μM) / (mM) 3PGA | Runs | 50.0 | 25.0 | 10.0 | 5.0 | 2.0 | 1.0 | 0.50 | 0.33 |
|---|---|---|---|---|---|---|---|---|---|
| | | A | B | C | D | E | F | G | H |
| 5.00 | 1 | 0.632 | 0.630 | 0.628 | 0.628 | 0.627 | 0.627 | 0.627 | 0.627 |
| 2.00 | 2 | 0.448 | 0.446 | 0.444 | 0.444 | 0.443 | 0.443 | 0.443 | 0.443 |
| 1.00 | 3 | 0.387 | 0.384 | 0.383 | 0.382 | 0.382 | 0.382 | 0.382 | 0.382 |
| 0.50 | 4 | 0.356 | 0.354 | 0.352 | 0.352 | 0.351 | 0.351 | 0.351 | 0.351 |
| 0.25 | 5 | 0.341 | 0.338 | 0.337 | 0.336 | 0.336 | 0.336 | 0.336 | 0.336 |
| 0.10 | 6 | 0.332 | 0.329 | 0.328 | 0.327 | 0.327 | 0.327 | 0.327 | 0.327 |
| 0.05 | 7 | 0.329 | 0.326 | 0.324 | 0.324 | 0.324 | 0.324 | 0.324 | 0.324 |
| 0.02 | 8 | 0.327 | 0.324 | 0.323 | 0.322 | 0.322 | 0.322 | 0.322 | 0.322 |

Table M.2.2.

Volume of 200 mM KCl (in mls) required to keep ionic strength of reactions 1A - 8H constant at ionic strength, I = 0.08.

| 23PGA (µM) / (mM) 3PGA | Runs | 50.0 | 25.0 | 10.0 | 5.0 | 2.0 | 1.0 | 0.50 | 0.33 |
|---|---|---|---|---|---|---|---|---|---|
| | | A | B | C | D | E | F | G | H |
| 5.00 | 1 | 0.000 | 0.006 | 0.009 | 0.010 | 0.011 | 0.011 | 0.011 | 0.011 |
| 2.00 | 2 | 0.252 | 0.258 | 0.261 | 0.262 | 0.263 | 0.263 | 0.263 | 0.263 |
| 1.00 | 3 | 0.360 | 0.366 | 0.369 | 0.370 | 0.371 | 0.371 | 0.371 | 0.371 |
| 0.50 | 4 | 0.405 | 0.411 | 0.414 | 0.415 | 0.416 | 0.416 | 0.416 | 0.416 |
| 0.25 | 5 | 0.428 | 0.433 | 0.437 | 0.438 | 0.439 | 0.439 | 0.439 | 0.439 |
| 0.10 | 6 | 0.441 | 0.447 | 0.450 | 0.451 | 0.452 | 0.452 | 0.452 | 0.452 |
| 0.05 | 7 | 0.446 | 0.451 | 0.455 | 0.456 | 0.457 | 0.457 | 0.457 | 0.457 |
| 0.02 | 8 | 0.448 | 0.454 | 0.457 | 0.458 | 0.459 | 0.459 | 0.459 | 0.459 |

Table M.2.3.

Extra determinations of $V_{max}$ at different $[Mg^{2+}]_{free}$ concentrations

Table in mls 24.53 mM $MgCl_2$

| Runs | 23PGA (μM) / (mM) 3PGA | A | B | C | D | E | F | G | H | $[Mg2^{+}]_{free}$ (mM) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 50.0 | 25.0 | 10.0 | 5.0 | 2.0 | 1.0 | 0.50 | 0.33 | |
| 9 | 2.00 | 0.456 | 0.454 | 0.452 | 0.451 | 0.451 | 0.451 | 0.451 | 0.451 | 2.898 |
| 10 | 1.00 | 0.359 | 0.357 | 0.356 | 0.356 | 0.355 | 0.355 | 0.355 | 0.355 | 2.332 |

cuvettes were placed in a Unicam SP 800 recording spectrophotometer (Unicam Instruments Ltd., Cambridge) which was thermostated at 30°C. When thermal equilibrium had been attained (approx. 20 min.) 0.01 ml. of diluted phosphoglycerate mutase solution (0.01 mls. stock PGLyM solution diluted with 3.0 mls. water and 1.0 mls. 50 mM Tris buffer, pH 7.4, to 4.01 mls. The solution contains $9.34 \times 10^{-3}$ units PGLyM/ml.) was added to the solution. The extinction at 340 nm measured against a similar cuvette, to which phosphoglycerate mutase had not been added, was recorded on the chart recorder set for a full scale deflection of 0-0.4 O.D. From the initial gradient of this plot the initial rate of reaction was calculated. All determinations were performed in duplicate.

Measurement of maximum velocities of reaction in forwards and reverse directions

The ratio of the maximum velocities of reaction in the forwards and reverse direction were determined in solutions of the same 3PGA and 2PGA concentration and containing the same concentrations of 23diPGA, $MgCl_2$, and enzyme. The concentrations were chosen so that the initial velocity of reaction in the forwards direction, 3PGA to 2PGA, was close to the $V_{max}$ in that direction. Two solutions were made up;

one containing 2.00 mM 3PGA, the other 2.00 mM 2PGA, together with 2 μM 23diPGA, 3.33 mM $MgCl_2$, and 16.67 mM tris buffer, pH 7.4, in a final volume of 2.99 mls. The solutions were incubated at 30°C till thermal equilibrium was reached and then 0.01 mls. of the same enzyme solution was added, 0.1 ml. samples withdrawn at various times and the 2PGA concentrations determined. The concentrations of 2PGA were plotted against time and the initial velocities determined from the initial slopes.

Radioactivity measurements

Determination of [$^{32}$P] 2PGA and [$^{32}$P] 3PGA

[$^{32}$P] 2PGA was measured by the enzymic conversion to [$^{32}$P] ATP, hydrolysis and precipitation of inorganic phosphate by the technique of Sugino and Miyoshi[18]. Samples (0.10 ml.) (containing [$^{32}$P] 2- and 3PGA) were taken from the reaction mixture and 2PGA converted to lactate and ATP as described for the chemical estimation of 2PGA. 0.6 mls. of the solutions were placed in a 15 ml. conical centrifuge tube containing 0.6 ml. 1.8 N $HClO_4$ and 0.05 ml. 10 mM sodium phosphate carrier pH 7.0. The stoppered tubes were placed in a boiling water bath for 10 min. to hydrolyse [$^{32}$P] ATP to AMP and [$^{32}$P] $PO_4$. Then 1.2 ml. of a freshly prepared solution containing equal parts of 57 mM ammonium

molybdate and 74 mM triethylamine were added and the tubes reheated to 100°C for 1½ minutes, with vigorous mixing of their contents. After cooling to room temperature the tubes were allowed to stand overnight at 4°C. The tubes were centrifuged at 2000 g for 10 min. to compact the phosphate precipitate and the supernatant which contained [$^{32}$P] 3PGA was discarded (see estimation of [$^{32}$P] 3PGA). The precipitates were washed three times with 1.0 ml. of a mixture of 57 mM ammonium molybdate, 74 mM triethylamine, 0.9 N $HClO_4$ and water (1:1:2:6 by volume). After each washing the tubes were recentrifuged for 5 min. before removing the supernatants. The precipitates were dissolved with gentle warming in 0.2 ml. of NNaOH and each solution was transferred quantitatively, together with three washings of 0.2 ml. of water, to a scintillation vial containing 15 ml. scintillation medium containing Cab-o-sil. The solutions were neutralised with 0.1 ml. of N HCl and the vials were counted as described previously.

[$^{32}$P] 3PGA was estimated by taking 0.4 ml. aliquots from the supernatants after centrifugation of the precipitated [$^{32}$P] Pi (see above) and adding to scintillation vials containing 15 ml. scintillation medium. Approximately 0.15 ml. of N NaOH was added to discharge the yellow colour and the vials counted as described previously.

The above methods were suitable for monophosphoglycerate concentrations up to 5.0 mM.

Determination of [$^{14}$C] 2PGA

The determination of [$^{14}$C] 2PGA was based on the method used by Britton and Clarke[27] to measure [$^{14}$C] G1P. Samples (0.1 ml.) were taken from the reaction mixture (containing [$^{14}$C] 2PGA and 3PGA) and 2PGA converted to ATP and lactate enzymatically as described previously. 1.0 ml. samples from the resulting solution were added 0.60 ml. $ZnSO_4$ solution (approx. 5% w/v) followed by 0.60 ml. $Ba(OH)_2$ (approx. 0.15 M) and the mixtures thoroughly agitated to ensure coprecipitation of 3PGA. After centrifugation at 2000 g. for 10 min. 0.6 ml. of the supernatants (containing the [$^{14}$C] lactate derived from 2PGA) were pipetted into 15 ml. of scintillation medium in scintillation vial and counted on a Packard tri-carb liquid scintillation counter as already described.

The concentrations of $Ba(OH)_2$ and $ZnSO_4$ were adjusted so that the addition of equal volumes of each gave a solution containing a slight excess of $Ba^{2+}$ ions as described by Nelson[15].

The method of determination of [$^{14}$C] 2PGA was suitable for concentrations of monophosphoglycerate up to about 5.0 mM.

For higher concentrations a smaller initial sample was taken. For example, 0.02 mls. in the experiment at 27.5 mM.

Total radioactivity in the reaction mixture was determined by the addition of 0.10 ml. samples to the scintillation mixture and counting as already described.

Determination of [$^{14}$C] 23diPGA

Samples (0.2 ml.) taken from the reaction mixture were quenched by the addition to 0.25 ml. of absolute ethanol and heating briefly to about 70°C to denature the enzyme. The quenched samples were applied to Whatman No. 4 paper previously washed for 12 hr. with 1N HCl containing 0.02% of the tetrasodium salt of EDTA (to minimise irreversible adsorption of salts to the chromatogram paper), and dried in a stream of cold air. Descending chromatography was carried out for 20 hr. at room temperature using the 2-methoxyethanol, methylethyl ketone, 3N $NH_4$ OH (7:2:3 by volume) system. The chromatograms were then dried and scanned with a radioactivity scanner (Packard Instruments Co.) and the relative proportions of radioactivity in the 23diPGA and the 2 + 3PGA peaks established by measuring the area under the peaks with a fixed arm planimeter (Allbrit Ltd., England).

Induced Transport tests

Rabbit muscle phosphoglycerate mutase solution, suitably diluted in a magnesium chloride-Tris buffer (16.7 mM Tris 3.33 mM $MgCl_2$, pH 7.40) was added in small volume to the reaction mixture containing 3PGA and 23diPGA in the same Tris buffer at 30°C. After 10 minutes, when equilibrium was nearly established, a small quantity of either $[^{32}P]$ 3PGA + 2PGA or $[^{14}C]$ 3PGA was added and the system incubated for a further 80 min. to allow it to come to complete equilibrium. The addition of radioactive substrate was delayed to reduce any induced transport of the labelled material which might occur during the initial equilibration. The total volume of solution was 3.00 ml. Four 0.10 ml. aliquots were withdrawn at known times in the last 15 min. of the incubation and assayed for radioactive 2PGA. At the end of the period non-radioactive 2PGA was added in small volume (0.4 ml.) and samples (0.10 ml.) taken for chemical and radioactive assay of 2PGA whilst the system relaxed to its new equilibrium. The concentration of Tris and $[Mg^{2+}]$ after the addition of 2PGA were 14.4 mM and 1.89 mM respectively.

Comparison of glyceric acid and phosphate fluxes at equilibrium

3PGA, and 23PGA were incubated with the enzyme in a tris

buffer, pH 7.4, until equilibrium was reached (approx. 120 mins.) [$^{14}$C] 3PGA and [$^{32}$P] 3PGA both free of 2PGA were then added in small volume (0.01-0.035 mls.) to give a final volume of solution of 3.00 mls. The final concentrations of tris and $MgCl_2$ were; 16.67 mM, and 3.33 mM respectively. Two sets of samples (0.1 mls.) were taken for assay of [$^{14}$C] 2PGA and [$^{32}$P] 2PGA. Samples were also taken for spectrophotometric assay of both 2PGA and 3PGA to check that equilibrium had been attained. The radioactive fluxes were calculated from the data as described for phosphoglucomutase.

Determination of $Mg^{2+}$ binding to 2PGA and 3PGA

The binding of magnesium to 2PGA and 3PGA was studied by the method of Burton[28] which depends upon measuring the change in the O.D. of 8-hydroxyquinoline at 360 nm upon the addition of $MgCl_2$ both in the presence and absence of the ligand.

Each solution for titration with magnesium contained in a final volume of 6.0 ml., 0.833 mM 8-hydroxyquinoline (freshly made up) and a tris 16.67 mM buffer, pH 7.40. In each experiment three solutions were made up, one contained 0.95 mM 3PGA, a second, 0.95 mM 2PGA, and a third contained enough 200 mM KCl to give a solution of the same ionic strength (I = 0.02). From each solution 3.0 ml. were taken

for titration and placed in a 10 mm. quartz cuvette, the remainder in a similar cuvette was used as a blank. The solutions were thermostated at 30°C in a Unicam SP 800 recording spectrophotometer. When thermal equilibrium had been reached (about 20 min.) aliquots (20 µl.) of approximately 60 mM $MgCl_2$ solution (standardised by titration with silver nitrate solution using dichlorofluorescein indicator) were added with an Agla micrometer syringe. The increase in the optical density at 360 nm was recorded with a chart recorder set to give full scale deflection at 0.4 O.D. The optical densities taken from the chart were corrected to the volume of the original solution. The optical density of 8-hydroxyquinoline fully saturated with $Mg^{2+}$ was determined with a solution containing 19.6 mM $MgCl_2$ (see results section R.4.).

Some additional experiments were carried out to study the binding of magnesium to 8-hydroxyquinoline over the pH range 6.77 to 8.78. The tris buffer was set at the appropriate pH in these experiments.

Throughout the investigations the initial pH of solution before the addition of $MgCl_2$ was measured on an E.I.L. pH meter.

# CHAPTER 3

# RESULTS

## SECTION 1. PHOSPHOGLUCOMUTASE SYSTEM. PRELIMINARY STUDIES: VALIDATION OF TECHNIQUES

### Standardization of chemical estimation of G1P

G1P solutions (conc. 5.0-0.2 mM) were made up by weighing assuming the G1P.$Na_2$.$4H_2O$ was 98% pure (Boehringer und Soehne). A typical calibration experiment is illustrated in Table R.1.1. and in Fig. R.1.1. The concentrations of unknown G1P solutions were determined by comparison of the optical densities at 830 nm with Fig. R.1.1.

Calibration was repeated with new Fiske-Subbarow reagent every three weeks as this reagent deteriorated after being stored for more than four weeks at $4^{o}C$[30]. All optical densities were measured against a water blank. Under the experimental conditions employed G6P (up to 10 mM) produced no colour.

### Enzyme protein concentration determination

The total enzyme protein present in the samples of rabbit muscle phosphoglucomutase supplied by Boehringer and Soehne was measured as described in the method section. 0.1 mls. of this solution was diluted to 1.42 mls. and placed

FIG. R.I.I.

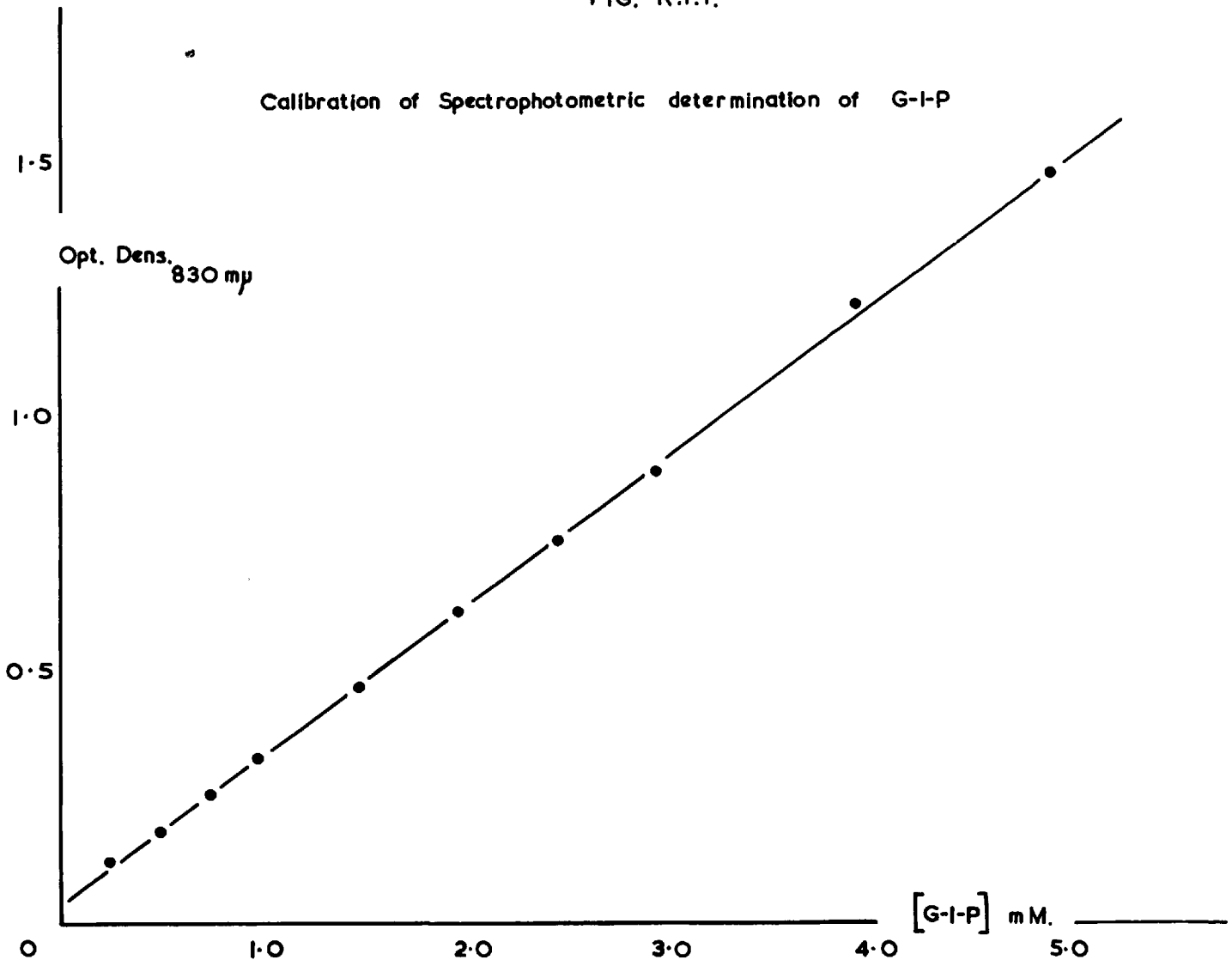

Calibration of Spectrophotometric determination of G-I-P
Opt. Dens. 830 mμ
1·5
1·0
0·5
0
1·0
2·0
3·0
4·0
5·0
[G-I-P] mM.

Table R.1.1.

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Conc. of G1P (by wt.) (mM) | 5.00 | 4.00 | 3.00 | 2.50 | 2.00 | 1.50 | 1.00 | 0.75 | 0.50 | 0.25 |
| Conc. of G1P (corrected) (mM) | 4.90 | 3.92 | 2.94 | 2.45 | 1.96 | 1.47 | 0.98 | 0.735 | 0.490 | 0.245 |
| Opt. Density 830 nm. | 1.479 | 1.218 | 0.890 | 0.752 | 0.616 | 0.466 | 0.330 | 0.255 | 0.181 | 0.124 |

in a 5.0 mm. quartz cuvette. The optical density of this solution measured at 278 nm. was 0.791. Assuming the extinction coefficient of the enzyme at this wavelength to be 7.70 the concentration of the enzyme in the stock solution was calculated to be 2.54 mg./ml. which was in good agreement with the manufacturers value of 2 mg./ml. quoted by Boehringer and Soehne.

Validation of [$^{14}$C] G1P determinations

[$^{14}$C] G1P was estimated in the presence of [$^{14}$C] G6P by preferentially hydrolysing [$^{14}$C] G1P to inorganic phosphate and [$^{14}$C] glucose. The phosphates were co-precipitated with $ZnSO_4$ and $Ba(OH)_2$ and the solution containing [$^{14}$C] glucose, was assayed for radioactivity. The details of the procedure finally adopted have been given in the methods section but in the development of this procedure a series of experiments were carried out which will be discussed below. These experiments were designed to optimize the conditions for co-precipitation of [$^{14}$C] G6P with $ZnSO_4$ and $Ba(OH)_2$. This was essential since the level of radioactivity in G6P was approximately 17.2 times that in G1P. If 1% of G6P remained in solution this would add a background to the G1P activity of about 17% and this additional activity, if variable, could obscure small systematic changes in the level of G1P

radioactivity occurring during induced transport experiments.

A. Effect of repeated additions of $ZnSO_4$ and $Ba(OH)_2$ upon precipitation of G6P, and effect of varying the quantity of $Ba(OH)_2$ added upon completeness of precipitation

The effect of varying the quantity of barium hydroxide on the completeness of precipitation of G6P was investigated by taking 0.1 mls. of a G6P solution, approx. 50 mM, containing [$^{14}$C] G6P and estimating the [$^{14}$C] G6P remaining in solution after $Ba(OH)_2/ZnSO_4$ precipitation steps by the method already described. The method, however, was modified in that the volume of $Ba(OH)_2$ reagent in the first precipitation step was varied and a third precipitation step was introduced. The results are shown in Table R.1.2. The table shows that the efficiency of precipitation falls off as the quantity of $Ba(OH)_2$ is reduced. A stoichiometric quantity of $Ba(OH)_2$ was present when 0.545 mls. of the reagent had been added to the G6P solution. The fall in efficiency was dramatic when the amount of $Ba(OH)_2$ becomes insufficient for stoichiometric reaction.

Table R. 1.2 also shows that successive additions of $Ba(OH)_2$ and $ZnSO_4$ increased the completeness of precipitation of [$^{14}$C] G6P. This procedure operated most effectively when $Ba(OH)_2$ was added prior to $ZnSO_4$.

In view of these findings 0.65 mls. $Ba(OH)_2$ was used during

Table R.1.2.

| $Ba(OH)_2$ added (mls) | Counts in solution (d.p.m.) | % G6P remaining | 2nd addition of $Ba(OH)_2$ $ZnSO_4$ counts left | % G6P remaining | 3rd addition of $Ba(OH)_2$ $ZnSO_4$ counts left | % G6P remaining |
|---|---|---|---|---|---|---|
| 0.538 | 73031 | 12.8 | 29669 | 5.2 | 20026 | 3.5 |
| 0.550 | 23435 | 4.1 | 14861 | 2.6 | 11245 | 2.0 |
| 0.555 | 48239 | 8.7 | 17743 | 3.2 | 10744 | 1.9 |
| 0.565 | 27827 | 4.9 | 11358 | 2.0 | 7223 | 1.3 |
| 0.580 | 25793 | 4.8 | 9135 | 1.7 | 5557 | 1.0 |
| 0.600 | 17631 | 3.3 | 9617 | 1.8 | 4495 | 0.82 |
| 0.650 | 4872 | 0.93 | 3667 | 0.70 | - | - |

Total Counts in solution = $5.70558 \times 10^5$ d.p.m.

$[Ba(OH)_2]$ = 0.287N.

the first precipitation step and one successive addition of $Ba(OH)_2$ and $ZnSO_4$ was adopted for the final procedure (see Methods section). Although further additions would have improved the completeness of precipitation the additional gain would have been small.

B. Effect of G1P concentration on precipitation of G6P

In the induced transport experiment the total monophosphate concentration is increased by a factor of two by the addition of non-radioactive G1P. G1P and G6P are present initially in equilibrium proportions of 1:17.2 so that the addition of G1P increases the concentration of G1P by a factor of about 20. If this G1P, or the products of its hydrolysis affected the precipitation of G6P a change would be observed in the apparent radioactivity in the G1P. An apparent rise in observed G1P radioactivity, due to impaired precipitation of G6P, could be especially misleading as the expected increase in G1P radioactivity as a result of counter-transport were small (see results section).

The experimental conditions chosen to look for possible changes in completeness of precipitation of G6P were those used for the detection of counter-transport, i.e. at very high glucose monophosphate concentrations. G6P and $[^{14}C]$ G6P were incubated at $30^{o}C$ with phosphogluco-mutase in the presence of

G16P (0.1155 mM) until equilibrium was reached. The concentration of G1P and G6P were then 2.465 mM and 42.4 mM respectively. The enzyme was denatured by heating to 60-70°C for two minutes and two 0.1 ml. samples were taken and analysed for [$^{14}$C] G1P as described previously. G1P was now added in small volume to the quenched enzyme solution to make the G1P concentration up to 47.1 mM. The G6P concentration was now 39.2 mM and G16P 0.1059 mM. A further two 0.1 ml. samples were taken and analysed for [$^{14}$C] G1P, and after making an allowance for dilution the results of the two sets of estimations were compared as shown below in Table R.1.3.

Table R.1.3.

| | Total [$^{14}$C] counts in G1P + G6P | Counts in G1P before addition of inactive G1P | Counts in G1P after addition of inactive G1P | Percentage charge |
|---|---|---|---|---|
| 10 min. counts | 115, 100 | 6358 | 6282 | -1.2% |
| | | 6285 | 6193 | -1.5% |

The counts in G1P actually fell by 1-2%. This may have been due to the phosphate released by hydrolysis from G1P acting as a carrier for G6P precipitation.

C. Modified Precipitation of G6P with addition of G6P carrier

The hydrolysis of 0.1 mls. of an approximately 50 mM solution of G6P containing [$^{14}$C] G6P and the precipitation of phosphate with 0.65 mls. $Ba(OH)_2$ was carried out as described in section A. The supernatant was then removed and inactive G6P added (conc. 100 mM) until the final G6P concentration was 10 mM. $H_2SO_4$ was added until the acid concentration was $\frac{N}{2}$ then 0.65 mls. $Ba(OH)_2$ was added followed by 0.2 mls. $ZnSO_4$. The solution was centrifuged and the supernatant assayed for radioactivity. Duplicated results showed that only 0.4% G6P was left in solution. This method, however, was not used as it was rather slow to operate and the original radioactive solution became very diluted.

D. Extent of G6P hydrolysis during boiling

The extent of hydrolysis of G6P in 0.5 N $H_2SO_4$ when boiled for various periods under the conditions described in section A was investigated. 0.1 mls. of G6P solution, approx. 50 mM, and containing [$^{14}$C] G6P was taken and [$^{14}$C] G6P was precipitated with 0.65 mls. $Ba(OH)_2$ and 0.2 mls. $ZnSO_4$, together with a further addition of 0.1 mls. $Ba(OH)_2$ and $ZnSO_4$ as already described. The radioactivity in the supernatant is compared with the time of boiling in Table R.1.4. below.

Table R.1.4.

| Boiling time | Experiment | Radioactive Counts left in solution | % G6P left |
|---|---|---|---|
| - | Total Counts, | 16,335 | 100 |
| 0 | Duplicated | 1544, 1527 | 0.94 |
| 5 | Determinations | 1582, 1748 | 1.02 |
| 7 | | 1565, 1744 | 1.01 |
| 15 | | 1671, 2002 | 1.12 |
| 20 | | 1727, 1974 | 1.13 |

The affect of boiling time on hydrolysis of G6P was virtually negligible; this was consistent with the high resistance of G6P to acid hydrolysis and consistent with the expected degree of hydrolysis, assuming $t_{\frac{1}{2}}$ at 100°C for hydrolysis = 1300 mins.[58].

E. Adsorption of [$^{14}$C] glucose to $Ba(OH)_2/ZnSO_4$ precipitate

The possibility that [$^{14}$C] glucose from the hydrolysis of [$^{14}$C] G1P was carried out of solution by adsorption onto the surface of the $Ba(OH)_2/ZnSO_4$ precipitate was investigated as follows.

A solution was made up containing 0.13 µc [$^{14}$C] D-glucose/ml. (S.A. 3.1 mC/mM) (Radiochemical Centre, Amersham, England), and 10 mM G6P. Two 0.1 ml. aliquots were taken from this solution boiled for 10 mins. in $\frac{N}{2}$ $H_2SO_4$ and assayed for [$^{14}$C] G6P as described in the method section. Two further samples of of 0.1 ml. were added untreated to scintillation solution and counted directly. The results are presented in the Table R.1.5. below.

Table R.1.5.

| Solutions not subject to precipitation [$^{14}$C] radioactivity | Solutions from which G6P was precipitated [$^{14}$C] radioactivity |
|---|---|
| 3722 d.p.m./ml. original solution | 3904 d.p.m./ml. original solution |
| 3668 | 3757 |

No significant decrease in the [$^{14}$C] glucose count after G6P precipitation was observed. Glucose is thus not carried down by the $Ba(OH)_2/ZnSO_4$ precipitate under the conditions of experiment.

Determination of [$^{32}$P] G1P

The separation of [$^{32}$P] G1P and [$^{32}$P] G6P was based upon standard methods[6, 17]. Two questions arise concerning the effectiveness of the separation; first, is all [$^{32}$P] $PO_4$ from the hydrolysis of G1P taken out of solutions by the precipitating reagent? Secondly, is any of the [$^{32}$P] G6P present in solution precipitated during separation? Excess phosphate precipitating reagents were present during the separation since addition of inorganic phosphate to the supernatant led to the formation of further precipitate. Thus phosphate precipitation would be expected to be complete. Further, in the experiments measuring the rate of transfer of [$^{32}$P] from G1P to G6P at equilibrium, the radioactivity in the supernatant ([$^{32}$P] G6P) tended to zero at zero time which implies that all [$^{32}$P] $PO_4$ from the hydrolysis of G1P was removed from solution by the precipitating reagent. Furthermore the base line of [$^{32}$P] G1P activity at the beginning and end of the induced transport experiments appeared to be constant despite the fact that the total quantity of monophosphates had been doubled. The addition of further precipitating reagents including phosphate carrier to supernatants containing [$^{32}$P] G6P at the end of induced-transport experiments did not increase the $^{32}$P-radioactivity in the precipitate by more than 2-3%.

Consequently there can be very little G6P precipitated by these reagents.

Enzyme assay studies

A. Enzyme activity

The activity of the rabbit muscle phosphoglucomutase supplied by Boehringer und Soehne was assayed at 30°C as already described. Reaction times were kept short so that less than 40% conversion of G1P to G6P occurred. Under these conditions there is little inhibition of the reactions by G6P and the reaction rate measured may be regarded as the true initial rate.

The specific activities of the enzyme under various conditions were calculated according to Najjar[31] as units activity/mg. protein. Typical specific activities obtained in the above experiment were approximately 60 units/mg. protein which compared well with the maximum activity of 105 units/mg. protein obtained by Ray and Roscelli[14] for their most active enzyme preparations. An old batch of enzyme (final date for use 29 August, 1967) and a more recent batch (final date for use 5 July, 1968) when assayed on 11 January, 1968 showed only a small difference in specific activity which suggested that the enzyme preparation was stable over many months when stored at 4°C in confirmation of the

manufacturers claim (no measurable decrease in activity when stored at $4^{o}C$ for 12 months). See Table R.1.6.

Tests for presence of G16P in G1P and G6P

The following tests were designed to indicate the presence of G16P in either G1P or G6P so that substrates could be prepared which were known to be free of G16P. It is important to have such substrates, for example, in any experiment in which it is proposed to investigate the G16P independent reaction of PGM noted by Ray and Roscelli[14], which accounts for 2% of the maximum turnover of the enzyme.

B. Rate of reaction in presence of G1P and G6P alone

The assay was carried out as described in section A, except that 0.2 mls. of substrate solution containing 10 mM G1P and 3.2 mM G6P only was added. As the removal of cofactor was expected to slow down the reaction considerably the conversion of G1P to G6P was allowed to proceed for 20 minutes before the reaction was quenched. Three samples were then taken for G1P assay and the average specific activity of the enzyme reported, in Table R.1.6, under the conditions of experiment. A value for the S.A. of 33.6 was obtained which was much higher than expected. This suggested that either or both G1P and G6P as supplied

Table R.1.6.

| Expt. No. | Enz. Batch | Enz. Conc. (M) | Assay Time Mins. | G1P mM | G6P mM | G16P µM | S.A. with G16P | S.A. without G16P |
|---|---|---|---|---|---|---|---|---|
| A | (Old) 6366108<br>(New) 6277310 | $7.7 \times 10^{-9}$ | 5<br>5 | 4.545<br>4.545 | –<br>– | 45.5<br>45.5 | 60.6<br>63.5 | –<br>– |
| B | 6366108 | $7.7 \times 10^{-9}$ | 20 | 4.545 | 0.892 | – | – | 33.6 |
| C | 6277310 | $7.7 \times 10^{-9}$ | 5<br>30<br>10 | 4.444<br>4.444<br>4.444 | –<br>–<br>1.42* | 10<br>–<br>10 | 56.3<br>–<br>32.4 | –<br>12.4<br>– |
| D | 6277310 | $7.7 \times 10^{-9}$ | 30<br>30<br>5 | 4.640*<br>5.365**<br>4.640* | –<br>–<br>– | –<br>–<br>10 | –<br>–<br>59.4 | 24.0<br>3.70<br>– |
| E | 6277310 | $1.54 \times 10^{-9}$ | 2<br>5<br>10<br>20<br>6<br>16<br>31<br>51 | 0.980**<br>0.980**<br>0.980**<br>0.980**<br>0.980**<br>0.980**<br>0.980**<br>0.980** | –<br>–<br>–<br>–<br>–<br>–<br>–<br>– | 2.04<br>2.04<br>2.04<br>2.04<br>–<br>–<br>–<br>– | 57.2<br>54.5<br>44.4<br>38.6<br>–<br>–<br>–<br>– | –<br>–<br>–<br>–<br>0<br>2.58<br>1.53<br>1.20 |
| G | 6277310 | $1.54 \times 10^{-9}$ | 5<br>10<br>25<br>40<br>10<br>30<br>60<br>100 | 0.980**<br>0.980**<br>0.980**<br>0.980**<br>0.980**<br>0.980**<br>0.980**<br>0.980** | 0.309*<br>0.309*<br>0.309*<br>0.309*<br>0.309*<br>0.309*<br>0.309*<br>0.309* | 2.04<br>2.04<br>2.04<br>2.04<br>–<br>–<br>–<br>– | 35.8<br>43.1<br>32.0<br>31.2<br>–<br>–<br>–<br>– | –<br>–<br>–<br>–<br>7.55<br>5.21<br>2.19<br>2.14 |

Code: * material chromatographed and recrystallized once.

** material chromatographed and recrystallized twice.

contained a trace of G16P cofactor which was catalysing the reaction.

C. Test for G16P in G1P

Reactions carried out with unpurified G1P and enzyme alone (rough estimations, not quoted here) suggested that G16P was present in G1P only to a small extent. A more accurate estimation (see Table R.1.6) gave a S.A. of 12.4 compared with 56.3 in the presence of added G16P, and confirmed that G1P did not contain sufficient G16P to be a major factor in activating the enzyme. This conclusion was confirmed by the Boehringe Corporation (London Ltd.) who supplied the substrate. However, G16P need only be present as a very minor contaminant to activate the enzyme as its Km is very low, $6.2 \times 10^{-8}$ M[14] so that complete removal of G16P from G1P by chromatography is as necessary to prevent activation of the enzyme.

In the complete absence of G16P using purified G1P Ray and Roscelli were able to reduce the specific activity to 2% of the maximum specific activity at enzyme concentrations less than $10^{-8}$ M. At higher enzyme concentrations the system quickly became self activating and at $10^{-7}$ M enzyme concentration the lowest possible specific activity was about 20% of the maximum value. As the concentration of enzyme

employed in the present series of experiments was $0.72 \times 10^{-8}$ M, a higher specific activity than approx. 1.5 (i.e. 2% of max. S.A. 60) indicated that G16P was present in one or both of the substrates.

D. Test for G16P in G1P purified by chromatography and recrystallization

G1P was purified as described in the method section, being recrystallized once before being assayed by the standard method for G1P analysis. The S.A. of phosphoglucomutase using the purified G1P as substrate was evaluated under the conditions described in Table R.1.6 both in the presence and absence of added G16P (10 μM). In the presence of G16P the S.A. was virtually the same as the maximum S.A. of the enzyme batch showing that G1P contained no inhibitors after purification. The S.A. in the absence of G16P during a 30 minute incubation was rather high (S.A. = 24.0) in duplicated experiments. This may have been due to chance contamination of the apparatus, as a further recrystallization of the G1P gave a new value the S.A. of 3.7, and it would be unlikely that recrystallization alone would affect such a dramatic improvement in purity when chromatography had apparently failed (see Table R.1.6). In further experiments once chromatographed and twice recrystallized G1P was used as

substrate for phosphoglucomutase and as this test did not categorically indicate that G1P was free of G16P it was repeated with one or two modifications (see below).

E. Repeat of test D and effect of incubation time upon the measured S.A.

In this series of experiments the concentration of recrystallized (x2) G1P was reduced to about 1 mM and the G16P concentration correspondingly reduced to 2 μM. The enzyme concentration was also reduced by a factor of five to $1.54 \times 10^{-9}$ M in order that in the absence of G16P the observed S.A. would relate even more specifically to the G16P independent reaction and not to the auto-catalytic reaction observed by Ray and Roscelli[14] at enzyme concentrations greater than $10^{-8}$ M. The experiments were carried out as follows:- 0.1 mls. of the diluted phosphoglucomutase solution (see section A) was incubated for 10 mins. with 0.4 mls. of the histidine/Tris/$Mg^{2+}$ buffer pH 7.4 previously described. The assay was initiated by the addition of 0.4 mls. of solutions containing either G1P alone (conc. 0.980 mM), or G1P (0.980 mM) and G16P (2.04 μM). 0.1 ml. samples were taken at the times indicated and the S.A's calculated from the extent of reaction, obtained by measuring the remaining G1P concentration. All determinations were duplicated and the results are shown in Table R.1.6.

The maximum S.A. of phosphoglucomutase in the presence of G16P recorded under these conditions showed no significant deviation from the values obtained with unpurified G1P (S.A. 60). The values recorded after 2 minutes incubation are liable to inaccuracy as the amount of G1P converted to G6P was small. The most accurate values were obtained after 5 minutes incubation. With further increases in the time of incubation inhibition by the G6P formed during the reaction became significant and the value of the S.A.'s began to fall. In the absence of added G16P the values recorded for the S.A.'s were all smaller than those recorded under the original conditions and showed that G1P was entirely free from G16P contamination as they were all less than 2% of the maximum S.A. with G16P (Ray and Roscelli[14]). That the values are lower than in experiment D may, at least in part, be attributed to the lower enzyme concentration which would have reduced the possibility of autoactivation virtually to zero. Moreover in the absence of G16P the S.A.'s were observed to fall with time. If this was regarded as significant, even for small amounts of G1P conversion, then it would be in accord with a reaction catalysed by an enzyme-phosphate complex which was slowly dissociating rather than an auto-catalysed reaction for which S.A's would be expected to increase with time.

To show that enzyme activity was still preserved 0.2 mg. G16P was added to the assay mixtures and after an hour a sample was withdrawn. Determination of the remaining G1P showed that the reaction had gone to completion demonstrating that enzyme activity was present at the end of assays.

F. Test for G16P in G6P

In test C it was shown that assay of enzyme activity with G1P alone gave a S.A. of 12.4. However, test B, carried out under almost identical conditions but with added G6P, gave a higher S.A. of 33.6. If the expected S.A. in test B is calculated from the value in test C by taking into account G6P inhibition (this can be done from the Km's of G6P and G1P[32]), then a value of approximately 12 is obtained. Consequently the G6P must have contained sufficient G16P to activate the enzyme.

G. Test for G16P in chromatographed G6P and effect of incubation time on the measured S.A.

On the basis of the results from test F it was decided to purify both G1P and G6P by column chromatography according to the method of Bartlett[2] which gave good separation of monophosphates and diphosphates. In the next two sections the results of these purifications (see methods section) have been analysed.

G6P which had been chromatographed and recrystallized as described in the methods section M.1, was dissolved in water and the concentration of the solution measured by the Anthrone method. To test for G16P in G6P it was first necessary to carry out an assay (see experiment C, Table R.1.6) in the presence of G6P and G16P to show that the G6P contained no inhibitors. Then to follow the effect of incubation time on the S.A. exactly the same procedure as in experiment E was used except that the final assay solution contained 0.309 mM G6P as well as G1P and G16P. Duplicated sets of estimations were performed both with and without G16P. The activity of the enzyme used in the absence of G16P was checked at the end of the experiment as before and it was shown that the enzyme had retained its activity.

As expected the S.A. of those assays containing both G16P and G6P did not fall as rapidly with time as those without G6P in experiment E, demonstrating competetive inhibition of the system by G6P. In the absence of added G16P higher S.A.'s were observed than for experiment E (without G6P) but a comparable fall in S.A. with time was recorded. The S.A.'s recorded for times greater than 30 mins. (see Table R.1.6) were in accordance with both G1P and G6P being completely free of G16P. The reason why the initial S.A.'s were as high as 5-10 units/mg. protein was not clear.

However, some reaction may have occurred if part of the enzyme was present initially as phosphoenzyme. Under the conditions of experiment this phosphoenzyme would be expected to dissociate to inorganic phosphate and inactive enzyme giving rise to the fall of S.A. with time observed in these experiments.

## SECTION 2. STUDIES ON THE PHOSPHOGLUCOMUTASE SYSTEM; EQUILIBRIUM CONSTANT; EFFECT OF MONOPHOSPHATE CONCENTRATION

A value of 17.2 has been quoted by various authors[33, 34] for the equilibrium constant of the phosphoglucomutase reaction at 30°C over the pH range 6.6-7.5 in the presence of 3.6 mM $Mn^{2+}$ ions. As $Mg^{2+}$ was used instead of $Mn^{2+}$ as an activator of phosphoglucomutase in the induced transport tests it was necessary to check that the value of equilibrium constant was the same under these conditions. It was also necessary to investigate whether increasing the monophosphate concentration affected the value of the equilibrium constant as both G1P and G6P were known to complex with magnesium. Moreover at the very high concentrations employed during counter-transport experiments the activity coefficients of G1P and G6P would be very different from unity and hence may also have affected the value of the equilibrium constant. The values quoted in Table R.2.1 below were measured at the end of equilibration periods during induced transport experiments at the substrate concentrations shown. In all cases the concentrations of the histidine, tris, $MgCl_2$ buffer were 27.7, 8.3 and 0.76 mM respectively.

Table R.2.1.

| Run No. | G1P (mM) | G6P (mM) | $Keq = \frac{G6P}{G1P}$ | G16P (μM) |
|---|---|---|---|---|
| 4 | 0.0868 | 1.517 | 17.48 | 4.6 |
| 3 | 0.8711 | 14.94 | 17.15 | 48.9 |
| 3 | 1.703 | 28.67 | 16.86 | 45.0 |
| 5 | 2.444 | 42.41 | 17.35 | 115.5 |
| 5 | 4.844 | 81.51 | 16.82 | 105.9 |

Average value = 17.13

The G1P concentrations measured by the phosphate method (see methods section) were corrected for the small increment due to hydrolysis of G16P.

Within the limits of experimental error there was no systematic variation in the value of the equilibrium constant. This was not entirely unexpected for although G1P and G6P do complex with magnesium, they do so to about the same extent; $K_{diss.}$ G1P-$Mg^{2+}$ = 11 mM, $K_{diss.}$ G6P-$Mg^{2+}$ = 16 mM[35], and the activity coefficients of both G1P and G6P should be very similar under most conditions of ionic strength as their size and charge are very similar. Confirmation that the activity coefficients are in fact very similar was given by depression of freezing point measurements which are discussed below.

Activity coefficients of G1P and G6P at various concentrations

The activity coefficients of solutions of $Na_2$G1P and $Na_2$G6P were calculated from the depression of freezing point measured with an 'Advanced Osmometer'. For comparison, similar measurements were also made in the histidine tris buffer pH 7.4 (29.85, 9.04 and 0.82 mM) used for the induced transport measurements. The osmotic pressures in mOsmoles were converted to depression of freezing point using Table R.2.2. below which correlated the freezing point depression with the osmotic pressure in mOsm/kg. for a NaCl/$H_2O$ standard[36].

Table R.2.2.

| NaCl gms/kg $H_2O$ | m Osm/kg. | Freezing point depression $^oC$ |
|---|---|---|
| 3.097 | 100 | 0.1858 |
| 6.265 | 200 | 0.3716 |
| 6.892 | 219.6 | 0.4080 |
| 8.692 | 275.8 | 0.5125 |
| 9.468 | 300 | 0.5574 |
| 10.206 | 323 | 0.6002 |
| 12.694 | 400 | 0.7432 |
| 15.932 | 500 | 0.9290 |

The freezing point depressions for solutions of G1P and G6P have been recorded in the following table (Table R.2.3).

Table R.2.3.

G1P and G6P in the presence of buffer pH 7.4

| No. | Substrate conc. mM | G1P mOsm | G1P $-\Delta T^{o}C$ | G6P mOsm | G6P $-\Delta T^{o}C$ |
|---|---|---|---|---|---|
| 1<br>2 | 152.3 | 374.8<br>375.4 | 0.696<br>0.697 | 367.6<br>365.0 | 0.683<br>0.678 |
| 3<br>4 | 89.55 | 211<br>211 | 0.392<br>0.392 | 212<br>208 | 0.394<br>0.386 |
| 5<br>6 | 44.78 | 153.5 | 0.285 | 151.6 | 0.282 |
| 7<br>8 | 12.7 | 74.8<br>74.6 | 0.1390<br>0.1386 | 71.4<br>71.3 | 0.1327<br>0.1325 |
| 9<br>10 | 5.97 | 59.5<br>59.6 | 0.1106<br>0.1107 | 57.6<br>563 | 0.1070<br>0.1046 |

G1P and G6P alone

| No. | Substrate conc. mM | G6P mOsm | G1P $-\Delta T^{o}C$ | G1P mOsm | G1P $-\Delta T^{o}C$ | Average G1P/G6P $-\Delta T^{o}C$ |
|---|---|---|---|---|---|---|
| 1<br>2 | 179.1 | 383.8<br>385.4 | 0.713<br>0.716 | 380.0<br>384.0 | 0.706<br>0.713 | 0.711 |
| 3<br>4 | 83.40 | 197.4<br>198.6 | 0.367<br>0.369 | 193.0<br>197.0 | 0.359<br>0.366 | 0.3647 |
| 5<br>6 | 41.70 | 97.0<br>97.2 | 0.180<br>0.1806 | 96.5<br>98.2 | 0.1793<br>0.1825 | 0.1804 |
| 7<br>8 | 22.39 | 48.0<br>48.5 | 0.0892<br>0.0901 | 47.0<br>47.0 | 0.0873<br>0.0873 | 0.0884 |
| 9<br>10 | 11.78 | 25.1<br>25.2 | 0.0466<br>0.0468 | 24.0<br>24.4 | 0.0446<br>0.0453 | 0.0459 |
| 11<br>12 | 5.00 | 14.0<br>12.4 | 0.0260<br>0.0230 | 13.2<br>13.8 | 0.0245<br>0.0256 | 0.0248 |

Average values of the freezing point depression for G1P and G6P alone in solution were used in the calculation of activity coefficients as the difference between G1P and G6P was so small. A standard equation was employed to relate freezing point depression ($\Delta T$) with activity coefficient ($\gamma_{\pm}$) of the monophosphates[37]

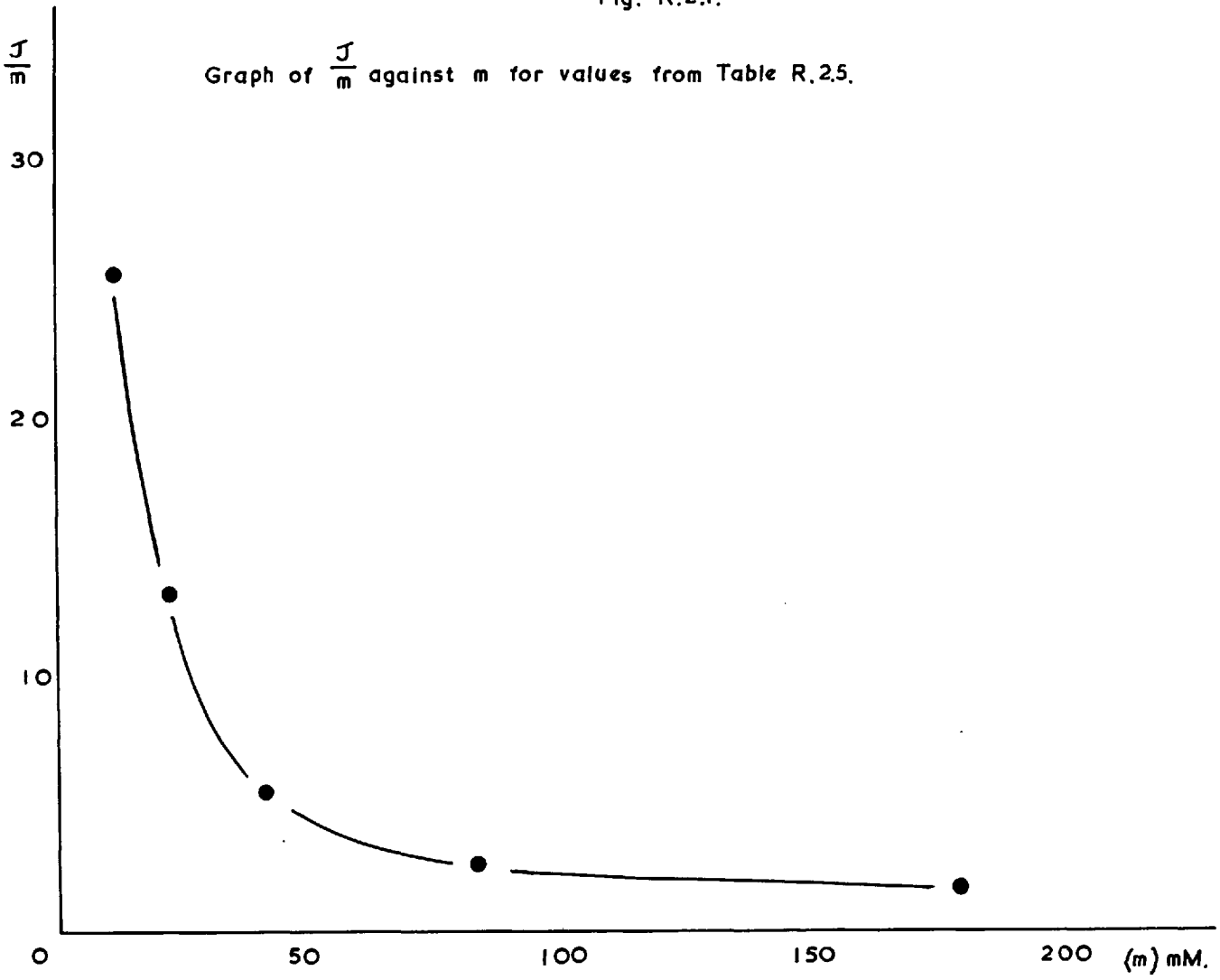
$$\ln \gamma_{\pm} = -J - \int_{o}^{m} \frac{J}{m}\, dm \qquad \text{where} \qquad = 1 - \frac{T}{vmk}$$

and ( v = total number of ions in solution per molecule substrate
( m = number of moles in 1000 g. solvent
( k = molal freezing point depression constant of water (1.858°C)
( $\gamma_{\pm}$ = mean activity coefficient
( $\Delta T$ = freezing point depression

In order to calculate the integral of $\frac{J}{m}\, dm$, values of $\frac{J}{m}$ were plotted against m and the area under the graph measured (see Fig. R.2.1.). J cannot be extrapolated below m = 11.78 the molality at which measurements were made because the area enclosed by the curve became very large. The activity coefficient at m = 11.78 was determined by the use of the Debye-Hückel expression.

Fig. R.2.1.

Graph of $\frac{J}{m}$ against m for values from Table R.2.5.



$$\log \gamma \pm = \frac{AZ_+ Z_- \sqrt{S}}{1 + db\sqrt{S}}$$

where A = 0.509 and db$\simeq$1, $Z_+$ = charge of positive group, $Z_-$ = charge of negative group. For a low value of m, the expression above becomes:-

$$\log \gamma \pm = \frac{0.509\ Z_+ Z_- \sqrt{S}}{1 + \sqrt{S}}$$

Thus for both $Na_2G1P$ and $Na_2G6P$ at a concentration of 11.78 mM the mean activity coefficient $\gamma \pm$ can be calculated to be 0.655. It is now possible to evaluate $\gamma \pm$ at higher concentrations of monophosphate by dividing up the integral $\int_0^m \frac{J}{m}\,dm$ as shown below.

$$\log \gamma \pm = -J - \int_0^{11.78} \frac{J}{m}\,dm - \int_{11.78}^{m} \frac{J}{m}\,dm$$

N.B. molal $\simeq$ molar at these cones.

$$= -J - (J_{11.78} + \log \pm 11.78) - \int_{11.78}^{m} \frac{J}{m}\,dm$$

Details of the calculations are shown in the following two tables.

Fig. R.2.2.

Graph of mean activity coeff. of G-1-P and G-6-P, $\gamma_{\pm}$, against concentration.

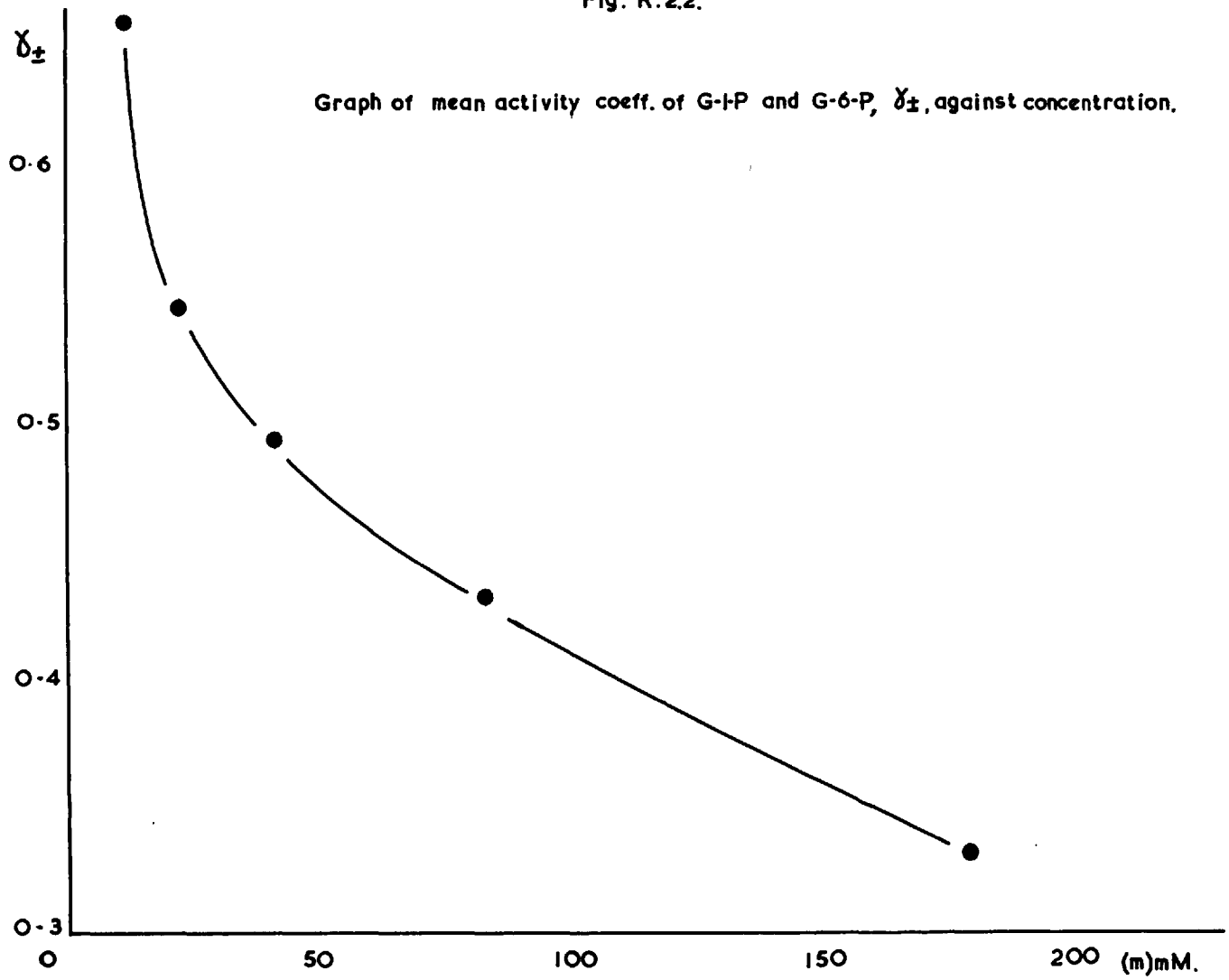


Table R.2.5.

Calculation of $\frac{J}{m}$ for various values of m

| Conc. of phosphates (mM) | $\Delta T^{o}C$ | $\frac{\Delta T}{vmk}$ | J | $\frac{J}{m}$ |
|---|---|---|---|---|
| 179.1 | 0.711 | 0.7122 | 0.2878 | 1.6069 |
| 83.4 | 0.3647 | 0.7845 | 0.2155 | 2.5839 |
| 41.7 | 0.1804 | 0.7761 | 0.2239 | 5.3693 |
| 22.39 | 0.0884 | 0.7083 | 0.2917 | 13.028 |
| 11.78 | 0.0459 | 0.69904 | 0.30096 | 25.548 |

Table R.2.6.

| Conc. of phosphates (mM) | $J-J_{11.78}$ | $\int_{11.78}^{m}\frac{J}{m}dm$ | $\ln\frac{\gamma\pm}{\gamma\pm 11.78}$ | $\log_{10}\frac{\gamma\pm}{\gamma\pm 11.78}$ | $\gamma\pm$ |
|---|---|---|---|---|---|
| 179.1 | +0.0132 | -0.6951 | -0.6819 | -0.2965 | 0.331 |
| 83.4 | +0.0855 | -0.5067 | -0.4212 | -0.1828 | 0.430 |
| 41.7 | +0.0771 | -0.3642 | -0.2871 | -0.1247 | 0.491 |
| 22.39 | +0.0093 | -0.1963 | -0.1870 | -0.0813 | 0.543 |
| 11.78 | 0.00 | -0.00 | 0.00 | 0.00 | 0.655 |

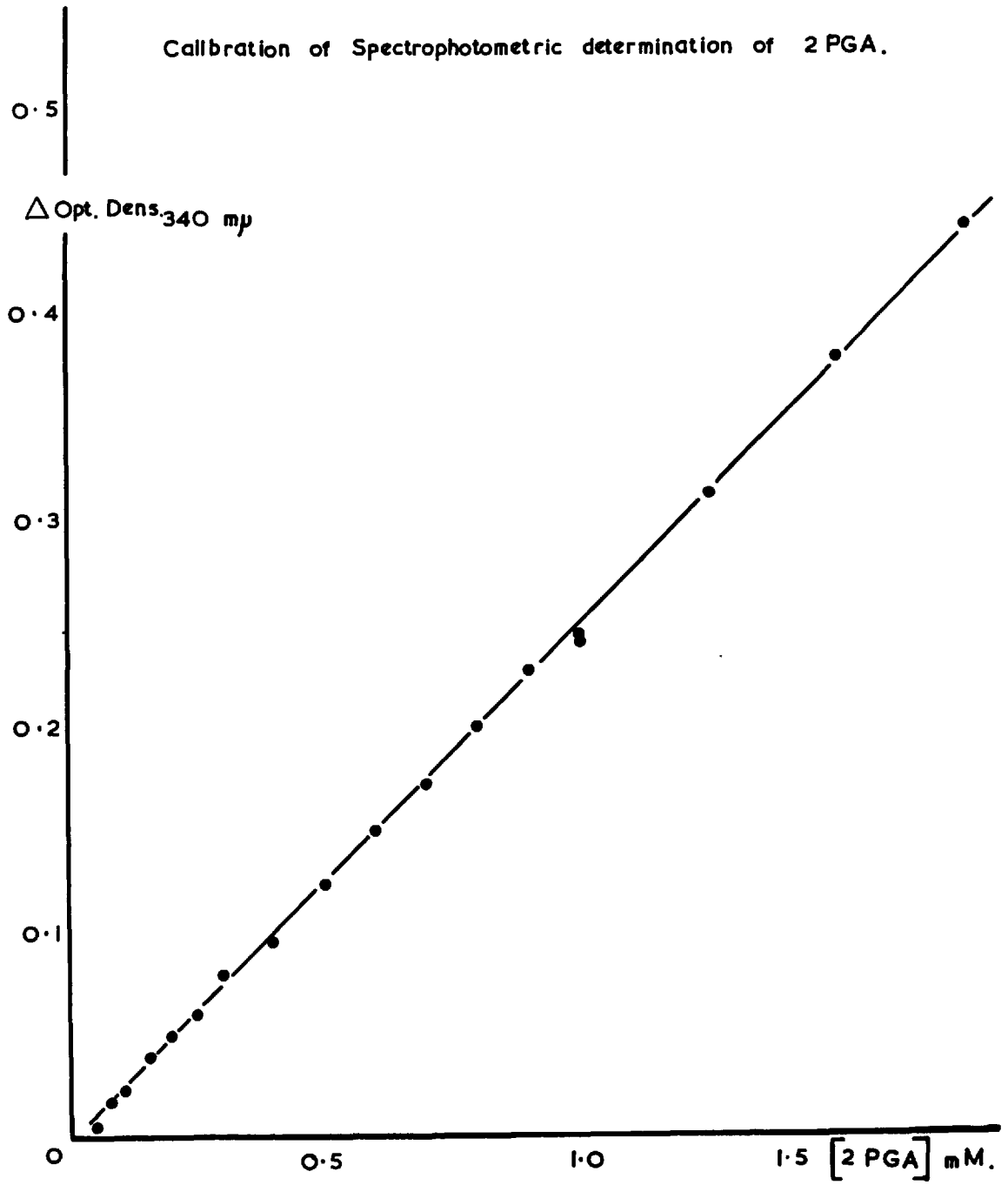
The fall in the mean activity coefficient $\gamma\pm$ of both monophosphates with increasing concentration has been shown graphically in Fig. R.2.2. The effect of this fall will be considered in the discussion section.

## SECTION 3. PHOSPHOGLYCERATE MUTASE SYSTEM; PRELIMINARY STUDIES:- VALIDATION OF TECHNIQUES

### Standardization of chemical estimation of 2PGA

The method of 2PGA estimation has been described previously and was calibrated as follows. Two standard solutions of 2PGA, 1.0 mM and 0.1 mM were made up by weighing. Various volumes were taken to simulate 0.1 ml. samples of various concentrations. The optical densities of the NADH solutions were measured against a water blank at 340 nm before addition of enolase, and again 13 minutes after the addition when the conversion of 2PGA to lactate was complete and a constant optical density was obtained. The initial optical density was adjusted for dilution and the difference between the two was then proportioned to the 2PGA concentration. Calculation of 2PGA concentration directly from the optical density showed that the purity of the trisdium salt $C_3H_4O_7PNa_3.6H_2O$ supplied by Boehringer and Soehne was 95%. This figure was in agreement with their own analysis of the salt. 3PGA ($Na^+$ salt) assayed in the same way (by the inclusion of phosphoglycerate mutase in the assay system), was also found to have a purity of 95%, again in agreement with the figure quoted by Boehringer and Soehne for $C_3H_4O_7PNa_3$. A calibration curve (Fig. R.3.1) was drawn up of the actual concentration of 2PGA against recorded difference in optical density at 340 nm. All subsequent

FIG. R.3.1.


Calibration of Spectrophotometric determination of 2 PGA.
0·5
Δ Opt. Dens. 340 mμ
0·4
0·3
0·2
0·1
0
0·5
1·0
1·5 [2 PGA] mM.

estimations of 2PGA concentration were interpolated from this graph. No change in optical density could be measured in experiments with solutions containing up to 20 mM 3PGA, showing that 3PGA does not interfere.

Enzyme protein concentration determination

The total enzyme protein concentration was measured from the optical density at 280 nm as already described. 0.05 ml. of the enzyme solution supplied by Boehringer and Soehne was diluted to 2.55 mls. with water. From the O.D. at 280 nm. of 0.123 and the extinction coefficient of $E^{1\%\ soln}_{1\ cm.\ cell} = 12.5$, the concentration of protein in the solution was calculated to be 5.02 mg./ml. in good agreement with the manufacturers value of 5 mg/ml.

Estimation of [$^{14}$C] 2PGA; preliminary experiments

As an initial procedure it was decided to convert [$^{14}$C] 2PGA to [$^{14}$C] lactate as already described and to attempt to remove [$^{14}$C] 3PGA with $ZnSO_4$ and $Ba(OH)_2$ as described for [$^{14}$C] G1P. This was done by making up solutions of [$^{14}$C] 3PGA at a concentration which contained different concentrations of 2PGA. Samples (0.1 mls.) were taken from these solutions and acidified with 0.1 mls. $NH_2SO_4$, followed

by 0.5 mls. (0.8 mg./ml.) lactate diluting solution, 0.65 mls. $Ba(OH)_2$ and 0.2 mls. $ZnSO_4$. The precipitate was centrifuged at 2000 g. for 10 mins. then a further 0.1 mls. $Ba(OH)_2$ was added followed by 0.1 mls. $ZnSO_4$. The solutions were recentrifuged and 0.8 mls. of the supernatant removed for estimation of radioactivity. The results are shown in Table R.3.1. below.

Table R.3.1.

Concentration of 3PGA = 2.30 mM. 0.01 mls. [$^{14}$C] 3PGA (44.5 µl/ml.) added.

| Conc. of 2PGA mM | Total Counts present/ 20 mins. | Remaining Counts after $Ba^{2+}/Zn^{2+}$ precipitation | | | Average Counts | % [$^{14}$C] 3PGA left |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | | |
| 2.50 | 12,048,000 | 7552 | 8121 | 8743 | 8139 | 0.68 |
| 1.00 | 12,048,000 | 14888 | 17072 | 16855 | 16271 | 1.35 |
| 0.40 | 12,048,000 | 24037 | 26281 | 25103 | 25140 | 2.09 |
| 0.10 | 12,048,000 | 22101 | 22835 | 26445 | 23793 | 1.97 |

The method thus precipitated 3PGA effectively but it was not investigated further because it became apparent that the

dilution of [$^{14}$C] lactate solution was excessive and that it would not be possible to add labelled material in sufficient amounts. This was particularly so in the measurement of radioactive fluxes at equilibrium as the initial equilibrium of non-radioactive substrate must not be significantly disturbed.

To reduce the dilution factor a systematic study of the effectiveness of the $ZnSO_4$ and $Ba(OH)_2$ precipitation was undertaken. The order of addition of the reagents was also examined since, as described below, under some conditions loss of lactate occurs. Equal volumes of the $ZnSO_4$ and $Ba(OH)_2$ reagents, described previously were used in various proportions to precipitate 3PGA from solution. The 3PGA remaining in solution was measured by the chromotropic acid method of Bartlett[13] and the experimental conditions and results reported in Table R.3.2 below.

Table R.3.2.

Chromotropic acid test:- 0.2 mls. solution + 5.8 mls. chromotropic acid. Heat to 100°C for 30 mins. Cool measure O.D. 695 mμ in 1 cm. cell.

Vol. of 3PGA soln. = 0.5 mls.

| Conc. of 3PGAmM | Test | mls. $ZnSO_4$ | mls. $Ba(OH)_2$ | Optical Density 695 mμ | OD-Blank | % 3PGA left in solution |
|---|---|---|---|---|---|---|
| 1.43 | $Ba(OH)_2$ first | 0.1 | 0.1 | 0.034 | 0.025 | 4.0 |
| 1.112 | then $ZnSO_4$ | 0.2 | 0.2 | 0.017 | - | 0 |
| 0.667 | | 0.5 | 0.5 | 0.021 | 0.002 | 1.1 |
| 1.43 | $ZnSO_4$ first | 0.1 | 0.1 | 0.021 | 0.002 | 0.53 |
| 1.112 | then $Ba(OH)_2$ | 0.2 | 0.2 | 0.026 | 0.007 | 2.4 |
| 0.667 | | 0.5 | 0.5 | 0.025 | 0.006 | 3.4 |
| 2.00 | Standard | - | - | 0.544 | 0.525 | - |
| 0.00 | Blank | - | - | 0.019 | - | - |

It will be seen that within the limits of experimental error $ZnSO_4$ and $Ba(OH)_2$ could be added in either order to precipitate 3PGA from the solution. As described in a later section addition of $Ba(OH)_2$ before $ZnSO_4$ leads to some loss of lactate whereas this does not occur if the reagents are added in the reverse order. This latter procedure was therefore adopted.

In view of the above results the precipitation of the phosphate esters was tested under the conditions which were to be used in the induced transport experiments. A solution was made up, volume 3.0 mls., containing 1.33 mM 2PGA, 10 μM 23PGA and $^{32}P$ monophosphates in $Mg^{2+}$/Tris buffer pH 7.4 ($MgCl_2$ = 3.33 mM, Tris = 16.7 mM) at 30°C. Phosphoglycerate mutase (0.25 μg) was added and the system allowed to come to equilibrium then 0.4 mls. of the solution were withdrawn and replaced by 0.4 mls. 10 mM 2PGA. Concentrations of reagents were then 2PGA, 1.47 mM, 3PGA 0.913 mM, 23PGA 9.64 μM. At various times after the addition of 2PGA, samples (0.1 mls.) were withdrawn and 0.1 mls. 0.25 N $HClO_4$ was added to denature the enzymes. 2PGA was converted to lactate as described in the method section. 0.5 mls. was taken from each solution and 0.3 mls. $ZnSO_4$ followed by 0.3 mls. $Ba(OH)_2$ (concentrations described previously) added to precipitate the phosphates. After centrifugation 0.4 mls. was taken from the supernatant for radioactive assay as shown in Table R.3.3. below.

Table R.3.3.

| Time after addition of 2PGA (mins.) | 2PGA conc. (mM) | Total [$^{32}$P] counts | Counts in supernatant after precipitation | % counts left |
|---|---|---|---|---|
| -1.0 | 0.142 | 16,430 | 155 | 0.94 |
| 1.0 | 1.47 | 16,430 | 221 | 1.34 |
| 6.0 | 1.336 | 16,430 | 134 | 0.82 |
| 11.0 | 1.190 | 16,430 | 164 | 1.00 |
| 25.0 | 0.820 | 16,430 | 119 | 0.72 |
| 50.0 | 0.457 | 16,430 | 144 | 0.88 |

These results indicated that a single precipitation step with the $ZnSO_2/Ba(OH)_2$ reagent was a very efficient method of removing all phosphate and phosphate esters from solution.

Conditions for retention of lactate in solution during $Ba(OH)_2/ZnSO_4$ precipitation

It was important for accurate estimation of [$^{14}$C] 2PGA that lactate was not adsorbed onto the $ZnSO_4/Ba(OH)_2$ precipitate during removal of 3PGA and other phosphates from solution. This possibility was first investigated using the phosphate precipitating conditions as described for precipitation of G6P.

To a solution containing 16.7 mM Tris, 3.33 mM $MgCl_2$, 10 μM 23PGA and 0.67 mM 3PGA at pH 7.4, 0.25 μg. of rabbit phosphoglycerate mutase was added and the reaction allowed to reach equilibrium. At this stage 0.01 mls. [$^{14}$C] pyruvate (0.1 mC/ml. S.A. 11.8 mC/mM). was mixed in and 0.1 ml. aliquots removed for conversion of 2PGA (and pyruvate) to lactate as described in the methods section. From the solution samples (0.5 mls.) were removed and phosphates precipitated as described in connection with Table R.3.1, but after each precipitation step with $Ba(OH)_2$ and $ZnSO_4$ a sample was taken from the supernatant for $^{14}$C-lactate assay. The results are shown in Table R.3.4. It will be seen that a substantial portion of $^{14}$C lactate was removed by the $Ba(OH)_2/ZnSO_4$ precipitation step.

It seemed possible that the removal of lactate was due to the fact that the precipitate was formed in alkaline ($Ba(OH)_2$) solution. An experiment was therefore carried out in which the order od addition of $ZnSO_4$ and $Ba(OH)_2$ was reversed.

The conditions chosen were those to be used in the induced transport experiments, as described in Table R.3.3, but [$^{14}$C] 3PGA rather than [$^{32}$P] 3PGA was added at the beginning of the equilibration phase of the experiment. Samples (0.1 mls.) were withdrawn at various stages

Table R.3.4.

| Total [$^{14}$C] lactate counts (20 mins) | [$^{14}$C]lactate counts/ 20 mins after 1st precipitation | % of lactate left after 1st precipitation | [$^{14}$C]lactate counts/ 20 mins after 2nd precipitation | % lactate remaining 2nd precipitation | [$^{14}$C]lactate counts/ 20 mins after 3rd precipitation | % lactate remaining 3rd precipitation |
|---|---|---|---|---|---|---|
| 1,397,503 | 1,256,355 | 89.9 | 1,194,865 | 85.5 | 1,172,505 | 83.9 |
| 1,250,285 | 1,076,495 | 86.1 | 1,053,990 | 84.3 | 975,222 | 78.0 |
| 1,358,828 | 1,259,634 | 92.7 | 1,205,280 | 88.7 | 1,112,880 | 81.9 |
| 1,329,387 | 1,153,908 | 86.8 | 1,151,249 | 86.6 | 1,015,652 | 76.4 |

throughout the reaction, added to 0.1 mls. 0.25 N $HlCO_4$ and the 2PGA converted to lactate. An aliquot (0.5 mls.) was withdrawn from each solution and 0.3 mls. of $ZnSO_4$ and $Ba(OH)_2$ added in that order. After centrifugation 0.2 mls. of the supernatant were taken for counting and a further, identical, precipitation step was carried out upon 0.5 mls. of the supernatant. The results are shown in Table R.3.5. below.

Table R.3.5.

Counts/ml. measured over 20 mins. and adjusted for dilution.

| Mins. after addition of 2PGA | Supernatant counts after 1st $ZnSO_4/Ba(OH)_2$ step | Supernatant counts after 2nd $ZnSO_4/Ba(OH)_2$ step | Supernatant radioactivity after 2nd step as % of activity remaining after 1st step |
|---|---|---|---|
| -1.0 | 64958 | 61969 | 95.4 |
| 1.0 | 81970 | 85117 | 103.8 |
| 8.0 | 74730 | 74237 | 99.3 |
| 25.0 | 68830 | 69110 | 100.4 |
| 90.0 | 70978 | 71595 | 100.9 |

Average counts left = 99.96%

These results indicated that lactate is not removed from solution with the phosphates if $ZnSO_4$ was added prior to $Ba(OH)_2$, and the procedure was therefore adopted to assay [$^{14}$C] 2PGA.

[$^{32}$P] 2PGA determinations: Validation of techniques

In the assay of [$^{32}$P] 2PGA, in the presence of [$^{32}$P] 3PGA, 2PGA is converted to lactate and [$^{32}$P] ATP, and the ATP is hydrolysed with acid to AMP and inorganic [$^{32}$P] phosphate. [$^{32}$P] 3PGA is resistant to hydrolysis under these conditions. Sufficient inorganic phosphate precipitating reagent[18] was then added to an aliquot of the solution to effect complete precipitation of inorganic phosphate leaving only [$^{32}$P] 3PGA in the supernatant. To check the efficiency of the procedure the following tests were made. A series of solutions of 3PGA (0.5 - 2.5 mM) were made up containing [$^{32}$P] 3PGA. The 3PGA in 0.1 ml. samples was converted to lactate and ATP (final volume 2.30 mls.) as described in the methods section (3PGA was converted to 2PGA by addition of 0.02 mls. 0.3 mM 23PGA and 0.01 mls. phosphoglycerate mutase (1.5 units)). The final solution contained a maximum of 0.76 µmoles of hydrolysable phosphates (corresponding to the 2.5 mM 3PGA solution). 0.5 mls. (0.165 µmoles $PO_4$) were taken and added to 0.4 mls. phosphate precipitating reagent

(which should be able to precipitate up to 1.66 µmole phosphate[18]). The reagents were placed for 10 minutes in a boiling water bath to hydrolyse the phosphate, then allowed to cool at 4°C for at least one hour. After centrifugation 0.4 mls. of the supernatant was taken for $^{32}P$ assay as recorded in Table R.3.6 below.

Table R.3.6.

| Conc of 3PGA mM | Original counts 0.1 mls/10 mins | Counts after precipitation 0.4 mls/10 mins | | Av. % 3PGA left in solution |
|---|---|---|---|---|
| | | 1 | 2 | |
| 2.50 | 29434 | 11365 | 14697 | 19.9 |
| 2.00 | 27188 | 24934 | 29928 | 45.4 |
| 1.00 | 28273 | 34533 | 35604 | 55.8 |
| 0.50 | 27474 | 35283 | 35630 | 58.0 |

These results indicated that a complete precipitation of $PO_4$ had not been obtained. This may have been caused by either one or a combination of the following factors:-

1. Impurities in [$^{32}P$] 3PGA particularly [$^{32}P$] 23PGA.
2. Incomplete conversion of 3PGA to lactate and ATP.
3. Incomplete precipitation of inorganic [$^{32}P$] phosphate

due to partial hydrolysis of [$^{32}$P] ATP.

4. Reduction of phosphomolybdate by ribose from ATP (which leads to a blue colour in solution).

The first three possibilities were investigated as follows. No evidence has been obtained in the present work that reduction of phosphomolybdate by ribose causes precipitation of phosphate to be significantly incomplete.

A. Check of purity of [$^{32}$P] 3PGA

The radioactive substrate [$^{32}$P] 3PGA was chromatographed on Whatman No. 4 paper using isopropylether, 90% formic acid (3:2 v/v) as solvent with 3PGA and 23PGA marker spots as described in the methods section. Scanning for radioactivity showed that less than 1% of the activity was associated with 23PGA. Thus the possibility that [$^{32}$P] 23PGA counts were being measured in the supernatants of samples after phosphate precipitation could be discounted.

B. Check of conversion of 3PGA to lactate and ATP

Conversion of [$^{32}$P] 3PGA to lactate and [$^{32}$P] ATP was carried out under the conditions described for Table R.3.6, except that the optical density change at 340 nm was measured.

The change in optical density was used to calculate the 3PGA concentration (see earlier sections) and the completeness of conversion estimated as shown in Table R.3.7. 0.5 mls. of the solution was then hydrolysed with 0.4 mls. of the phosphate precipitating reagent by heating in a boiling water bath for 10 mins. and after cooling for 1 hr. or more at $4^{o}C$ and centrifugation the supernatant was assayed for $[^{32}P]$. It will be seen from Table R.3.7 that the conversion of 3PGA to lactate was complete but there was still a high percentage of $[^{32}P]$ activity left in the supernatant after phosphate precipitation. As a further check that 3PGA had been completely converted to lactate and ATP and that all $[^{32}P]$ activity was associated with ATP, a sample (0.1 mls.) of the solution before the addition of the phosphate precipitating reagent was applied to the origin of a Whatman No. 4 paper chromatogram together with 3PGA, 2PGA, 23PGA, ADP, ATP and $PO_4$ markers. The chromatogram was developed for about 16 hours with Butan-1-ol, acetic acid, water (12:3:5 by volume) and then dried. The marker spots were developed and the "active strip" was scanned for $[^{32}P]$ radioactivity. A small peak was found at the position of the phosphate marker but the main peak was associated with ATP. No activity was found to correspond with 2PGA or 3PGA. These results confirmed that conversion of $[^{32}P]$ to $[^{32}P]$ ATP was complete and indicated

Table R.3.7.

| Conc. of 3PGA mM | Original OD340 mμ | OD340 T= 6 min | OD340 T=30 min | OD original adj. for diln | $\Delta$ $OD_{T=6}$ 340 mμ | $\Delta$ $OD_{T=30}$ 340 mμ | *Conc. calc. for 3PGA |
|---|---|---|---|---|---|---|---|
| 0.00 | 0.851 | 0.844 | 0.819 | 0.847 | 0.003 | 0.028 | - |
| 2.20 | 0.904 | 0.302 | 0.280 | 0.900 | 0.598 | 0.620 | 2.20 |
| 2.00 | 0.883 | 0.362 | 0.536 | 0.879 | 0.517 | - | 1.92 |
| 1.00 | 0.941 | 0.643 | 0.627 | 0.937 | 0.294 | 0.310 | 1.05 |
| 0.50 | 0.842 | 0.691 | 0.676 | 0.838 | 0.147 | 0.162 | 0.508 |

T = time after addition of enzymes
OD340 = optical density at 340 mμ measured against water blank.
* = concentration calculated from Av of (OD340 - Blank) at T = 6, and 30 mins.

| Conc. of 3PGA mM | Original Counts in 0.1 mls/10 min. | Counts in 0.4 mls after precipitation | % "3PGA" left in solution |
|---|---|---|---|
| 2.20 | 75487 | 38162 | 22.8 |
| 2.00 | 72071 | 65562 | 40.9 |
| 1.00 | 69850 | 66614 | 42.9 |
| 0.50 | 69960 | 64586 | 41.6 |

that the most probable cause of incomplete precipitation of radioactive phosphate was incomplete hydrolysis of [$^{32}$P] ATP.

C. Hydrolysis of [$^{32}$P] ATP

Complete hydrolysis of ATP could be achieved by hydrolysis in 1 M $HClO_4$ at 100°C for 10 minutes[18]. In previous experiments, therefore, hydrolysis may have been incomplete since the acid was only 0.2 M. Moreover the acid was added in the presence of both triethanolamine buffer (pH 7.6) and triethylamine (from the phosphate precipitating solution) which would be expected to increase the pH of the acid solution (which was, however, less than pH 2 after the addition of all reagents). To improve these conditions for hydrolysis the concentration of the $HClO_4$ used was doubled to 1.8 M, giving a final acid concentration of 0.4 M. The results of hydrolysis of ATP in lactate solutions using this increased acid concentration has been shown in Table R.3.8. Although more than 80% of [$^{32}$P] ATP was converted to [$^{32}$P] Pi, a distinct improvement on the previous results, yet hydrolysis was still incomplete.

Table R.3.8.

| Conc. of 3PGA in original solution (mM) | Counts/10 mins before precipitation | Counts/10 mins after precipitation | % counts left % ATP unhydrolysed |
|---|---|---|---|
| 2.20 | 73660 | 29606 | 19.1 |
| 2.00 | 70241 | 24867 | 16.9 |
| 1.00 | 72089 | 31871 | 21.0 |
| 0.50 | 71847 | 28739 | 19.0 |

Eventually it was decided to separate the stages of hydrolysis of ATP and precipitation. 2PGA was converted to lactate as described in connection with Table R.3.6. and 0.2 mls. of the solution were added to 0.2 mls. 1.8 M $HClO_4$. The solution was heated to 100°C for 10 minutes to hydrolyse ATP then 0.4 mls. of equal parts by volume of 57 mM ammonium molybdate and 74 mM triethylamine were added together with 0.02 mls. $Na_2HPO_4$ (5 μ moles/ml.) as carrier. This solution was heated to 100°C for 1½ minutes then cooled to 4°C for at least one hour. After centrifugation 0.4 mls. supernatant was taken for counting. The results which are presented in Table R.3.9. below showed that very few counts were present in the solution. Hydrolysis of ATP and

precipitation of phosphate were thus virtually complete. The separation of [$^{32}$P] 2PGA from 3PGA was therefore based on these conditions.

Table R.3.9.

| Conc. of 3PGA mM | Original counts in 0.1 mls/10 mins | Counts left after precipitation/ 10 mins | % ATP left in solution |
|---|---|---|---|
| 2.00 | 44632 | 3114 | 3.6 |
| 1.00 | 46585 | 235 | 0.25 |
| 0.50 | 46184 | 1075 | 1.19 |

Precipitation of 3PGA

A final test was carried out to ensure that no 3PGA was carried down by the precipitate of inorganic phosphate. The precipitation was carried out on solutions containing chromatographed [$^{14}$C] 3PGA, conc. 2.3 mM, and various concentrations of 2PGA. Samples (0.1 mls.) were taken and 2PGA converted to lactate and phosphate as described in the methods section. After precipitation of the phosphate and centrifugation 0.4 mls. of the supernatant were taken for $^{14}$C-assay. The [$^{14}$C] 3PGA remaining in solution was calculated from the results as shown in Table R.3.10 below.

Table R.3.10

| Conc. of 2PGA mM | Initial $^{14}C$ counts x diln factor (10 mins) | Counts after precipitation | | Average counts/ 10 mins | % 3PGA recovery |
|---|---|---|---|---|---|
| | | 1 (10 mins) | 2 (10 mins) | | |
| 2.50 | 29367 | 30185 | 30801 | 30493 | 103.8 |
| 1.00 | 40662 | 41975 | 41965 | 41970 | 103.2 |
| 0.40 | 40662 | 42345 | 43094 | 42720 | 105.1 |
| 0.10 | 40662 | 43626 | 43533 | 42930 | 105.6 |

Since the recovery of [$^{14}C$] 3PGA in the supernatant was complete, within experimental error, no [$^{14}C$] 3PGA was taken down by the inorganic phosphate precipitating reagents.

## SECTION 4. MEASUREMENTS OF THE BINDING CONSTANTS OF 2PGA, 3PGA, AND 8-HYDROXYQUINOLINE WITH $Mg^{2+}$

### Introduction

During the course of kinetic studies of rabbit-muscle phosphoglyceratemutase (E.C.2.7.5.3.) it became apparent that binding of the substrates, 2PGA and 3PGA by magnesium was sufficiently extensive to affect the apparent Km's and the equilibrium constant of the reaction. Wold and Ballou (1957)[38] reported a value of 280 $M^{-1}$ for the binding constant of the 1:1 magnesium-$2PGA^{3-}$ complex at 25°C ($2PGA^{2-}$ was not considered to bind), based on titration studies. These results were valid over the range of ionic strength, I = 0.1 to 0.4. However, there appears to be no published value for the 1:1 magnesium-$3PGA^{3-}$ complex although Pizer in a private communication has given a value of 480 $M^{-1}$.

In view of the limited information available about the magnesium binding constants and the fact that measurements had not been made under the present conditions of temperature (30°C) and ionic strength (I = 0.02), it was decided to redetermine these constants by the method of Burton (1959)[28] which uses 8-hydroxyquinoline as a competitive ligand for magnesium. The majority of the measurements were made at 30°C in a tris buffer at pH 7.4 as this was used in the enzyme studies, but a few observations were made at different pH's.

In the method of Burton[28] the formation of the magnesium-8-hydroxyquinoline complex is followed spectrophotometrically in both the presence and absence of 2PGA or 3PGA as $MgCl_2$ is added to the solution. Burton followed the change in optical density at 360 nm whereas Milstein (1961)[35] in a later investigation employed a wavelength of 253 nm since the extinction coefficient for the $Mg^{2+}$-8-hydroxyquinoline complex is approx. ten times that at 360 nm (Milstein, 1960)[68]. More recently Ray and Roscelli (1966)[6] used a wavelength of 255 nm as they found that in the absence of $Mg^{2+}$ the optical adsorption was more stable than at 253 nm. In the present work it was found that drifting of the adsorption in this region was too great to permit accurate measurements to be made and 360 nm has therefore been used. Though the cause of the drift was not investigated it is clear that the optical adsorption at short wavelengths would be sensitive to slight turbidity in solution.

To minimise complexing of $Mg^{2+}$ Burton used triethanolamine as a buffer, but Milstein (1961) showed that there was negligible complexing between $Mg^{2+}$ and tris at pH 7.4. It was therefore decided to use the latter buffer since it was used in the enzyme studies. Measurements carried out during the course of this study confirmed that

at pHs less than pH 8.0 there is negligible binding of $Mg^{2+}$ by tris.

In the course of this investigation it was noticed that in solutions with pHs greater than pH 7.4 the optical density increased more rapidly as $MgCl_2$ was added than could be accounted for by the formation of the 1:1 $MgOx^{2+}$ complex alone. This has been interpreted as evidence for the formation of $Mg(Ox)_2$ in more alkaline solution. At neutral pHs little of the $MgCl_2$ which was added to the solution complexed with 8-hydroxy-quinoline. Consequently in order to calculate the binding constant of $Mg^{2+}$ with 2PGA or 3PGA the amount of binding need only be known approximately (see Theory section). Under more alkaline conditions, however, a substantial amount of magnesium is bound by the 8-hydroxyquinoline and it is therefore essential to know the amount of binding more accurately. For this reason the formation of $Mg(Ox)_2$ must be considered and the method of Burton has been modified (see theory section) to take this into account. In solutions more alkaline than pH 8 , however, the extent of magnesium binding becomes so great that the method becomes very inaccurate, even if the formation of $Mg(Ox)_2$ is taken into account. The situation is further complicated by the tendency of $Mg(Ox)_2$ to polymerize. On standing solutions at alkaline pH show a characteristic slow

decrease in optical density. Under extreme conditions a white polymeric precipitate of $Mg(Ox)_2$ is formed.

Burton employed two methods of analysing the data. In one curves were drawn for the increase in optical density at 360 nm against added $MgCl_2$ for the buffer solution alone, and then for the buffer in the presence of ligand. Lines of constant optical density were drawn and the intercepts on the curves determined. From this information the binding constant of the ligand with $Mg^{2+}$ was calculated. In the second method the binding constant was determined from the initial slopes of the graphs. This latter method was adopted for the present work as small but measurable pH changes occurred in the solution on the addition of $MgCl_2$ and extrapolation to zero $Mg^{2+}$ ion concentration minimized this error. Moreover, the ionic strength of solution was known most accurately under these conditions, and at high pH the formation of polymerized $Mg(Ox)_2$ was least likely at low $Mg^{2+}$ ion concentrations. On the other hand, however, initial slopes give no indication of the extent of formation of Mg $(ligand)_2$ complexes. However such complexes are unlikely to make a significant contribution to magnesium binding at the concentrations of reagents employed as the binding constants of the 1:1 complexes are relatively small. A preliminary investigation of the data using Burton's intercept method gave no evidence for the formation of such complexes. The

initial slope of the curves of O.D. (compensated for increasing dilution) plotted against the total concentration of $Mg^{2+}$ ions were calculated by fitting an empirical polynomial of the form shown in equation R.4.1. to the experimental data.

$$f(x) = p_1(1 - e^{-p_3 x}) + (a - p_1)(1 - e^{-p_2 x}) \quad \ldots \quad \text{R.4.1.}$$

In equation R.4.1. a represents the final optical density when all the 8-hydroxyquinoline is bound by $Mg^{2+}$, and this was determined as described below. $p_1$, $p_2$, $p_3$ are arbitrary constants which were evaluated for each curve by an Atlas 360 computer (University of London Computer Centre) using programme BMDX 85 for curve fitting.

8-hydroxyquinoline-magnesium binding; determination of a

Table R.4.1 shows calculations based upon the binding constant of $4.613 \times 10^{-6}$ (at I = 0.02) for 8-hydroxyquinoline-$Mg^{2+}$ binding obtained by Näseänen[29]. It will be seen that even in approximately 20 mM $MgCl_2$ binding of 8-hydroxyquinoline is incomplete below pH 8.5. Consequently it was necessary to determine a in solutions with a greater pH than this. Measurement of a in solutions with the pH at approx. 8.8 at various tris buffer concs. shows that the value determined

Table R.4.1.

| pH | $[Ox]_{Free}$ (mM) | Percent $[Ox]$ bound by $Mg^{2+}$ at $MgCl_2$ = 19.6 mM | $\frac{OD_{max}}{OD_{19.6mM\ MgCl_2}} = \frac{[Ox]_{Tot}}{([Ox]_{Tot} - [Ox]_{Free})}$ |
|---|---|---|---|
| 6.50 | 0.624 | 32.8 | 3.05 |
| 7.35 | 0.2523 | 69.9 | 1.43 |
| 7.40 | 0.2269 | 73.1 | 1.37 |
| 7.93 | 0.08120 | 89.9 | 1.11 |
| 8.55 | 0.02075 | 97.5 | 1.03 |
| 8.78 | 0.01245 | 98.4 | 1.016 |
| 9.27 | 0.00407 | 99.5 | 1.005 |
| 9.45 | 0.00269 | 99.7 | 1.003 |

Fig. R. 4.2.

Graph of $O.D._{360\,nm.\,max.}$ against Tris conc. at high pH.

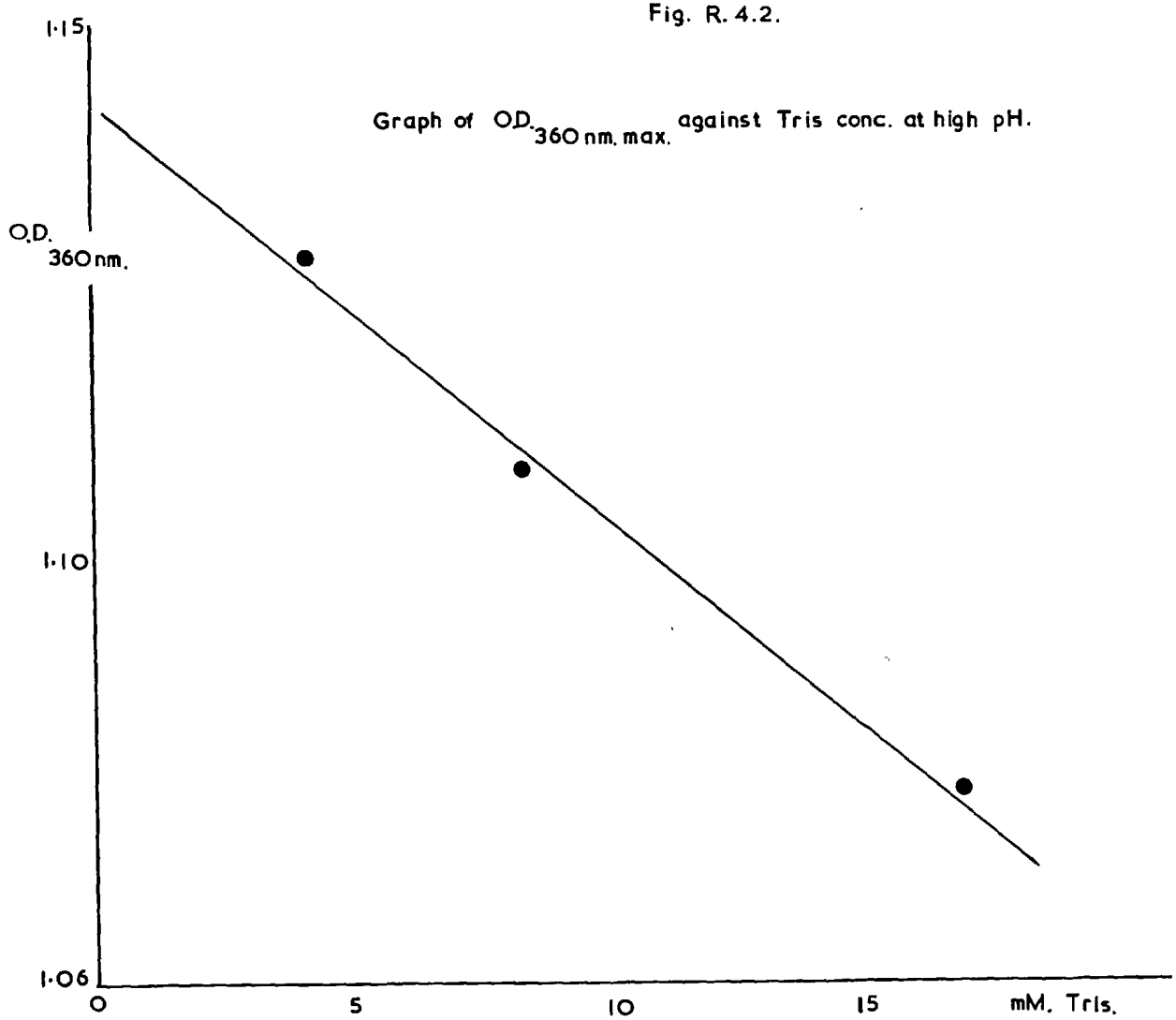


Table R.4.2.

Variations in optical density at 360 nm with different Tris concentrations for solutions containing 19.6 mM $MgCl_2$, and 0.833 mM 8-hydroxyquinoline at pH's above 8.5.

| pH | Tris conc. (mM) | O.D. 360 nm |
|---|---|---|
| 8.55 | 4.17 | 1.128 |
| 8.78 | 8.33 | 1.108 |
| 9.02 | 16.67 | 1.068 |

depends upon tris concentration (Table R.4.2), probably as a result of some $Mg^{2+}$-Tris binding. Consequently a was measured over a range of tris concentrations and the true value of a at zero tris concentration determined graphically (Fig. R.4.2). Small changes of pH in this pH region should have virtually no effect on the maximum optical density and consequently these changes were ignored on drawing the graph shown in Fig. R.4.2. In this way the optical density for complete complexing of 0.833 mM 8-hydroxyquinoline by $Mg^{2+}$ was determined to be 1.142. This value was used in all subsequent calculations. Measurements of (a) were made immediately after mixing the reagents in order to minimize errors due to the slow formation of polymerized $Mg(Ox)_2$.

8-hydroxyquinoline-magnesium binding: titration with $MgCl_2$

$MgCl_2$ was added to 0.833 mM 8-hydroxyquinoline buffered with tris and with KCl added to maintain the ionic strength at 0.02. Most measurements of O.D. were made at pH 7.35 and the initial gradients calculated by the extrapolation method described previously by differentiation of eq. R.4.1 (see Table R.4.4.). To analyse the data equation R.4.2 below was used. The equation (see theory section) is derived assuming that an Mg(Ox) complex is formed with a binding constant $K_1$ and a $Mg(Ox)_2$ complex with an association constant

Table R.4.3.

Comparison of initial gradients of optical density titrations at different concentrations of tris.

8-hydroxyquinoline = 0.833 mM

KCl = 12.0 mM

At $Mg^{2+}$ = 0.0 mM, ionic strength = 0.02.

| Initial gradient $\frac{O.D.}{Mg^{2+}}$ (x $10^{-2}$) | pH of expt. | Initial gradient adjusted to pH 7.93 $\frac{O.D.}{Mg^{2+}}$ (x $10^{2}$) | Tris conc. (mM) |
|---|---|---|---|
| 3.60 | 7.91 | 3.85 | 16.67 |
| 3.42 | 7.87 | 3.90 | 8.33 |
| 3.34 | 7.86 | 3.87 | 4.17 |

Table R.4.4.

Initial gradients calculated from equation R.4.1.
Titrations of $MgCl_2$ (58.8 mM) with 0.833 mM 8-hydroxyquinoline; KCl = 12.0 mM.

| pH at $Mg^{2+} = 0$ | Titration No. | Tris (mM) | Initial Gradient $(\times 10^3)$ $\frac{O.D.}{\mu l}$ | Initial Gradient $\frac{O.D.}{Mg^{2+}} \times (10^{-2})$ |
|---|---|---|---|---|
| 7.35 | A | 16.67 | 1.9776 | 1.00898 |
| | B | | 1.9663 | 1.00321 |
| | C | | 2.0010 | 1.02092 |
| | D | | 2.0511 | 1.04648 |
| | E | | 1.7895* | 0.913021* |
| | F | | 2.0639 | 1.05301 |
| | G | | 2.0398 | 1.04071 |
| 6.50 | H | | 0.30984 | 0.158097 |
| | I | | 0.31245 | 0.159413 |
| 7.93 | J | | 6.8700 | 3.5051 |
| | K | | 7.0560 | 3.6000 |
| 7.91 | L | 16.67 | 7.05 | 3.597 |
| 7.87 | M | 8.33 | 6.70 | 3.418 |
| 7.86 | N | 4.17 | 6.55 | 3.342 |

* Initial gradient calculated for a = 0.7135 (different 8-hydroquinoline solution)

Initial gradients of L-N were obtained by direct measurement.

of $K^1$. It is assumed that the optical absorption of $Mg(Ox)_2$ is twice that of Mg(Ox), that $Mg(Ox)_2$ is a monomer, and that other ternary complexes, such as MgtrisOx do not contribute to $Mg^{2+}$ binding (also that no $Mg^{2+}$-tris binding occurs (see later) ).

$$\frac{OD_{360nm}}{[Mg^{2+}]} = A = \frac{aK_1(1+2K^1[Ox][H^+]^{-1})}{[H^+](1+K_1[Ox][H^+]^{-1} + K_1K^1[Ox]^2[H^+]^{-2})} \quad \text{..... R.4.2.}$$

Ox = total 8-hydroxyquinoline concentration.

If $K^1 = 0$ the treatment becomes that of Burton[28]. With this simplified treatment, however, the data was not found to be self-consistent. Thus the mean initial slope at pH 7.35 of $1.029 \times 10^2$ ($OD/Mg^{2+}$) gives a value for K of $4.35 \times 10^{-6}$. This value would indicate that the initial slope at pH 7.8-7.9 should be 5.55 and not 3.49 as found.

To solve eq. R.4.2. when $K^1 \neq 0$ it was necessary to measure the initial gradients over a range of pH. Measurements (Table R.4.4.) were made over the range 6.5 to 8.0, maximum sensitivity occurring in the region of pH 7.4. Attempts to extend the range of observations to include higher pH's were not successful as the initial gradients

Table R.4.5.

Solutions of equation R.4.2 for titrations A - K in terms of $K^1$, $K_1$.

$OD_{max}$ = 1.142.

$$\alpha K_1 K^1 + \beta K_1 - \gamma = 0 \quad \text{.....} \quad \text{R.4.2.}$$

At pH 7.35

| Titration | $(\alpha)(x10^{-11})$ | $(\beta)(x10^{-6})$ | $\gamma$ |
|---|---|---|---|
| A | 3.5923 | 9.264 | 39.47 |
| B | 3.5931 | 9.268 | 39.24 |
| C | 3.5907 | 9.255 | 39.93 |
| D | 3.5872 | 9.237 | 40.93 |
| E | 3.5786 | 9.190 | 43.42 |
| F | 3.5863 | 9.232 | 41.19 |
| G | 3.5880 | 9.241 | 40.71 |

At pH 6.50

| Titration | $\alpha(x10^{-10})$ | $\beta(x10^{-6})$ | $\gamma$ |
|---|---|---|---|
| H | 5.2384 | 9.885 | 43.77 |
| I | 5.2382 | 9.884 | 44.14 |

At pH 7.93

| Titration | $\alpha(x10^{-12})$ | $\beta(x10^{-6})$ | $\gamma$ |
|---|---|---|---|
| J | 12.366 | 7.443 | 36.06 |
| K | 12.317 | 7.374 | 37.04 |

became too sensitive to small pH changes (see eq. R.4.2) for accurate measurements to be made. The data obtained was fed into eq. R.4.2 and a series of eqs. in $K_1$ and $K^1$ at different pH's obtained (Table R.4.5). Equations containing average values of the coefficients $\alpha$, $\beta$ and $\gamma$ were calculated for each pH and then the equations were solved in pairs to calculate average value of $K_1$ of $4.425 \pm 0.099 \times 10^{-6}$ and $K^1$ of $7.7 \pm 2.5 \times 10^{-8}$.

Although the analysis just given is subject to assumptions concerning the nature of $Mg(Ox)_2$ it is felt that these should be at least approximately true (see discussion), and that it was more accurate to analyse the data with these assumptions than to ignore the formation of $Mg(Ox)_2$. At pH 7.4 and below less than 0.2% of the bound $Mg^{2+}$ is present as $Mg(Ox)_2$ so that the formation of this complex represents only a small factor in the calculation of the binding constants of the ligands.

Tris-$Mg^{2+}$ binding at pH 7.93

In the previous section it has been assumed that $Mg^{2+}$ binding by tris could be ignored. To test for binding an alkaline pH was used and the curves of O.D. vs $Mg^{2+}$ concentration were determined for various tris buffer concentrations at a single pH, 7.9 (see Table R.4.3).

It was found that when measured immediately before titration there appeared to be small differences in pH between solutions of different tris concentration. Consequently the gradients could not be compared directly for evidence of tris binding. However, the effect of small changes in pH upon the gradients can be allowed for by calculating the initial gradients at a common pH using equation R.4.2. To use this equation it has to be assumed that over the pH range involved the extent of $Mg^{2+}$-tris binding remained the same. This assumption appears to be largely justified as calculation showed that the concentration of the tris base form changed by only 6% over the total pH range involved (7.86-7.91). The initial gradients adjusted to pH 7.93 by equation R.4.2, are shown in Table R.4.3. (This pH was chosen so that the gradients could be compared in the presence and absence of 2PGA and 3PGA - see later).

As there was no significant change in the initial gradients over the range of concentration studied the binding constant of tris with $Mg^{2+}$ should be less than $20M^{-1}$, since for this value a 10% change in the initial gradient would be observed over the range of tris concentration studied. It should be noted that if the correction for small pH differences were not applied the limit for the apparent binding would be even lower.

The unionized rather than the ionized form of tris may be expected to bind $Mg^{2+}$. The pK of tris is 7.935 and at pH 7.93 approximately half will be present as the ionized form. At pH's lower than 8 therefore, when the proportion of ionized form will be higher it would seem that binding of $Mg^{2+}$ is negligible. This confirms the result of Milstein[35].

Determination of $2PGA^{3-}$- and $3PGA^{3-}$-$Mg^{2+}$ binding constants

Titrations of 0.833 mM 8-hydroxyquinoline with 58.8 mM $MgCl_2$ solution were carried out in the presence of 0.95 mM 2PGA or 3PGA in 16.67 mM tris buffer, pH 7.40; the ionic strength of solutions was 0.02. The initial gradients of 5 titrations for each ligand were determined by the curve fitting technique already described and the results are shown in Table R.4.6. Since careful measurement with a glass electrode showed slight differences in pH between solutions it was considered preferable to adjust the values to a common pH rather than to make direct comparison of the initial gradients in the presence and absence of phosphoglycerates. For solutions containing 2PGA, 3PGA, and KCl the respective pH's at the start of titration were: 7.38, 7.37, and 7.35. Eq. R.4.2 describes the pH dependence of the initial gradients for 8-hydroxyquinoline in this region of pH. Eq. R.4.3 below was derived from this equation by including a term for

Table R.4.6.

Initial gradients calculated from equation R.4.1 for binding of $Mg^{2+}$ by 3PGA. Titrations of $MgCl_2$ (58.8 mM) with 0.833 mM 8-hydroxyquinoline in the presence of 0.95 mM 3PGA.

| Titration No. | pH at $[Mg^{2+}] = 0$ | Initial gradient $\frac{OD}{\mu l Mg^{2+}}$ (x $10^3$) | Initial gradient $\frac{OD}{Mg^{2+}}$ (x $10^{-2}$) |
|---|---|---|---|
| A | | 1.7645 | 0.90026 |
| B | | 1.7373 | 0.88638 |
| C | 7.37 | 1.7355 | 0.88546 |
| D | | - | 0.65727* |
| E | | - | 0.66899* |

Initial gradients calculated from equation R.4.1 for binding of $Mg^{2+}$ by 2PGA titrations of $MgCl_2$ (58.8 mM) with 0.833 mM 8-hydroxyquinoline in the presence of 0.95 mM 2PGA.

| Titration No. | pH at $[Mg^{2+}] = 0$ | Initial gradient $\frac{OD}{\mu l Mg^{2+}}$ (x $10^3$) | Initial gradient $\frac{OD}{Mg^{2+}}$ (x $10^{-2}$) |
|---|---|---|---|
| A | | 1.7473 | 0.89148 |
| B | | 1.7348 | 0.88510 |
| C | 7.38 | 1.7599 | 0.89791 |
| D | | - | 0.74308* |
| E | | - | 0.74818* |

* Initial gradient calculated for different 8-hydroxyquinoline solution, conc. 0.685 mM.

magnesium-phosphoglycerate binding. This equation assumes that only the triply charged forms of 2- and 3PGA bind $Mg^{2+}$ significantly. The work of Wold and Ballou[38] appears to justify this assumption. However, since only very small differences in pH are involved, little error would be introduced in the apparent binding at pH 7.4 if the assumption were not true

Contributions from ternary complexes such as $[MgPGAOx]^{2-}$ and $[MgPGA_2]^{4-}$ have not been considered as the order of magnitude of the 1:1 binding constants suggest that the extent of formation of these ternary complexes would be negligible.

$$\frac{O.D._{360nm}}{[Mg^{2+}]} = B = \frac{K_1 a(1+2K^1[Ox][H^+]^{-1})}{[H^+]\left(1+\frac{K_1[Ox]}{[H^+]}\left(1+\frac{K^1[Ox]}{[H^+]}\right)+\frac{K_2K_i[PGA]_T}{(K_i+H^+)}\right)} \quad \text{.....} \quad \text{R.4.3.}$$

B is the initial gradient of the titration curve, $K_2$ is the binding constant of the triply charged form of the phosphoglycerate, $K_i$ it's third acid dissociation constant, and $[PGA]_T$ the total concentration in solution.

From eq. R.4.3 a binding constant for magnesium with

$2PGA^{3-}$ of 582 ± 39 $M^{-1}$, and for magnesium with $3PGA^{3-}$ of 302 ± 20 $M^{-1}$ were obtained (see Table R.4.7). The previous measurements of the $Mg^{2+}$-$2PGA^{3-}$ binding constant at 25°C by Wold and Ballou (1957)[38] using a potentiometric titration technique gave a value of 280 $M^{-1}$ at ionic strength 0.1, and 180 $M^{-1}$ at ionic strength 0.4. No evidence was found by them for binding of magnesium by $2PGA^{2-}$. The higher value obtained in the present study may reflect the lower ionic strength, I = 0.02, used. The effect of carrying out measurements at 30°C instead of 25°C is not certain, but it is probably not large. In the present study both 2PGA and 3PGA were used as their trisodium salts but it is unlikely that alkali metal binding of the phosphate esters is sufficiently extensive to influence measurement of the magnesium binding constants. Wold and Ballou (1957)[38] reported that at ionic strength I = 0.1, and 25°C the binding constant of 2PGA and potassium was 15 $M^{-1}$. Should sodium have a similar binding constant with the phosphate esters less than 3% of the esters would have been bound.

Pizer in a private communication reported a magnesium-3PGA binding constant of 480 $M^{-1}$ but as the conditions of experiment were not reported no comparison of values can be made.

Table R.4.7.

Binding constants for $Mg^{2+}$ with 2PGA

| Titration No. | pH | 2PGA mM | $K_{2app}$ |
|---|---|---|---|
| A | | 0.95 | 602 |
| B | | " | 623 |
| C | 7.38 | " | 581 |
| D | | " | 563 |
| E | | " | 543 |
| Average value at pH 7.38 | | $582 \pm 39$ | |

$pK_3$ 2PGA = 7.41 at I = 0.02

Binding constants for $Mg^{2+}$ with 3PGA

| Titration No. | pH | 3PGA mM | $K_{2app}$ |
|---|---|---|---|
| A | | 0.95 | 295 |
| B | | " | 321 |
| C | 7.37 | " | 323 |
| D | | " | 289 |
| E | | " | 278 |
| Average value at pH 7.37 | | $301 \pm 20$ | |

$pK_3$ 3PGA = 6.67 at I = 0.02

## SECTION 5. STUDIES OF THE EQUILIBRIUM CONSTANT OF THE PHOSPHOGLYCERATE MUTASE REACTION

### Introduction

Though many investigations have been carried out to determine the equilibrium constant of the phosphoglycerate-mutase reaction agreement between authors is poor. In Table R.5.1 the value of the apparent equilibrium constant, $K_{eq\ app}$, obtained by different authors has been listed together with the experimental conditions employed. Earlier investigations were based on the different specific optical rotations of 2PGA and 3PGA in acid or neutral molybdate solution. The equilibrium constants for some authors (Meyerhof and Schulz, 1938[39]; Meyerhof and Oesper, 1949[40]), have been amended in the light of a more recent determination of the specific rotation of 2PGA by Ballou and Fischer (1954)[41]. Later measurements of $K_{eq\ app}$ were carried out using an enolase coupled assay to measure the 2PGA concentration at equilibrium. In one study $K_{eq\ app}$ was shown to be comparatively temperature independent (Meyerhof and Schultz, 1938[39]). A fuller review of this data has been made by Pizer (1962)[48].

Differences in the values of $K_{eq\ app}$ may have been due to the different experimental conditions under which the constant was measured. Both the pH of solution and the

Table R.5.1.

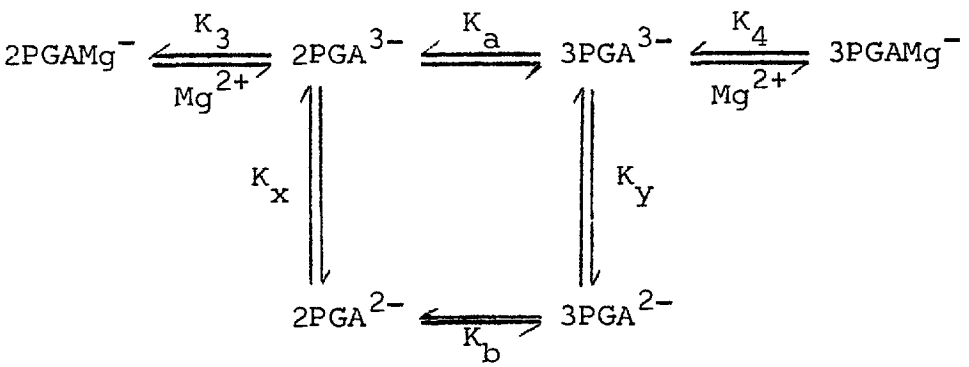
Equilibration between 2PGA and 3PGA catalysed by rabbit muscle phosphoglycerate mutase

| Authors/Ref. | Method of Measurement | Temp. °C | pH | $K_{eq\ app.}$ |
|---|---|---|---|---|
| O. Meyerhof, W. Schulz[39] (1938) | Optical rotation in neutral molybdate | 20 | ~7.6 | 10.8 |
| O. Meyerhof, P. Oesper[40] (1949) | Optical rotation in acid solution 1M HCl | 24 | ~7.6 | 3.65 |
| C.E. Ballou, M.O.L. Fischer[41] (1954)* | x 1. Optical rotation, neutral molybdate | 100 | 0.0 | 4.1 |
| | 2. Optical rotation in acid solution | | | 4.0 |
| R.W. Cowgill, L.I. Pizer[42] (1956) | ++Optical rotation in neutral molybdate | 37 | 6.8 | 5.0 |
| M. Chiba, E. Sugimoto[43] (1959) | Optical rotation in neutral molybdate | 25 | 5.0-7.2 | 5.8 |
| V.W. Rodwell, J.C. Towne, S. Grisolia[44] (1957) | **Enolase conversion of 2PGA to PEP | 30 | 4.6-6.5 | 6.3 |
| N. Ito, S. Grisolia[45] (1959) | +Enolase conversion of 2PGA to PEP | 30 | possibly ~7.6 | 6-10 |

* Equilibration between 2PGA and 3PGA catalysed by 1 N HCl
** Equilibration catalysed by yeast PGM
\+ Equilibration catalysed by Wheat germ PGM
++ Equilibration catalysed by rabbit-muscle PGM
x Equilibration catalysed by PGM (probably muscle enzyme)

concentration of $Mg^{2+}$ can affect the apparent equilibrium constant and in Fig. R.5.1 the full system for phosphoglycerate-mutase is set out.

Fig. R.5.1.




The equilibrium scheme is represented as shown as there is no direct evidence to suggest that rabbit-muscle phosphoglycerate-mutase catalyses the interconversion of the triply-ionized species of 2PGA and 3PGA, but not the conversion of the doubly-ionized species[48]. From a thermodynamic point of view the pathway of interconversion is unimportant and the equilibrium constant $K_b$ must be linked to the equilibrium constant $K_a$ by the following relationship if the 2nd law of thermodynamics is to be obeyed.

$$K_b = K_a \cdot \frac{K_x}{K_y} \qquad \text{..... R.5.1.}$$

With the symbols shown in Fig. R.5.1. it follows that:-

$$K_{eq\ app.} = K_a \frac{1 + K_4[Mg^{2+}]_{free} + \frac{[H^+]}{K_y}}{1 + K_3[Mg^{2+}]_{free} + \frac{[H^+]}{K_x}} \qquad \text{..... R.5.2.}$$

$K_a$ (and $K_b$) will be independent of pH as each relates to a particular ionized species of the substrates. They should also be independent of ionic strength as the sizes and charge distributions of $2PGA^{3-}$ and $3PGA^{3-}$ (and of $2PGA^{2-}$ and $3PGA^{2-}$), will be very similar.

It was not possible to calculate $K_a$ from the data of authors in Table R.5.1 as the magnesium ion concentration was generally not recorded. An attempt to correlate the data better by correcting the $K_{eq\ app.}$'s to a common pH resulted in a wider scatter of values so that no reliable comparison could be made.

In equation R.5.2 the constants $K_x$ and $K_y$ (Table R.5.2) and probably $K_3$ and $K_4$ are affected by the ionic strength of solution. Consequently $K_{eq\ app.}$ also becomes dependent on

Table R.5.2.

| Substrate constant | Ionic strength I = 0.0 | Ionic strength I = 0.02 | Ionic strength I = 0.15 | Units | Ref. |
|---|---|---|---|---|---|
| $K_x$, 2PGA | $3.311 \times 10^{-8}$ | $3.890 \times 10^{-8}$ | $1.148 \times 10^{-7}$ | $M^{-1}$ | (46) |
| $K_y$, 3PGA | $1.820 \times 10^{-7}$ | $2.138 \times 10^{-7}$ | $6.310 \times 10^{-7}$ | $M^{-1}$ | (46) |
| $K_3$, 2PGA | - | $582 \pm 39$ | - | $M^{-1}$ | (47) |
| $K_4$, 3PGA | - | $302 \pm 20$ | - | $M^{-1}$ | (47) |

ionic strength and at high ionic strength significant changes may occur. For this reason measurement of $K_{eq\ app.}$ by polarimetric methods must be suspect since high phosphoglycerate concentrations, of the order of 30.0 mM are necessary to obtain sufficient accuracy of measurement. The same criticism must be made of the experimental determinations with the enolase coupled assays as the phosphoglycerate concentrations were greater than 20 mM. Furthermore in this latter method the determination requires a knowledge of the extinction coefficient of PEP at 240 nm and the equilibrium constant of the enolase reaction. As both of these are dependent on the $Mg^{2+}$ ion concentration (Wold and Ballou, 1957)[38], which was not reported, the calculation must be regarded as approximate.

In the light of these observations it seemed necessary to redetermine the value of the equilibrium constant by a more sensitive method which would permit measurements to be made at low ionic strength. The $NAD^+$/NADH coupled assay of 2PGA described in the methods section (M.2) fulfilled these conditions. Measurements were made under standard conditions of ionic strength, pH, and free magnesium ion concentration.

Determination of the equilibrium constant of the phosphoglycerate-mutase reaction

Measurements of the equilibrium constant for the

phosphoglycerate-mutase were made using the technique for estimating the 2PGA and 3PGA concentration described in the method section. Equilibrium was set up between 2PGA and 3PGA at various concentrations by incubation at 30°C with 3 Units of phosphoglycerate-mutase (0.01 mls from stock solution) in 16.67 mM tris buffer pH 7.4 in the presence of 10 μM 23diPGA. Equilibration proceeded for 30-60 mins. The conditions employed were very similar to those of the induced transport tests (in which plots of 2PGA conc. vs. time showed that equilibrium was attained in 100-150 mins.) except that 50-100 times the quantity of enzyme was added. Consequently equilibration should be complete at the end of the period allowed. Samples were then withdrawn from the reaction vessel and analysed for 2PGA and 3PGA by the $NAD^+$/NADH coupled method. The volume of the two samples were chosen so that the changes in optical density at 340 nm were approx. equal thus reducing errors from reading the calibration curve (optical density vs conc. of phosphoglycerates). The values of the apparent equilibrium constants $K_{eq\ app.}$ for the various conditions, together with the calculated value of $K_2$ are recorded in Table R.5.3. An average value of $K_a$ of $20.45 \pm 0.40$ was obtained, where $K_a$ is defined by equation R.5.2 (see Introduction).

As $[2PGA]^{3-}$ and $[3PGA]^{3-}$, and similarly $[2PGA]^{2-}$ and

Table R.5.3.

Equilibrium Constant of Phosphoglycerate mutase reaction

| Expt. No. | Estimation of | Measured Conc. (mM) | pH | Ionic strength I | $Mg^{2+}$ total (mM) | $Mg^{2+}$ free (mM) | $K_{eq\ app}$ | K |
|---|---|---|---|---|---|---|---|---|
| 1<br>2<br>3 | 2PGA<br>2PGA<br>2- + 3PGA | 0.6767<br>0.6767<br>8.193 | 7.4 | 0.085 | 4.525 | 1.973 | 11.11 | 19.77 |
| 4<br>5<br>6 | 2PGA<br>2PGA<br>2- + 3PGA | 0.3295<br>0.3306<br>4.171 | 7.4 | 0.059 | 4.525 | 2.999 | 11.64 | 20.97 |
| 7<br>8 | 2PGA<br>2- + 3PGA | 0.2043<br>2.531 | 7.4 | 0.038 | 3.333 | 2.441 | 11.39 | 20.40 |
| 9<br>10 | 2PGA<br>2- + 3PGA | 0.2043<br>2.523 | 7.4 | 0.038 | 3.333 | 2.441 | 11.35 | 20.32 |
| 11<br>12 | 2PGA<br>2- + 3PGA | 0.2036<br>2.511 | 7.4 | 0.038 | 3.333 | 2.441 | 11.33 | 20.29 |
| 13<br>14 | 2PGA<br>3PGA | 0.1988<br>2.3345 | 7.4 | 0.030 | 3.333 | 2.435 | 11.74* | 21.02 |
| 15<br>16<br>17 | 2PGA<br>2PGA<br>2- + 3PGA | 0.1676<br>0.1698<br>2.075 | 7.4 | 0.046 | 4.525 | 3.624 | 11.30 | 20.48 |
| 18<br>19 | 2PGA<br>2- + 3PGA | 0.1172<br>1.442 | 7.4 | 0.032 | 3.333 | 2.786 | 11.31 | 20.33 |

* Average result from 6 induced transport experiments

Average K = 20.45 $\pm$ 0.40 standard deviation

As $[2PGA]^{3-}$ and $[3PGA]^{3-}$, and similarly $[2PGA]^{2-}$ and $[3PGA]^{2-}$ have the same charge and will be of very similar size their activity coefficients in a solution of given ionic strength will be virtually the same. However, there will be a difference between the activity coefficients of the doubly and triply charged species of each phosphoglycerate. Consequently the value of $K_{eq\ app.}$ will depend upon the relative changes of $K_x$ and $K_y$ (the third acid eq app. dissociation constants of 2PGA and 3PGA respectively) with ionic strength. From Table R.5.2. it can be seen that both $K_x$ and $K_y$ change by exactly the same amount over the range I = 0-0.15. Thus over this range the ratio of $[2PGA]^{2-}$ to $[2PGA]^{3-}$ will remain the same as the ratio of $[3PGA]^{2-}$ to $[3PGA]^{3-}$ and hence $K_{eq\ app.}$ will be virtually independent of ionic strength.

## SECTION 6. MEASUREMENT OF $K_m$'s of 3PGA AND 23PGA, AND $V_{max}$ IN THE FORWARD AND REVERSE DIRECTIONS FOR PHOSPHOGLYCERATEMUTASE AT 30°C

### Introduction

The measurements of the $K_m$ for 3PGA and the ratio of the maximum velocities of reaction in the forwards and reverse directions, under conditions similar to those employed for the induced transport experiments, were necessary in order to establish the order of magnitude of the rate constants for the interconversion of the two forms of the enzyme. The precise interpretation of the induced transport curves depends upon whether or not this step is rate limiting. Though the $K_m$'s for 3PGA and 23PGA have been measured by other authors for the chicken breast enzyme[54] no measurements have been made using rabbit-muscle phosphoglycerate-mutase. Furthermore it is possible that the $K_m$'s reported were erroneous as no account was taken of changes in the free magnesium ion concentration due to binding with substrate over the range of substrate concentrations which were employed. Finally, the change with ionic strength in the pattern of radioactive labelling suggests that the $K_m$'s may be markedly affected by ionic strength.

An investigation was therefore carried out to determine

the $K_m$'s for 3PGA and 23PGA keeping constant; the pH, the ionic strength, the free $Mg^{2+}$ concentration, and the temperature. The ratio of the maximum velocities in the forwards and reverse directions was also determined and this enabled an estimate of the $K_m$ for 2PGA to be made by the use of the Haldane relationship.

Initial velocity of reaction studies for conversion of 3PGA to 2PGA

The ideal concentration ranges for both 3PGA and 23PGA for maximum accuracy of evaluation of their $K_m$'s is $\frac{1}{3}$ $K_m$ to 3 $K_m$ [50]. However, the range used was extended to lower substrate concentrations as the previously reported $K_m$'s were not necessarily a good guide to the true value. Furthermore, potentiation of the rate of reaction, possibly due to the formation of a new conformation of the enzyme with a higher turnover number, is reported to occur (although no experimental evidence was given) when the concentration of 23PGA was above 10 μM and that of 3PGA above 1 mM [51]. As these values were similar to the recorded $K_m$'s of 23PGA ($1.5 \times 10^{-6}$M) and 3PGA ($1.4 \times 10^{-4}$M) [54] it was considered advisable to carry out initial rate measurements at the lowest possible concentration of substrates. The range of concentration of the various reagents have been recorded in Tables M.2.1,

M.2.2, and M.2.3 in the methods section. A free magnesium ion concentration of 2.250 mM was chosen for most observations so that it was comparable with that in the induced transport tests.

3PGA purified by chromatography was used in this investigation as sufficient 23PGA was present in the unpurified material for reaction to occur without the need to add additional 23PGA (see methods section). The purified 3PGA was assayed for residual 23PGA as follows. To 2.00 mM 3PGA in 3.33 mM $MgCl_2$ and 16.67 mM tris buffer pH 7.4 (in the presence of 0.17 mM ADP, 0.1 mg/ l. NADH and 0.01 mls. each of the stock solutions of PK, ENOL and LDH) rabbit muscle phosphoglycerate mutase ($1.89 \times 10^{-4}$ moles) was added to give a total volume of 3.0 ml. The fall in O.D. ($1.99 \times 10^{-4}$ O.D./min.) at 340 nm was observed over a period of 1 hr. and from this a rate of reaction of $0.96 \times 10^{-4}$ μM/min. was calculated. This corresponded to only 0.013% of the initial velocity in a similar experiment in which 0.02 μM 23PGA was added. The 23PGA in the purified 3PGA had thus been reduced to adequately low levels. There also appeared to be effectively no residual reaction in the absence of 23PGA.

Initial velocity measurements

The sensitivity of the measurement of 2PGA concentration

by the NADH/$NAD^+$ linked assay system was sufficient to measure the production of $10^{-2}$ μ moles of 2PGA (in 3.0 mls.), and it was possible to measure the first 2% of the conversion of 3PGA to 2PGA at high 3PGA concentrations, and the first 30% of conversion at the lowest 3PGA concentration. Rodwell, Towne and Grisolia reported that only 2% of the conversion of 3PGA to 2PGA occurred at a linear rate[44] with the enolase coupled assay, but in this assay 2PGA accumulates. As the equilibrium constant $K_{eq}$ = 11.3 (under conditions of experiment was in favour of 3PGA and the $K_m$ for 2PGA is of the order of 30 μM (see later) it would be expected that the back reaction would soon form a substantial contribution to the net rate of reaction. In the present case the back flux is not present since 2PGA is immediately converted to ATP and lactate. The steady state concentration of 2PGA should be less than 2.3 μM (see later). Consequently the initial rate of reaction should be constant over a greater extent of the reaction.

Before the initial velocity measurements were carried out the system was checked to ensure that under no circumstances could the conversion of 2PGA to ATP and lactate be rate limiting. This was particularly necessary as a three enzyme assay system was being used although the fact that pyruvate kinase is effectively irreversible makes the requirements less stringent than for a reversible system. In the experiments

0.2 units of enolase, 1.5 units of pyruvate kinase and 9.0 units of lactic dehydrogenase, the units relating to the appropriate direction of reaction required in each case, were used compared with 0.0347 units of phosphoglycerate mutase activity. The number of units of each successive enzyme was made greater to offset the progressive slowing down of reaction due to the fall in the substrate concentrations. Experiments carried out at high substrate concentrations showed that the assay system was capable of measuring initial velocities up to at least six times as fast as the highest velocity (5.7 μM/min.) recorded during the measurements of $K_m$'s As the lowest substrate concentration used in measurement of initial velocities, 0.02 mM 3PGA, was below the $K_m$'s of enolase for 2PGA ($0.58 \times 10^{-4}$ M), pyruvate kinase for PEP ($8.6 \times 10^{-5}$ M), and lactic dehydrogenase for pyruvate ($1.0 \times 10^{-3}$ M, Bovine M.4 enzyme[69]) it was thought necessary to check the rate of conversion of low concentrations of 2PGA to lactate and ATP by the assay system and to determine the lowest concentration of 2PGA at which this rate remained constant. The initial reaction rate for the conversion of 2PGA, 0.02 mM to lactate under the experimental conditions was 11.8 μM/min., approximately fifteen times as fast as the observed initial rate of

conversion of 0.02 mM 3PGA to 2PGA, and twice as fast as the highest recorded velocity 5.7 μM/min. (see above).

The reaction rate was linear until the concentration of 2PGA remaining was 2.3 μM, about 7% of the $K_m$ for 2PGA (see below) which corresponds to 12% of the original 2PGA conc. In the light of these findings it was concluded that the assay system should give a true estimate of the rate of conversion of 3PGA to 2PGA over the range of concentrations studied.

The initial rates of reaction were measured under the experimental conditions previously described (see methods section). The concentration of 3PGA was varied from 0.02 - 5 mM, and 23PGA from 0.33 - 50 μM. In one set of determinations, ammonium sulphate, derived from the enzyme solutions, gave a sulphate concentration of 7.9 mM. The results of these experiments are recorded in Table R.6.1. The concentration of free $Mg^{2+}$ ion was 2.154 mM in runs 1A-8H, 2.898 mM in runs 9A-9H, and 2.332 mM in runs 10A-10H. The latter runs were a brief investigation of the effect of varying the free $Mg^{2+}$ ion concentration. Sulphate ion was found to have an appreciable effect upon the reaction and a further series of runs were carried out in the absence of sulphate (Table R.6.3) to investigate the kinetics and determine the $K_m$'s for 3PGA and 23PGA.

Table R.6.1.

Initial velocity of reaction for various 3PGA and 23PGA concentrations at 7.9mM $SO_4^{2-}$.

| Run No. | 23PGA (μM) | | A: 50.0 | | B: 25.0 | | C: 10.0 | | D: 5.0 | | E: 2.0 | | F: 1.0 | | G: 0.5 | | H: 0.33 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 23PGA $^{-1}$ ($M^{-1}$) | | 2.0 x $10^4$ | | 4.0 x $10^4$ | | 10.0 x $10^4$ | | 20.0 x $10^4$ | | 50.0 x $10^4$ | | 100 x $10^4$ | | 200 x $10^4$ | | 300 x $10^4$ | |
| | 3PGA(mM) | 3PGA $^{-1}$ | VμM/min | $\frac{1}{V}$ | Vx$10^2$ | $\frac{1}{V}$ | Vx$10^2$ | $\frac{1}{V}$ | Vx$10^2$ | $\frac{1}{V}$ | Vx$10^2$ | $\frac{1}{V}$ | Vx$10^2$ | $\frac{1}{V}$ | Vx$10^2$ | $\frac{1}{V}$ | Vx$10^2$ | $\frac{1}{V}$ |
| 1 | 5.00 | 200 | 4.537 | 22.05 | 4.019 | 24.89 | 4.797 | 20.85 | - | - | 2.507 | 39.89 | 0.7939 | 126.0 | 0.450 | 222.2 | - | - |
| | | | 4.683 | 21.36 | 3.953 | 25.30 | 4.552 | 21.97 | - | - | 2.471 | 40.47 | 0.835 | 119.8 | 0.407 | 245.7 | - | - |
| 2 | 2.00 | 500 | 4.458 | 22.43 | 4.962 | 20.15 | 5.606 | 17.84 | - | - | 3.505 | 28.53 | 1.514 | 66.06 | 0.759 | 131.8 | 0.4905 | 203.9 |
| | | | 4.724 | 21.17 | 4.932 | 20.27 | 5.721 | 17.48 | - | - | 2.772 | 29.86 | 1.455 | 68.73 | 0.7495 | 133.4 | 0.482 | 207.6 |
| 3 | 1.00 | 1,000 | 4.087 | 24.47 | 4.508 | 22.14 | 4.563 | 21.92 | - | - | 3.768 | 26.54 | 1.494 | 66.92 | 1.041 | 96.06 | 0.6243 | 160.2 |
| | | | 4.261 | 23.46 | 4.353 | 22.97 | 5.191 | 19.27 | - | - | 3.480 | 28.74 | 1.594 | 62.72 | 1.057 | 94.60 | 0.6488 | 154.1 |
| 4 | 0.50 | 2,000 | 3.250 | 30.77 | 3.194 | 31.31 | 3.810 | 26.25 | - | - | 3.237 | 30.89 | 1.803 | 55.47 | 1.213 | 82.45 | 0.8763 | 114.2 |
| | | | 3.053 | 32.76 | 3.286 | 30.43 | 3.607 | 27.73 | - | - | 3.181 | 31.44 | 1.781 | 56.15 | 1.258 | 79.49 | 0.8521 | 117.4 |
| 5 | 0.25 | 4,000 | 1.945 | 51.42 | 2.237 | 44.70 | 2.233 | 44.78 | - | - | 2.279 | 43.87 | 1.608 | 62.21 | 1.123 | 89.06 | 0.8462 | 118.2 |
| | | | 2.173 | 46.01 | 2.188 | 45.70 | 2.300 | 43.48 | - | - | 2.273 | 43.99 | 1.594 | 62.77 | 1.121 | 89.21 | 0.8409 | 118.9 |
| 6 | 0.10 | 10,000 | 1.027 | 97.40 | 1.175 | 85.09 | 1.329 | 75.27 | - | - | 1.242 | 80.52 | 1.070 | 93.46 | 0.883 | 113.3 | 0.7616 | 131.3 |
| | | | 1.110 | 90.09 | 1.100 | 90.91 | 1.340 | 74.63 | - | - | 1.268 | 78.86 | 1.092 | 91.56 | 0.920 | 108.7 | 0.7568 | 132.2 |
| 7 | 0.05 | 20,000 | 0.532 | 188.0 | 0.678 | 147.5 | 0.748 | 133.7 | - | - | 0.793 | 126.1 | 0.681 | 146.8 | 0.589 | 169.8 | 0.4677 | 213.8 |
| | | | 0.611 | 163.7 | 0.665 | 150.4 | 0.771 | 129.7 | - | - | 0.799 | 125.2 | 0.657 | 152.2 | 0.586 | 170.6 | 0.4823 | 207.4 |
| 8 | 0.02 | 50,000 | 0.256 | 390.6 | 0.317 | 315.5 | 0.321 | 311.5 | 0.362 | 276.2 | 0.360 | 277.8 | 0.354 | 282.5 | 0.318 | 320.5 | 0.3005 | 332.8 |
| | | | 0.298 | 335.6 | 0.294 | 340.1 | 0.311 | 321.5 | 0.368 | 271.7 | 0.353 | 283.3 | 6.360 | 277.8 | 0.321 | 311.5 | 0.3024 | 330.7 |
| 9 | 2.00 | 500 | 5.701 | 17.54 | 5.142 | 19.45 | 4.299 | 23.26 | 3.562 | 28.07 | 1.336 | 74.86 | 0.805 | 124.2 | 0.416 | 240.4 | 0.301 | 332.2 |
| | | | 5.688 | 17.58 | 5.395 | 18.54 | 4.466 | 22.39 | 3.349 | 29.86 | 1.494 | 66.91 | 0.779 | 128.4 | 0.419 | 238.7 | 0.298 | 335.6 |
| 10 | 1.00 | 1,000 | 5.142 | 19.45 | 4.932 | 20.27 | 4.180 | 23.92 | 4.087 | 24.46 | 1.891 | 52.88 | 1.138 | 87.89 | 0.657 | 152.2 | 0.437 | 228.8 |
| | | | 4.567 | 21.90 | 5.035 | 19.85 | 3.993 | 25.04 | 3.934 | 25.42 | 1.864 | 53.64 | 1.117 | 89.56 | 0.657 | 152.2 | 0.433 | 230.9 |

Kinetics in the presence of 7.9 mM Ammonium sulphate

Lineweaver-Burke plots of $\frac{1}{V}$ vs $\frac{1}{3PGA}$ at different 23PGA concentrations, and of $\frac{1}{V}$ vs $\frac{1}{23PGA}$ at different 3PGA concentrations are shown in Figs. R.6.1-4. At low concentrations parallel lines were obtained in both plots (Figs. R.6.2, R.6.4). This is the result expected of a phosphoenzyme mechanism provided that the rate of breakdown of the phosphoenzyme is not appreciable when compared with the rate of dissociation of 23PGA. However, the sequential pathway may also give the same pattern (see theory section). Higher concentrations of 3PGA and 23PGA were inhibitory (Figs. R.6.1, R.6.3), suggesting that 3- and 23PGA can compete for the same binding site.

An estimate of the $V_{max}$ and $K_m$'s was obtained from appropriate secondary plots. The intercepts on the $\frac{1}{V}$ axis taken from the $\frac{1}{V}$ vs $\frac{1}{23PGA}$ graphs were plotted against $\frac{1}{3PGA}$. The results are shown as dotted lines on Figs. R.6.4 and R.6.2 respectively. From Fig. R.6.2 a value for $V_{max}$ of $7.75 \pm 0.54 \times 10^{-2}$ µmoles/min. and from Fig. R.6.4 a value of $12.24 \pm 0.86 \times 10^{-2}$ µmoles/min. were obtained giving an average value of $10.0 \pm 0.59 \times 10^{-2}$ µmoles/min. or $52,900 \pm 3,100$ moles 3PGA/mole enzyme/min. This figure is similar to the $V_{max}$ of 60,000 moles 3PGA/mole enzyme/min.

Fig. R.6.1.

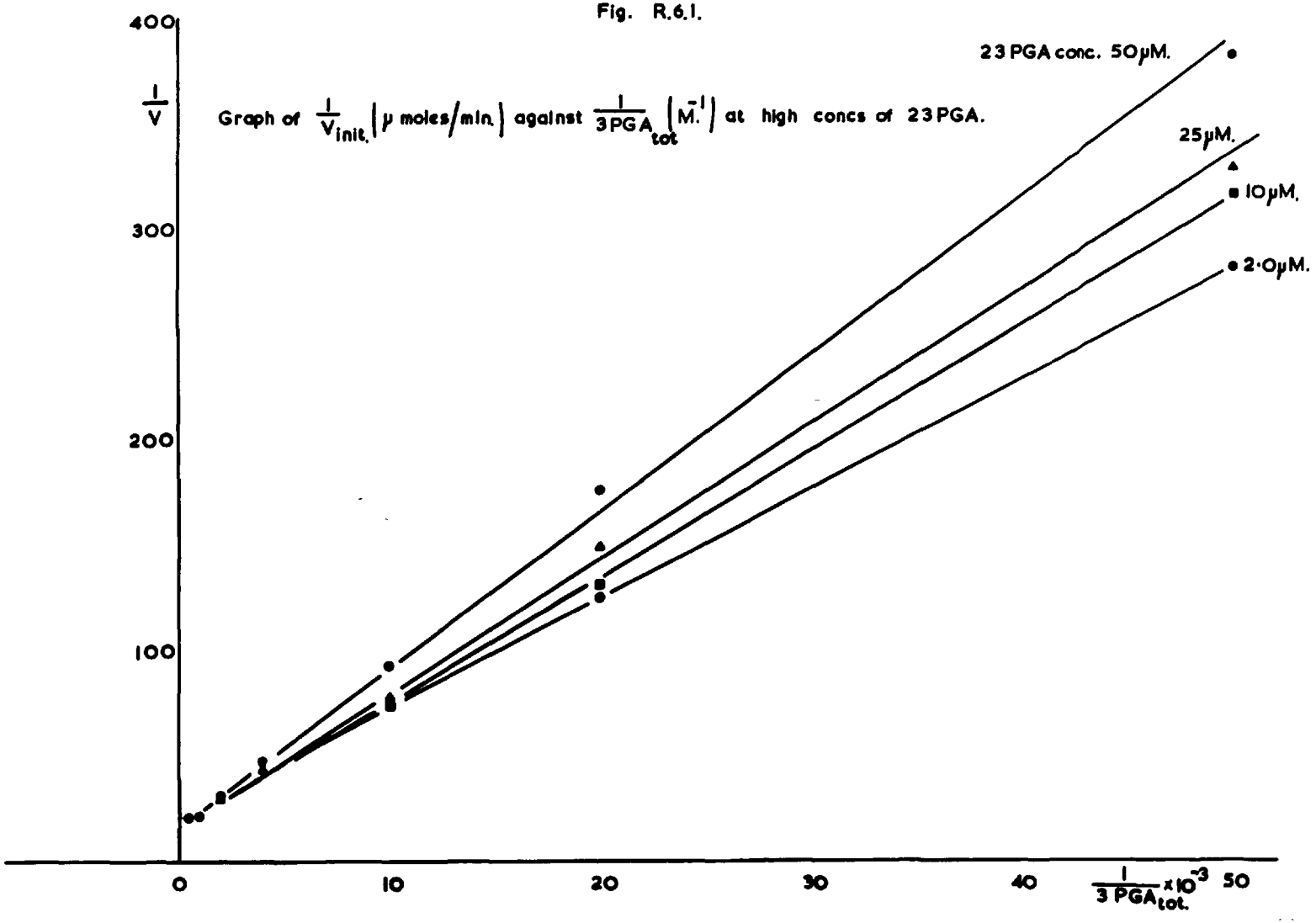

400
300
200
100
1/V
Graph of 1/V_init. (μ moles/min.) against 1/3PGA_tot (M.^-1) at high concs of 23PGA.
23PGA conc. 50μM.
25μM.
10μM.
2·0μM.
0
10
20
30
40
50
1/3PGA_tot. x10^-3

Fig. R.6.2.

Graph of $\frac{1}{V_{init.}}$ (μ moles/min) against $\frac{1}{3PGA_{tot.}}$ ($M.^{-1}$) at low concs. of 23 PGA.

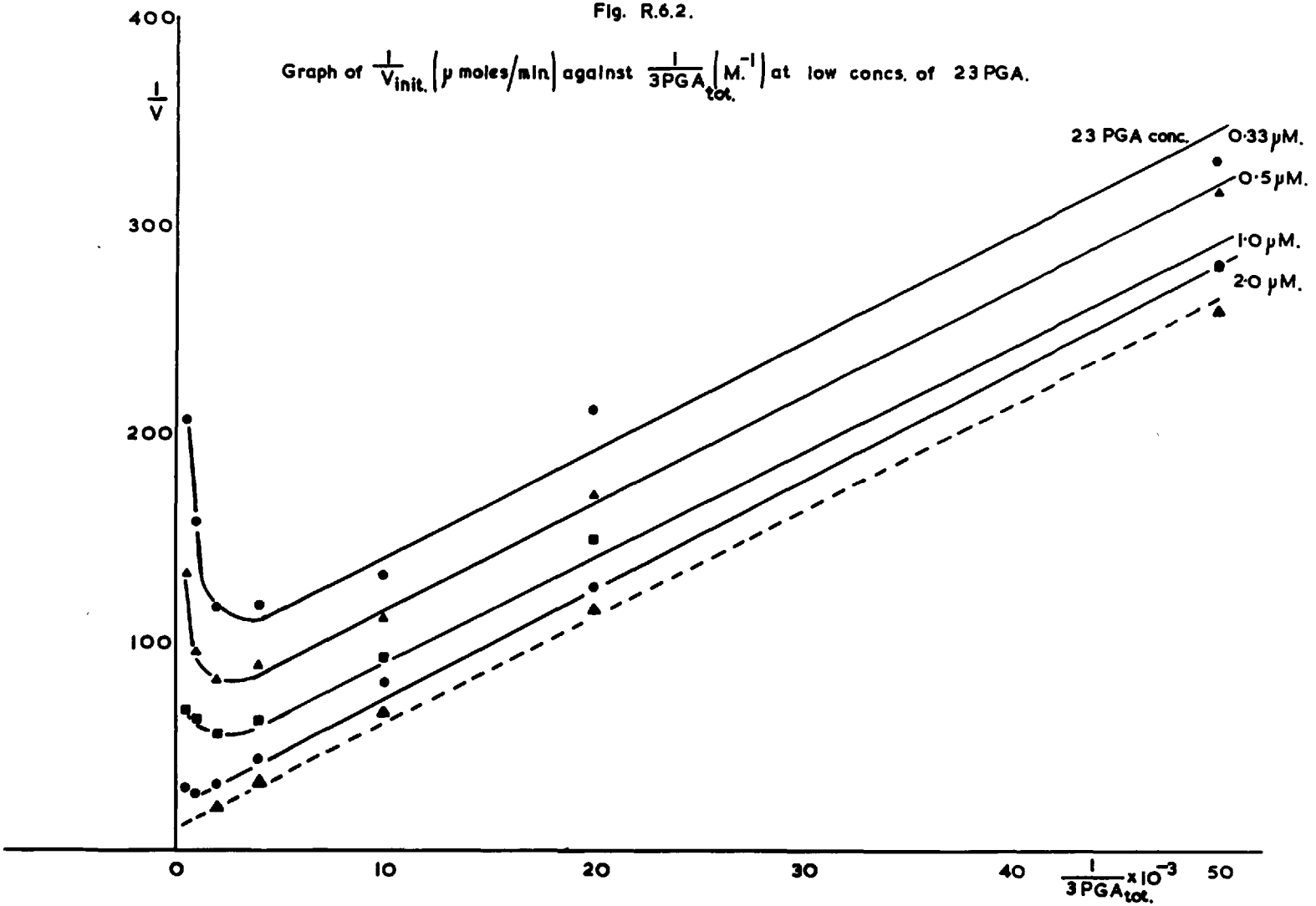


Fig. R.6.3.

Graph of $\frac{1}{V_{init.}}$ $(\mu$ moles/min.$)$ against $\frac{1}{23PGA_{tot.}}$ $(M.^{-1})$ at high concs. of 3PGA.

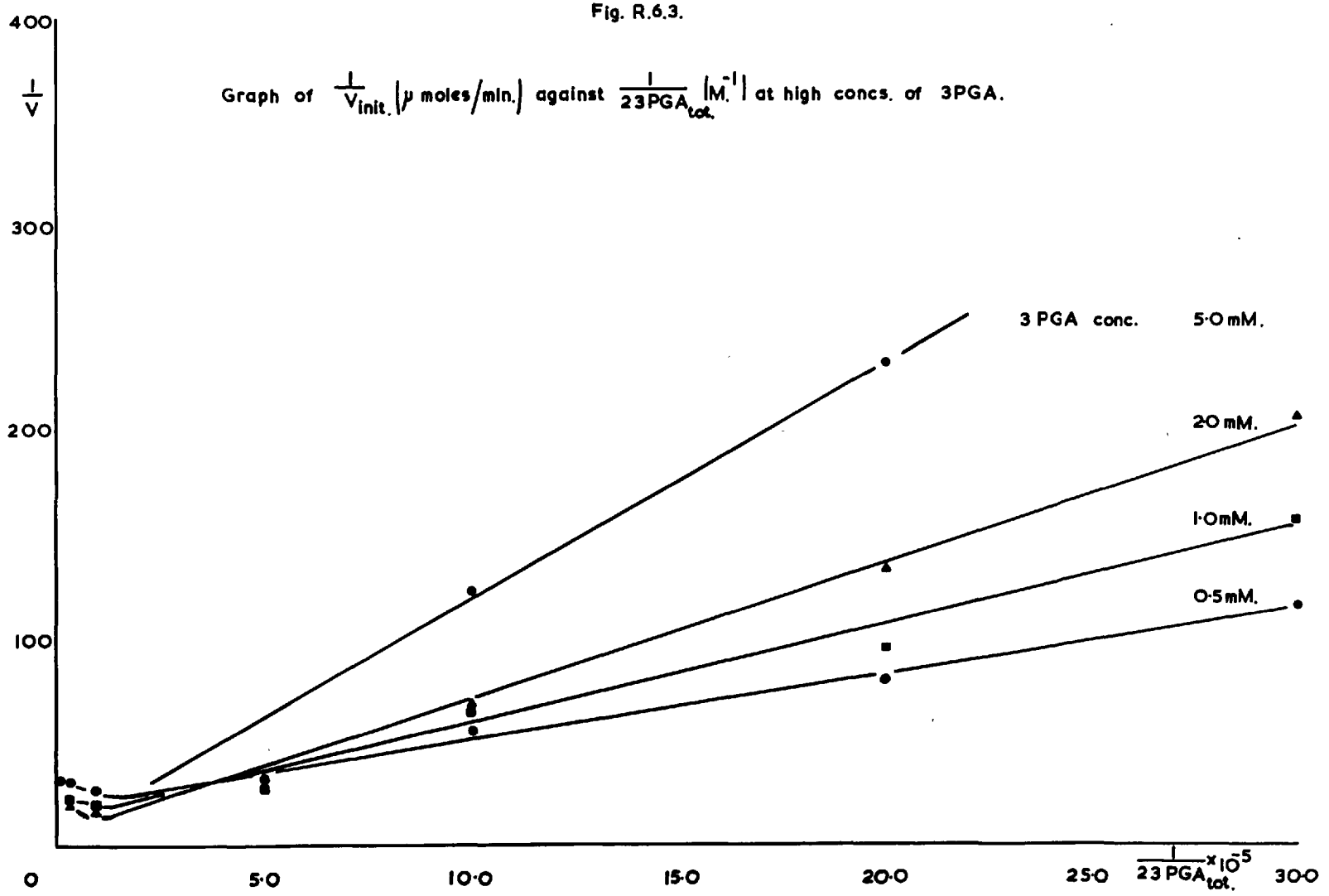


Fig. R. 6.4.

Graph of $\frac{1}{V_{init.}}$ (μ moles/min.) against $\frac{1}{23PGA_{tot.}}$ ($M^{-1}$) at low concs. of 3 PGA.

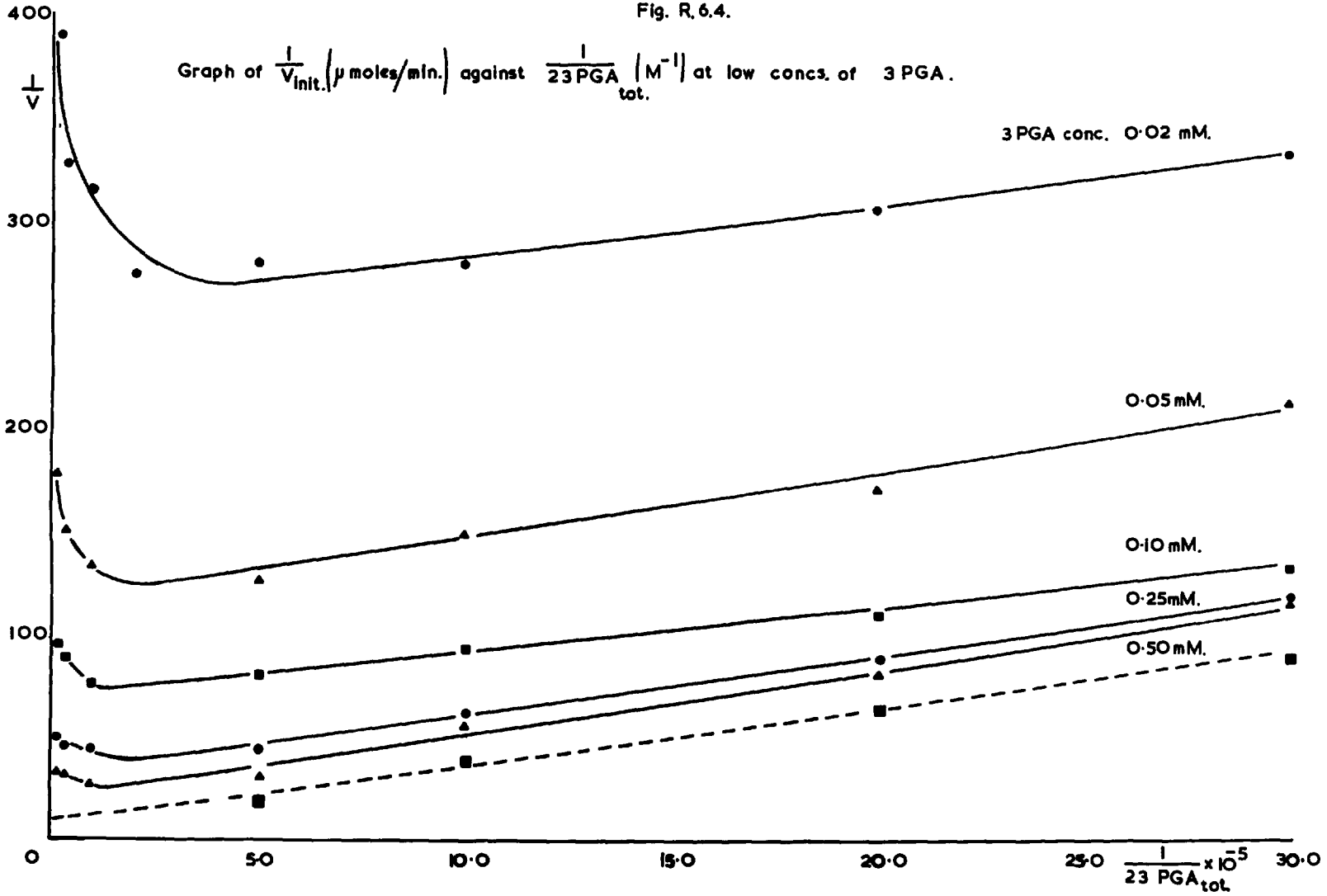
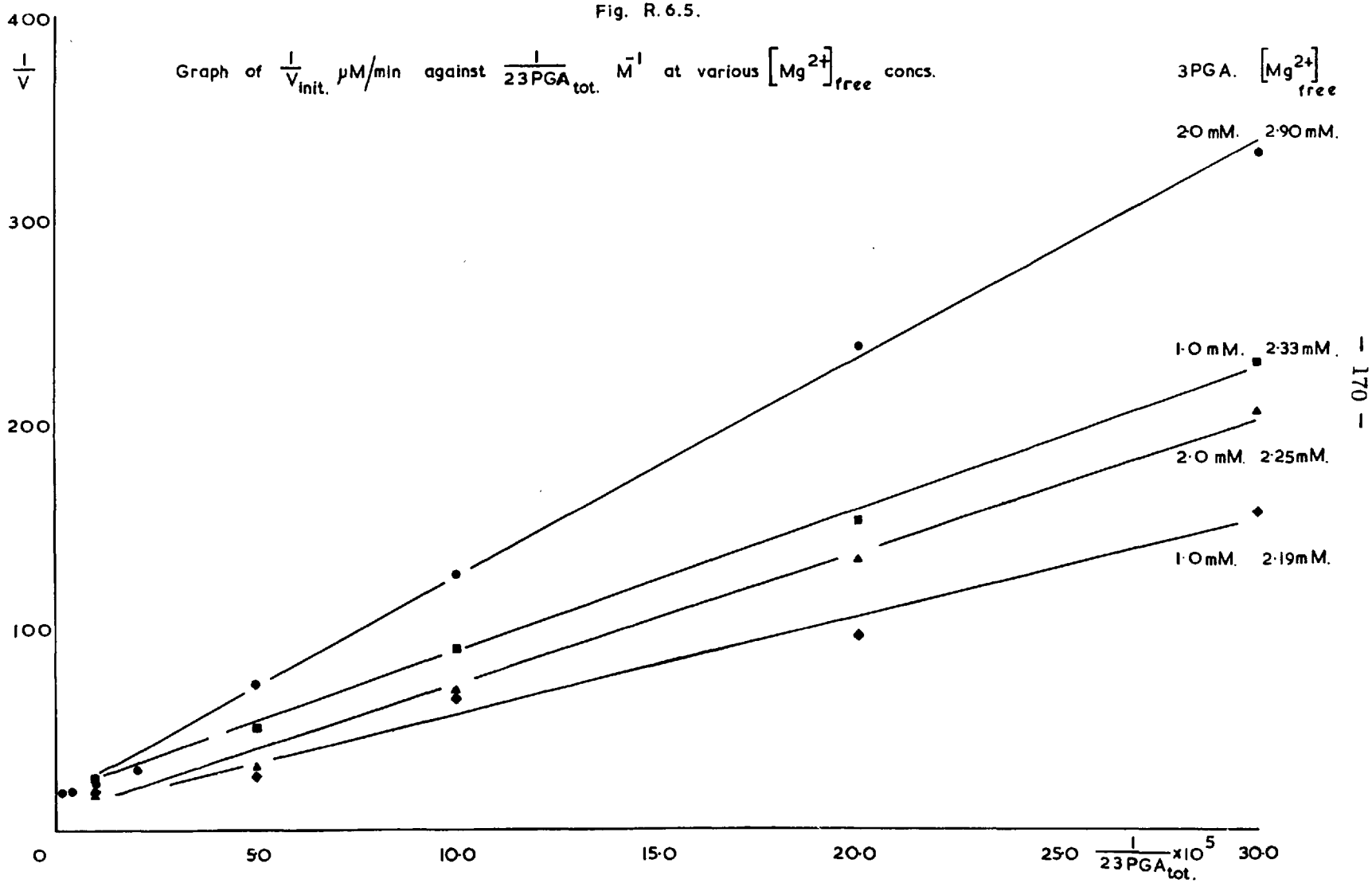


Fig. R.6.5.


Graph of $\frac{1}{V_{init.}}$ μM/min against $\frac{1}{23PGA_{tot.}}$ $M^{-1}$ at various $[Mg^{2+}]_{free}$ concs.
3PGA. $[Mg^{2+}]_{free}$
2·0 mM. 2·90 mM.
1·0 mM. 2·33 mM.
2·0 mM. 2·25 mM.
1·0 mM. 2·19 mM.
$\frac{1}{V}$
400
300
200
100
0
5·0
10·0
15·0
20·0
25·0
30·0
$\frac{1}{23PGA_{tot.}} \times 10^5$

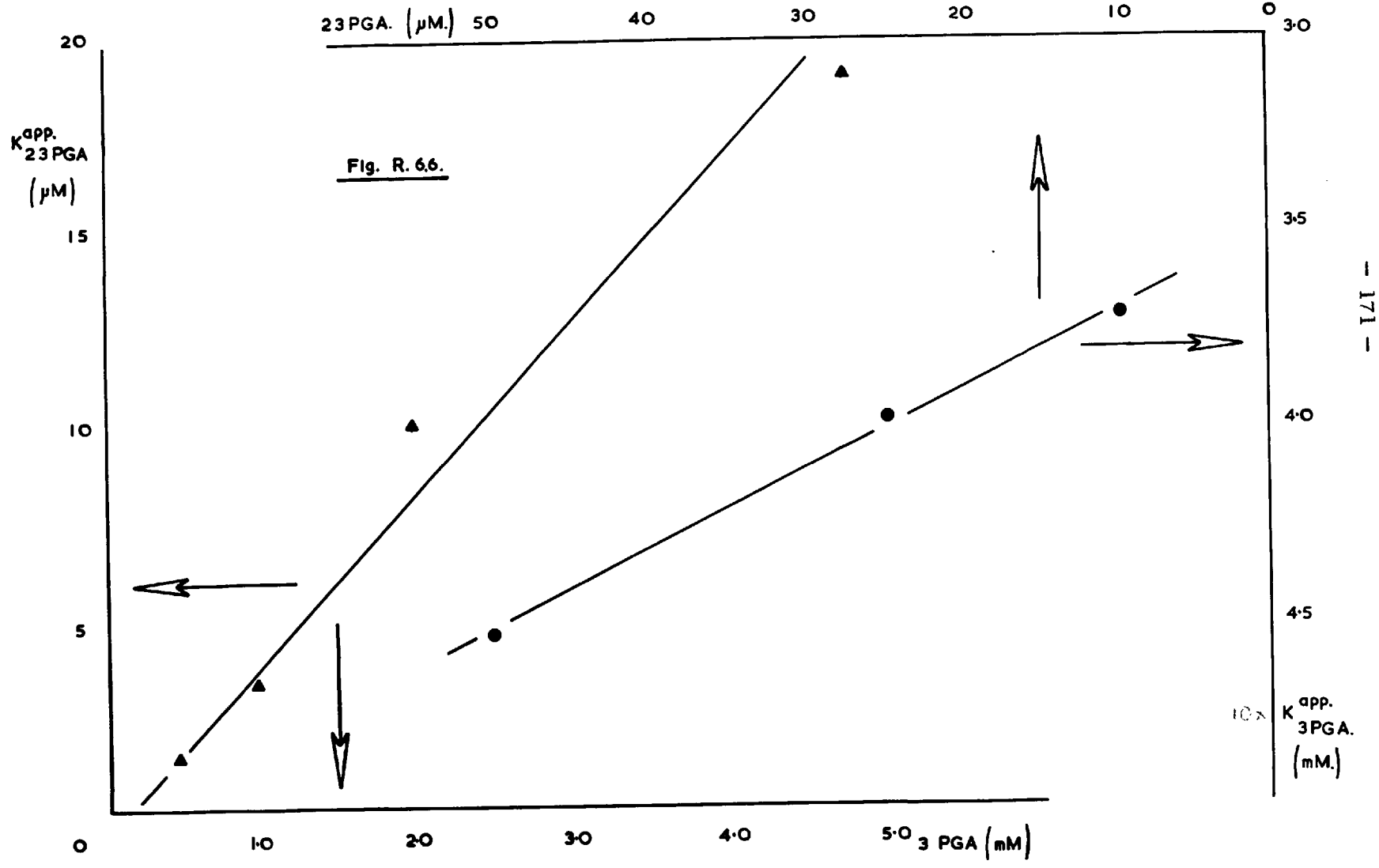

23PGA. (μM.) 50 40 30 20 10 0
20
15
10
5
0
$K^{app.}_{23PGA}$ (μM)
3·0
3·5
4·0
4·5
10× $K^{app.}_{3PGA.}$ (mM.)
1·0 2·0 3·0 4·0 5·0 3 PGA (mM)

Fig. R. 6.6.

Table R.6.2.

Determination of $K_{i23PGA}$

| Run Nos. | 23PGA (μM) | Intercept $\frac{1}{V_{max}}$ | Gradient (mins) x $10^3$ | $K^{app}_{3PGA}$ (mM) |
|---|---|---|---|---|
| 1A - 8A | 50.0 | 16.5 (mins) | 7.47 | 0.453 |
| 1B - 8B | 25.0 | 16.0 | 6.39 | 0.399 |
| 1C - 8C | 10.0 | 16.0 | 5.96 | 0.377 |
| 1D - 8D* | 2.0 | 20.0 | 5.20 | 0.260 |

Determination of $K_{i3PGA}$

| Run Nos. | 3PGA (mM) | Intercept $\frac{1}{V_{max}}$ | Gradient $10^3$ x (mins) | $K^{app}_{23PGA}$ (μM) |
|---|---|---|---|---|
| 1A - 1H | 5.00 | 6.0 (mins) | 0.1127 | 18.8 |
| 2A - 2H | 2.00 | 6.5 | 0.0652 | 10.0 |
| 3A - 3H | 1.00 | 13.5 | 0.0467 | 3.46 |
| 4A - 4H | 0.50 | 19.5 | 0.0317 | 1.62 |

* Values from these sets of runs were not used to determine $K_{i23PGA}$ as the apparent $K_{m3PGA}$ was approximately equal to the true $K_{m3PGA}$.

quoted by Mantle and Garfinkel[51] (in a computer simulation study of the chicken breast enzyme) for the turnover number of phosphoglycerate mutase using a ping-pong mechanism for the analysis of the data, although Torralba and Grisolis gave a slightly lower value of 42,000 moles/mole enzyme/minute[70] for the same enzyme. The $K_m$ for $[3PGA]_{Tot}$ was determined from the gradient of the dotted line, Fig. R.6.2. and a value of 0.36 $\pm$ 0.05 mM was obtained. Similarly from Fig. R.6.4 the $K_m$ of $[23PGA]_{Tot}$ was calculated to be 3.27 $\pm$ 0.32 μM.

The inhibition constants for 3PGA and 23PGA were calculated by measuring the apparent $K_m$'s of 3PGA and 23PGA from graphs R.6.1 and R.6.3 respectively for which the concentration of the non-varied substrate was sufficient to produce competetive inhibition. The apparent values of the $K_m$'s for each substrate were plotted against the concentration of the other substrate, see Table R.6.2 and Fig. R.6.6., the gradient of the straight line graph obtained was related to the inhibition constant required by equation R.6.1 shown below.

$$K_A^{app} = K_A\left(1 + \frac{B}{K_{iB}}\right) \qquad \text{.....} \quad \text{R.6.1.}$$

where $K_A^{app}$ = apparent $K_m$ of A.

$K_A$ = $K_m$ of A.

$K_{iB}$ = inhibition const. of B.

B = concentration of B.

As the kinetics were of the ping-pong pattern A and B were interchangeable; see equation R.6.2.

In this way approximate values for $K_{i23PGA}$ = $1.9 \pm 0.8 \times 10^{-5}$ M and $K_{i3PGA} = 0.83 \pm 0.53$ mM were obtained. This method of estimating inhibition constants was only valid at substrate concentrations $\gg K_m$ and only initial velocity plots corresponding to this condition were used. However, it was not possible strictly to adhere to these conditions as at high 23PGA and 3PGA concentration potentiation of the $V_{init}$ of the enzyme may occur and there was evidence for the occurrence of this phenomenon even under the conditions of the experiments (see later). In the case where no potentiation occurs the initial velocity of reaction v is described by eq. R.6.2 below, using the kinetic nomenclature of Cleland[53].

$$v = \frac{V_{max}AB}{K_A B\left(1+\frac{B}{K_{iB}}\right) + K_B A\left(1+\frac{A}{K_{iA}}\right) + AB} \qquad \text{.....} \quad \text{R.6.2.}$$

Kinetics in the presence of 0.145 mM Ammonium sulphate

Sulphate was present in the experiments just described since the enzyme which formed the assay system (PK, ENOL, LDH) were used in the form of a suspension in 2.37 M $(NH_4)_2SO_4$. In the experiments described below the $(NH_4)_2SO_4$ was largely removed from the enzymic suspension by centrifugation at 25,000 r.p.m. (55,000 g.) at 4°C for 30 mins. After removing the supernatant the precipitated enzymes were dissolved in water and the solution made up to its original volume. When assayed for sulphate by the barium chloroanilate method[71] the solution was found to contain 0.0435 M $(NH_4)_2SO_4$. Assay of the enzymes by comparing the initial velocities of conversion of 2.0 mM 2PGA to lactate showed that about three quarters of the original activity of the enzyme assay system was still present. When the enzyme solution was used for the initial velocity measurements (described in methods section M.2), the concentration of sulphate in the assay solutions was 0.145 mM.

Observations were made at low concentrations of both 3PGA and 23PGA to determine the basic kinetic pattern and to determine the $K_m$'s and $V_{max}$. The data is given in Table R.6.3. Lineweaver-Burke plots of $\frac{1}{V}$ vs $\frac{1}{3PGA}$ and $\frac{1}{23PGA}$ (Figs. R.6.7 and R.6.8 respectively) both showed parallel line patterns.

Table R.6.3.

Initial velocity of reaction for various 3PGA and 23PGA concentrations at 0.15 mM. $SO_4^{2-}$.

| Run No. | | | A | | B | | C | | D | | E | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 23PGA ( μM ) | | 2.00 | | 1.00 | | 0.467 | | 0.333 | | 0.20 | |
| | $^{-1}$23PGA ( $M^{-1}$ ) | | $50 \times 10^4$ | | $100 \times 10^4$ | | $214 \times 10^4$ | | $300 \times 10^4$ | | $500 \times 10^4$ | |
| | 3PGA(mM) | $^{-1}$3PGA | V uM/min | $\frac{1}{V}$ | $V \times 10^2$ | $\frac{1}{V}$ | $V \times 10^2$ | $\frac{1}{V}$ | $V \times 10^2$ | $\frac{1}{V}$ | $V \times 10^2$ | $\frac{1}{V}$ |
| 1 | 0.50 | 2000 | - | - | 3.165 | 31.60 | 2.621 | 38.16 | 2.285 | 44.95 | 1.723 | 58.04 |
| | | | - | - | 3.173 | 31.52 | 2.621 | 38.16 | 2.284 | 43.78 | 1.708 | 58.56 |
| 2 | 0.25 | 4000 | 2.906 | 34.41 | 2.745 | 36.43 | 2.512 | 39.81 | 2.284 | 43.78 | 1.899 | 52.65 |
| | | | 2.934 | 34.08 | 2.766 | 36.15 | 2.431 | 41.13 | 2.217 | 45.11 | 1.841 | 54.32 |
| 3 | 0.10 | 10000 | 2.306 | 43.37 | 2.157 | 46.47 | 1.954 | 51.17 | 1.808 | 55.33 | 1.524 | 65.62 |
| | | | 2.292 | 43.63 | 2.119 | 47.19 | 1.939 | 51.57 | 1.819 | 54.99 | 1.499 | 66.68 |
| 4 | 0.05 | 20000 | 1.414 | 70.71 | 1.474 | 67.84 | 1.301 | 76.87 | 1.203 | 83.14 | 1.122 | 89.14 |
| | | | 1.423 | 70.28 | 1.458 | 68.59 | 1.332 | 75.08 | 1.243 | 80.46 | 1.084 | 92.23 |
| 5 | 0.02 | 50000 | 0.714 | 140.1 | 0.665 | 150.4 | 0.646 | 154.8 | 0.609 | 164.2 | 0.554 | 180.5 |
| | | | 0.716 | 139.7 | 0.669 | 149.5 | 0.621 | 161.0 | 0.564 | 177.3 | 0.572 | 174.8 |

Fig. R. 6.7.

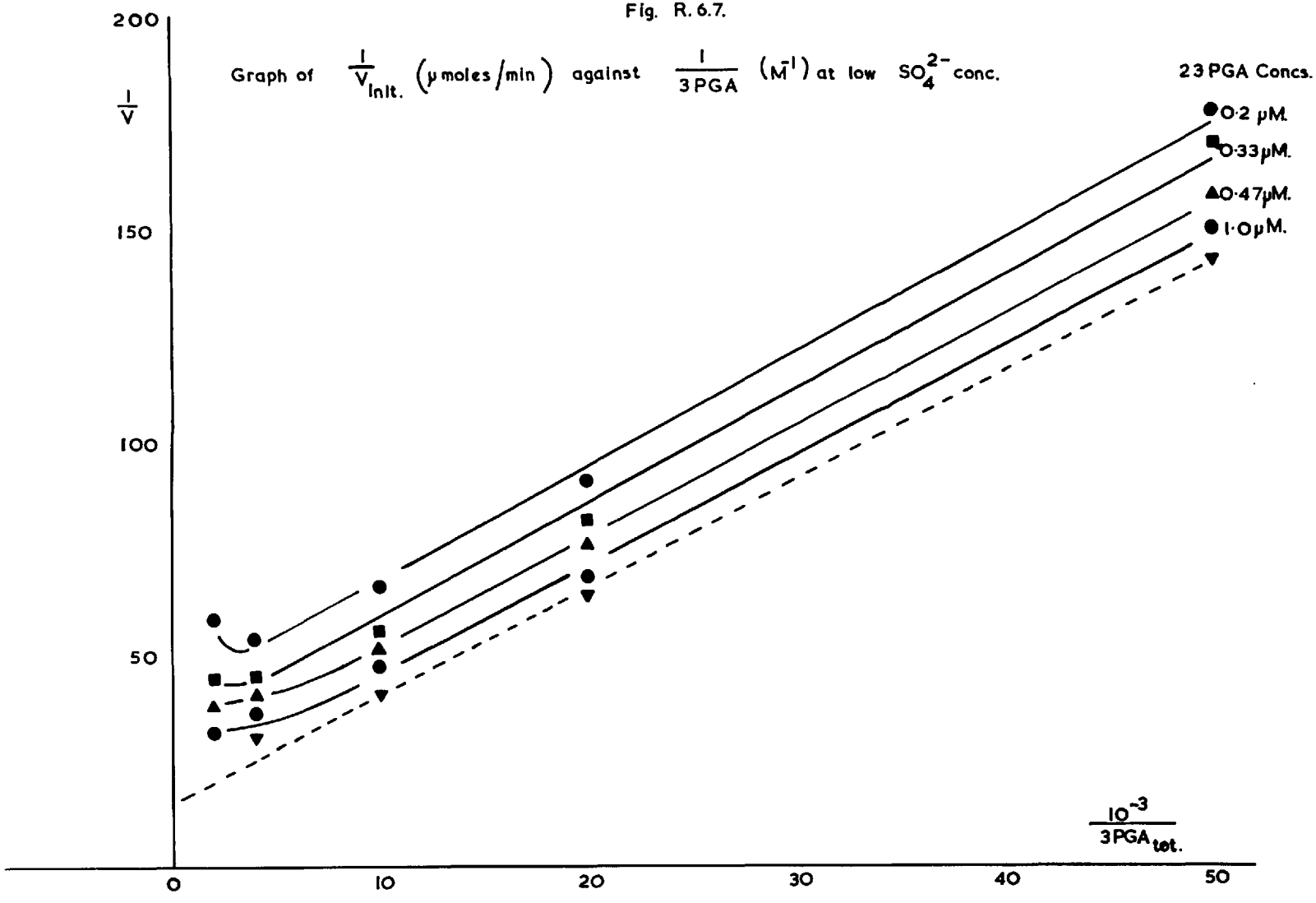
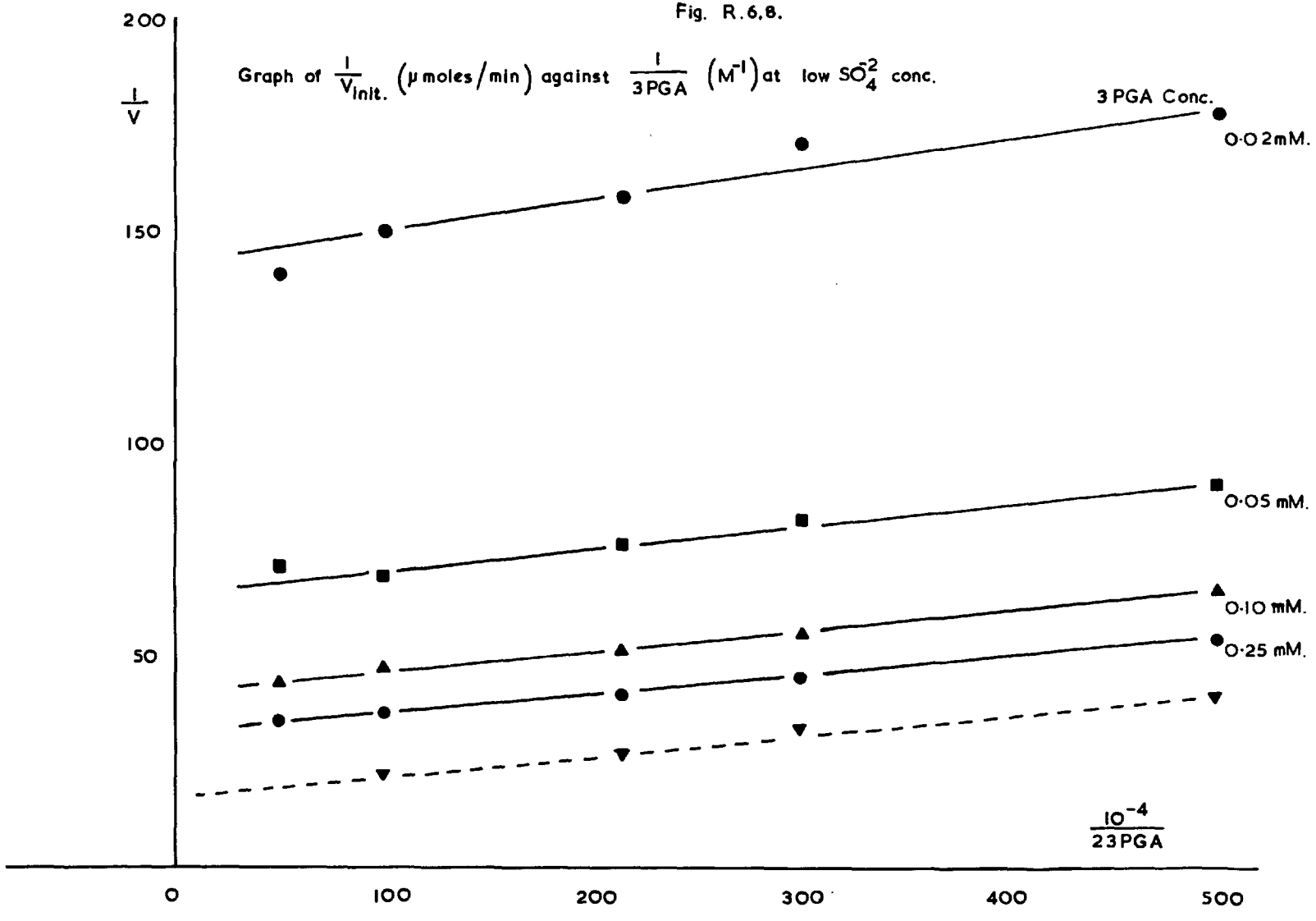

Graph of $\frac{1}{V_{Init.}}$ (μ moles/min) against $\frac{1}{3PGA}$ ($M^{-1}$) at low $SO_4^{2-}$ conc.
2·3 PGA Concs.
0·2 μM.
0·33 μM.
0·47 μM.
1·0 μM.
$\frac{1}{V}$
200
150
100
50
0
10
20
30
40
50
$\frac{10^{-3}}{3PGA_{tot.}}$

Fig. R.6.8.


Graph of $\frac{1}{V_{Init.}}$ (μ moles/min) against $\frac{1}{3PGA}$ $(M^{-1})$ at low $SO_4^{-2}$ conc.
200
150
100
50
$\frac{1}{V}$
3 PGA Conc.
0·02mM.
0·05 mM.
0·10 mM.
0·25 mM.
$\frac{10^{-4}}{23PGA}$
0
100
200
300
400
500

From the secondary plots described in the previous section (shown as dashed lines in Figs. R.6.7 and R.6.8 a $K_m$ for 23PGA of 0.33 $\pm$ 0.02 μM, and for 3PGA of 0.17 $\pm$ 0.02 mM, and a $V_{max}$ of 34,900 $\pm$ 210 moles/mole enzyme/min. were obtained. The $V_{max}$ was appreciably less than the value previously determined. This may have been due to some inactivation of the enzyme since it had been stored at 4°C for 12 weeks.

Inhibition of phosphoglycerate-mutase by sulphate ion

The larger $K_m$'s found for both 3PGA and 23PGA at the higher concentration of $(NH_4)_2SO_4$ suggest that sulphate is a competitive inhibitor for both. If the inhibition should be entirely competitive the inhibition constants may be calculated from eq. R.6.1 to be 6.8 mM for 3 PGA and 0.73 mM for 23 PGA. That sulphate should be highly inhibitory for 23PGA is not unexpected. Grisolia and Cleland[54] showed that 100 mM $(NH_4)_2SO_4$ markedly inhibited the chicken breast enzyme in the presence of 6.6 μM 23PGA but that the inhibition was almost entirely relieved by increasing the concentration of 23PGA to 3.3 mM. Calculations based on the present date suggest that if the chicken breast enzyme is similar to the rabbit muscle enzyme the apparent $K_{23PGA}$ should be 0.34 mM in 100 mM $(NH_4)_2SO_4$, and the sulphate should give little inhibition in the presence of 3.3 mM 23PGA.

The data of Grisolia and Cleland[54] for the chicken breast enzyme suggests that inhibition by sulphate is only in small part due to the effect of its presence upon ionic strength. To test whether this was also true of the rabbit muscle enzyme the following experiments were carried out. The initial velocity of reaction in the presence of 20 μM 23PGA (60 x $K_m$) and 2.0 mM 3PGA (12 x $K_m$) was determined under the conditions described in the methods section. The measurements were then repeated with KCl added to give a concentration of 19.3 mM, or $(NK_4)_2SO_4$ added to give a concentration of 6.43 mM. All experiments were carried out in duplicate. Although the KCl and $(NH_4)_2SO_4$ gave the same increment in ionic strength ($\Delta I = 0.019$), the velocities in the presence of KCl were within 3-5% of the values in its absence, whereas in the presence of the $(NH_4)_2SO_4$ the velocities were reduced by approximately 50%. It is concluded, therefore, that $(NH_4)_2SO_4$ is a specific inhibitor for the rabbit muscle enzyme.

With the inhibition constants just obtained the $K_m$'s for 3PGA and 23PGA in the absence of sulphate may be calculated to be 0.166 $\pm$ 0.02 mM for 3PGA and 0.275 $\pm$ 0.02 μM for 23PGA. The results from the above sections are summarized in Table R.6.4.

Table R.6.4.

Summary of results of determinations of $K_m$'s and $V_{max.}$

$MgCl_2$ = 2.154 mM  I = 0.08

| | $K_m$ $[SO_4^{2-}]$ = 0.0 mM | $K_m$ $[SO_4^{2-}]$ = 0.145 mM | $K_m$ $[SO_4^{2-}]$ = 7.9 mM | $K_{ib}$ 2nd substrate inhibition | $K_{iSO_4^{2-}}$ sulphate inhibition |
|---|---|---|---|---|---|
| 3PGA | 0.166±0.02 mM | 0.17±0.02 mM | 0.36±0.05 mM | 0.83±0.53 mM | 6.8 mM |
| 23PGA | 0.275±0.02 | 0.33±0.02 µM | 3.27±0.32 µM | 19±8 µM | 0.73 mM |
| $V_{max.}$ | - | 34,900±210 moles/mole/min | 52,900±3,100 | - | - |

Measurement of the ratio of the maximum velocities of reaction in the forward and reverse direction and the $K_m$ for 2PGA

The ratio of the maximum forward to backward velocities of reaction $V_f/V_r$ was measured at 2.00 mM concentrations of 3PGA and 2PGA (i.e. at 10 $K_{3PGA}$ and 60 $K_{2PGA}$ respectively and at 2 μM 23PGA (i.e. at 10 $K_{23PGA}$) in solutions containing a very low sulphate ion concentration (13 μM). Exactly the same amount of the same enzyme solution was used in each determination of $V_f$ and $V_r$ and all experiments were carried out in 16.67 mM Tris buffer pH 7.4, in the presence of 3.3 mM $MgCl_2$ (see methods section M.2). The velocity in the forward direction was determined from the rate of disappearance of 2PGA over the first 10-30% of the reaction, since the reaction rate is essentially linear over this range[99]. These determinations gave an average value of $2.835 \times 10^{-2}$ μM/min.

The velocity in the reverse direction was more difficult to measure as the unfavourable equilibrium constant allows only a limited amount of conversion of 3PGA to 2PGA and the reaction rate is linear for conversion of only 2-3% 3PGA into 2PGA. However, to obtain accurate estimations of the 2PGA formed four aliquots were taken at equal time intervals for up to 30% conversion. The velocity in the reverse direction was then determined as the intercept of an unweighted

regression line on a 2PGA/time versus time plot[32, 111]. Three determinations of the $V_f/V_r$ ratios were carried out and ratios of 2.11, 2.38 and 2.00 were obtained giving an average value of $2.2 \pm 0.3$. At the concentration chosen for this estimation any potentiation of the phosphoglycerate mutase by high substrate concentrations should not be important[51].

By the use of the Haldane relationship[112] it was possible to calculate the $K_m$ for 2PGA. In the absence of sulphate ion inhibitor the $K_m$ was calculated to be $32.3 \pm 0.2$ μM , at 0.145 mM $[SO_4^{2-}]$ $33.1 \pm 0.2$ μM, and at 7.9 mM $[SO_4^{2-}]$ $70.1 \pm 0.2$ μM.

Effect of $[Mg^{2+}]_{free}$ concentration upon reaction rate

In the preceding kinetic investigation the $MgCl_2$ and KCl concentrations were varied to keep $[Mg^{2+}]_{free}$ and the ionic strength constant. The proportions of 3PGA and 23PGA complexed with $Mg^{2+}$ thus remained fixed. Consequently the results are consistent with either both the free and the bound forms acting as substrates for the enzyme (providing the $K_m$'s are unaltered by $Mg^{2+}$ binding) or with only one of the forms acting as substrate. In the latter case the free form would presumably be the active species since the enzyme does not require $Mg^{2+}$ for activity[48]. A brief

investigation was therefore carried out on the effect of varying $[Mg^{2+}]_{free}$ on plots of $\frac{1}{V}$ vs $\frac{1}{23PGA}$ at fixed concentrations of 3PGA and at constant ionic strength. The graph obtained from experiments 2A - 2H at 2.25 mM $[Mg^{2+}]_{free}$ was compared with the graph from experiments 9A - 9H at 2.90 mM $[Mg^{2+}]_{free}$. Similarly experiments 3A - 3H at 2.19 mM $[Mg^{2+}]_{free}$ were compared with experiments 10A - 10H (see Table R.6.1). The Lineweaver-Burke plots are shown in Fig. R.6.5. In each case increasing the $[Mg^{2+}]_{free}$ concentration caused inhibition of $V_{init}$ as might be expected if the $Mg^{2+}$ complexes were not substrates. Though a wide range of $Mg^{2+}$ concentrations was not investigated it seems that the $V_{max}$ is unaffected by the free $Mg^{2+}$ concentration (see Fig. R.6.5). These results suggest that only $Mg^{2+}$ free 3PGA and 23PGA can combine with the enzyme and the $Mg^{2+}$ complexes cannot bind to form dead end inhibition complexes.

In order to investigate quantitatively $Mg^{2+}$ inhibition $K^{app}_{23PGA}$'s have been calculated from Fig. R.6.5 (see Table R.6.5) with the assumption (column 3) that both the free and complexed forms of 23PGA are equally active as substrates or alternatively with the assumption (column 4) that only the uncombined form of 23PGA can act as a substrate. The free 23PGA concentration was calculated

Table R.6.5.

$K^{app}_{23PGA}$ determinations at various $[Mg^{2+}]_{free}$ concentrations

| Expt. | Concs. of $Mg^{2+}$ | $K^{app}_{23PGA}$ (μM) $Mg^{2+}$ complexes react with enzyme | $K^{app}_{23PGA}$ (μM) $Mg^{2+}$ complexes do not react with enzyme | Theoretical ratio $\frac{[K^{app}_{23PGA}]\ Mg^{2+}\ 1}{[K^{app}_{23PGA}]\ Mg^{2+}\ 2}$ | $K^{app}_{23PGA}$ ratio of $\frac{\text{Expt. 1}}{\text{Expt. 4}}$ $Mg^{2+}$ complexes react with enzyme | $K^{app}_{23PGA}$ ratio of $\frac{\text{Expt. 1}}{\text{Expt. 4}}$ $Mg^{2+}$ complexes do not react with enzyme |
|---|---|---|---|---|---|---|
| 1 | 3PGA=2.0 mM $Mg^{2+}$=2.90 mM | 6.48 | 1.53 | 0.83 | Theoretical 1.35 | Theoretical 1.23 |
| 2 | 3PGA=2.0 mM $Mg^{2+}$=2.25 mM | 5.74 | 1.64 | | | |
| | | 1.13 | Ratio 0.93 | | | |
| 3 | 3PGA=1.00 mM $Mg^{2+}$=2.33 mM | 3.94 | 1.10 | 0.96 | Experimental 1.71 | Experimental 1.38 |
| 4 | 3PGA=1.00 mM $Mg^{2+}$=2.19 mM | 3.80 | 1.11 | | | |
| | | 1.04 | Ratio 0.99 | | | |

assuming that the Mg-23PGA binding constant, K, was 1111 $M^{-1}$ (5). The experimental ratios of $K^{app}_{23PGA}$ thus obtained were compared with theoretical ratios (either assuming that both free and complexed forms of 3PGA and 23PGA are equally active or assuming that only the free forms are active) determined from eq. R.6.3 below. This equation was derived from the general eq. R.6.2 for a ping-pong mechanism and can be used to predict the ratios of $K^{app}_{23PGA}$'s at different 3PGA concentrations for both models.

$$\frac{(K^{app}_{23PGA})_1}{(K^{app}_{23PGA})_2} = \frac{(K_{iB} + B_1)(B_2 + K_B)\ B_1\ (K[Mg^{2+}]_2 + 1)}{(K_{iB} + B_2)(B_1 + K_B)\ B_2\ (K[Mg^{2+}]_1 + 1)} \quad \ldots \quad R.6.3.$$

1 and 2 refer to the two free magnesium ion concentrations $[Mg^{2+}]$, A is 23PGA, and B, the free or total concentration of 3PGA depending upon which model is taken. The appropriate value of $K_{i3PGA}$ for the model in which magnesium complexes are inhibitory was calculated to be 0.536 mM from the value (0.83 mM) obtained previously assuming magnesium complexes were not inhibitory.

Using this equation the theoretical ratios of $K^{app}_m$ between experiments 1 and 2, and between experiments 3 and 4 were calculated. The results are shown in Table R.6.5.

On the basis of these calculations it seems that the ratio of the $K^{app}_m$'s of 23PGA measured experimentally fit more closely the

theoretical ratios in the case of the model in which magnesium complexes are considered not to combine with the enzyme. However, this conclusion must remain tentative since the difference in the theoretical ratios (col. 5, Table R.6.5) is comparable with the expected experimental error in determination of $K^{app}_{23PGA}$'s. Discrimination can be improved by a comparison of the $K^{app}_{23PGA}$'s for the most disparate $[Mg^{2+}]_{free}$ concentrations (experiments 1 and 4, last two columns in Table R.6.5). This comparison more clearly demonstrates that better agreement between the theoretical and experimental ratios is obtained if the magnesium complexes are considered not to combine with the enzyme. However, an unequivocal distinction between these two possibilities cannot be made on the available evidence nor was it practicable to consider models in which only one of the two magnesium complexes does not combine with the enzyme. Nevertheless it seemed most likely that above conclusions are correct and that inhibition due to $Mg^{2+}$ is analagous to that in the phosphoglucomutase system[6].

If $Mg^{2+}$ inhibition were to be investigated systematically the effect of varying the magnesium ion concentration on both $K^{app}_{23PGA}$ at $3PGA \gg K_{i3PGA}$ and $\gg K_{3PGA}$, and $K^{app}_{3PGA}$ at $23PGA \gg K_{i23PGA}$ and $\gg K_{23PGA}$ should be measured over a wide

range of magnesium ion concentration. Under these circumstances variations in the apparent $K_m$'s of both substrates would depend only upon the formation of magnesium complexes with the varied substrate, and consequently interpretation of the results would be easier (see Ray and Roscelli[6]).

The possibility that chloride from the $MgCl_2$ also competitively inhibits 3PGA and 23PGA is thought to be unimportant in the present system although Ray and Roscelli[6] have shown its importance in the phosphoglucomutase system. In this investigation the ionic strength of solution was buffered at I = 0.08 by the addition of 200 mM KCl solution. Consequently the changes in chloride concentration will not be as large as those occurring for magnesium. Although chloride is known to be an inhibitor of the reaction[54] the changes in its concentration are not expected to contribute significantly to the overall inhibition, indeed addition of 19.3 mM KCl only decreased the initial velocity by only 3-5% (with 2.0 mM 3PGA and 20 μM 23PGA, see earlier).

In the determination of $K_m$'s in the previous two sections there were small changes in $[Mg^{2+}]_{free}$ concentration (2.121 - 2.188 mM) over the range of substrate concentrations used. The results from this section indicate

that over this range of $[Mg^{2+}]_{free}$ concentration the attendant variations of $K_m^{app}$'s should be well within the overall experimental error. Thus these experiments could be considered as having been carried out at a single $[Mg^{2+}]_{free}$ concentration of 2.154 mM.

Potentiation of enzyme reaction rate

Under most circumstances the rate of conversion of 3PGA to 2PGA was initially constant and slowed down as the 3PGA was consumed. However, under certain circumstances, when the 3PGA concentration was greater than 1 mM and the 23PGA concentration greater than 10 μM at the same time (experiments 1A - 3C), an initial acceleration phase starting with a finite velocity and lasting 1-2 mins. was observed. This was followed by a linear phase of more rapid reaction. The increase in reaction rate (i.e. ratio of initial velocity to constant and maximum velocity of reaction) was much more marked at the higher concentrations of 3PGA and 23PGA in experiment 1A than in the lower ones of 1C. When such an acceleratory period was observed the $V_{init}$ recorded in Table R.6.1 and R.6.2 was that of the subsequent linear phase of reaction corresponding to the maximum velocity of reaction. These two phases of the reaction were easily separable.

To investigate the nature of this potentiation phenomenon a series of experiments were carried out in which the enzyme was incubated with either 23PGA or 3PGA alone and the reaction

initiated by the subsequent addition of the second substrate. It was hoped that these experiments would show whether the acceleratory phase was caused by a co-operative potentiation effect between 3PGA and 23PGA or was caused primarily by the interaction between one of the substrates and the enzyme. Such experiments were carried out under the conditions described in Table R.6.1 and the results are shown in Table R.6.6. Preincubation with either 23PGA or 3PGA led to a partial activation of the enzyme and in this respect 23PGA was more effective. A potentiation phase still occurred however, when the second substrate was added, and although the percentage increase in rate during the potentiation phase was smaller after preincubation the final rate achieved appeared to be slightly greater. A similar acceleratory period was reported by Grisolis and Cleland[54] for the chicken breast enzyme and this may well be connected with the potentiation observed here.

The acceleration period is not due to a slow build up of 2PGA to sufficient concentration to allow the enzyme assay system to operate at a rate greater than the phosphoglycerate-mutase. During this period sufficient time elapses to allow the 2PGA concentration to exceed by a factor of 5-10 times the 2PGA concentration required to fully activate the assay system ($\lesssim$ 2.3 μM 2PGA) even at the initial rate of reaction.

Table R.6.6.

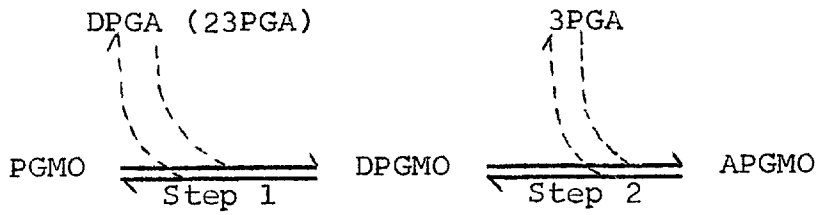
$[Mg^{2+}]_{free}$ = 2.247 mM. I = 0.08

| 23PGA μM | 3PGA mM | Substrate $S_1$ added first | Incubation time with $S_1$ | Init. vel. of reaction $10^2$ μM/min | Vel. of reaction $10^2$ μM/min | Ratio $\frac{V_{init}}{V_{steady}}$ | Time to steady velocity of reaction |
|---|---|---|---|---|---|---|---|
| 50.0 | 2.00 | 23PGA | 10 mins. | 3.926 | 5.544 | 0.708 | 1.5 mins |
| " | " | 3PGA | 10 " | 2.112 | 5.407 | 0.391 | 1.8 " |
| " | " | Both | - | 1.196 | 4.724 | 0.253 | 2.1 " |
| " | " | Both | - | 1.123 | 4.458 | 0.252 | 2.4 " |

Moreover the acceleration would be expected over a much wider range of conditions than is observed.

Mantle and Garfinkel[51] suggested a model for potentiation of the enzyme by incorporating a "black box modifier" (Fig. R.6.9) in their kinetic model for phosphoglycerate-mutase at high concentrations of 3PGA ($\geqslant$ 5 mM) and 23PGA ($\geqslant$ 10 µM). However, no information about the speed of the activation process nor any experimental evidence was given.

Fig. R.6.9.




ordinary enzyme potentiated enzyme

Obligatory sequential addition of 23PGA then 3PGA.

PGMO is the ordinary and APGMO the "activated" form of phosphoglycerate-mutase.

The present results suggest that the addition of 23PGA or 3PGA in such a "black box modifier" must be random (since

preincubation with either leads to partial activation) and do not support the model of Mantle and Garfinkel. They may also suggest that in the activation process 23PGA binds more slowly to the enzyme than 3PGA. It was not possible to deduce anything further from this preliminary investigation except to note that there may be a connection between the time taken to reach the first steady reaction rate and the number of turnovers completed by the enzyme. The implications involved were unclear without further experimental evidence.

## SECTION 7. INDUCED TRANSPORT EXPERIMENTS; PHOSPHOGLUCOMUTASE SYSTEM

In all induced transport experiments the reaction was carried out at 30°C using enzyme pre-activated by incubation for 10 mins. with histidine-tris buffer, pH 7.4, as described by Ray and Roscelli[1]. G16P was present in excess. Calculations from the data of Ray and Roscelli[1, 32] indicated that its concentration exceeded the effective $K_m$ for G16P by five to ten-fold. The experimental determinations of radioactivity and conditions under which induced transport test were conducted are described in methods section M.1.

### Induced transport tests with $^{14}C$-labelled substrates

The results of a series of induced-transport tests at different substrate concentrations with $^{14}C$-labelled substrates have been illustrated in Figs. R.7.1-3. Where single experiments only are shown the results were confirmed by duplicate experiments not illustrated. In individual experiments the time course of the reaction varied slightly. The time scale of each reaction was therefore adjusted so that the course of the reaction was represented by the curve shown. At low concentrations of hexose phosphates Fig. R.7.1A, B, C the $^{14}C$ activity in G1P was measured for the following conditions. For curve A non-radioactive G1P was added in a

Fig. R.7.1.

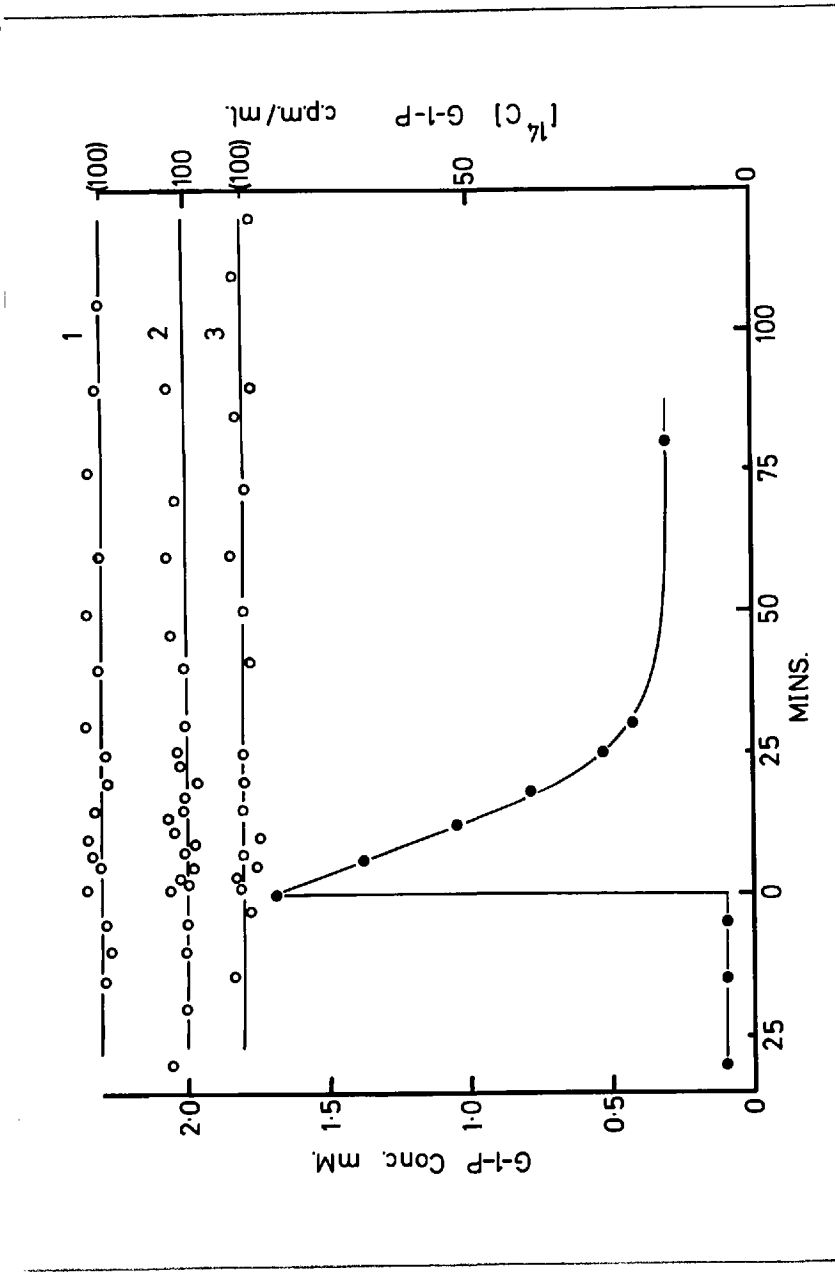

G-1-P Conc. mM.
2·0
1·5
1·0
0·5
0
25
0
25
50
75
100
MINS.
[14C] G-1-P c.p.m./ml.
(100)
100
(100)
50
0
1
2
3

Fig. R.7.2.

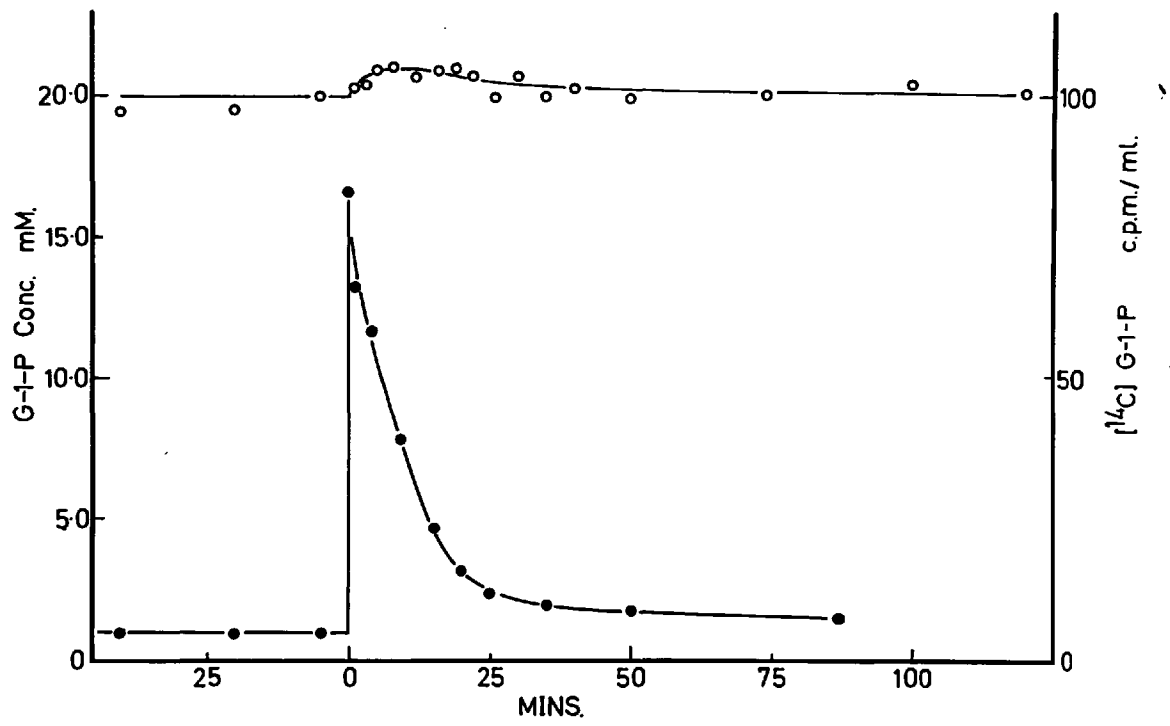
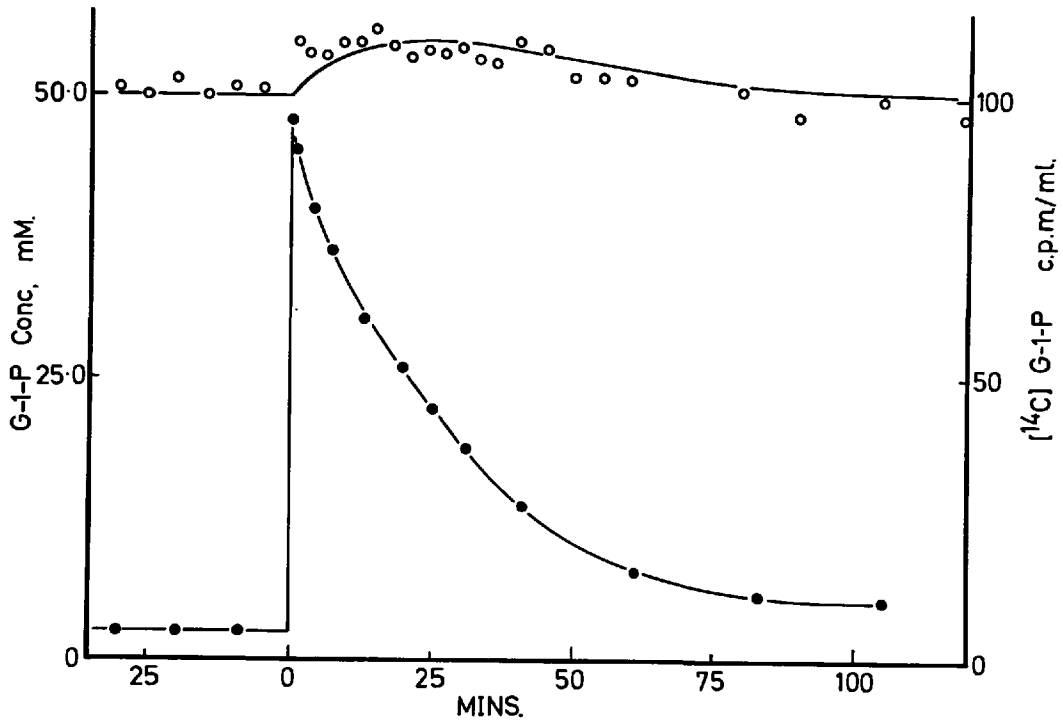

20·0
15·0
10·0
5·0
0
G-1-P Conc. mM.
100
50
0
[14C] G-1-P c.p.m./ml.
25
0
25
50
75
100
MINS.

Fig. R.7.3.



volume of 0.4 mls. to 4.6 mls. of solution (see method section M.1. for all relevant conditions). Concentration of hexose phosphates before addition were: G1P, 0.0404 mM; G6P, 0.695 mM; G16P, 2.13 μM; concentrations immediately after addition: G1P, 1.044 mM; G6P, 0.630 mM; G16P, 2.0 μM. For curve B non-radioactive G1P was added in a volume of 0.4 mls. to 4.95 mls. of solution. Concentrations before addition: G1P, 0.087 mM; G6P, 1.51 mM; G16P, 4.6 μM; concentrations immediately after addition: G1P, 1.58 mM; G6P, 1.39 mM; G16P 4.2 μM. The points shown represent the means of duplicated or triplicated observations. For curve C, non-radioactive G1P was added in a volume of 0.4 mls. to 4.6 mls. of solution. Concentrations before addition: G1P, 0.0808 mM; G6P, 1.39 mM; G16P, 4.26 μM; concentrations immediately after addition: G1P, 2.088 mM; G6P, 1.26 mM; G16P, 4.0 μM.

The induced transport test at an intermediate concentration of hexose phosphates are shown in Fig. R.7.2. At zero time 0.4 mls. of non-radioactive G1P was added to 4.95 mls. of reaction mixture. Concentrations before addition of non-radioactive G1P: G1P, 0.869 mM; G6P, 14.92 mM; G16P, 0.0489 mM; concentrations immediately after addition: G1P, 16.53 mM; G6P, 13.74 mM; G16P, 0.0450 mM.

Finally, the induced transport tests at high concentration of hexose phosphates are shown in Fig. R.7.3. The points

represent duplicated or triplicate determinations. At zero time non-radioactive G1P in 0.4 mls. was added to 4.95 mls. of reaction mixture. Concentrations of hexose phosphates before addition: G1P, 2.465 mM; G6P, 4.24 mM; G16P, 0.1155 mM; concentrations immediately after addition: G1P, 47.1 mM; G6P, 39.2 mM; G16P, 0.1059 mM.

At low concentrations of hexose phosphate (Figs. R.7.1A, B, C) the $^{14}C$ radioactivity in G1P remained substantially unchanged during the reaction, but at intermediate concentrations (Fig. R.7.2) a small rise in the radioactivity probably occurred. At high concentrations (Fig. R.7.3) there was a pronounced rise of about 10% while the reaction was in progress. The increase in radioactivity in Fig. R.7.3 did not appear to be an artefact since it occurred in all experiments, and no rise took place in control experiments in which the enzyme was inactivated by brief heating (to approx. 80°C) immediately before the addition of non-radioactive G1P. At the end of the reaction the radioactivity in the G1P always returned substantially to the value existing before the addition of the non-radioactive G1P.

It was possible that after the addition of non-radioactive G1P some radioactivity was transferred from the G16P to the G1P (which was now of low specific activity), but any contribution from this source must have been very small (see later).

Consequently a change in the radioactivity in the G1P must very largely have reflected a corresponding change in the radioactivity in the G6P. Thus the experiments shown in Figs. R.7.1-3 provide strong evidence for a lack of induced transport of $^{14}C$-labelled substrates at low substrate concentration and counter-transport at high concentrations (see theory section). If isotope effects and the non-ideal behaviour of solutions, which are probably relatively unimportant are ignored (see discussion section) it follows that at low substrate concentrations the glucose fluxes must obey the independence relationship, but that at high concentrations the flux ratio deviates from this relationship in the direction of counter-transport (equivalent to exchange-diffusion). This result is clearly compatible with either mechanism 1 or mechanism 2 (theory section) for which the equations for induced transport are identical, and the continuous lines drawn through the experimental points in Figs. R.7.1-3 are the theoretical lines expected for $\alpha = 0.637 \times 10^{-2}\ mM^{-1}$. The constant $\alpha$, which has been defined in the theory section, measures the extent to which the isomerization of the phosphoenzyme is rate limiting. It is zero when the isomerization is rapid. With the value of $\alpha$ given above and assuming that the behaviour of the solution is

ideal and that for the isomerization step the forward and reverse rate constants are identical ($k_{+3} = k_{-3}$), the rate constant for isomerization is approximately $4.5 \times 10^7$ $sec^{-1}$ (see theory section).

The results in Figs. R.7.1-3 exclude mechanism 3, since this mechanism at low concentrations of substrate should show co-transport of [$^{14}$C] G1P. Thus the theoretical curves 3 and $1_3$ in Fig. R.7.1 show the calculated results if the whole reaction had proceeded via mechanism 3, or if 20% of the flux from G1P to G6P had proceeded via mechanism 3, assuming that the value of $\alpha$ was small enough to be ignored (see theory section). These curves clearly diverged from the experimental results. If the value of $\alpha$ were large the predicted amount of co-transport would have been less and the theoretical lines closer to the experimental results. However, if this were the case, the nett reaction should have shown product inhibition of a partly non-competitive nature. Ray and Roscelli[32] were unable to detect any inhibition of this kind so that it was concluded that these results unambiguously excluded even a small contribution of mechanism 3 to the reaction.

The contribution to the [$^{14}$C] G1P radioactivity from impurities in, and incomplete precipitation of [$^{14}$C] G6P was estimated from the radioactivity remaining in the supernatant

of samples which had been subjected to $Ba(OH)_2/ZnSO_4$ precipitation of phosphates (see methods section) without prior hydrolysis of [$^{14}$C] G1P. This contribution was subtracted from the total [$^{14}$C] G1P, and usually amounted to 7-10% of it.

As has been mentioned before, the possibility that radioactivity was transferred transiently from the G16P to the G1P when non-radioactive G1P was added, must be considered, because of the very low specific activity of the G1P immediately after the addition. To test for this possibility samples were taken at various times during the reaction and chromatographed on DEAE-cellulose paper with ammonium formate to separate the G16P from the monophosphates (see methods section M.1). The chromatograms were then scanned for radioactivity and the areas of the peaks corresponding to G16P measured with a planimeter. The areas of these peaks were then compared with the areas of peaks in calibration chromatograms in which known quantities of [$^{14}$C] G6P were run and the scan of radioactivity for a typical chromatogram is presented in Fig. R.7.4. (The results obtained under the conditions of experiment 3 (Fig. R.7.3) are summarized in Table R.7.1.) The method does not give very accurate estimates of $^{14}$C-labelling in G16P because of the proximity of the peak of $^{14}$C-labelled

Table R.7.1

Calibration of chart area in terms of [$^{14}$C] G6P (3.1 mc/mM) for radioactive chromatogram scans. The same chart speed and sensitivity of detection were used in all cases.

| Moles of G6P carrier added to chromatogram | Total d.p.s. of [$^{14}$C] G6P present ($x10^{-1}$) | Area of graph ($cm^2$) |
|---|---|---|
| $9.50 \times 10^{-8}$ | 9.25 | 45.9 |
| $4.75 \times 10^{-8}$ | 4.63 | 22.8 |
| $2.38 \times 10^{-8}$ | 2.31 | 12.1 |

Assay of $^{14}$C-labelling in G16P from expt. 3 after chromatography.

Total monophosphate concentration = 44.87 mM (equilibrium period).

Specific activity of monophosphates at end of equilibrium period, time zero = $1.59 \times 10^8$ d.p.s./mole.

| Time (mins) after addition of G1P | Area ($cm^2$) of G16P peak | d.p.s. in G16P peak ($x10^{-1}$) | Specific activity of G16P (d.p.s./mole $x10^{-8}$) |
|---|---|---|---|
| -1.0 | 13.4 | 2.68 | 1.27 |
| 1.0 | 18.1 | 3.62 | 1.71 |
| 5.0 | 12.2 | 2.44 | 1.15 |
| 10.0 | 8.8 | 1.75 | 0.83 |
| 15.0 | 12.6 | 2.51 | 1.19 |
| 20.0 | 15.5 | 3.09 | 1.46 |
| 50.0 | 9.9 | 1.96 | 0.93 |
| 120.0 | 9.1 | 1.80 | 0.85 |

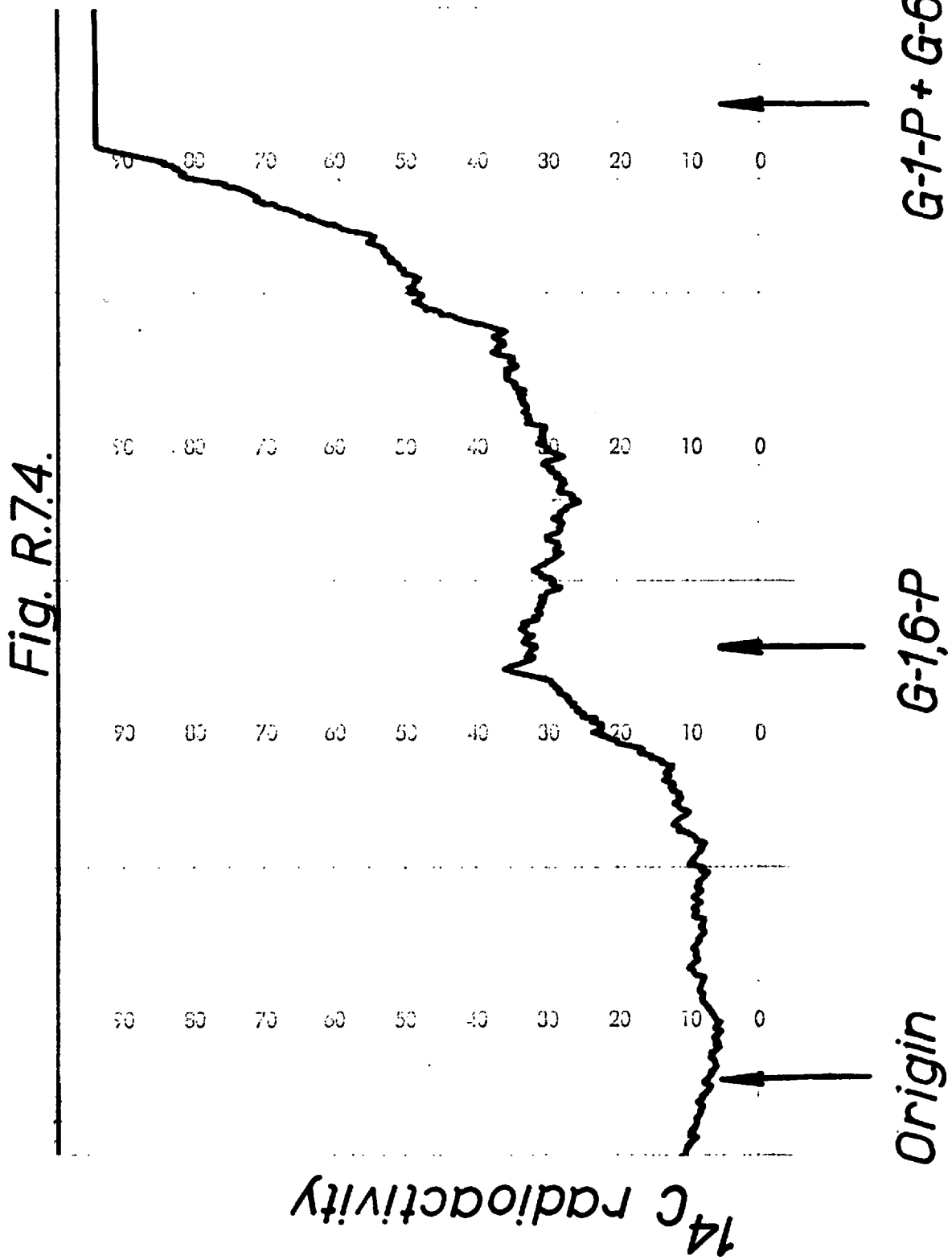

G-1-P + G-6-P
G-1,6-P
Origin
14C radioactivity
90
80
70
60
50
40
30
20
10
0

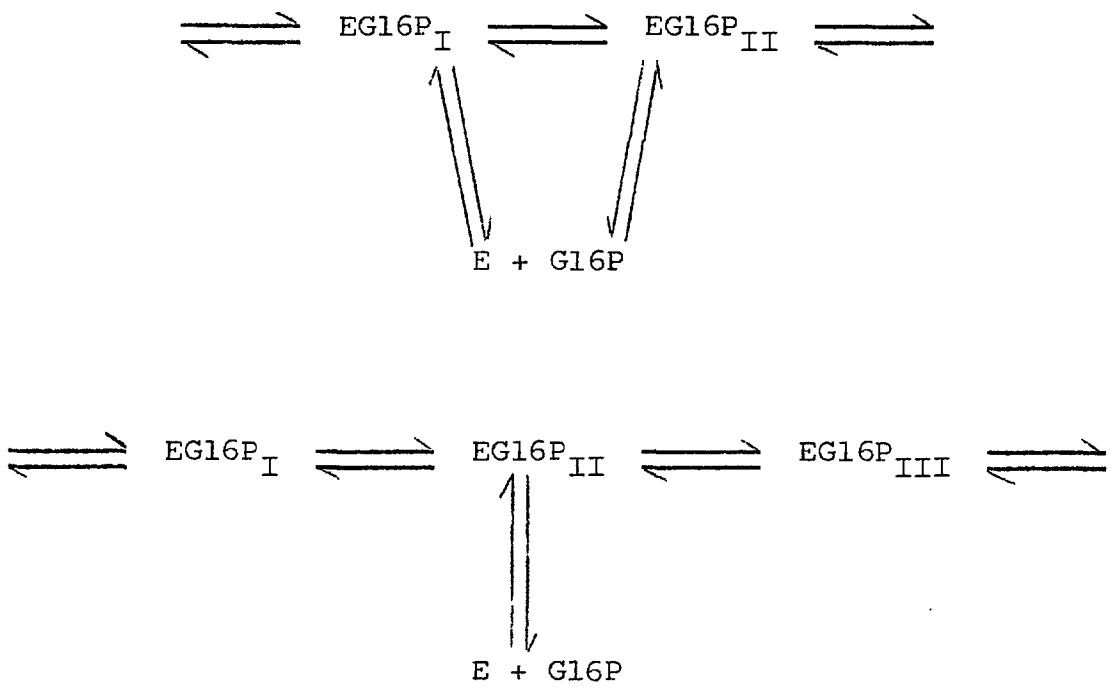
Fig. R.74.

monophosphates. Nevertheless it was adequate to show whether there were large changes in the radioactivity in G16P during the induced transport experiments.

The G16P had approximately the same specific radioactivity as the substrates at the end of the equilibration period. (The fact that the S.A. of the G16P was slightly higher than the substrates was probably a result of some G16P impurity in G6P contributing to the overall concentration of G16P which would thus be a little in excess of the value used in the calculations.) When the non-radioactive G1P was added the specific radioactivity of G16P fell progressively to reach the expected value of approximately one half. Moreover, at the time when the radioactivity in the G1P was elevated (within the first few minutes after the addition of inactive G1P) Fig. 3, the radioactivity in the G16P had fallen by less than 25%. The total radioactivity in the G16P at the end of the equilibration period was only about 5% at the lower, and 2.5 % at the higher concentrations of the radioactivity in G1P. Thus the G16P could only have contributed at most only 1.3 - 2.5% of the radioactivity in the G1P. Further, it was extremely unlikely that all the radioactivity disappearing from the G16P would be transferred to the G1P. For example, using the rate constants for the maximum

velocities in the forwards (G1P to G6P) and reverse direction (750 $sec^{-1}$ and 242 $sec^{-1}$ respectively) obtained by Ray and Roscelli[1, 32] it may be calculated that only about one quarter of the radioactivity could be thus transferred. This latter calculation, however, may not be entirely valid since it assumes that there is only one intermediate enzyme-substrate complex, and that the slow steps in the reaction are the breakdown of the central enzyme-substrate complex to either G1P and EP or G6P and EP. From initial-rate studies with G1P Ray and Roscelli[1, 32] obtained an apparent $K_m$ for G16P of $6.2 \times 10^{-8}$M, whereas they obtained a value of $1.1 \pm 0.5 \times 10^{-8}$M from initial-rate studies with G6P under similar experimental conditions. Such a difference could be most simply explained by assuming two intermediate enzyme-substrate complexes as shown in the Fig. R.7.5 below. Alternatively if there were three intermediate EG16P complexes it is only necessary for G16P to dissociate from the central complex (Fig. R.7.5) in order for the apparent $K_m$ for G16P to depend upon the direction in which the initial rate of reaction studies were carried out.

Fig. R.7.5.




These two reactions are homioikinetic for both nett fluxes and for undirectional fluxes. Should the interconversion of these enzyme-substrate complexes be rate limiting for the overall enzyme reaction then the former calculations would be invalid. Nevertheless the G16P cannot have contributed more than approximately one quarter of its radioactivity to the G1P which represents only about 0.6% of

the initial radioactivity in G1P. Consequently any radioactivity appearing in the G1P must have been largely derived from the G6P. At the end of the reaction there was a small contribution from the G16P to the G1P, but this was less than 0.1% of the initial radioactivity.

Ray and Roscelli[1], using $^{32}P$-labelled phosphoenzyme, concluded that the enzyme substrate complex dissociate into G16P on about 5% of occasions, but the present observations suggest that the frequency of dissociation must be rather smaller. This conclusion was arrived at in the following manner. At zero time, after the addition of inactive G1P in Experiment 3 most of the enzyme substrate complex was formed from the largely inactive G1P ($K_{mG1P} = 8.5 \times 10^{-6}M$, $K_{mG6P} = 5 \times 10^{-5}M$; G1P = 47.1 mM, G6P = 39.2 mM). Consequently the rate of incorporation of non-radioactive G16P into the G16P pool would be 5% of the initial reaction rate. Since there is no nett synthesis of G16P the flux of G1P to G16P must be balanced by the flux from G16P. If, at time zero, the specific radioactivity of G1P was considered to be negligible then the rate of loss of radioactivity by G16P could be calculated, assuming that all the active enzyme was bound by G1P.

From Table R.7.1 and expt. 3.

Initial rate of incorporation of non-radioactive G1P into G16P. $\simeq$ 0.05 x 9.55 μMoles/min.

$\therefore$ Initial rate of loss of radioactivity in G16P $\simeq$ 0.478 x 1.71 x $10^2$ d.p.s./min.

Vol. of solution = 5.35 mls; G16P conc. = 0.1059 mM.

Hence initial rate of loss of specific activity of G16P $\simeq$ $\frac{0.478 \times 1.71}{0.1059 \times 5.35} \times 10^2$ d.p.s./min./μmole

$\simeq$ 1.44 x $10^2$ d.p.s./min./μmole

By measurement, initial rate of loss of S.A. by G16P $\simeq$ 0.14 x $10^2$ d.p.s./min./μmole.

Thus the dissociation of the enzyme substrate complex into G16P and free enzyme must have occurred at a rate, approximately an order of magnitude slower than that proposed by Ray and Roscelli[1].

Hashimoto and Handler[55] studied the rate of incorporation of $^{32}$P-labelled phosphate into G16P with [$^{32}$P] G1P as substrate and also obtained results that indicated a low rate of dissociation of the enzyme-substrate complex. Further, in later experiments Ray *et al.*[17] found negligible incorporation of radioactivity into G16P in

equilibrium flux measurements with $^{32}P$-labelled substrates. It was therefore possible that in the original experiments of Ray and Roscelli[1] the frequency of dissociation was overestimated. This might have arisen if there were some [$^{32}P$] G16P rather tightly bound to the phosphoenzyme; alternatively, there may have been some alteration in the enzyme during the mixing procedures, perhaps as a result of transient electrolyte changes. The latter might also explain the relatively rapid incorporation of $^{32}P$-labelled phosphate into G16P that occurred in the initial stages of the experiments of Hashimoto and Handler. That such enzyme changes might have occurred was suggested by the work of Cascales and Grisolia[57] on phosphoglyceromutase in which the rate of incorporation of $^{32}P$-labelled phosphate into 23PGA from the substrates was greatly increased when the ionic strength of the solution was raised.

Induced transport tests with $^{32}P$-labelled substrates

Induced transport tests (experiments 4 and 5, Table R.7.2) with $^{32}P$-labelled substrates are illustrated in Figs. R.7.6-7. These tests were carried out at low concentration of hexose phosphates (see methods section). For experiment 4 the concentrations of the substrates were then: G1P, 0.0404 mM; G6P, 0.695 mM; G16P, 2.13 μM. At

Table R.7.2.

Summary of conditions for induced-transport tests. Measurements were made in histidine-tris buffer, pH 7.4 at 30°C.

| Expt. No. | Conditions at equilibrium time zero | | | Conditions after addition of G1P, at time zero | | | Enz. conc. µg/ml. | Results % change in G1P counts |
|---|---|---|---|---|---|---|---|---|
| | G1P (mM) | G6P (mM) | G16P (µM) | G1P (mM) | G6P (mM) | G16P (µM) | | |
| 1A | 0.0404 | 0.695 | 2.13 | 1.044 | 0.630 | 2.0 | 0.44 | No induced transport |
| 1B | 0.087 | 1.510 | 4.60 | 1.580 | 1.390 | 4.2 | 0.42 | Independence |
| 1C | 0.0808 | 1.390 | 4.26 | 2.088 | 1.260 | 4.0 | 1.08 | Relationship obeyed |
| 2 | 0.869 | 14.92 | 48.9 | 16.53 | 13.74 | 45.0 | 3.64 | Counter-transport 3% rise |
| 3 | 2.465 | 42.4 | 115.5 | 47.1 | 39.2 | 105.9 | 8.65 | Counter-transport 10% rise |
| 4 | 0.0404 | 0.695 | 2.13 | 1.044 | 0.630 | 2.0 | 0.44 | Co-transport fall to 39% |
| 5 | 0.808 | 1.39 | 4.26 | 2.088 | 1.26 | 4.0 | 1.08 | Co-transport fall to 38.5% |

Fig. R.7.6.

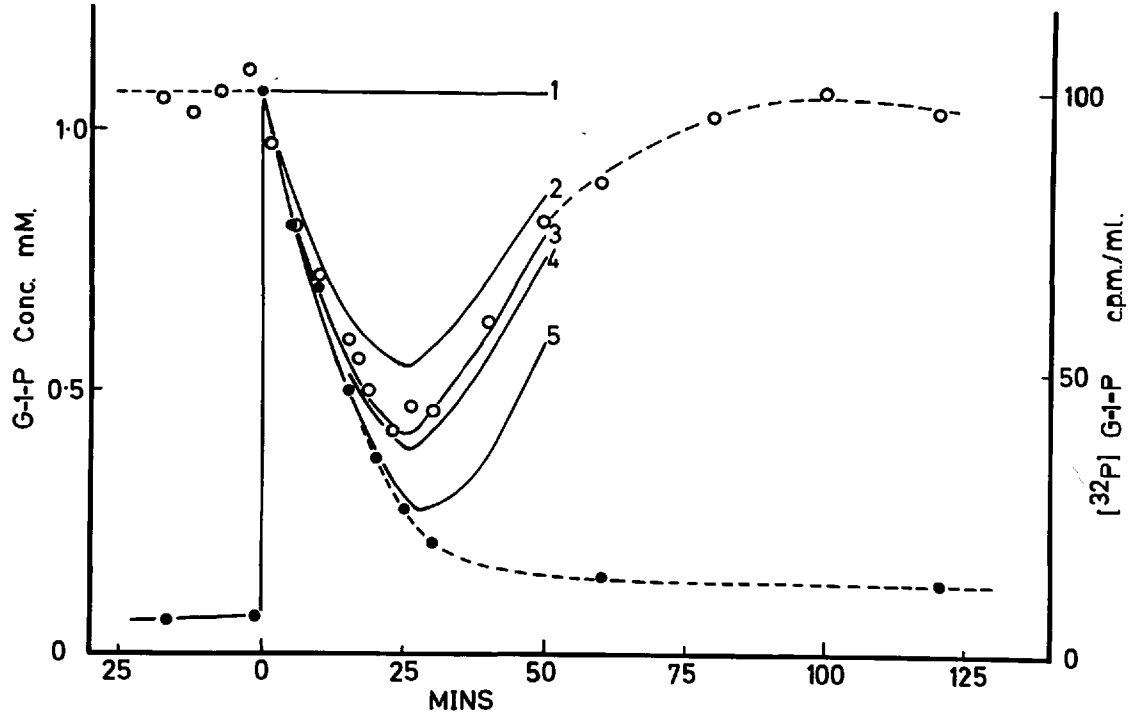
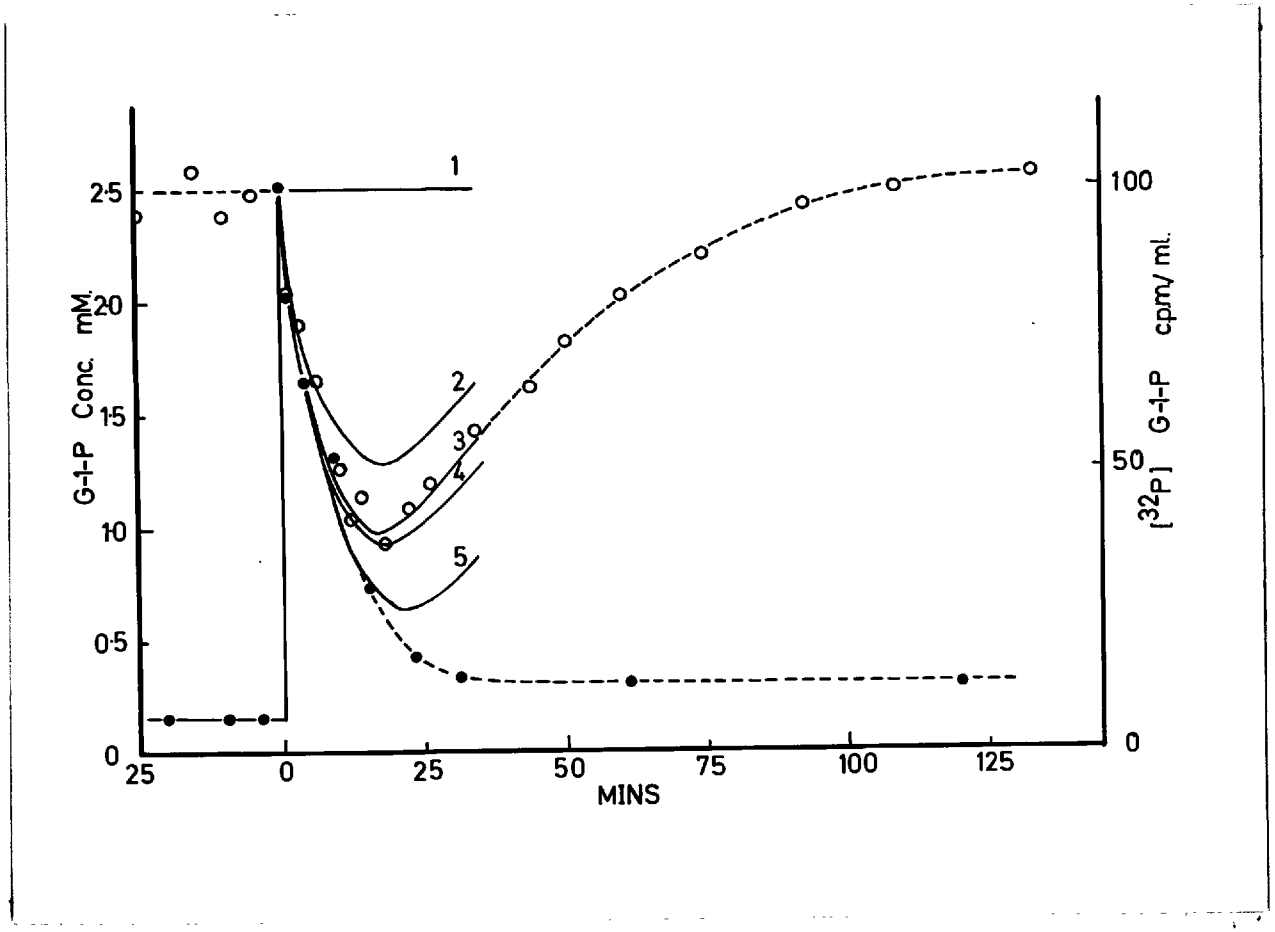

G-1-P Conc. mM.
1·0
0·5
0
25
0
25
50
75
100
125
MINS
100
50
0
[32P] G-1-P c.p.m./ml.
1
2
3
4
5

Fig. R.7.7.


G-1-P Conc. mM.
2·5
2·0
1·5
1·0
0·5
0
1
2
3
4
5
25
0
25
50
75
100
125
MINS
100
50
0
[32P] G-1-P cpm/ml.

zero time non-radioactive G1P was added in a volume of 0.4 mls. to 4.6 mls. of the mixture. The concentrations of substrates immediately after addition were: G1P 1.044 mM; G6P 0.630 mM; G16P 2.0 μM. The chemical reaction and the changes of radioactivity in G1P were then assayed as described in the methods section M.1. The results are shown in Fig. R.7.6. The radioactivity in the G1P fell after the addition of the non-radioactive G1P and the initial rate of fall was similar to the rate of chemical reaction. Therefore under these conditions the nett flux must have been virtually equal to the flux of [$^{32}$P] G1P to G6P, and the flux of [$^{32}$P] G6P to G1P must have been very small. As the reaction progressed, however, the two curves diverged, and the radioactivity in G1P reached a minimum of 39% and then returned to its initial value. Fig. R.7.6 also shows for comparison the results of the comparable experiment (1A) with $^{14}$C-labelled substrates. A second experiment under the condition s of experiment 4 yielded a very similar result with the same minimum of $^{32}$P[G1P] activity of 39%.

In Fig. R.7.7. the results of experiment 5 are shown. The experimental conditions were similar to those in experiment 4, but the substrate concentrations were doubled. Concentrations of substrates before addition of non-radioactive G1P were: G1P, 0.0808 mM; G6P, 1.39 mM;

G16P, 4.26 μM, and immediately after addition of G1P, 2.088 mM; G6P, 1.26 mM; G16P, 4.0 μM. The radioactivity in the G1P fell in a similar manner to Experiment 4 and the minima for the radioactivity in the G1P for the two experiments from which the data in Experiment 5 was obtained were 37 and 40%. As before, the results of a comparable experiment (1C) with $^{14}C$-labelled substrates are shown in Fig. R.7.7 for comparison.

Clearly in the Experiments 4 and 5 considerable co-transport of the $^{32}P[G1P]$ occurred, but, as in the experiments with the $^{14}C$-labelled substrates, the contribution from the G16P to the radioactivity in the G1P has to be considered. This is discussed later, but the effect must have been slight and amounted to a probable overestimate of the amount of co-transport by rather less than 1% of the initial radioactivity in the G1P.

The fact that co-transport occurred indicated that the phosphate flux ratio must show the chemical analogue of the long pore effect. This observation therefore qualitatively supports either mechanism 1 or mechanism 3 and excludes mechanism 2 (see Theory Section). It was possible to calculate quantitatively the amount of co-transport for the different mechanisms and for various combinations of them. This was done by using a computer to integrate the general

equation for induced transport (Theory Section) under a variety of conditions which simulated various degrees of co- or counter-transport. A series of theoretical curves was obtained corresponding to particular boundary conditions of equilibrium constant and substrate concentrations. The appropriate theoretical curves which were the same at both concentrations, have been drawn as continuous lines in Figs. R.7.6 and R.7.7. The experimental results correspond closely to the curve for mechanism 1. The theoretical minimum for radioactivity in the G1P is 39.1% which agreed well with the minima for the radioactivity in the individual experiments. The curves for mechanisms 2 and 3 differed widely from the experimental results, so that these mechanisms could be excluded as major reaction pathways. The curve for 20% of the flux from G1P to G6P proceeding via mechanism 2 and 80% via mechanism 1 also differed considerably from the experimental results, so that the possibility of any substantial but minor contribution from mechanism 2 was also excluded. The results did not exclude the possibility of a small part of the reaction proceeding via mechanism 3; however, this possibility has already been excluded by the induced transport tests using $^{14}C$-labelled substrates.

In the calculation of the induced transport curves for

Experiments 4 and 5 α (related to the isomerization of the phosphoenzyme, see $^{14}C$ induced-transport tests) was assumed to be small and of the order of that found in the induced-transport tests with $^{14}C$-labelled substrates. The justification for this assumption has already been discussed in relation to these experiments.

The occurrence of isotope effects and non-ideal behaviour of the solutions have been ignored, but these effects should be unimportant (see discussion section).

To consider the contribution from the G16P to the radioactivity found in the G1P it is necessary to remember that the method of estimation of $^{32}P$ in G1P also determines $^{32}P$ in the 1-position of G16P. An allowance was made for the radioactivity contributed by the G16P and other sources by comparing the radioactivity found in the G1P at the end of the equilibration period with the radioactivity calculated to be present in this substrate using an equilibrium constant for the reaction of 1/17.2[32, 3]. (Chemical determinations of G1P yielded equilibrium constants within 2% of this value at all concentrations.) The difference between the measured $^{32}P$ labelling in G1P and the calculated value amounted to about 15-20% of the total radioactivity found in the G1P, and represented not only $^{32}P$-labelling in the 1-position of G16P but also the contribution from partial hydrolysis of

[$^{32}$P] G6P and $^{32}$P impurities in G1P. This correction was valid after the addition of non-radioactive G1P only if the amount of radioactivity in the 1-position of G16P remained constant. However, immediately after the addition of the non-radioactive G1P, the enzyme substrate complex was most frequently formed by reaction of the phosphoenzyme with G1P, and since the specific radioactivity of the G1P fell by a large factor the enzyme-substrate complex and any G16P formed by dissociation of this complex, subsequent to the addition of G1P must have had little radioactivity in the 1-position. Thus the contribution of labelling in the 1-position of G16P to the measured activity of [$^{32}$P] G1P would decrease giving rise to an overestimate of the amount of co-transport. The data obtained by measurement of the extent of $^{14}$C-labelling in G16P after separation from monophosphates by chromatography (Table R.7.1) suggested that not more, and probably less, than 50% of the G16P present at the time at which the minima occurred in Experiments 4 and 5 (Figs. R.7.6, R.7.7) had been formed after the addition of the non-radioactive G1P. Thus as the phosphate in the 1-position of the G16P represented about 5% of the total acid-labile phosphate, before the addition of non-radioactive G1P, it follows that the fall in the amount of labelling in the 1-position of the G16P must have represented not more than 2.5% of the initial

radioactivity in the G1P. A proportion of the radioactivity lost from the 1-position of G16P must have been transferred to the G1P and this part would not have contributed to the error. As discussed in the previous section the ratio of the maximum velocities in the forwards and reverse directions suggests that about one quarter of the activity would be thus transferred. Consequently less than 2% of the fall in G1P radioactivity would be due to transfer of radioactivity from the 1-position of G16P to G6P.

Comparison of glucose and phosphate fluxes at chemical equilibrium

The fluxes of [$^{14}$C] G6P and [$^{32}$P] G1P to G1P and G6P respectively at chemical equilibrium were compared at 30°C in histidine-tris-$Mg^{2+}$ buffer pH 7.4 (concentrations: histidine, 37.5 mM; tris, 11.2 mM; $MgCl_2$, 1.03 mM and gelatin 47 µg/ml.), as described in the methods section M.1. The fluxes were calculated from the rate of change of radioactivity in G1P or G6P. The slope of the best straight line fit of the logarithms to the base 10 of the differences between the actual radioactivity in the individual samples and the final equilibrium value was plotted against time and the fluxes calculated by multiplying the slope by the expression shown in equation R.7.1. (see theory section for derivation):

$$\text{Flux} = \frac{2.303V}{\left(\frac{1}{[G1P]} + \frac{1}{[G6P]}\right)E} \text{ (Slope)} \qquad \ldots\ldots \quad \text{R.7.1.}$$

where V is the volume of solution, E is the total quantity of enzyme and [G1P] and [G6P] represent concentrations. No allowance was made for radioactivity appearing in the G16P, since Ray et al.[17] found a negligible transfer of radioactivity into this material. Furthermore, the concentration of the G16P was only about 5% of the G1P concentration.

The results of equilibrium flux-ratio measurements at two different substrate concentrations have been shown in Table R.7.3. The ratio of the glucose flux to the phosphate flux determined with $^{14}C$- and $^{32}P$-labelled substrates was close to 2:1 at both concentrations. This result excluded mechanism 2, which would have given a 1:1 ratio, but was in accordance with mechanism 1 provided that $\alpha$ was small, as was indicated by the small amount of counter-transport in the induced-transport experiments with $^{14}C$-labelled substrates and by the fact that Ray and Roscelli[32] could not detect any product inhibition that was not strictly competitive. For mechanism 3, the flux ratio would be 3:2 if $\alpha$ were small, but would approach 2:1 if $\alpha$ were large. Thus the

Table R.7.3.

Fluxes of glucose and phosphate at chemical equilibrium determined by the use of $^{14}C$- and $^{32}P$-labelled substrates

| Hexose phosphates conc. (mM) | Conc. of enzyme (µg/ml.) | Glucose flux/µg of enzyme (µ moles/min/µg) | Phosphate flux/µg enzyme (µ moles/min/µg) | Glucose flux / Phosphate flux | |
|---|---|---|---|---|---|
| G1P 0.055<br>G6P 0.945<br>G16P 0.003 | 0.686 | 25.2 | 12.0 | 2.09 | mean 1.99 |
| | | 32.7 | 17.3 | 1.89 | |
| G1P 0.138<br>G6P 2.362<br>G16P 0.077 | 1.65 | 32.2 | 17.6 | 1.82 | mean 1.95 |
| | | 48.7 | 23.6 | 2.07 | |

equilibrium flux ratios were only compatible with mechanism 1 as the major pathway. If a minor part of the reaction proceeded by mechanism 2 or mechanism 3 the effect would be to decrease the flux ratio, but the equilibrium flux ratio is not a very sensitive test for such pathways. For example, if 20% of the glucose flux proceeded by mechanism 2 or mechanism 3 the flux ratios would become 1.8:1 and 1.9:1 respectively.

Summary of induced transport experiment results

The induced transport tests with $^{14}C$-labelled substrates were compatible with mechanisms 1 and 2, but excluded even a minor contribution from mechanism 3. The induced transport tests with $^{32}P$-labelled substrates, on the other hand gave the expected result for mechanism 1 and excluded any contribution from mechanism 2, together with any large contribution from mechanism 3. The equilibrium flux-ratio measurements again indicated that mechanism 1 must be the only major pathway. Thus, of the three, mechanism 1 appeared to be the only possible mechanism for the reaction.

The occurrence of counter-transport with $^{14}C$-labelled substrates at high concentrations provided, in addition, evidence for a rate limiting isomerization of the phosphoenzyme.

## SECTION 8. PHOSPHOGLYCERATE-MUTASE SYSTEM: INDUCED-TRANSPORT EXPERIMENTS

In all induced transport experiments reaction was carried out at 30°C in $MgCl_2$/tris buffer, pH 7.4. During the reaction 23PGA was present in excess. Calculations from the data presented in results section R.6 indicated that its concentration exceeded the effective $K_m$ for 23PGA by three to five times under the conditions of experiment. Consequently the presence of 2PGA and 3PGA caused the enzyme reaction to be inhibited only to a small extent by competition with 23PGA. Details of the method of radioactivity assay and conditions under which the induced transport tests were carried out have been described in methods section M.2.

### Induced transport tests with $^{14}C$-labelled substrates

The results of a series of induced transport tests at different substrate concentrations and different ionic strengths with $^{14}C$-labelled substrates are illustrated in Figs. R.8.1-5. Duplicated experiments have been shown in all cases. In individual experiments the time course of reaction varied slightly. The time scale of each reaction was therefore adjusted so that the course of the reaction was represented by the curve shown. The conditions under which the induced transport experiments were carried out have been

Fig. R.8.1.

Induced-transport tests at intermediate substrate concentrations and low ionic strength with $^{14}C$-labelled substrates showing absence of induced-transport. [$^{14}C$] 3PGA was incubated at 30°C with rabbit-muscle phosphoglycerate-mutase in a $MgCl_2$/Tris buffer, pH 7.4, containing 23PGA until equilibrium was reached. Samples were then taken from the reaction mixture and at zero-time non-radioactive 2PGA and 23diPGA added. •, Concn. of 2PGA; o, [$^{14}C$] 2PGA radioactivity (disintegrations/min/ml.) corrected for dilution caused by addition of 2PGA. Non-radioactive 2PGA and 23diPGA were added in a volume of 0.4 ml. to 2.6 ml. of solution. Concentrations before addition: 2PGA, 51.5 μM; 3PGA, 582 μM; 23diPGA, 5.0 μM; concentrations immediately after addition: 2PGA, 678 μM; 3PGA, 504 μM; 23diPGA, 4.8 μM; ionic strength = 0.028. The points shown represent means of duplicate or triplicate observations. In individual experiments the time-course of the reaction varied slightly, consequently the time-scales of each experiment was adjusted so that the course of reaction was represented by the curve shown. The continuous lines are theoretical curves: curve 1 for mechanism 1 or 2, assuming $\alpha = 1.33 \times 10^{-2}$ $mM^{-1}$; curve 2 for 80% of the glyceric acid flux from 2PGA to 3PGA proceeding by mechanism 1 or 2 and 20% proceeding by mechanism 3; curve 3 for mechanism 3.

Fig. R.8.1.

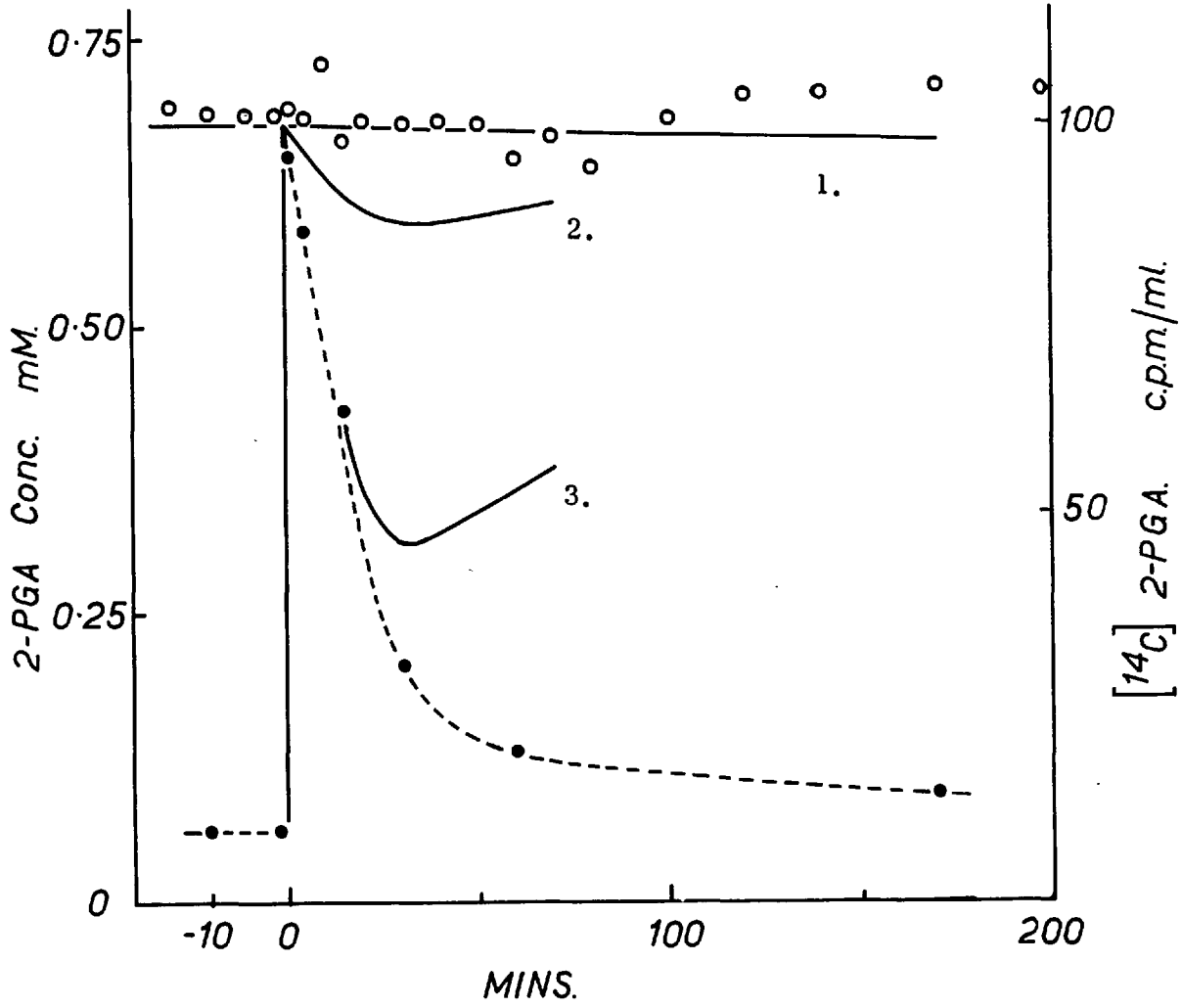

0·75
0·50
0·25
0
2-PGA Conc. mM.
100
50
[14C] 2-PGA. c.p.m./ml.
1.
2.
3.
-10
0
100
200
MINS.

Fig. R.8.2.

Induced-transport test with $^{14}C$-labelled substrates at intermediate substrate concentrations and low ionic strength. For symbols and experimental procedure see the legend to Fig. R.8.1. Concentrations before addition of non-radioactive 2PGA and 23diPGA: 2PGA, 0.130 mM; 3PGA, 1.164 mM; 23diPGA, 10 μM; concentrations immediately after addition: 2PGA, 1.356 mM; 3PGA, 1.009 mM; 23diPGA, 9.7 μM; ionic strength = 0.036. The continuous line is the theoretical curve for mechanism 1 or mechanism 2 assuming $\alpha = 1.33 \times 10^{-2}$ $mM^{-1}$.

Fig. R.8.2.

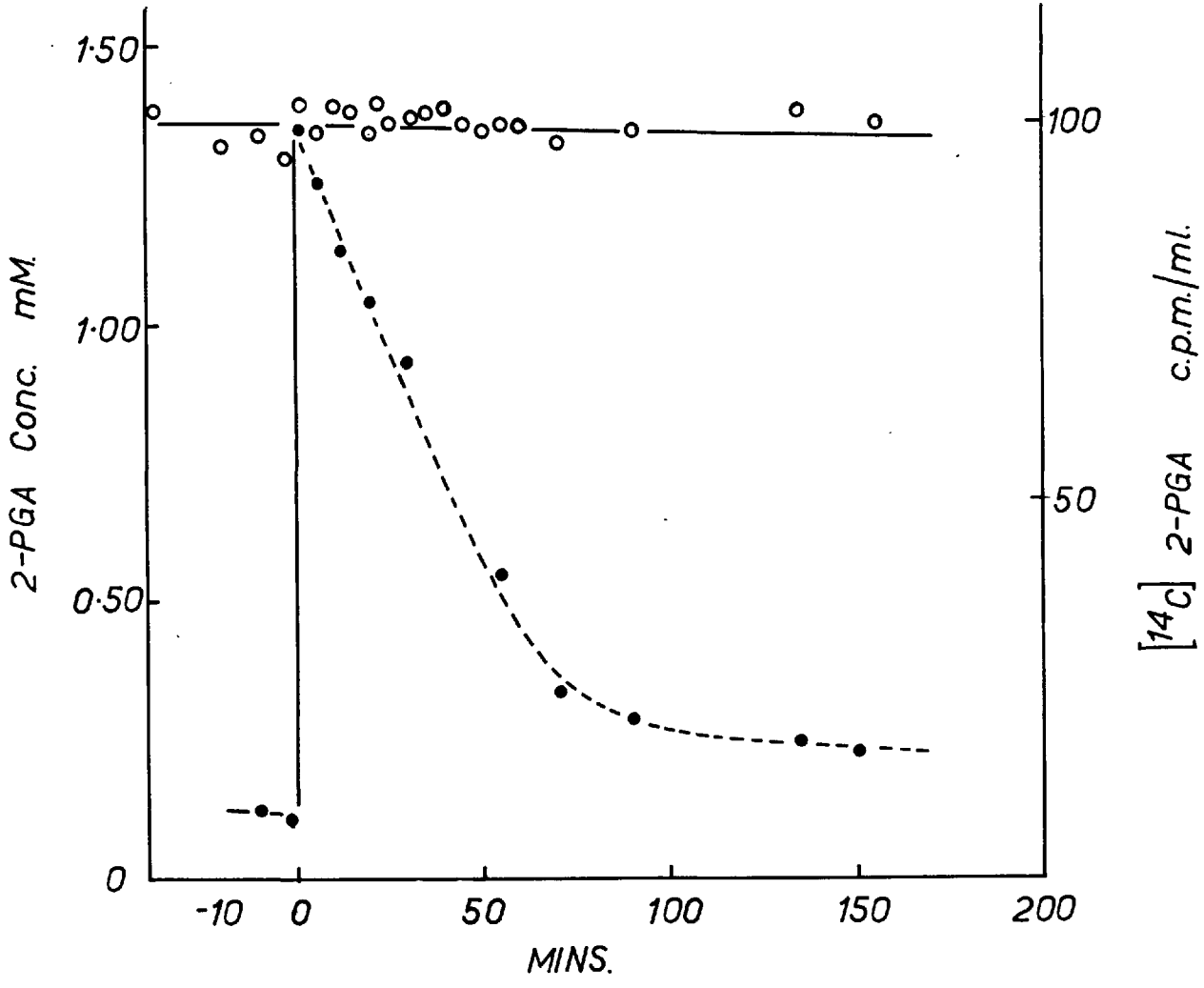

1·50
1·00
0·50
0
2-PGA Conc. mM.
100
50
[14C] 2-PGA c.p.m./ml.
-10
0
50
100
150
200
MINS.

Fig. R.8.3.

Induced-transport test with $^{14}C$-labelled substrates at high substrate concentrations and high ionic strength. For symbols and experimental procedure see the legend to Fig. R.8.1. Concentrations before addition of non-radioactive 2PGA and 23diPGA: 2PGA, 1.140 mM; 3PGA, 12.88 mM; 23 diPGA, 0.110 mM; concentrations immediately after additicn: 2PGA, 15.01 mM; 3PGA, 11.16 mM; 23diPGA, 0.106 mM; ionic strength = 0.184. The continuous line is the theoretical curve for mechanism 1 or mechanism 2 assuming $\alpha = 1.33 \times 10^{-2}$ $mM^{-1}$.

Fig. R.8.3.

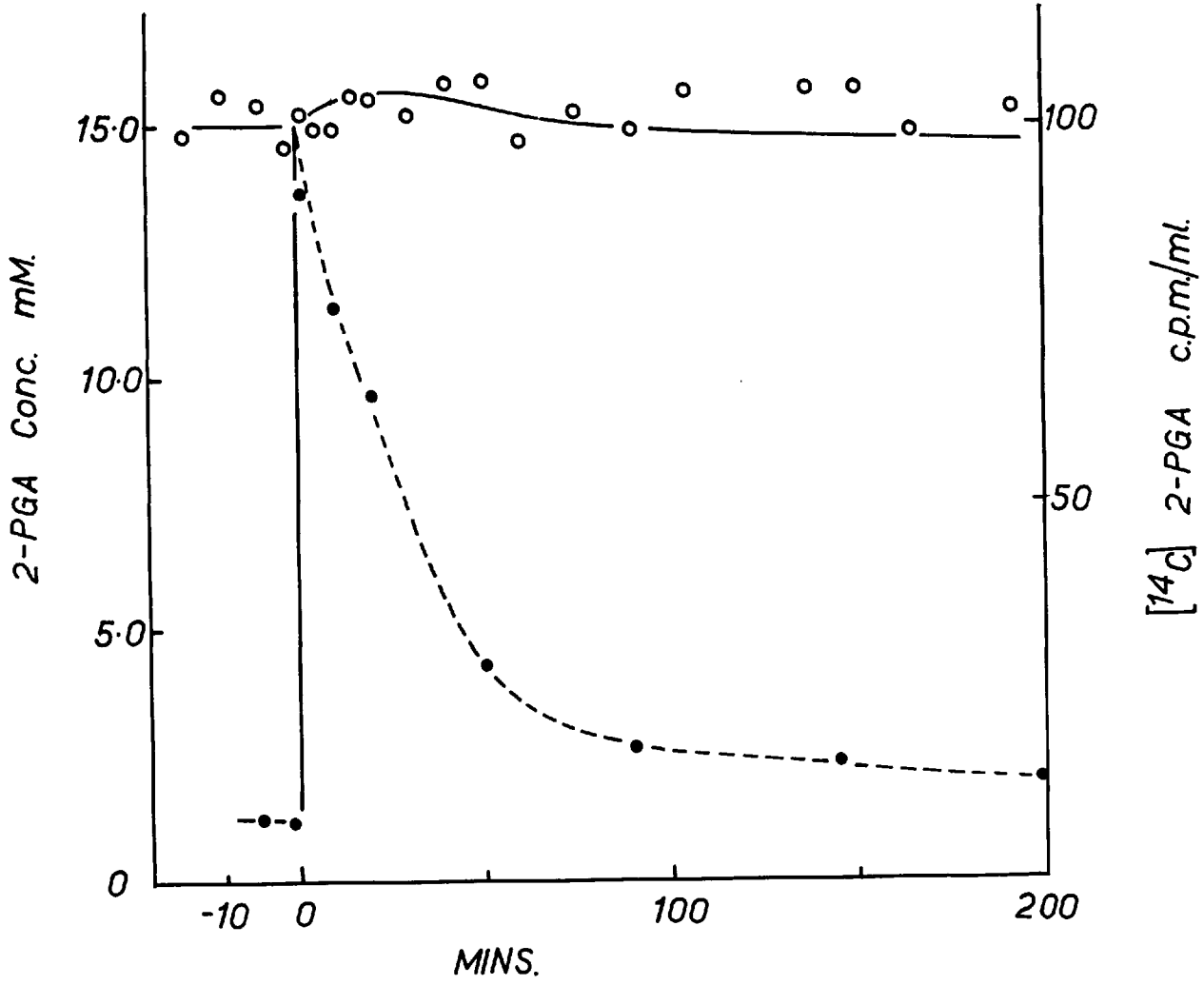


Fig. R.8.4.

Induced-transport test with $^{14}C$-labelled substrates at intermediate substrate concentrations and high ionic strength. For symbols and experimental procedure see the legend to Fig. R.8.1. Concentrations before addition of non-radioactive 2PGA and 23diPGA: 2PGA, 0.130 mM; 3PGA, 1.164 mM; 23diPGA, 10.0 μM; concentrations immediately after addition: 2PGA, 1.356 mM; 3PGA, 1.009 mM; 23diPGA, 9.7 μM; ionic strength 0.166. The continuous line is the theoretical curve for mechanism 1 or mechanism 2 assuming $\alpha = 1.33 \times 10^{-2}$ $mM^{-1}$.

Fig. R.8.4.

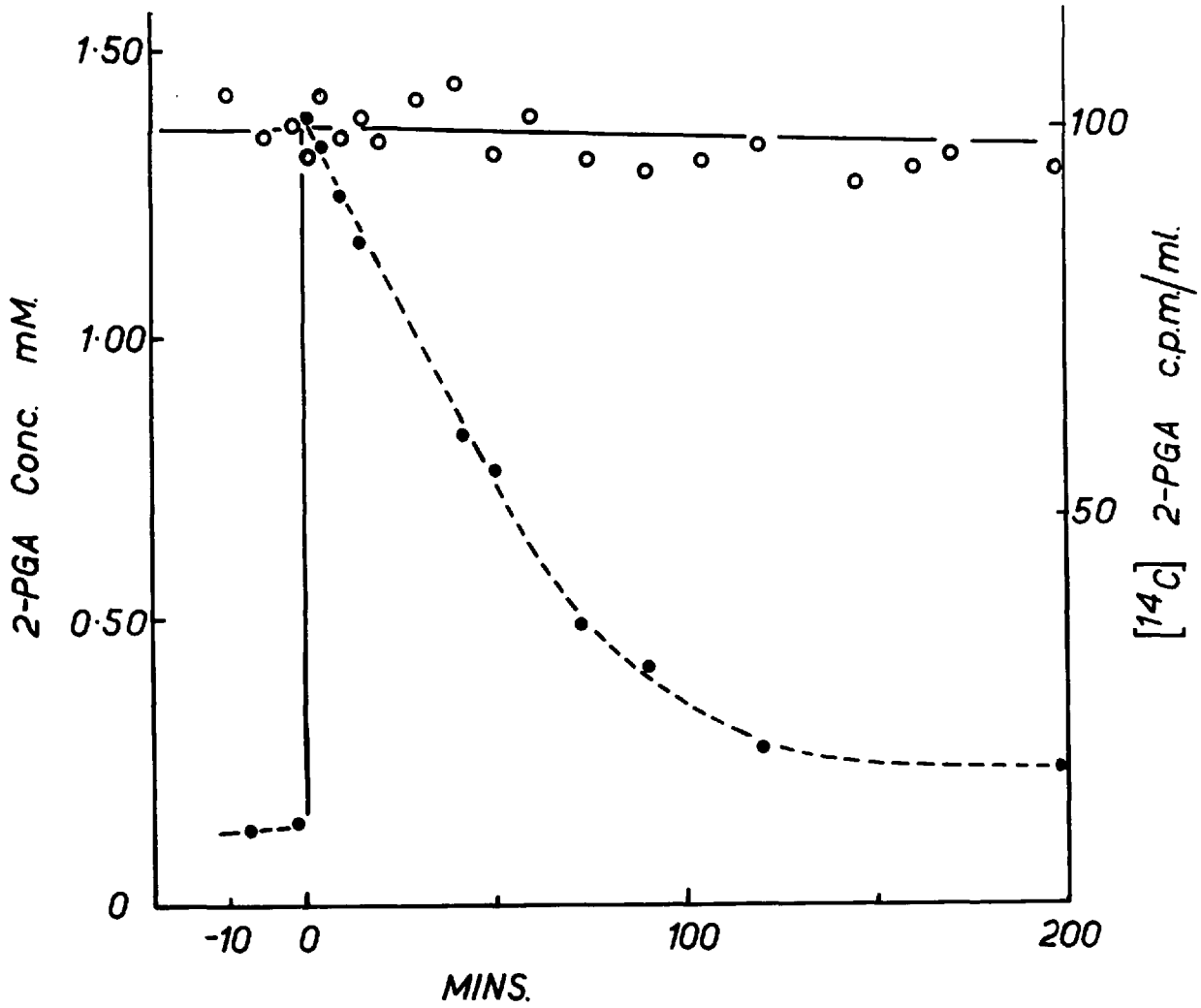

1·50
1·00
0·50
0
2-PGA Conc. mM.
100
50
[14C] 2-PGA c.p.m./ml.
-10
0
100
200
MINS.

Fig. R.8.5.

Induced-transport tests with $^{14}C$-labelled substrates at low substrate concentrations and low ionic strength. For symbols and experimental procedure see the legend to Fig. R.8.1. Concentrations before addition of non-radioactive 2PGA and 23diPGA: 2PGA, 10.8 μM; 3PGA, 122.5 μM; 23diPGA, 1.33 μM; concentrations immediately after addition: 2PGA, 143 μM; 3PGA, 106.2 μM; 23diPGA, 1.29 μM; ionic strength, 0.023. The continuous lines are theoretical curves: curve 1 for mechanism 1 or 2, assuming $\alpha = 1.33 \times 10^{-2}$ $mM^{-1}$, and curve 2 for mechanism 3, assuming $\alpha = 6.99 \times 10^{2}$ $mM^{-1}$.

Fig. R. 8.5.

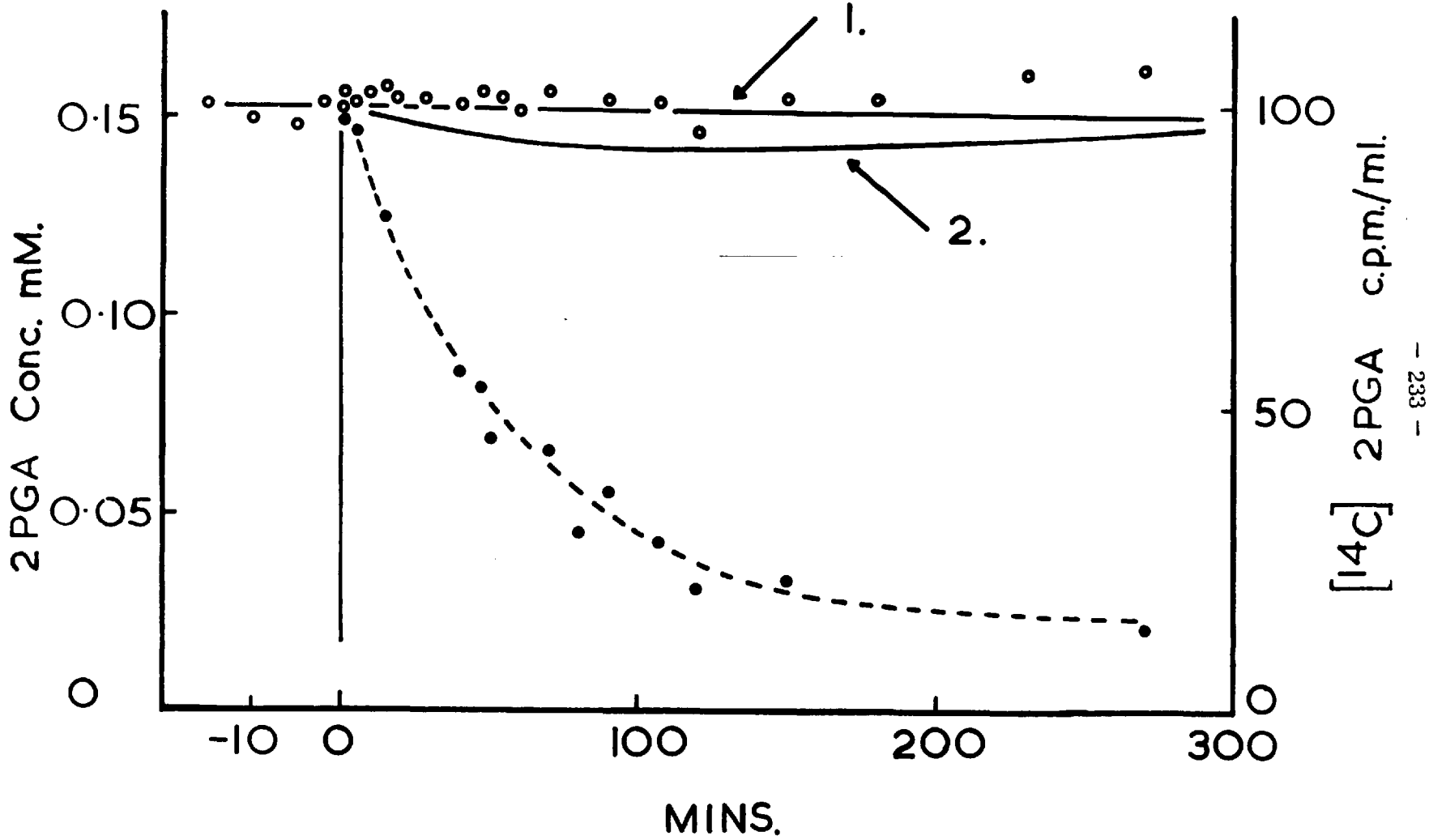

1.
2.
0·15
0·10
0·05
0
2PGA Conc. mM.
100
50
0
[14C] 2PGA c.p.m./ml.
-10
0
100
200
300
MINS.

Table R.8.1.

Summary of conditions for $^{14}C$-induced transport experiments.

Measurements were made in $Mg^{2+}$/Tris buffer, pH 7.4, at 30°C.

$MgCl_2$ = 3.33 mM. Tris = 16.67 mM.

| Expt. No. | Conditions at equilibrium time zero | | | Conditions after addition of non-radioactive 2PGA at time zero | | | Enz. conc. (μg/ml.) | Ionic strength |
|---|---|---|---|---|---|---|---|---|
| | 2PGA (mM) | 3PGA (mM) | 23PGA (μM) | 2PGA (mM) | 3PGA (mM) | 23PGA (μM) | | |
| 1 | 0.0515 | 0.582 | 5.0 | 0.678 | 0.504 | 4.8 | 0.0417 | 0.028 |
| 2 | 0.130 | 1.164 | 10.0 | 1.356 | 1.009 | 9.7 | 0.0833 | 0.036 |
| 3 | 1.140 | 12.88 | 110.0 | 15.01 | 11.16 | 106.0 | 0.914 | 0.184 |
| 4 | 0.130 | 1.164 | 10.0 | 1.356 | 1.009 | 9.7 | 0.0833 | 0.165 |
| 5 | 0.0108 | 0.1225 | 1.33 | 0.143 | 0.1062 | 1.29 | 0.0238 | 0.023 |

summarized in Table R.8.1. In each experiment radioactively labelled substrate was allowed to equilibrate in the presence of the enzyme, then non-radioactive 2PGA was added in 0.4 mls. to 2.6 mls. of the solution to initiate the induced transport test. A small quantity of non-radioactive 23PGA was added simultaneously with the non-radioactive 2PGA in order to keep the relative concentrations of 23PGA to the monophosphates approximately constant. As will be discussed later there must have been little transfer of radioactivity from 23PGA to 2PGA after the addition of non-radioactive 2PGA. Consequently any change in the radioactivity of the 2PGA must have largely reflected a corresponding change in the radioactivity of 3PGA. In the experiments shown in Figs. R.8.1-5 as the level of radioactivity in 2PGA remained substantially unchanged throughout the reaction, within the limits of experimental error, about $\pm$2-3%, there is good evidence for a lack of induced transport of $^{14}$C-labelled substrates over the range 0.249 mM to 26.17 mM total monophosphate concentration. The continuous lines in the figures represent the expected result if the fluxes obeyed the Independence relationship (see Theory section), and the close correspondence of the experimental points shows that over the substrate concentration range investigated the glyceric

acid fluxes must have obeyed the Independence relationship. Unlike the phosphoglucomutase system there was no initial rise in the 2PGA radioactivity at high substrate concentrations due to counter-transport as would have been expected if there was a rate-limiting isomerization of the "phosphoenzyme". These results were compatible with either mechanism 1 or mechanism 2 if $\alpha$ (see Theory section) was small, but could also be compatible with mechanism 3 if $\alpha$ was large enough to prevent the appearance of co-transport (see Theory section).

If $\alpha$ were large there would be marked product inhibition of a partly non-competitive nature due to a slow "phosphoenzyme" isomerization but kinetic evidence of this nature was not available as it had been for phosphoglucomutase. As is shown below, however, the apparent value of the $K_m$ for 3PGA under the conditions of the experiment and the ratio $V_F/V_R$ for the enzyme reaction set a limit to the maximum possible value for $\alpha$. Thus, as is shown in the theory section it can be deduced that:-

$$\alpha \lesssim \frac{K_{eq}}{K_{3PGA}} \left( 1 + \frac{V_R}{V_F} \right) \qquad \text{.....} \qquad \text{R.8.1.}$$

Substituting for $K_{3PGA}$ and $K_{eq}$ in equation R.8.1, and using the ratio $V_R/V_F = 1/2.2$ gives a maximum value for $\alpha$ of $9.2 \times 10^4\ M^{-1}$.

The induced transport curve for mechanism 3, with $\alpha A$ (see Theory section) large enough to give only a 5% fall in the 2PGA radioactivity was computed and if the theoretical curve is compared with the results of the experiments carried out at the lowest phosphoglycerate concentration, Fig. R.8.5, it will be seen that any co-transport was appreciably less than in the computed curve. Thus if mechanism 3 were involved $\alpha A$ must have been greater than the value of $\alpha A$ for the theoretical curve shown in Fig. R.8.5. Consequently $\alpha$ must be greater than $6.99 \times 10^5$ $M^{-1}$. Since the value for $\alpha$ is very much greater than the maximum permitted value of $\alpha$ from eq. R.8.1 the results exclude mechanism 3. If, however, 20% of the reaction proceeds via mechanism 3 with large $\alpha$ and the rest via mechanism 1 or 2 (with $\alpha \geqslant 0$), the value of $\alpha$ required to give a 5% fall in the theoretical induced-transport curve will be of the same order of magnitude as the maximum permitted value of $\alpha$ from equation R.8.1. Consequently mixed mechanisms in which 20% or less of the reaction proceeds via mechanism 3 cannot be excluded on the basis of this test unless $\alpha \simeq 0$ for both mechanisms. Thus these results are compatible only with mechanism 1 or 2, though a minor contribution from mechanism 3 (of 20% or less) cannot necessarily be excluded, and they also show that $\alpha$ must be small. An upper limit to the value of $\alpha$ may be derived

by considering what is the greatest degree of counter-transport which would remain undetected at the highest substrate concentration used, Fig. R.8.3. The continuous line superimposed on Fig. R.8.3 shows the theoretical counter-transport curve for $\alpha = 13.3\ M^{-1}$. This rise in 2PGA radioactivity could not be reliably detected by the method of analysis, so that if the non-ideal behaviour of the solution is ignored the apparent lower limiting rate constant for the isomerization step (if $k_3 = k_{-3}$) from eq. T.1.48 will be about $2.71 \times 10^6\ sec^{-1}$.

The $[^{14}C]$ 2PGA determined by the assay method will include radioactivity due to $^{14}C$ impurities (mainly $[^{14}C]$ glyceric acid) present in the reaction solutions. This contribution to $[^{14}C]$ 2PGA was determined by estimation of the radioactivity remaining in the supernatant of samples which had been subjected to $ZnSO_4/Ba(OH)_2$ precipitation of phosphates (see methods section) without previous conversion of $[^{14}C]$ 2PGA to ATP and $[^{14}C]$ lactate. This contribution was subtracted from the total $[^{14}C]$ 2PGA and usually amounted to 15-20% of it. To check the method the ratio of the $[^{14}C]$ 2PGA to $[^{14}C]$ 3PGA activity was determined. The values obtained agreed well with the equilibrium constant of 11.3.

As mentioned previously, the possibility that there was a transient transfer of radioactivity from the 23PGA to the

2PGA after the addition of the non-radioactive 2PGA has to be considered, because of the low specific radioactivity of the 2PGA immediately after the addition. To test for this possibility an induced transport experiment was carried out under identical conditions to experiment 2 in Table R.8.1 (i.e. at 2.365 mM total monophosphates concentration) except that the samples (0.1 mls.) taken were quenched in 0.25 mls. of absolute alcohol and then chromatographed on Whatman No. 4 paper using the 2-methoxyethanol:butanone:ammonia system to separate the 23PGA from the monophosphates. The chromatograms were then scanned for radioactivity and the relative sizes of the 23PGA and monophosphate peaks measured. The results are presented in Table R.8.2. The 23PGA had approximately the same specific radioactivity as the monophosphates at the end of the equilibration period. After the addition of the inactive 2PGA the specific radioactivity of 23PGA fell to about one-half of the initial radioactivity after a period of about 15 mins.

As the total radioactivity in the 23PGA was less than 10% of the 2PGA radioactivity in all induced transport experiments the 23PGA could have contributed at most 5% to the radioactivity in 2PGA. Moreover, it was extremely unlikely that all the radioactivity disappearing from 23PGA would appear in the 2PGA. According to the kinetic data for

Table R.8.2.

Assay of $^{14}C$-labelling in 23PGA under the conditions employed in experiment 2 (see Table R.8.1).

Specific activity of monophosphates at the end of the equilibrium period = $1.03 \times 10^9$ d.p.s./mole.

| Time, mins (after addn. of 2PGA) | Area ($cm^2$) of monophosphate peak | Area ($cm^2$) of 23PGA peak * | % radioactivity in 23PGA | Ratio of $\frac{\text{23PGA specific activity}}{\text{2+3PGA specific activity}}$ |
|---|---|---|---|---|
| -1.0 | 0.283 | 0.162 | 0.57 | 0.74 |
| 1.0 | 0.283 | 0.129 | 0.46 | 1.12 |
| 5.0 | 0.279 | 0.101 | 0.36 | 0.88 |
| 15.0 | 0.239 | 0.047 | 0.20 | 0.49 |
| 25.0 | 0.278 | 0.073 | 0.26 | 0.63 |
| 35.0 | 0.297 | 0.078 | 0.26 | 0.63 |

* The sensitivity of scanning was increased by a factor of $10^2$ for 23PGA radioactivity.

$V_F/V_R$ from Results section R.6 it is probable that only about one-third would do so (see corresponding discussion for phosphoglucomutase). Thus the overall contribution from the 23PGA to the initial radioactivity of the 2PGA must be less than 1.6%. An increase in the radioactivity of 2PGA of this order of magnitude would not be detected as the error in any determination was about $\pm$ 3%.

Induced transport with $^{32}$P-labelled substrates

Induced transport tests with $^{32}$P-labelled substrates were carried out under the conditions listed in Table R.8.3 and the results are illustrated in Figs. R.8.6-8. In these graphs the individual experiments have been adjusted to fit an average time course for the chemical reaction. $^{32}$P-labelled 2- and 3PGA were incubated in Tris/$MgCl_2$ buffer (Tris, 16.67 mM, $MgCl_2$, 3.333 mM), pH 7.4 at 30$^{o}$C in the presence of rabbit muscle phosphoglycerate mutase and 23PGA cofactor until equilibrium was reached (see legends of Figs. R.8.6-8 for details). At zero time non-radioactive 2PGA and 23PGA were added in a volume of 0.4 mls. to 2.6 mls. of the mixture. Samples were taken from the reaction vessel before this addition and afterwards. The subsequent chemical reaction and the changes of radioactivity in the

Table R.8.3.

Summary of conditions for $^{32}P$-induced transport experiments

Measurements were made in $Mg^{2+}$/Tris buffer, pH 7.4, at 30°C

| Expt. No. | Conditions at equilibrium time zero | | | Conditions after addition of non-radioactive 2PGA at time zero | | | Enz. conc. (μg/ml) | Ionic strength | % of initial 2PGA radioactivity present at minimum |
|---|---|---|---|---|---|---|---|---|---|
| | 2PGA (mM) | 3PGA (mM) | 23PGA (μM) | 2PGA (mM) | 3PGA (mM) | 23PGA (μM) | | | |
| 1<br>2<br>3 | 0.0515 | 0.582 | 5.0 | 0.678 | 0.504 | 4.8 | 0.0417 | 0.028 | 46.5)<br>43.9) 47.3 %<br>51.5) |
| 4<br>5<br>6 | 0.130 | 1.164 | 10.0 | 1.365 | 1.009 | 9.7 | 0.0833 | 0.036 | 46.1)<br>48.5) 47.3 %<br>47.3) |
| 7<br>8 | 0.130 | 1.164 | 10.0 | 1.365 | 1.009 | 9.7 | 0.0833 | 0.166 | 48.5)<br>50.1) 49.3 % |

Fig. R.8.6.

Induced-transport test with $^{32}P$-labelled substrates at intermediate substrate concentrations and low ionic strength. [$^{32}P$] 2PGA and [$^{32}P$] 3PGA were incubated at 30°C with rabbit-muscle phosphoglycerate mutase in a $MgCl_2$/Tris buffer, pH 7.4, until equilibrium was reached. Samples were taken from the reaction mixture and at zero time non-radioactive 2PGA and 23diPGA were added in a volume of 0.4 ml. to 2.6 ml. of the mixture. ●, Concn. of 2PGA; o, [$^{32}P$] 2PGA radioactivity (disintegrations/min/ml.) corrected for dilution after addition of the non-radioactive substrates. Concentrations before addition: 2PGA, 51.5 μM; 3PGA, 582 μM; 23diPGA, 5.0 μM. concentrations immediately after addition: 2PGA, 678 μM; 3PGA, 504 μM; 23diPGA, 4.8 μM, ionic strength = 0.028. The continuous lines are theoretical curves for $^{32}P$-labelled substrates; curve 1 for mechanism 1; curve 2 for mechanism 3; and curve 1a for 80% of flux from 2PGA to 3PGA proceeding via mechanism 1 and 20% proceeding via mechanism 2.

Fig. R.8.6.

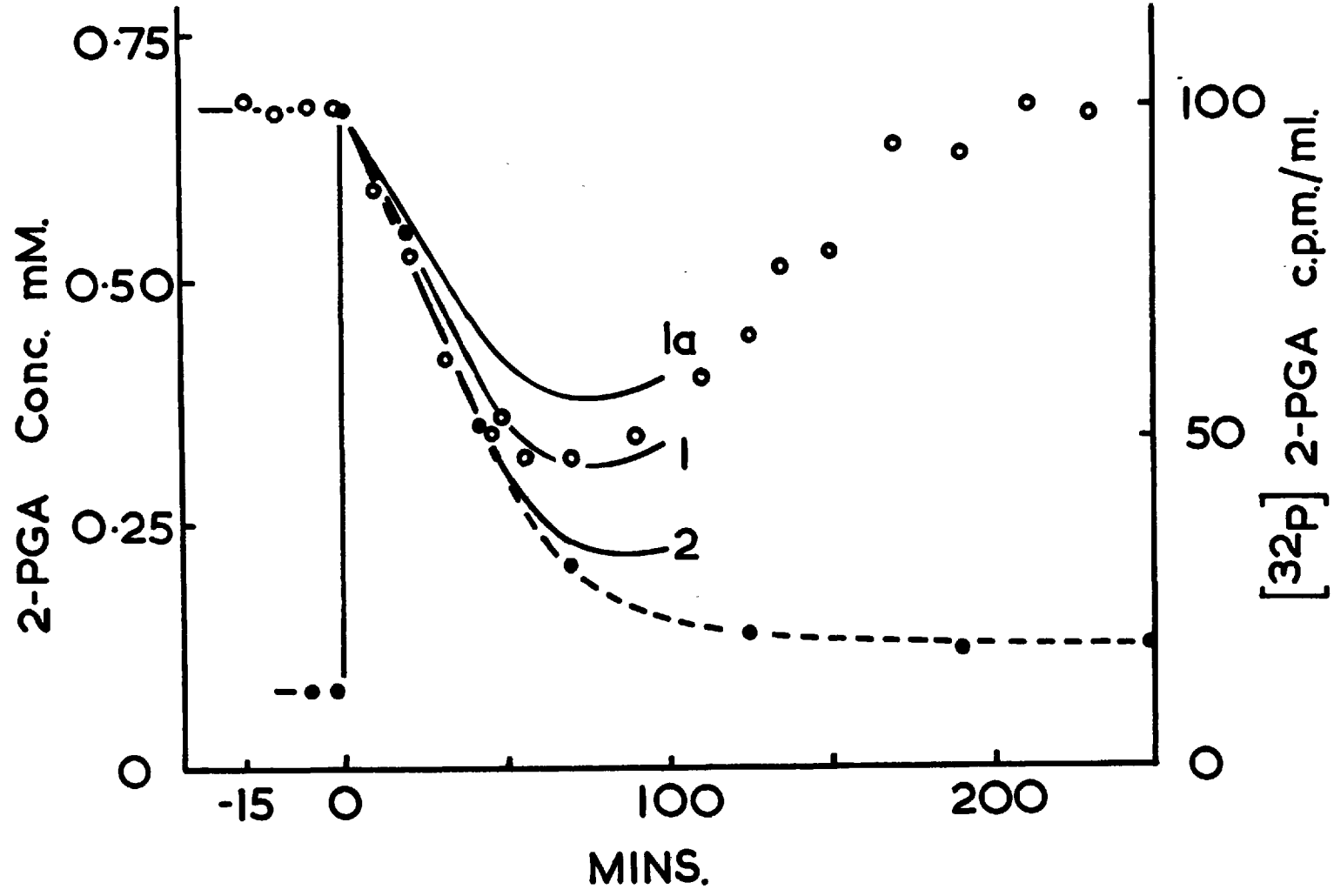

0·75
0·50
0·25
0
2-PGA Conc. mM.
100
50
0
[32P] 2-PGA c.p.m./ml.
-15
0
100
200
MINS.
1a
1
2

Fig. R.8.7.

Induced-transport test with $^{32}$P-labelled substrates at intermediate substrate concentrations and low ionic strength. For symbols and experimental procedure see the legend to Fig. R.8.6. Concentrations before addition of non-radioactive substrates: 2PGA, 0.130 mM; 3PGA, 1.164 mM; 23diPGA, 10 μM. Concentrations after addition: 2PGA, 1.365 mM; 3PGA, 1.009 mM; 23diPGA, 9.7 μM; ionic strength = 0.036. The continuous lines are the theoretical curves for $^{32}$P-labelled substrates: curve 1 for mechanism 1; curve 2 for mechanism 3; curve 1a for 80% of flux from 2PGA to 3PGA proceeding via mechanism 1 and 20% proceeding via mechanism 2; curve 1b for 80% of the flux from 2PGA to 3PGA proceeding via mechanism 1 and 20% proceeding via mechanism 3.

Fig. R. 8.7.

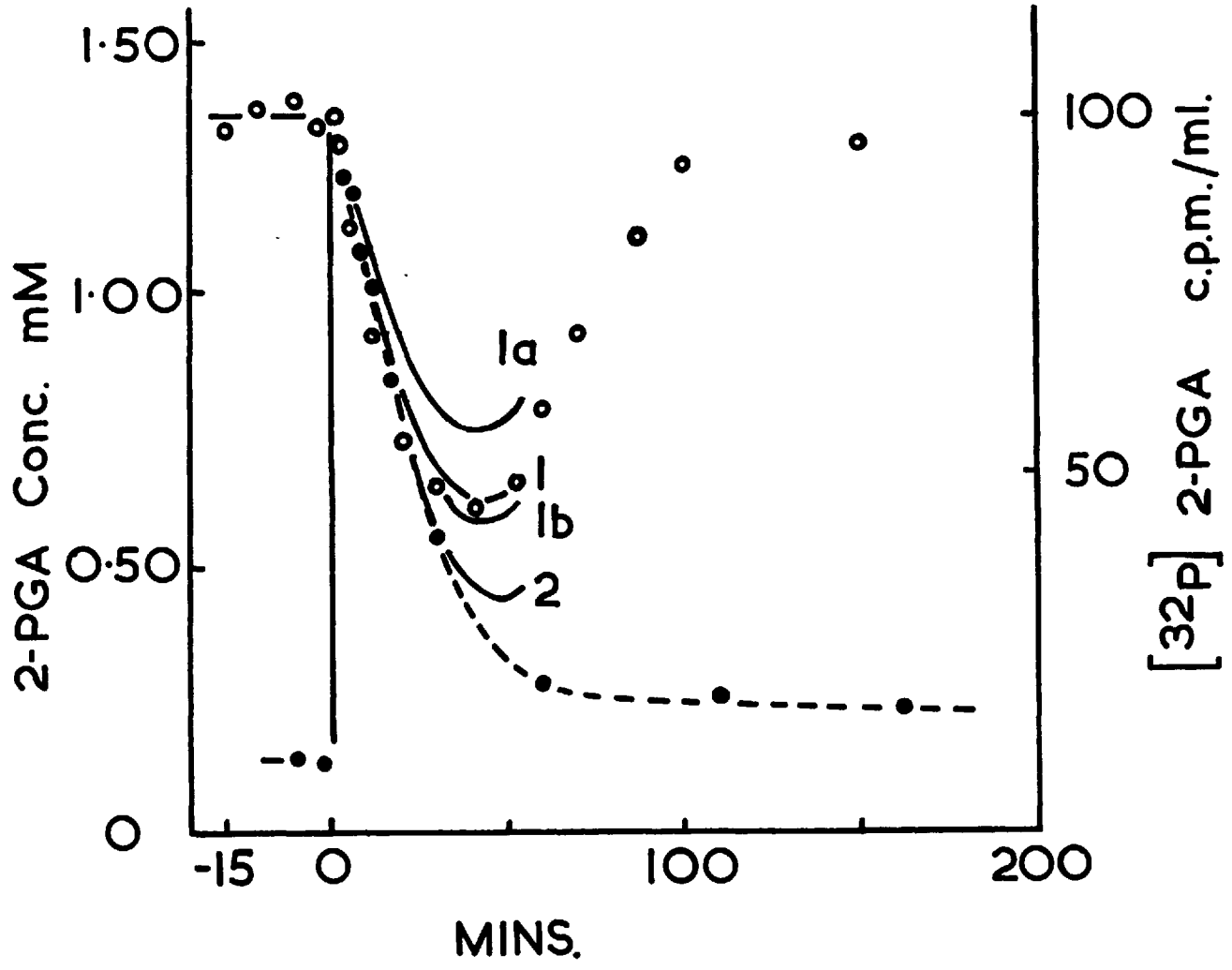
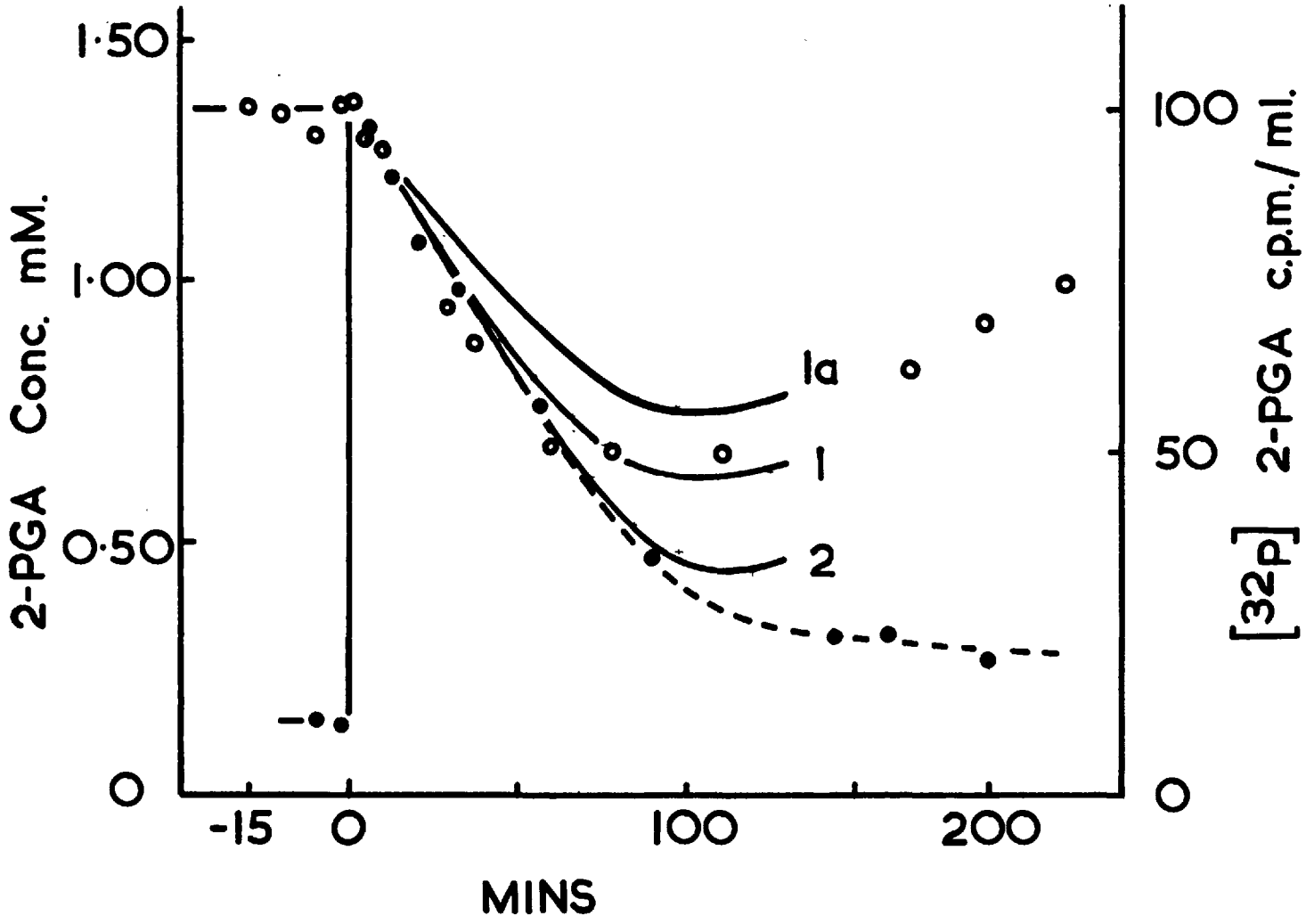

1·50
1·00
0·50
0
2-PGA Conc. mM
100
50
[32P] 2-PGA c.p.m./ml.
-15
0
100
200
MINS.
Ia
I
Ib
2

Fig. R.8.8.

Induced-transport test with $^{32}P$-labelled substrates at intermediate substrate concentrations and high ionic strength. For symbols and experimental procedure see Fig. R.8.6. Concentrations of substrates before addition of non-radioactive substrates: 2PGA, 0.130 mM; 3PGA, 1.164 mM; 23diPGA, 10 μM. Concentrations after addition: 2PGA, 1.365 mM; 3PGA, 1.009 mM; 23diPGA, 9.7 μM; ionic strength 0.166.

Fig. R.8.8.


1·50
1·00
0·50
0
2-PGA Conc. mM.
100
50
0
[32P] 2-PGA c.p.m./ml.
-15
0
100
200
MINS
1a
1
2

2PGA were assayed as described in the methods section M.2. For each set of experiments the rate of fall of the radioactivity in 2PGA immediately after the addition of the non-radioactive 2PGA was approximately the same as the rate of chemical reaction. Therefore under these conditions the nett flux must have been virtually equal to the flux of $[^{32}P]$ 2PGA to 3PGA, and the flux of $[^{32}P]$ 3PGA to 2PGA must have been small. As the reaction progressed, however, the two curves diverged and eventually the radioactivity in 2PGA reached a minimum value before returning to its initial value. The average minimum values of the radioactivity in 2PGA after subtracting the background correction (see later) was 47.3% for Experiments 1-3 Fig. R.8.6, 47.3% for Experiments 4-6, Fig. R.8.7, and 49.3% for Experiments 7 and 8, Fig. R.8.8.

In these experiments, therefore, a considerable degree of co-transport of the $^{32}P$ labelled substrates occurred. This constitutes qualitative evidence supporting mechanism 1 or mechanism 3 but excluding mechanism 2. The amounts of co-transport expected for the different mechanisms, and combinations of them may be calculated from the general equation for induced transport by the use of a computer (see Theory Section). The theoretical curves, are the same at both concentrations of phosphoglycerates, despite the differences in $Mg^{2+}$ binding since the effects upon the

equilibrium constant of reaction are very small.

In the calculation of the induced-transport curves $\alpha$ (see Theory section) was assumed to be small and less than the upper limit for $\alpha$ calculated from the induced transport tests with $^{14}C$-labelled substrates. These curves (assuming $\alpha = 0$) are shown as continuous lines in Figs. R.8.6-8. In each case the experimental results corresponded closely to the curve for mechanism 1, for which the theoretical minimum radioactivity in the 2PGA was 46.3% in agreement with the minima of the individual experiments. The curves for mechanisms 2 and 3 differ widely from the experimental results as shown in Figs. R.8.6-8, no fall in 2PGA radioactivity being expected for mechanism 2 and a fall to a minimum of 33% being expected for mechanism 3. The curve for 20% of the flux from 2PGA to 3PGA proceeding via mechanism 3 and 80% proceeding via mechanism 1 had an expected minimum of 56.4% which was sufficiently different from the experimental results to exclude the possibility of all but a minor contribution from mechanism 2. The difference would be even greater if $\alpha \neq 0$ for Scheme 2. The results do not exclude the possibility of a small contribution to the reaction (up to 20%) proceeding via mechanism 3. The possibility of such a contribution has already been discussed in connection with the induced transport tests with $^{14}C$-labelled substrates.

Isotope effects and the non-ideal behaviour of the solutions have been ignored, but are discussed in the Discussion section. These effects should be relatively unimportant.

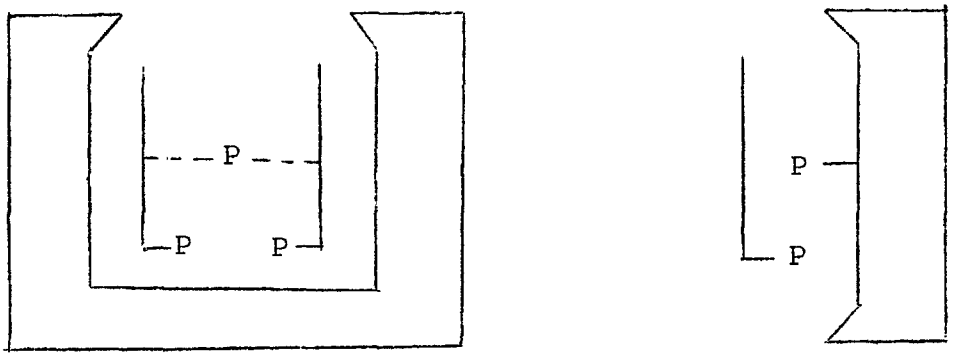
There was a contribution to the measured radioactivity of [$^{32}$P] 2PGA from hydrolysis of [$^{32}$P] 3PGA and (mainly) from $^{32}P_i$ present as an impurity in the radioactive substrates. Allowance was made for this contribution by comparing the radioactivity found in 2PGA at the end of the equilibration period with that calculated to be present by using the equilibrium constant (cf. estimation of blank for [$^{32}$P] G1P). Usually this amounted to about 20% of the total [$^{32}$P] 2PGA radioactivity.

After the addition of unlabelled 2PGA to the reaction mixture the specific activity of 2PGA was very low and consequently there was a flow of radioactively labelled phosphate from 23PGA in order to reduce the difference in specific activity. The correction that needs to be applied to the percentage fall in the radioactivity of 2PGA at its minimum value due to a contribution from 23PGA can be estimated as follows. The change in 2PGA radioactivity closely parallels the time course of the reaction and the specific activity of 2PGA will be virtually unchanged

until the minimum level of $^{32}P$-activity is reached. Consequently it is possible to calculate that the maximum contribution of radioactivity from 23PGA to 2PGA would reduce the fall in 2PGA activity by about 7%, assuming that only one phosphate group of the 23PGA exchanges. However, from the chromatographic results obtained in the experiments with $^{14}C$-labelled substrate, not more, and probably less than 70% of the 23PGA present at the time of the minimas in Figs. R.8.5-7 had been formed after the addition of the non-radioactive 2PGA. Thus the fall in the labelling in 23PGA must have represented not more than 5% of the initial radioactivity in the 2PGA provided that the rate at which $^{14}C$ and $^{32}P$ exchange between 2PGA and 23PGA is the same. Whereas this is true if the active enzyme intermediate is a true phosphoenzyme it is not necessarily quite true if it is an E.23PGA complex. In this case the central enzyme complex (see Fig. R.8.9 below) may contain a symmetrical arrangement of groups in which distortion of 2PGA and 23PGA is sufficient to make them indistinguishable. As a result when 23PGA containing $^{32}P$ and $^{14}C$ combine with E.3PGA to form an E.23PGA.PGA complex the 2PGA which may subsequently dissociate will not always contain $^{14}C$ whereas it will always contain $^{32}P$. Thus the rate of accumulation of $^{32}P$ in 2PGA is higher than that of $^{14}C$. However it is unlikely that the

Fig. R.8.9.

Alternative central complexes




E.23PGA PGA

EP.2PGA

$$\frac{\text{Flux } {}^{32}P_{23PGA \rightarrow 2PGA}}{\text{Flux } {}^{14}C_{23PGA \rightarrow 2PGA}} \gtrsim 1$$

$$\frac{\text{Flux } {}^{32}P_{23PGA \rightarrow 2PGA}}{\text{Flux } {}^{14}C_{23PGA \rightarrow 2PGA}} = 1$$

difference will be large. Even if it were large the conclusions in the above section would remain relatively unchanged. Furthermore it is extremely unlikely that all the radioactivity from 23PGA would appear in 2PGA. According to the kinetic data for $V_F/V_R$ obtained in section R.6 only about one-third would do so (see corresponding discussion for phosphoglucomutase). Thus the

total contribution of radioactivity from the 23PGA to the radioactivity in 2PGA at its minimum value should be about 1.6% or less of the initial radioactivity in 2PGA.

Comparison of glyceric acid and phosphate fluxes at equilibrium

The fluxes of [$^{14}$C] 3PGA and [$^{32}$P] 3PGA to 2PGA at chemical equilibrium were compared under identical conditions at 30°C in tris/$MgCl_2$ buffer, pH 7.4 as described in the methods section M.2. The radioactive fluxes were calculated from the rate of change of the radioactivity in 2PGA. The best straight lines were fitted to plots of the $\log_{10}$ of the differences between the actual radioactivity in individual samples and the final value. The fluxes were obtained from the slopes of these lines by substitution of values in equation R.7.1. The concentration of 23PGA was only about 5% of the 2PGA concentration.

The results of equilibrium flux-ratio measurements are shown in Table R.8.4 from which it will be seen that the ratio of the glyceric acid to phosphate fluxes with $^{14}$C- and $^{32}$P-labelled substrates was close to 2:1. This result excludes mechanism 2, for which the ratio should have been 1:1, but was in accordance with mechanism 1 provided that $\alpha$ was small. For mechanism 3 the flux ratio should be 3:2 if $\alpha$ were small, but would approach 2:1 for large $\alpha$ (see theory section).

Table R.8.4.

Radioactive fluxes at chemical equilibrium, at 30°C and pH 7.4.

Tris = 16.67 mM; $MgCl_2$ = 3.33 mM.

| Conc. of phosphates (mM) | Conc. of enzyme µg/ml. | Experimentally determined $K_{eq\ app.}$ | Glyceric acid flux/µg of enzyme (µmoles/min/µg) | Phosphate flux/µg of enzyme (µmoles/min/µg) | Ratio $\frac{\text{Glyceric acid flux}}{\text{Phosphate flux}}$ |
|---|---|---|---|---|---|
| 2PGA = 0.203mM | 0.0417 | 11.5 | 0.207 | / | / |
| 3PGA = 2.297mM | 0.0417 | 11.3 | 0.177 | 0.0894 | 1.98 |
| | | | | | Average = 2.05 |
| 23PGA = 0.01mM | 0.0417 | 11.9 | 0.147 | 0.0700 | 2.11 |

This latter possibility has been excluded and the $^{14}C$-induced transport tests at high substrate concentrations (Fig. R.8.3) give an upper limit for $\alpha$ of 13.3 $M^{-1}$. Assuming $\alpha$ has this limiting value the flux ratio for mechanism 3 becomes 1.50037. Consequently the ratio of the two radioactive fluxes at equilibrium was compatible only with mechanism 1 as the major reaction pathway. If a minor part of the reaction proceeded by mechanism 2 or mechanism 3 the effect would be to decrease the flux ratio but as the equilibrium flux ratio is not a very sensitive test for mixed mechanisms the effect will be small. For example, if 20% of the glyceric acid flux proceeded by mechanism 2 or 3 the flux ratios would decrease to 1.8:1 and 1.9:1 respectively, provided that $\alpha$ for the reaction was small.

In the above experiments a sample was assayed for 2PGA and 3PGA spectrophotometrically at time zero to check that chemical equilibrium had been reached. The equilibrium constants thus obtained agreed very well with the value of 11.3 determined previously, showing that the reactions had, in fact, been carried out at equilibrium.

# CHAPTER 4

## THEORY SECTION

In this chapter the background concepts and the derivation of equation used in the interpretation of the results are presented. The chapter has been divided up into a series of sections each dealing with a particular aspect of the theory involved. Details of computer programmes employed have not been included.

### SECTION T.1. THEORY OF INDUCED TRANSPORT TESTS

These tests were based upon the analysis of three mechanisms of reaction which were considered possible for the interconversion of G1P and G6P by phosphoglucomutase and of 2PGA and 3PGA by phosphoglycerate mutase in the light of the available evidence. The mechanisms have been presented schematically in Figs. and diagrams T.1.1-3 for the phosphoglucomutase system. Exactly analogous schemes for the phosphoglycerate mutase system can be obtained by substituting the appropriate substrates and cofactors (2PGA for G1P, 3PGA for G6P, and 23PGA for G16P). Mechanism 1, Fig. T.1.1, represents direct transfer of the glucose (or glyceric acid) moiety from G1P to G6P, but indirect transfer of

Fig. T.1.1.

$$G^*1P^* + EP_I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP^*_{II} + G^*6P$$

$$EP^*_{II} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP^*_I$$

$$G1P + EP^*_I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP_{II} + G6P^*$$

$$EP_{II} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP_I$$

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

Mechanism 1 for phosphoglucomutase system.

Transfer of labelled glucose and labelled phosphate from G1P to G6P occurs by a mechanism involving the transfer of phosphate from the substrate to the phosphoenzyme. ES represents the enzyme-substrate complex; $EP_I$ and $EP_{II}$ represent different forms of the phosphoenzyme, and E represents the dephosphoenzyme. $k_1$, $k_2$ etc. are rate constants. G* and P* represent labelled molecules. The labelled glucose is transferred directly whereas two G1P molecules have to react in succession with the enzyme to transfer the phosphate.

## Scheme T I.1.

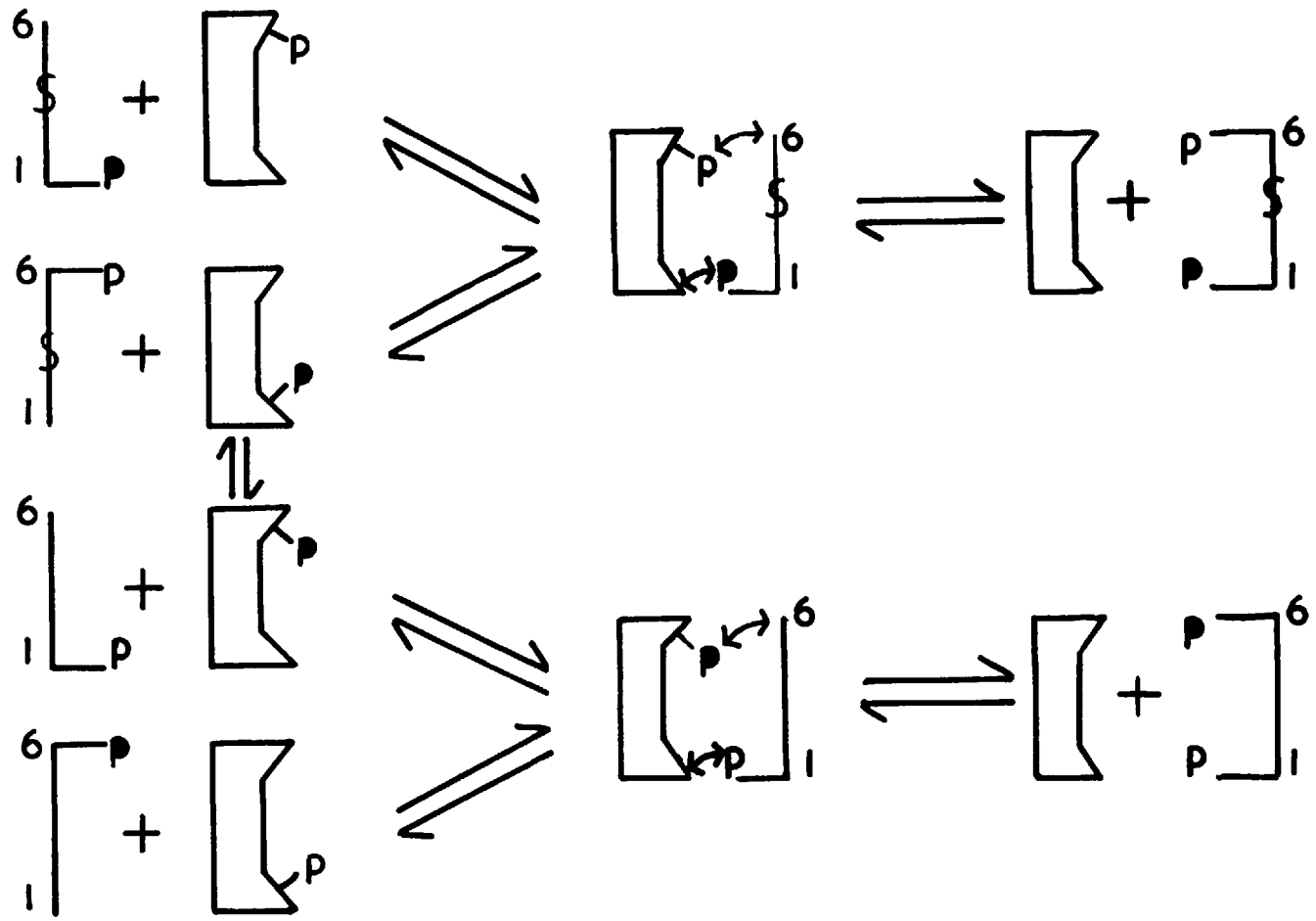


Fig. T.1.2.

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

$$EP_{II} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP_I$$

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

Mechanism 2 for phosphoglucomutase system

Transfer of labelled glucose and labelled phosphate from G1P to G6P occurs by a mechanism involving intramolecular transfer of phosphate. ES represents the enzyme substrate complex; $EP_I$ and $EP_{II}$ represent different forms of the phosphoenzyme, and E represents the dephosphoenzyme. $k_1$, $k_2$ etc. are rate constants. G* and P* represent labelled molecules. Both the labelled glucose and the labelled phosphate are transferred directly from G1P to G6P and vice versa.

## Scheme T.1.2.

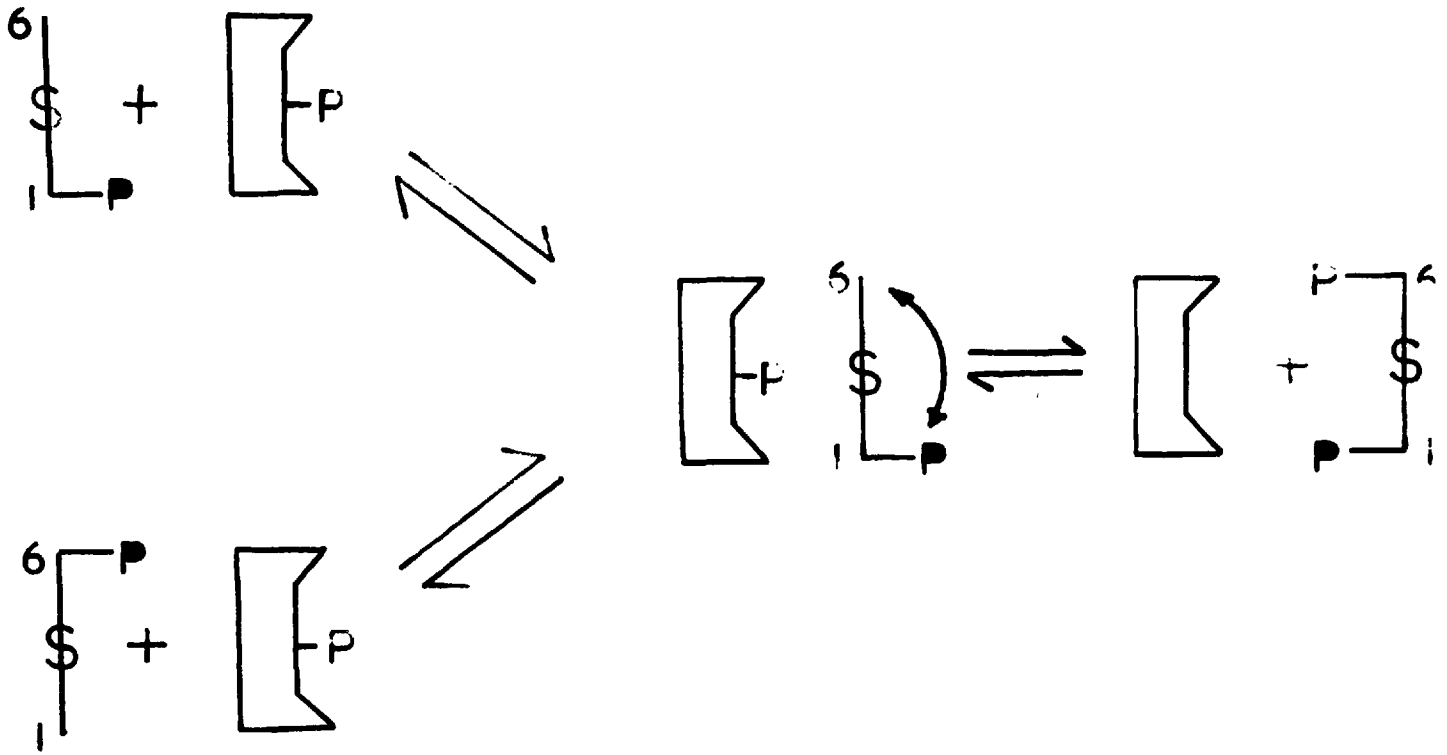


Fig. T.1.3.

$$G^*1P^* + EG1P6P_I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EG^*1P^*6P_{II} + G6P$$

$$EG^*1P^*6P_{II} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EG^*1P^*6P_I$$

$$G1P + EG^*1P^*6P_I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EG1P6P^*_{II} + G^*6P$$

$$EG1P6P^*_{II} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EG1P6P^*_I$$

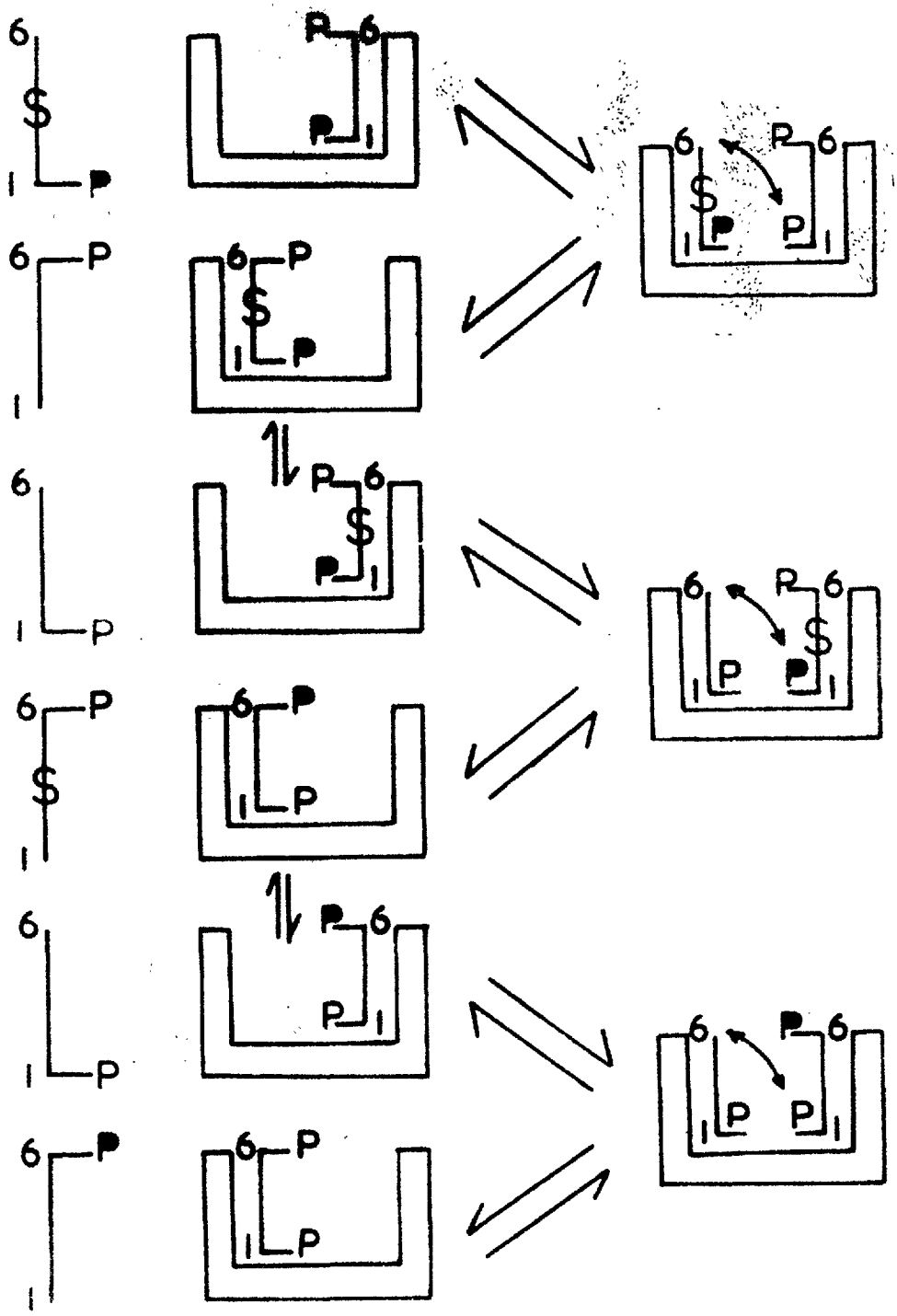
$$G1P + EG1P6P^*_I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EG16P_{II} + G6P^*$$

$$EG1P6P_{II} \underset{}{\overset{k_3}{\rightleftharpoons}} EG1P6P_I$$

Mechanism 3 for phosphoglucomutase system

Transfer of labelled glucose and labelled phosphate from G1P to G6P occurs by a mechanism involving intermolecular transfer of phosphate from G1P6P to G1P. $EG1P6P_I$ and $EG1P6P_{II}$ represent different forms of the enzyme-G1P6P complex. G* and P* represent labelled molecules. $k_1$, $k_2$ etc. are rate constants. The reaction is supposed to take place in the presence of excess G1P6P, so that dissociation of the enzyme-G1P6P complex can be ignored. Two molecules of G1P must react in succession with the enzyme to transfer the labelled glucose. At least three molecules of G1P have to react to transfer the phosphate.

# Scheme T.1.3.



phosphate via a phosphoenzyme involving one phosphate intermediate between G1P and G6P. The possible existence of two forms of the phosphoenzyme is also considered. Mechanism 2, Fig. T.1.2, represents direct transfer of both glucose (or glyceric acid) and phosphate, again the existence of two forms of the phosphoenzyme have been postulated. Such a scheme for reaction was considered possible as the enzyme cofactor (G16P or 23PGA) could play an indirect role in enzyme activity, perhaps by the formation of a pyrophosphate bond. A direct transfer of phosphate might explain why phosphoglucomutase still showed some activity in the absence of G16P. Mechanism 3, Fig. T.1.3, represents involvement of the cofactor in enzyme catalysis in which both transfer of glucose (or glyceric acid) and phosphate are indirect. In this case the transfer of glucose from G1P to G6P involves one intermediate and the transfer of phosphate two intermediates. The existence of two forms of the enzyme-cofactor complex is also proposed.

The term "intermediate", mentioned above, is not intended to indicate an intermediate in the kinetic sense of a transitionally stable state of the enzyme-substrate complex but is intended to describe the stable entity or entities which are created by the first, or subsequent, reaction of the enzyme with a molecule of substrate. The intermediates must

be formed before transfer of label to the product can occur. Thus the transfer of $^{32}P$ from G1P to G6P by mechanism 1, Fig. T.1.1, must occur in two stages since it is necessary for the entity $EP^{32}$ to be formed in the first stage of reaction before reaction can occur with a second G1P molecule to transfer the label to G6P. Consequently transfer of $^{32}P$-label by this mechanism is described as having one "intermediate". These "intermediates" affect the flux ratios whereas intermediates in the sense of different states of the enzyme substrate complex do not affect flux rates and cannot be detected by flux measurements[63]. From arguments given in the introduction all three mechanisms would seem possible for either of the mutases. The flux approach of Britton[59, 60, 61, 62, 63] offers a method of distinguishing between the possibilities and also provides a very sensitive method of detecting isomerization of the free enzyme.

In the following, the term flux is used in the way defined by Britton[61]. The flux of phosphate from G1P to G6P is the rate of transfer of phosphate groups from the 1-position to the 6-position of the glucose in unit time under particular conditions of concentrations, temperature etc., and the flux of glucose from G1P to G6P is the rate of transfer of glucose from G1P to G6P. Similar definitions

apply to the fluxes of glyceric acid and phosphate in the phosphoglycerate mutase system.

Flux equation for mechanism 1

Flux equations for reactions of the type $RX + R^1 \rightleftharpoons R + R^1X$ where the group transferred X forms a stable intermediate, EX, with the enzyme have been calculated by Britton[61]. Thus the initial reaction is a transfer of X from RX to the enzyme forming an EX intermediate complex. This then isomerizes to a form that can transfer X to $R^1$ to yield $R^1X$. Conversion of the nomenclature to that used by Britton shows that; RX = A, $R^1$ = B, R = C, $R^1X$ = D. By comparison with mechanism 1 it is then possible to put A = B = G1P and C = D = G6P. As A = B and C = D the fluxes of glucose and phosphate from G1P to G6P will be twice the fluxes given by equations 24-29 for the case considered by Britton[61], hence flux of phosphate from G1P to G6P = 2x flux of A to D; flux of phosphate from G6P to G1P = 2x flux of D to A; flux of glucose from G1P to G6P = 2x flux from A to C; flux of glucose from G6P to G1P = 2x flux of C to A; also $(EP_1) = 2E_A = 2E_B$ and $(EP_{11}) = 2E_C = 2E_D$, where $(EP_1)$, $(EP_{11})$, $E_A$, $E_B$, $E_C$ and $E_D$ represent the quantities of the enzyme species. Furthermore $[G1P] = A = B$ and $[G6P] = C = D$, where the symbols represent concentrations;

and $k_1 = a = g$, $k_{-1} = b = h$, $k_2 = c = k$, $k_{-2} = d = l$, $k_3 = e = m$, $k_{-3} = f = n_o$. Substituting these values in the appropriate equations (24-29):-

$$\text{Flux of phosphate from G1P to G6P} = \frac{k_1^2 k_2^2 k_3 [G6P]^2 (EP_1)}{D_n} \quad \text{..... T.1.1.}$$

$$\text{Flux of phosphate from G6P to G1P} = \frac{k_{-1}^2 k_{-2}^2 k_{-3} [G6P]^2 (EP_{11})}{D_n} \quad \text{..... T.1.2.}$$

where the denominator, Dn, is given by the expression

$$D_n = \left[ k_1 k_2 k_3 (k_{-1} + k_2)[G1P] + k_{-1} k_{-2} k_{-3} (k_{-1} + k_2)[G6P] + k_1 k_2 k_{-1} k_{-2} [G1P][G6P] \right]$$

$$\text{Flux of glucose residues from G1P to G6P} = \frac{k_1 k_2 [G1P](EP_1)}{(k_{-1} + k_2)} \quad \text{..... T.1.3.}$$

$$\text{Flux of glucose residues from G6P to G1P} = \frac{k_{-1} k_{-2} [G6P](EP_{11})}{(k_{-1} + k_2)} \quad \text{... T.1.4.}$$

Further, under steady state conditions, from Britton and Clarke (1968)[27]:

$$(EP_1) = \frac{(E)\left( k_3(k_{-1} + k_2) + k_{-1}k_{-2}[G6P]\right)}{D_1} \quad \text{.....} \quad \text{T.1.5.}$$

and

$$(EP_{11}) = \frac{(E)\left(k_{-3}(k_{-1} + k_2) + k_1k_2[G1P]\right)}{D_1} \quad \text{.....} \quad \text{T.1.6.}$$

where the denominator $D_1$ is given by the expression below, and (E) is the total quantity of enzyme

$$D_1 = k_1(k_2 + k_3)[G1P] + k_{-2}(k_{-1} + k_{-3})[G6P] +$$

$$k_1k_{-2}[G1P][G6P] + (k_3 + k_{-3})(k_{-1} + k_2) +$$

$$\frac{k_4}{k_{-4}[G16P]}\left( k_3[G1P] + k_{-3}[G6P] + k_1k_{-2}[G1P][G6P]\right)$$

The rate constants must also be related by the expression:-

$$K\, k_1\, k_2\, k_3 = k_{-1}\, k_{-2}\, k_{-3} \quad \text{.....} \quad \text{T.1.7.}$$

where K is the overall equilibrium constant of the reaction.

The ratio of fluxes in the forwards and backwards direction of reaction for phosphates can be derived from

equations T.1.1, 2, 5, 6, 7 to obtain the expressions shown below:

$$\frac{\text{Flux of phosphate from G1P to G6P}}{\text{Flux of phosphate from G6P to G1P}}$$

$$= \frac{[G1P]^2 (EP_1)^{k_{-3}}}{[G6P]^2 K^2 (EP_{11})^{k_3}}$$

$$= \frac{[G1P]^2 (1 + \alpha K [G6P])}{[G6P]^2 (1 + \alpha [G1P])} \qquad \text{..... T.1.8.}$$

Similarly for the ratio of glucose fluxes an expression can be derived from equations T.1.3-7

$$\frac{\text{Flux of glucose residues from G1P to G6P}}{\text{Flux of glucose residues from G6P to G1P}}$$

$$= \frac{[G1P](EP_1)^{k_{-3}}}{[G6P]K(EP_{11})^{k_3}}$$

$$= \frac{[G1P](1 + \alpha K [G6P])}{[G6P]K(1 + \alpha [G1P])} \qquad \text{..... T.1.9.}$$

At chemical equilibrium the ratio of the glucose to phosphate fluxes can be derived from equations T.1.1, 3, 7.

$$\frac{\text{Flux of glucose residues from G1P to G6P}}{\text{Flux of phosphate from G1P to G6P}} = 2 + \alpha[\text{G1P}] \qquad \text{..... T.1.10.}$$

In the above expressions $\alpha$ is given by equation T.1.11.

$$\alpha = \frac{k_1 k_2}{k_{-3}(k_{-1} + k_2)} \qquad \text{..... T.1.11.}$$

Since $\alpha$ is inversely related to $k_{-3}$, $\alpha$ tends to zero as the isomerization of the enzyme becomes less rate limiting. Such flux ratio equations could also be obtained if the active enzyme agent were an enzyme-cofactor complex, as in mechanism 3, rather than a phosphoenzyme, provided that the mechanism of the reaction remained the same.

Flux equations for mechanism 2

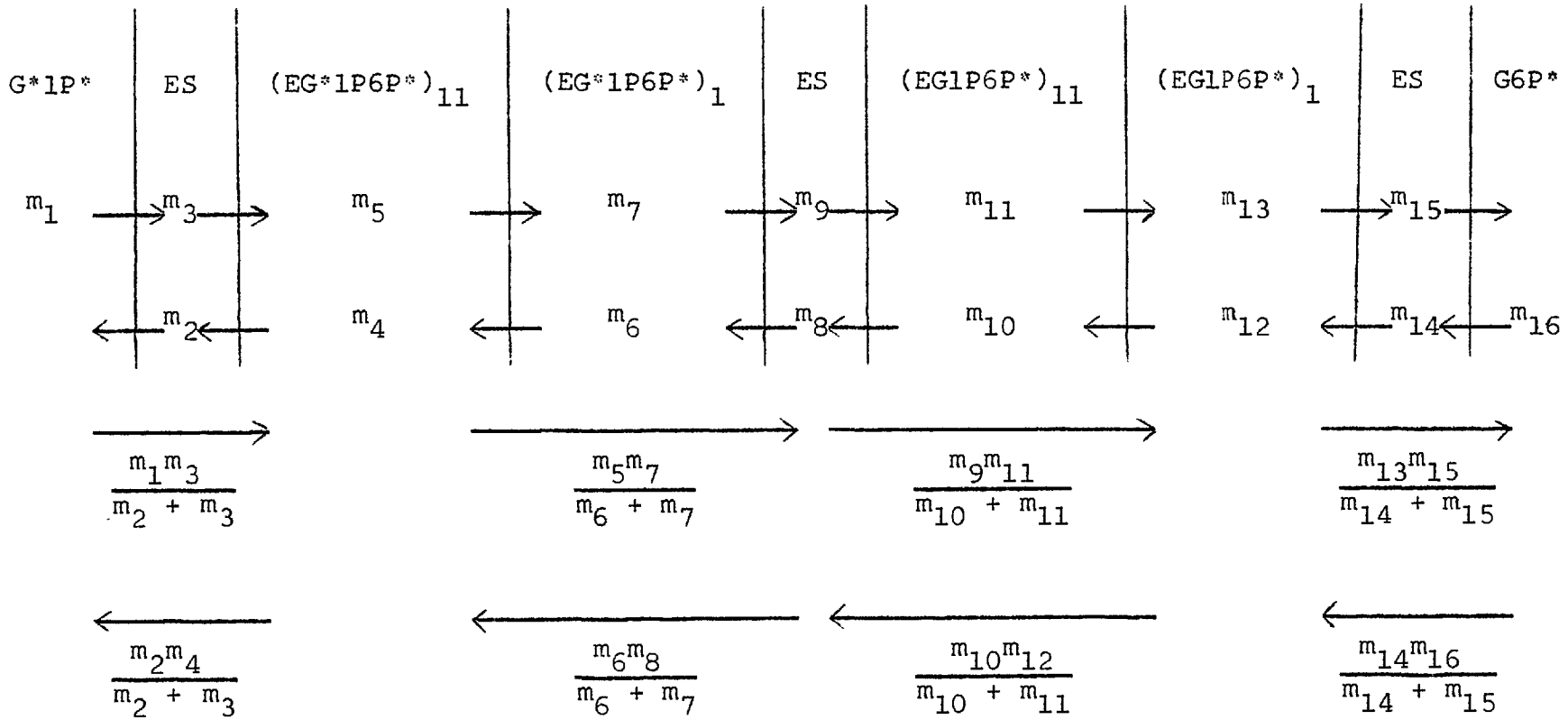
In this mechanism there is a direct transfer of phosphate as well as glucose from G1P to G6P. Consequently the expression for both the glucose and phosphate fluxes will be identical to the expression, shown in equation T.1.9, for the glucose flux of mechanism 1. The ratio of the glucose flux to

the phosphate flux at chemical equilibrium will therefore be unity.

Flux equations for mechanism 3

The flux equations for mechanism 3 were derived by an analogous method to those of mechanisms 1 and 2. For mechanism 3, see Fig. T.1.3, there are two intermediate phosphate groups and one intermediate glucose groups in the transfer of labelled material from G1P to G6P. Under steady state conditions the method of Britton[64], in which enzyme substrate complexes may be regarded as intermediate substrate pools, can be used to calculate the overall fluxes. In calculating the overall flux, a system of substrate pools appropriate to mechanism 3 was drawn up (see next page) and the overall flux calculated from the individual fluxes ($m_i$) between the various adjacent pools as shown. The process of adding together the fluxes across a substrate pool was continued until a single expression was obtained for the overall flux. The values of the individual fluxes in terms of the rate constants in mechanism 3 (see below) were then substituted into the final expression for the overall flux and thus the equations for the phosphate fluxes, equations T.1.12 and T.1.13, were obtained.

## Substrate pools for mechanism 3



Values of individual fluxes $m_{1,\ 2}$ etc. for Scheme T.1.3, mechanism 3.

$$m_1 = m_7 = m_{13} = k_1[G1P]\ [EG16P]_1$$

$$m_2 = m_8 = m_{14} = k_{-1}[ES]$$

$$m_3 = m_9 = m_{15} = k_2[ES]$$

$$m_4 = m_{10} = k_{-2}[G6P]\ [EG16P]_{11}$$

$$m_5 = m_{11} = k_3[EG16P]_{11}$$

$$m_6 = m_{12} = k_{-3}[EG16P]_1$$

$$\text{Flux of phosphate from G1P to G6P} = \frac{k_1^3 k_2^3 k_3^2 [G1P]^3 (EG16P_1)}{D_2} \quad \text{.....} \quad \text{T.1.12}$$

$$\text{Flux of phosphate from G6P to G1P} = \frac{k_{-1}^3 k_{-2}^3 k_{-3}^2 [G6P]^3 (EG16P_{11})}{D_2} \quad \text{.....} \quad \text{T.1.13.}$$

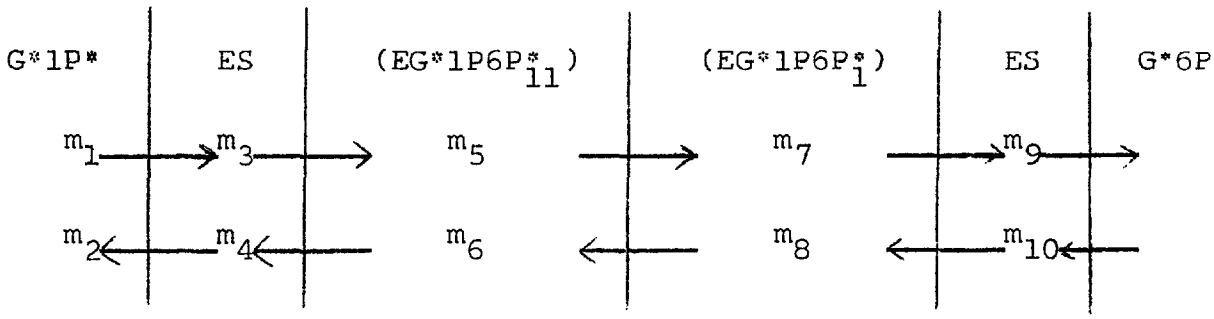
Where $D_2$ is given by the expression:-

$$D_2 = k_1^2 k_2^2 k_3^2 (k_{-1} + k_2)[G1P]^2 + k_{-1}^2 k_{-2}^2 k_{-3}^2 (k_{-1} + k_2)[G6P]^2 +$$

$$k_1 k_2 k_3 k_{-1} k_{-2} k_{-3} (k_{-1} + k_2)[G6P]\ [G1P] +$$

$$k_1 k_2 k_{-1}^2 k_{-2}^2 k_{-3} [G1P]\ [G6P]^2 + k_1^2 k_2^2 k_3 k_{-1} k_{-2} [G1P]^2 [G6P]$$

The fluxes of glucose from G1P to G6P and vice versa can be derived in an exactly similar fashion by consideration of the substrate pool system shown below:




$$\text{Flux of glucose from G1P to G6P} = \frac{k_1^2 k_2^2 k_3 [G1P]^2 (EG16P_1)}{D_n} \quad \text{..... T.1.14}$$

$$\text{Flux of glucose from G6P to G1P} = \frac{k_{-1}^2 k_{-2}^2 k_{-3} [G6P]^2 (EG16P_{11})}{D_n} \quad \text{.. T.1.15.}$$

In addition in the presence of excess G16P, and using equations 59, 60, 3 from Britton, (1966)[61]

$$(EG16P)_1 = \frac{(E)\left(k_3(k_{-1} + k_2) + k_{-1}k_{-2}[G6P]\right)}{D_3} \quad \text{..... T.1.16}$$

$$(EG16P)_{11} = \frac{(E)\left(k_{-3}(k_{-1} + k_2) + k_1 k_2 [G1P]\right)}{D_3} \quad \text{..... T.1.17.}$$

Where (E) is the total quantity of enzyme and $D_3$ is derived from $D_1$, by putting $[G16P] = \infty$ Hence:-

$$D_3 = k_1(k_2 + k_3)[G1P] + k_{-2}(k_{-1}+k_{-3})[G6P] + k_1k_{-2}[G1P][G6P] + (k_3 + k_{-3})(k_{-1} + k_2)$$

As the rate constants for mechanism 3 must also be related according to equation T.1.7, the ratio of the phosphate fluxes can be deduced from equations T.1.12, 13, 16, 17 and 7.

$$\frac{\text{Flux of phosphate from G1P to G6P}}{\text{flux of phosphate from G6P to G1P}} = \frac{[G1P]^3(EG16P_1)k_{-3}}{[G6P]^3(EG16P_{11})k_3}$$

$$= \frac{[G1P]^3(1 + \alpha K[G6P])}{[G6P]^3K^3(1 + \alpha[G1P])} \quad \ldots\ldots \text{T.1.18}$$

Again the flux ratio for the glucose fluxes can be deduced from equations T.1.7, 14-17.

$$\frac{\text{Flux of glucose from G1P to G6P}}{\text{Flux of glucose from G6P to G1P}}$$

$$= \frac{[G1P]^2 (EG16P_1) k_{-3}}{[G6P]^2 (EG16P_{11}) k_3}$$

$$= \frac{[G1P]^2 (1 + \alpha K [G6P])}{[G6P]^2 K^2 (1 + \alpha [G1P])} \qquad \text{..... T.1.19.}$$

At chemical equilibrium the ratio of the glucose to phosphate fluxes can be obtained from equations T.1.12, 14 and 17.

$$\frac{\text{Flux of glucose from G1P to G6P}}{\text{Flux of phosphate from G1P to G6P}} = \frac{3 + 2\alpha [G1P]}{2 + \alpha [G1P]} \qquad \text{... T.1.20}$$

Where $\alpha$ is defined as in equation T.1.11, but with rate constants referring to mechanism 3.

$$\alpha = \frac{k_1 k_2}{k_{-3}(k_{-1} + k_2)} \qquad \text{..... T.1.11.}$$

These equations can be used to define the flux equations for the phosphoglyceratemutase if G1P is replaced by 2PGA, G6P by 3PGA, and G16P by 23PGA.

Conditions affecting flux equations

At high concentrations of substrates or in solutions of high ionic strength the activity coefficients of G1P and G6P (or 2PGA and 3PGA) should be introduced into the flux equations. The activity coefficients of the different enzymic species should also be included in relation to the appropriate rate constants, but if the activity coefficients of all the enzymic species are the same these coefficients cancel. The latter assumption may not necessarily be true if the electrical charge on the enzyme changes on the addition of the substrate, and as a consequence the apparent rate constants may change in solutions of high electrolyte strength. However, except for the numerical value of $\alpha$, the flux-ratio equations will be unaltered.

The flux-ratio equations for mechanisms 1 and 2 are independent of G16P or 23PGA concentration. For mechanism 3 this will also be true provided that an ordered addition of substrates occurs at all cofactor concentrations. If the reaction is not ordered the equation remains of the same form but the significance of the rate constants may change somewhat if for example at low cofactor concentrations an appreciable part of the reaction takes place by the substrate combining with the free enzyme and the cofactor then subsequently adding to the enzyme-substrate complex. This is unlikely to

occur if the cofactor dissociates only occasionally from the enzyme except at very low cofactor concentrations. Such a change from an effectively obligatory order to a random order of addition of substrates could also occur at high substrate concentrations or high ionic strength should a labilization of the cofactor occur under the influence of these conditions.

In deriving the flux equations only fluxes between G1P and G6P (or 2PGA and 3PGA) have been considered, and the contribution of fluxes into and out of the cofactor pool have been ignored. Thus it has been assumed that either the free cofactor in solution exchanges very rapidly and comes into a steady state or that the rate of exchange with the cofactor is negligible. The experimental situation lies in between these two extremes, so that some contribution from these fluxes might be expected, but as the quantity of cofactor present was always small the effect is relatively unimportant. This is discussed at greater length in the Results section R.7 and R.8.

When the isomerization of the enzyme is not rate limiting an alternative way of deriving the flux-ratio equations is by the probability method of Hodgkin and Keynes[65].

Significance of the Flux Ratios

If G1P molecules are converted to G6P molecules independently of each other or the reverse reaction then the

flux ratio of the forwards to backwards reaction should obey the Independence relationship[59, 66]. Thus:-

$$\frac{\text{Flux of G1P to G6P}}{\text{Flux of G6P to G1P}} = \frac{f_{G1P}[G1P]}{f_{G6P}[G6P]K} \qquad \text{..... T.1.21}$$

where $f_{G1P}$ and $f_{G6P}$ represent the activity coefficients of G1P and G6P respectively and the other symbols are defined in the same way as before. A similar equation for 2PGA and 3PGA interconversion is made by appropriate substitutions.

Consider the case where there is a nett conversion of G1P and G6P. For mechanism 1 the glucose flux ratio, defined by equation T.1.9 should be less than that expected from the Independence relationship, and the chemical equivalent of exchange diffusion[59, 61, 72] should occur unless $\alpha[G1P]$ and $\alpha K[G6P]$ are very small, in which case the Independence relationship should be obeyed. The same will be true for the phosphate flux ratio if there is a direct transfer of phosphate as in mechanism 2. However, for mechanism 1 the phosphate flux ratio, defined by equation T.1.8, should exceed that to be expected from the Independence relationship, so that the chemical analogue of the long-pore effect[65] should be found. For mechanism 3 both the glucose flux ratio, equation T.1.19, and the phosphate flux ratio, equation T.1.18, should show the

long-pore effect. However, if $\alpha$ becomes very large both the phosphate flux ratio of mechanism 1 and the glucose flux ratio of mechanism 3 should obey the Independence relationship. Examination of the reaction for the chemical analogues of exchange diffusion and the long-pore effect should thus provide a method of distinguishing between the models. The ratio of the glucose flux to the phosphate flux at chemical equilibrium will provide a further method of making a distinction, since the ratio will be 1:1 for mechanism 2, direct transfer of both phosphate and the remaining part of the substrate, but will be greater than 1:1 for both mechanisms 1 and 3. For mechanism 1, equation T.1.10, the ratio will be 2 for small values of $\alpha$ and greater than 2 at high values of $\alpha$. For mechanism 3, equation T.1.20, the ratio will be $\frac{3}{2}$ for small values of $\alpha$ and 2 for high values of $\alpha$.

In contrast, the expressions for the nett fluxes for all three models are identical in the presence of excess cofactor. At lower concentrations of cofactor the expressions for the nett fluxes for mechanisms 1 and 2 remain identical but for mechanism 3 the effect of a low cofactor concentration will depend upon the relative values of the rate constants for the affinity of the enzyme and the enzyme-substrate complex for the cofactor. If the free enzyme has a much greater affinity for the cofactor than the enzyme-substrate complex then

mechanism 3 will have the same nett fluxes as mechanisms 1 and 2 at all but very low cofactor concentrations. Under these latter conditions the reaction scheme will more nearly approximate to a sequential (as opposed to a ping-pong) pathway. This is discussed at greater length in section T.2.

Induced transport test of reaction mechanism

Consider a reaction of the type $A + B \rightleftharpoons C + D$ and the flux ratio $\frac{f_{A \rightarrow C}}{f_{C \rightarrow A}}$. If the fluxes obey the Independence Relationship[66] then the flux ratios will be given by eq. T.1.22[59].

$$\frac{f_{A \rightarrow C}}{f_{C \rightarrow A}} = \frac{a_A \cdot a_B}{a_C \cdot a_D} \cdot \frac{1}{K} e^{-\left(\frac{nEF}{RT}\right)} \qquad \ldots\ldots \text{ T.1.22}$$

where $a_A$ etc. are activities, K is the equilibrium constant of reaction, E is the electrical potential difference between A and B, C and D, n is the nett number of charges transferred from left to right and RT and F have their usual significance. It would be necessary to include the term E should the reaction involve uptake across a cell membrane or similar barrier. Deviations from this relationship indicate an interaction between the fluxes and the terms Exchange diffusion and the Long-pore effect are applied according to the

particular type of interaction.

Consider a nett reaction from A to C. If an interaction between the fluxes is such that $\frac{f_{A \longrightarrow C}}{f_{C \longrightarrow A}}$ is less than the right hand term in equation T.1.22 then exchange diffusion[73] is said to occur. On the other hand if the flux ratio is greater than the right hand term in equation T.1.22, it is termed the long-pore effect[65]. In exchange diffusion the conversion of a molecule of A to C facilitates the conversion of a further molecule (or molecules) of C to A, whereas in the long-pore effect the conversion of one molecule of A to C facilitates the conversion of another molecule (or molecules) of A to C.

In the present situation studies of the enzyme reaction were carried out <u>in vitro</u> so that A, B, C and D are in the same solution and consequently the potential difference E is zero. Furthermore there is only one species of substrate and one species of product (i.e. the reaction can be represented as, $A \rightleftharpoons C$). Under these circumstances co- and counter-transport are closely related to the long-pore effect and exchange diffusion respectively[59].

To test for co- and counter-transport appropriately labelled substrate is allowed to come to equilibrium with its product in the presence of the enzyme. Then unlabelled

substrate (usually the one not favoured by the equilibrium constant) is added and the system allowed to relax towards equilibrium. If activity coefficients and isotope effects are ignored[27], then, if the flux ratio obeys the Independence relationship (i.e. if there is no induced transport), the distribution of radioactivity between the substrate and product will remain unchanged during the reaction. If the reaction shows the chemical analogue of exchange diffusion (i.e. if the flux ratio is less than that given by the Independence relationship) there will be a transient flow of labelled material in a direction opposite to the nett flow; by analogy with permeability studies this may be called Counter-transport. Finally, if the reaction shows the chemical analogue of the long-pore effect (i.e. if the flux ratio is greater than that given by the Independence relationship), there will be a transient flow of labelled material in the same direction as the nett flow; this may be termed Co-transport. These movements are against the free energy gradients and the term Induced-transport is therefore used. This test is also referred to as an Induced-transport test. The influence of isotopic effects and non-ideal behaviour of solutions on these tests is considered in a later section.

For a reaction A $\rightleftharpoons$ B the expected rate of accumulation

of an isotopically labelled species A* during conversion of A to B can be calculated for various flux ratios as follows.

Ignoring isotopic effects, the nett rate of accumulation of an isotopically labelled A is given by:-

$$V\frac{d[A^*]}{dt} = \frac{[B^*]}{[B]}(\text{flux } B \longrightarrow A) - \frac{[A^*]}{[A]}(\text{flux } A \longrightarrow B) \quad \ldots \quad T.1.23$$

where $[A]$ and $[B]$ represent total concentrations and $[A^*]$ and $[B^*]$ the concentrations of labelled A and B, t is time and V is the volume of the solution.

$$\text{Let:-} \quad \frac{\text{Flux of A to B}}{\text{Flux of B to A}} = r \qquad \ldots\ldots \quad T.1.24$$

and as:-

$$\text{Nett flux} = (\text{flux of A to B}) - (\text{flux of B to A})$$

$$\text{flux A to B} = \frac{r}{(r-1)} \quad (\text{nett flux of A to B}) \qquad \ldots\ldots \quad T.1.25$$

similarly:-

$$\text{flux of B to A} = \frac{1}{(r-1)} \quad (\text{nett flux of A to B}) \qquad \ldots\ldots \quad T.1.26$$

also:-

$$\text{nett flux of A to B} = -V\frac{d[A]}{dt} \qquad \text{..... T.1.27.}$$

Substituting equations T.1.24-26 into equation T.1.22

$$\frac{d[A^*]}{d[A]} = \frac{[A^*]r}{[A](r-1)} - \frac{[B^*]}{[B](r-1)} \qquad \text{..... T.1.28.}$$

If the amount of labelling associated with the cofactor is negligible in comparison with $[A^*]$ or $[B^*]$, or is constant it is possible to write:-

$$[A^*] + [B^*] = N \qquad \text{..... T.1.29}$$

where N is the total concentration of labelled substrate.

Furthermore:

$$[A] + [B] = T \qquad \text{..... T.1.30}$$

where T is the total concentration of substrates.

Equation T.1.28 can be used in conjunction with

equations T.1.29 and T.1.30 to calculate the changes in [A*] during the reaction for the particular flux ratios, r, associated with the various proposed mechanisms of reaction. The following relationships for r were introduced into equation T.1.28, where [A] = [G1P] or [2PGA] and [B] = [G6P] or [3PGA].

For direct transfer of label (either glucose, glyceric acid or phosphate) from equation T.1.9

$$r = \frac{[A](1 + \alpha K[B])}{K[B](1 + \alpha[A])} \quad \ldots\ldots \text{T.1.31.}$$

For indirect transfer of labelling with one intermediate group, from equation T.1.8 or T.1.19:-

$$r = \frac{[A]^2(1 + \alpha K[B])}{K^2[B]^2(1 + \alpha[A])} \quad \ldots\ldots \text{T.1.32.}$$

If $\alpha$ is small and low concentrations of A and B are employed this expression can be simplified to:-

$$r = \frac{[A]^2}{K^2[B]^2} \quad \ldots\ldots \text{T.1.33.}$$

For indirect transfer of labelling with two intermediate groups, from equation T.1.18:-

$$r = \frac{[A]^3(1 + \alpha K[B])}{K^3[B]^3(1 + \alpha[A])} \quad \ldots\ldots \text{T.1.34}$$

which can be simplified at low concentrations of A and B and for small $\alpha$ to:-

$$r = \frac{[A]^3}{K^3[B]^3} \quad \ldots\ldots \text{T.1.35.}$$

It is also possible to derive values of r for mixed pathways. If a fraction (1 - p) of the flux of A to B has one intermediate group for the transfer (eq. T.1.32) and a fraction p of the flux has direct transfer (eq. T.1.31), and it is also assumed that $[A]$, and $[B]$, and $\alpha$ are small then:-

$$r = \frac{[A]^2}{(1 - p)K^2[B] + pK[A][B]} \quad \ldots \text{T.1.36.}$$

Again if a fraction (1 - p) of the flux A to B had one intermediate for the transfer (eq. T.1.32) and a fraction p had two intermediates for the transfer (eq. T.1.34), making

## Fig. T.1.4.

### Phosphoglucomutase system

Integration of eq. T.1.28 for various values of the flux ratio, r, was carried out for the following conditions: $A_o$ = 1.044 mM, $B_o$ = 0.695 mM, total radioactivity in $A_o$ = 1.0, total radioactivity in $B_o$ = 17.2, and equilibrium constant K = 17.2. The concentration of A was changed from 1.044 mM to 0.084 mM in steps of 0.005 mM, integration of dA*/dA being performed for each step.

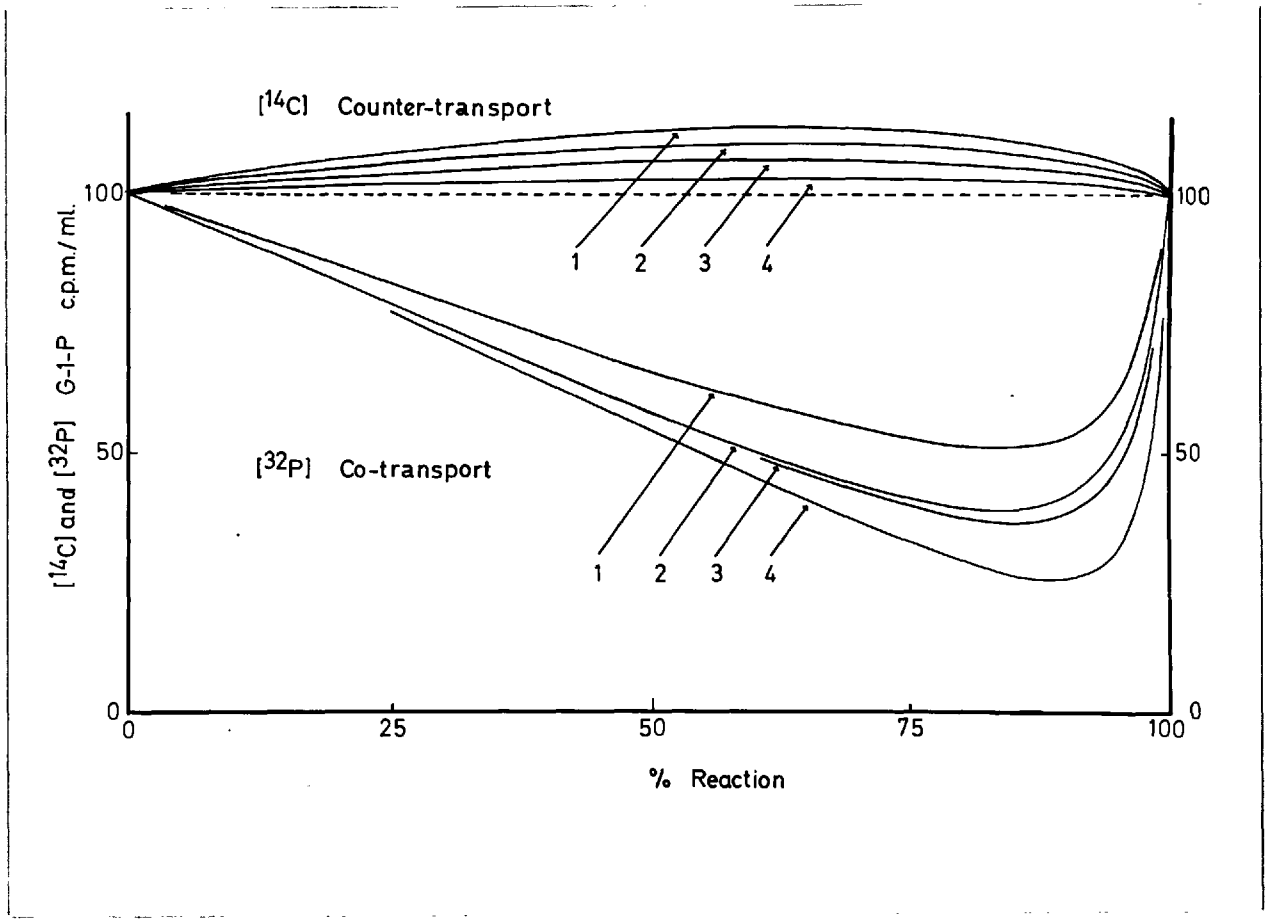
The $[^{14}C]$ Counter-transport curves, 1-4 obtained when eq. T.1.28 is integrated with the flux ratio, r, defined by eq. T.1.31. The values of $\alpha A$ used are: 0.1 (curve 4), 0.2 (curve 3), 0.3 (curve 2), 0.4 (curve 1), and give maximum values of A* of: 1.032, 1.067, 1.098, and 1.132 respectively.

The dotted line is the expected curve for direct transfer of radioactivity when the Independence Relationship is obeyed and there is no Induced-transport.

The $[^{32}P]$ Co-transport curves 1 and 2 are obtained when eq. T.1.28 is integrated with the flux ratio, r, defined by eq. T.1.36. In this expression a fraction (1-p) of the reaction occurs *via* one intermediate, and a fraction p *via* direct transfer. For curve (2), p = 0, and for curve (1), p = 0.2, which give minimum values of A* of 0.391, and 0.511 respectively.

The $[^{32}P]$ Co-transport curves 3 and 4 are obtained when eq. T.1.28 is integrated with the flux ratio, r, defined by eq. T.1.37. In this expression a fraction (1-p) of the reaction occurs *via* one intermediate and a fraction p *via* two intermediates. For curve (4), p = 1.0, and for curve (3), p = 0.2, which give minimum values of A* of: 0.254, and 0.366 respectively.

Fig. T.1.4.


[14C] Counter-transport
[32P] Co-transport
[14C] and [32P] G-1-P c.p.m./mL.
100
50
0
0
25
50
75
100
% Reaction
1
2
3
4

the same assumption as in equation T.1.36.

$$r = \frac{[A]^3}{(1 - p)K^2[A][B]^2 + pK^3[B]^3} \quad .. \quad T.1.37.$$

$[B]$ and $[B^*]$ were eliminated from equation T.1.28 using equations T.1.29 and T.1.30. Then the series of equations resulting when equations T.1.31, 32, 36 and 37 were substituted into equation T.1.28 were integrated numerically for the phosphoglucomutase system with an ICL 1900 series computer using the initial conditions given in Fig. T.1.4. It can be seen that $\alpha$ was closely proportional to the magnitude of the counter-transport over the range of conditions calculated for eq. T.1.31.

For the phosphoglycerate mutase system the calculations of induced transport is more complicated since the equilibrium constant does not quite retain the same value throughout the reaction. This change in the equilibrium constant arises as a result of the different binding constants of 2PGA and 3PGA with the $Mg^{2+}$ ions which are needed to keep the enzyme in its active form. The equilibrium constant and the free $Mg^{2+}$ ion concentration are related by equation T.1.38, below (see later for details of derivation, and see section on $Mg^{2+}$ binding).

$$K_{eq}^{app} = K_a \frac{\left(1 + K_4[Mg^{2+}] + \frac{[H^+]}{K_y}\right)}{\left(1 + K_3[Mg^{2+}] + \frac{[H^+]}{K_x}\right)} \quad \ldots\ldots \text{T.1.38}$$

Where $K_{eq}^{app}$ is the apparent equilibrium constant, $K_a$ the true equilibrium constant defined by equation T.1.39, below, $[Mg^{2+}]$ the free magnesium ion concentration, $K_x$ and $K_y$ are the third acid dissociation constants of 2PGA and 3PGA respectively, and $K_3$ and $K_4$ the respective association constants of the (3-) complexes with magnesium. In these equations it has been assumed that magnesium only binds the completely ionized forms of 2PGA and 3PGA. However, since the apparent binding was measured at pH 7.4 and since the assumption that only the $3^-$ forms bind $Mg^{2+}$ was also made in the original calculations of $K_3$ and $K_4$ no error will be introduced if the $2^-$ forms also bind $Mg^{2+}$.

$$K_a = \frac{[3PGA^{3-}]}{[2PGA^{3-}]} \quad \ldots\ldots \text{T.1.39.}$$

The free magnesium ion concentration for any one pair of 2PGA and 3PGA concentrations was calculated from the following equation, T.1.40, the derivation of which is shown in a subsequent section.

$$K_3K_4[Mg^{2+}]^3_{Free} +$$

$$\left\{K_3+K_4+K_3K_4\left(\frac{[B][H^+]}{([H^+]+K_y)} + \frac{[A][H^+]}{([H^+]+K_x)} - [Mg^{2+}]_I\right)\right\}[Mg^{2+}]^2_{Free}$$

$$+ \left\{1 + \frac{K_4[B][H^+]}{([H^+]+K_y)} + \frac{K_3[A][H^+]}{([H^+]+K_y)} - (K_3+K_4).[Mg^{2+}]_I\right\}[Mg^{2+}]_{Free}$$

$$- [Mg^{2+}]_I = 0 \qquad \text{.....} \quad \text{T.1.40}$$

where $[Mg^{2+}]_I$ is the total magnesium ion concetration, and A and B are the total concentrations of 2PGA and 3PGA respectively.

For each step in the integration of the flux equations equation T.1.40 was solved for $[Mg^{2+}]_{free}$ and the appropriate equilibrium constant obtained from equation T.1.38. The flux equations were obtained by substitution of equations T.1.31, 32, 36, 37 and 38 into equation T.1.28 and were integrated numerically with an Atlas 360 computer starting from the initial conditions described in Fig. T.1.5. A series of curves for [2PGA*] against percentage reaction were derived each of which was characteristic of a particular mechanism of group transfer (see Fig. T.1.5).

Fig. T.1.5.

Phosphoglycerate-mutase system

Integration of eq. T.1.28 for various values of the flux ratio, r, was carried out for the following initial conditions, $A_o$ = 1.356 mM, $B_o$ = 1.09 mM, $Mg^{2+}$ = 2.89 mM, total radioactivity in $A_o$ = 1.0, total radioactivity in $B_o$ = 11.33. The concentration of A was changed from 1.356 mM to 0.192 mM in steps of 0.005 mM, integration of $dA^*/dA$ being performed for each step. As the equilibrium constant, K, depends slightly upon the concentration of $[Mg^{2+}]_{free}$ and hence upon the relative proportions of A and B it was recalculated from eqs. T.1.38 and T.1.40 after each integration step and the new value used in the subsequent integration.

Curves $a_1$ - $a_4$ show the extent of Counter-transport which occurs when eq. T.1.28 is integrated with the flux ratio, r, defined by eq. T.1.31. The values of $\alpha A$ are; 0.1 ($a_1$), 0.2 ($a_2$), 0.3 ($a_3$), 0.4 ($a_4$), and give maximum values of A* of; 1.016, 1.045, 1.075, and 1.104 respectively.

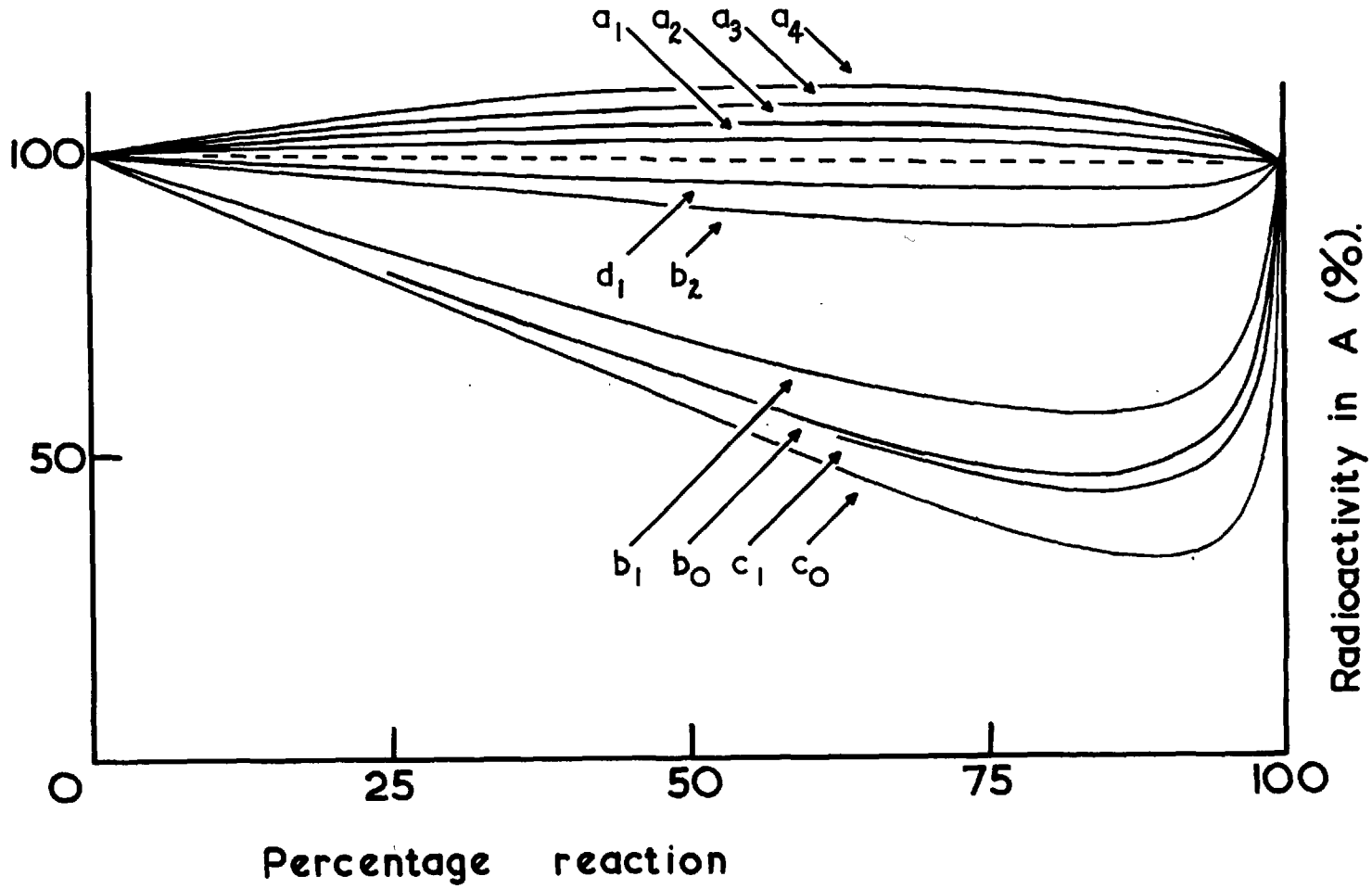
The dotted line is the expected curve for direct transfer of radioactivity when the Independence Relationship is obeyed and there is no Induced-transport.

The curves $b_o$ - $b_2$ show the extent of Co-transport which occurs when eq. T.1.28 is integrated with the flux ratio, r, defined by eq. T.1.36. In this expression a fraction (1-p) of the reaction occurs via one intermediate, and a fraction p via direct transfer. For curve $b_o$, p = 0. , for curve $b_1$, p = 0.2, and for curve $b_2$, p = 0.8, which give minimum values of A* of; 0.463, 0.564, and 0.874 respectively.

The curves $c_o$ and $c_1$ show the extent of Co-transport which occurs when eq. T.1.28 is integrated with the flux ratio, r, defined by eq. T.1.37. In this expression a fraction (1-p) of the reaction occurs via one intermediate, and a fraction p via two intermediates. For curve $c_o$, p = 1.0, and for curve $c_1$, p = 0.2, which give minimum values of A* of; 0.325, and 0.437 respectively.

The curve $d_1$ shows the extent of Co-transport which occurs when eq. T.1.28 is integrated with the flux ratio, r, defined by eq. T.1.32. In this expression reaction occurs by one intermediate but a high value of $\alpha A$ = 100 is assumed, which gives a minimum value of A* of 0.934.

Fig. T. 15.


a1
a2
a3
a4
100
d1
b2
50
b1
b0
c1
c0
0
25
50
75
100
Percentage reaction
Radioactivity in A (%).

If the solutions show non-ideal behaviour activity coefficients should be introduced into the expressions for the flux ratio r. However, when the activity coefficients for A and B are equal, as in the present case for G1P and G6P (see results section) the coefficients in equations T.1.31-37 cancel, but the numerical value of $\alpha$ in equations T.1.31, 32 and 34 is changed (see later).

In equations T.1.36 and 37 for simplicity it has been assumed arbitrarily that the fluxes of A to B via the different pathways remain in the same proportion throughout the reaction. However, if two enzymes are involved, for example, it might be more realistic to assume that the nett fluxes remain in constant proportion. A consideration of equation T.1.25 shows that the induced transport curves will remain of the same form for the first part of the reaction and that the differences in the latter parts of the curve will be relatively small.

The initial conditions of [A] and [B] concentrations etc. in the above analysis were chosen arbitrarily. It may be that these induced transport tests would benefit by a systematic investigation of the effect of varying the relative initial concentrations of [A] and [B], and [A*] and [B*] upon the expected degree of co- or counter-transport for the various mechanisms. For example a large quantity of non-

radioactive A could be added at the beginning of the reaction in proportion to the quantity of A present at the end of the initial equilibrium phase of the experiment. Alternatively dispensing with the initial equilibrium phase may enhance the sensitivity of the test. Suppose the induced transport experiment was initiated by the addition of the enzyme to a mixture of non-radioactive A and radioactive B, then if no induced-transport occurred the radioactivity in A would show a linear increase from zero to its equilibrium value when plotted against the extent of reaction. If, however, some co- or counter-transport occurred, provided the expected degree of induced transport was the same as in the case of the pre-equilibrated experiments than an enhancement of sensitivity in measurement might be expected as the percentage change observed in the radioactivity of A* would be greater.

Such an experimental approach might find useful application in the study of very slowly reacting enzyme systems, for example the conversion of G1P to G6P by phosphoglucomutase in the absence of G16P cofactor. It is hoped that a systematic investigation of the means of increasing the sensitivity of induced transport tests will be carried out as a logical extension of the work.

Determination of the magnitude of $\alpha$

The interpretation of the induced transport tests as set out above may not be entirely unambiguous. This is because there will be no induced transport (see Figs. T.1.4, T.1.5) of the glucose moiety for mechanism 3 and the phosphate for mechanism 1 if the isomerization of the phosphoenzyme is rate limiting (i.e. when $\alpha$ is large): and the phosphate induced transport curves for mechanism 3 will resemble the phosphate induced transport curve for mechanism 1. Generally speaking a system with n intermediates will behave as if it had only $n^{-1}$ intermediate if $\alpha$ is large. This complication did not arise with phosphoglucomutase since counter-transport of the glucose occurred at high substrate concentrations and this cannot be brought about by mechanism 3.

There are two possible tests which can be used to show whether $\alpha$ is large or small. The first relies on a kinetic approach in which the presence of partial uncompetitive inhibition due to a rate limiting isomerization of the "phosphoenzyme" is used to measure $\alpha$ or to define its upper limit. This technique was used by Ray and Roscelli[32] in an effort to detect such a rate limiting step for the phosphoglucomutase reaction. The ratios of the initial velocity of reaction to the average velocity for the conversion of 80% of the substrate was measured in both

directions over a wide range of substrate concentrations. If these ratios varied with substrate concentration then there was a product inhibition due to a rate limiting isomerization of the phosphoenzyme. In the case of phosphoglucomutase the ratios remained constant showing that there was no detectable isomerization step. However, assuming that there was such a step it was possible to calculate a lower limit for the rate of interconversion. The author concluded that, ignoring the non-ideal behaviour of solutions that the rate constant for the phosphoenzyme isomerization was greater than $10^7$ $sec^{-1}$. This corresponded to an upper limit for $\alpha$ of 1.42 $M^{-1}$, which was calculated from equation T.1.48 (see below for derivation).

$$\frac{\alpha \, K_{G1P} \, E}{V_{max}} = \frac{1}{k_3} + \frac{1}{k_{-3}} \qquad \text{..... T.1.48.}$$

With a value of $\alpha$ of this order of magnitude the terms containing $\alpha$ are quite negligible in the flux ratio equations and an unambiguous interpretation of the induced transport data is possible.

The value of $\alpha$ which would be necessary to change a co-transport curve for n intermediates into one which could not be distinguished experimentally from a co-transport curve of order $n^{-1}$ can be obtained by substituting a variety of values

of $\alpha$ into eq. T.1.31 and integrating. For example the value of $\alpha$ A was determined which would bring the induced transport curve (for 1 intermediate) within 5% of the curve for no induced transport over the whole course of the chemical reaction (see Fig. T.1.5). The value of $\alpha$ necessary to fulfill this condition increases when the concentration of the substrate is lowered.

The upper limit to the value of $\alpha$ can be set since the lowest values possible of $k_3$ and $k_{-3}$ are related by the maximum velocities of the overall reaction in forwards and reverse direction respectively as follows:- $V_{Fmax} < Ek_3$ and $V_{Rmax} < Ek_{-3}$. Hence by substitution in equation T.1.48.

$$\frac{\alpha K_{G1P} E}{V_{Fmax}} < \frac{E}{V_{Fmax}} + \frac{E}{V_{Rmax}} \qquad \text{.....} \quad \text{T.1.43}$$

$$\text{or} \quad \alpha < \frac{1}{K_{G1P}} \left( 1 + \frac{V_{Fmax}}{V_{Rmax}} \right) \qquad \text{.....} \quad \text{T.1.43.}$$

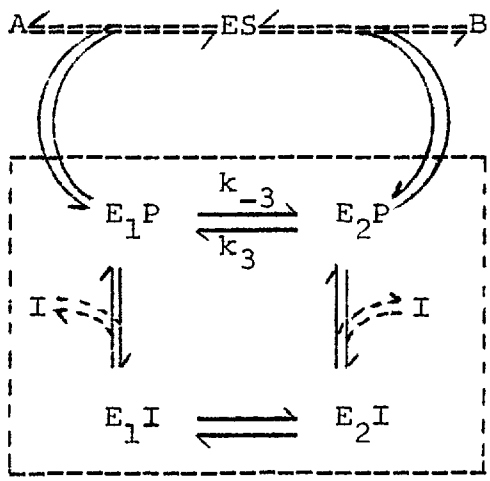
Using the Haldane relationship equation T.1.43 can alternatively be stated as:-

$$\alpha < \frac{K_{eq}}{K_{G6P}} \left( 1 + \frac{V_{Rmax}}{V_{Fmax}} \right) \qquad \text{.....} \quad \text{T.1.44}$$

In the present experiments small traces of sulphate are present which appears to act as a competitive inhibitor. Chloride may also act in the same way though less effectively. The influence of such inhibitors on the significance of eqs. T.1.43 and T.1.44 should therefore be considered.

Let the inhibitor be denoted as I, then the enzyme reaction may be set out schematically (ignoring the dissociation of the diphosphate) as follows:-

Scheme T.1.4




If the concentration of free I is held constant, all the rate constants within the dotted rectangle also become constant. Consequently in the steady state the reactions

within the rectangle may be replaced by $E^{1}P_{1} \underset{k_3^1}{\overset{k_{-3}^1}{\rightleftharpoons}} E_2^{1}P$

where the primes indicate that they are apparent quantities. It follows from this argument that eq. T.1.43 and T.1.44 still apply in the presence of competitive inhibitors, but that the appropriate $K_m$ is the apparent value determined under the conditions of the induced transport experiment (see also section T.2), and eq. T.1.48 may thus be rewritten as:-

$$\frac{\alpha K_{G1P}^{app} E^{1}_{t}}{V_{max}} = \frac{1}{k_3} + \frac{1}{k_{-3}} \qquad \text{..... T.1.48a.}$$

Calculation of the Rate Constants for the isomerization of the "phosphoenzyme"

The rate constants for the isomerization of the "phosphoenzyme" were calculated from the degree of counter-transport obtained at high concentrations of $^{14}C$-labelled substrates. Theoretical induced transport curves with $\alpha A$ = 0.1, 0.2 etc. were calculated for both the phosphoglucomutase and phosphoglycerate mutase systems (see Figs. T.1.4, T.1.5) by integration of equation T.1.31 and the results were compared with the experimentally obtained counter-transport curves. From the theoretical curve with

the closest fit the value of $\alpha$ was obtained. $\alpha$ is related to the kinetic constants of the reaction by equation T.1.11.

$$\alpha = \frac{k_1 k_2}{k_{-3}(k_{-1} + k_2)} \qquad \text{..... T.1.11.}$$

All terms apart from $k_3$ and $k_{-3}$, the rate constants for the isomerization of the phosphoenzyme, can be expressed in terms of the Michaelis constants, and the maximum velocities of the reaction. The initial velocity of reaction in presence of excess G16P from equations T.1.3 and T.1.5 is

$$V_f = \frac{\frac{k_2 k_3}{(k_2 + k_3)}(E)}{1 + \frac{(k_3 + k_{-3})(k_{-1} + k_2)}{k_1(k_2 + k_3)} \times \frac{1}{[G1P]}} \qquad \text{..... T.1.45}$$

By analogy with the Michaelis-Menten velocity equation:-

$$\frac{V_{max}}{E} = \frac{k_2 k_3}{(k_2 + k_3)} \qquad \text{..... T.1.46}$$

and

$$K_{mG1P} = \frac{(k_3 + k_{-3})(k_{-1} + k_2)}{k_1(k_2 + k_3)} \qquad \text{..... T.1.47}$$

where $V_{max}$ is the maximum initial velocity of reaction and

$K_{mG1P}$ is the Michaelis constant for G1P in the presence of excess G16P. Substituting equations T.1.46 and T.1.47 in equation T.1.11 it follows that:-

$$\frac{\alpha \ K_{G1P} \ E}{V_{max}} = \frac{1}{k_3} + \frac{1}{k_{-3}} \qquad \text{.....} \quad \text{T.1.48}$$

From this equation it was possible to determine the rate constant of isomerization of the "phosphoenzyme" as described in the results section.

Calculation of Fluxes at Equilibration

The fluxes at equilibration were calculated for the reaction $A \underset{}{\overset{k_1}{\rightleftharpoons}} B$ by measuring the rate of accumulation of radioactivity in B when a small quantity of radioactive A was added to the solution. A graph of the logarithm to the base of 10 of the differences between the actual radioactivity at time t in B and the final equilibrium value was plotted against time and the best straight line drawn through the points. The fluxes were obtained from the slope of the line by using equation T.1.53, whose derivation is shown below.

In a system $A \rightleftharpoons B$ at equilibrium where A = KB let k be the flux of A to B, a be the specific activity of A, b the specific activity of B and N the total radioactivity per

unit volume in the system such that

$$aA + bB = N \qquad \text{..... T.1.49}$$

Flux of radioactivity from A to B = $k(a - b)$ ..... T.1.50

Substituting from equation T.1.49:-

Fall in Specific activity of A = $\frac{-da}{dt} = \frac{k}{A}(a-b) = \frac{k}{A}\left(a-\frac{(N-aA)}{B}\right)$

$$\therefore \frac{-1}{k}\frac{da}{dt} = a\left(\frac{1}{A} + \frac{1}{B}\right) - \frac{N}{AB} \qquad \text{..... T.1.51}$$

Integrating equation T.1.45

$$kt = \frac{1}{\left(\frac{1}{A} + \frac{1}{B}\right)} \cdot \log \left( \frac{N}{AB} - \left(\frac{1}{A} + \frac{1}{B}\right)a\right)$$

$$k = \frac{2.303}{\left(\frac{1}{A} + \frac{1}{B}\right)}\left[\frac{\log_{10}\left(1 - \frac{a(A + B)}{N}\right)}{t}\right] \qquad \text{..... T.1.52}$$

where $\frac{N}{A + B} = a$ at $t = \infty$

Hence the flux per mole of enzyme, concentration E, in a solution of volume V, is given by equation T.1.53 in which the (slope) is the gradient of the graph described above:-

$$\text{Flux} = \frac{2.303\ V}{\left(\frac{1}{[A]} + \frac{1}{[B]}\right)[E]}\ (\text{Slope}) \qquad \ldots\ldots \text{T.1.53}$$

Induced Transport: Influence of non-ideal behaviour of solutions and isotope effects

In the following discussion the influence of isotope effects and the non-ideal behaviour of solutions upon the relationship between the Independence relationship and induced transport is considered for the following reaction:

$$A \rightleftharpoons B$$

Suppose that the concentrations of the unlabelled species at equilibrium are $[A]_o$ and $[B]_o$, that their activity coefficients are $f_A^o$ and $f_B^o$ and that the equivalents for the labelled species are $[A^*]_o$, $[B^*]_o$ etc. The equilibrium constants will be defined by the following equations:-

$$\frac{f_A^o[A]_o}{f_A^o[B]_o} = K \qquad \ldots\ldots \text{T.1.54}$$

$$\frac{f_{A^*}^o[A^*]_o}{f_{B^*}^o[B^*]_o} = K^* \qquad \ldots\ldots \text{T.1.55}$$

If the flux of unlabelled A to B is $m_{AB}$ and the flux of unlabelled B to A is $m_{BA}$ then it is possible to write:-

$$\text{Flux of labelled A to B} = p_o\, m_{AB}\, \frac{f^o_{A^*}[A^*]_o}{f^o_A[A]_o} \qquad \text{.....} \quad \text{T.1.56.}$$

$$\text{Flux of labelled B to A} = q_o\, m_{BA}\, \frac{f^o_{B^*}[B^*]_o}{f^o_B[B]_o} \qquad \text{.....} \quad \text{T.1.57.}$$

Where $p_o$ and $q_o$ are factors to allow for isotope effects at chemical equilibrium.

Since there is equilibrium:-

$$p_o\, m_{AB}\, \frac{f^o_{A^*}[A^*]_o}{f^o_A[A]_o} = q_o\, m_{BA}\, \frac{f^o_{B^*}[B^*]_o}{f^o_B[B]_o} \qquad \text{.....} \quad \text{T.1.58}$$

From equations T.1.54-55, and T.1.58:-

$$\frac{p_o}{q_o} = \frac{K}{K^*} \qquad \text{.....} \quad \text{T.1.59}$$

Suppose, as in the induced transport experiments, that unlabelled A is now added to the equilibrium mixture. Then:-

$$\text{Flux of labelled A to B} = p\, m_{AB} \frac{f_{A^*}[A^*]_o}{f_A[A]} \qquad \ldots\ldots \quad T.1.60$$

$$\text{Flux of labelled B to A} = q\, m_{BA} \frac{f_{B^*}[B^*]_o}{f_B[B]_o} \qquad \ldots\ldots \quad T.1.61.$$

Where $f_A$, $f_B$ etc. are the activity coefficients after the addition of non-radioactive A and p and q are isotope factors for the particular non-equilibrium conditions.

$$\text{Let,} \qquad \frac{p.p_o}{q.q_o} = n. \qquad \ldots\ldots \quad T.1.62.$$

Provided that the forwards and reverse fluxes of the labelled materials are equal no induced transport will occur. Thus from equations T.1.55, T.1.59, T.1.61 and 62, the conditions for no induced transport are as shown below in equation T.1.63.

$$\frac{n.m_{AB}}{m_{BA}} = \frac{f_A[A]f_{B^*}f^o_{A^*}}{f_B[B]_o\ K\ f_{A^*}f^o_{B^*}} \qquad \ldots\ldots \quad T.1.63.$$

Counter-transport occurs for the labelled material when:-

$$\frac{n.m_{AB}}{m_{BA}} > \frac{f_A[A]f_{B*}f^{o}_{A*}}{f_B[B]_o \; K \; f_{A*}f^{o}_{B*}} \qquad \text{..... T.1.64.}$$

Co-transport of the labelled material occurs when:-

$$\frac{n.m_{AB}}{m_{BA}} < \frac{f_A[A]f_{B*}f^{o}_{A*}}{f_B[B]_o \; K \; f_{A*}f^{o}_{B*}} \qquad \text{..... T.1.65.}$$

Though the fluxes $m_{AB}$ and $m_{BA}$ are the fluxes of the unlabelled material in a normal experimental situation the isotopically labelled species will only be present in trace amounts so that to a good approximation these fluxes represent the total fluxes.

Influence of isotope effects

In the equations defining the Independence relationship and the conditions for co- and counter-transport (eqs. T.1.63-65) the equilibrium constant for the isotopic species K* does not appear. Consequently the criteria for induced transport will be independent of any difference between K and K*. Furthermore, the factor n will also be unity even

though isotope effects occur provided that the reaction has only one pathway, since labelled and unlabelled molecules must take part in exactly the same reactions and the ratio p/q will always remain constant. Should alternative pathways exist n will not necessarily be unity as the isotopic species may preferentially follow one pathway and consequently be influenced by the flux ratio for this pathway rather than that of the overall reaction. Nevertheless the occurrence of induced transport will still give rise to the thermodynamic necessity of postulating a mechanism to account for the transfer of energy from the unlabelled to the labelled species[59, 60] and may therefore provide evidence for certain steps in the reaction.

Although isotope effects will often have no effect upon the criteria for the occurrence of induced transport they will affect the magnitude of any induced transport that occurs. Thus for a reaction $A \rightleftharpoons B$ in which isotope effects are present, the nett rate of accumulation of isotopically labelled A, from equations T.1.56, 57 will be:-

$$V \frac{d[A^*]}{dt} = q \frac{f_{B^*}[B^*]m_{BA}}{f_B[B]} - p \frac{f_{A^*}[A^*]m_{AB}}{f_A[A]} \quad \ldots \quad T.1.61$$

where p and q are factors allowing for the isotope effects and

V is the volume of the solution (cf. equation T.1.23). Thus comparing with equation T.1.23 and T.1.28 in the case where the activity coefficients are unity the extent of the induced transport is given by the integral of equation T.1.67,

$$\frac{d[A^*]}{d[A]} = \frac{p[A^*]r}{[A](r-1)} - \frac{q[B^*]}{[B](r-1)} \quad \ldots\ldots \quad \text{T.1.67}$$

where r is the flux ratio $m_{BA}/m_{AB}$ (see theory section T.1. eq. T.1.28).

If p and q should tend towards zero (i.e. if the enzyme discriminates against the labelled species) for example the amount of induced transport would also tend towards zero.

Influence of non-ideal behaviour of solutions

If the ratio $f_{A^*}/f_{B^*}$ is uninfluenced by the addition of non-radioactive A, then in equations T.1.63-65 the activity coefficients of the labelled species cancel out. If n is also assumed to be unity the Independence relationship becomes identical with equation T.1.21 and co- or counter-transport occur when the flux ratio deviates from this relationship. Isotope effects may be expected to have negligible influence upon the activity coefficients, and in most cases it would seem to be sufficient to demonstrate that

the ratio $f_A/f_B$ is uninfluenced by the addition of A in order to assume that the activity coefficients for the labelled species cancel.

From the above it is seen that it should also be possible to use an alternative substrate rather than labelled material in the induced transport test to test whether the Independence relationship is obeyed. The magnitude of any induced transport, however, might well be different.

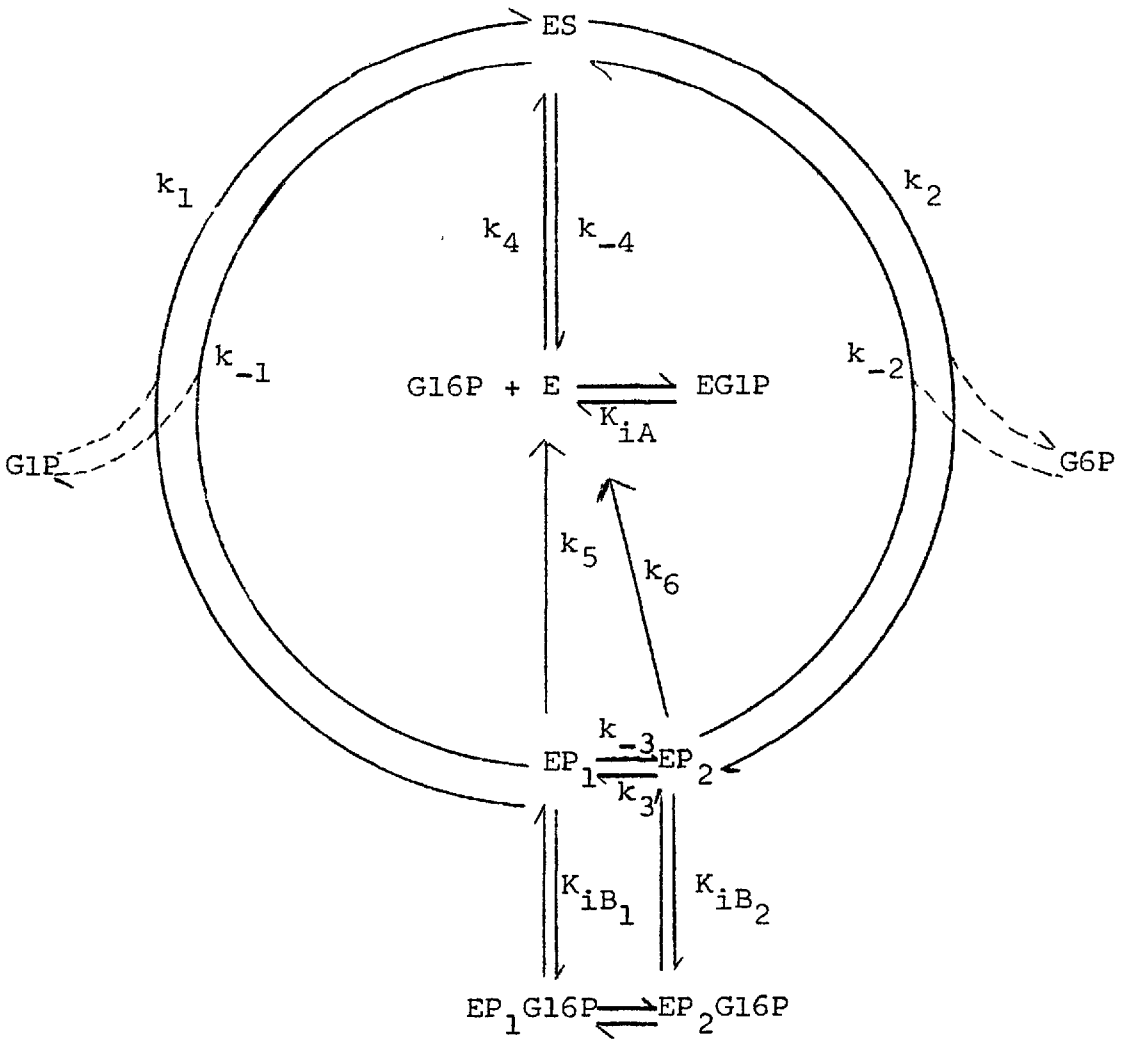
## SECTION 2. KINETICS OF REACTION

Two mechanisms of reaction have been proposed for the phosphoglucomutase and phosphoglycerate mutases, one involves a phosphoenzyme whilst in the other both the substrate and cofactor are present on the surface of the enzyme at the same time. It has been said that a simple phosphoenzyme leads to a parallel line initial velocity pattern whereas a simple sequential mechanism leads to an intersecting-line pattern and this has formed the basis of a kinetic test to distinguish between the mechanisms. However, a more general consideration of each mechanism must be undertaken before the test may be regarded as unambiguous.

### Phosphoenzyme mechanism

In the case of the phosphoenzyme mechanism it would seem necessary to take into account the effect of a slow hydrolysis of the phosphoenzyme upon the initial velocity pattern. Scheme 1 shows such a mechanism for phosphoglucomutase which has been extended to take into account the formation of inhibition complexes; EG1P and EP.G16P.

Scheme 1


ES
$k_1$
$k_2$
$k_4$
$k_{-4}$
$k_{-1}$
$k_{-2}$
G16P + E ⇌ EG1P
$K_{iA}$
G1P
G6P
$k_5$
$k_6$
$k_{-3}$
$EP_1$ ⇌ $EP_2$
$k_3$
$K_{iB_1}$
$K_{iB_2}$
$EP_1G16P$ ⇌ $EP_2G16P$

For convenience of analysis the influence upon the initial velocity pattern of including an EP isomerization step and hydrolysis of EP in the general kinetic treatment will be considered separately. Otherwise unwieldy equations are obtained which are more difficult to interpret. On the same basis the effect of including inhibition steps will be considered with EP hydrolysis rather than with EP isomerization. As the initial velocity pattern is under investigation $[G6P] = 0$, consequently there will be no terms involving $k_{-2}$ or $[G6P]$.

For EP hydrolysis and inhibition by G1P and G16P it is assumed there is only one form of EP, and EP hydrolyses at a rate $k_5$. Also:-

$$K_{iB} = \frac{[EP][G16P]}{[EPG16P]} \quad \text{and} \quad K_{iA} = \frac{[E][G1P]}{[EG1P]}$$

and that the inhibition complexes are always at equilibrium with the enzyme species being inhibited. Under steady state conditions the rate of breakdown and formation of all enzyme species is the same.

Hence for ES:-

$$k_{-4}(E)[G16P] + k_1(EP)[G1P] = (ES)(k_4 + k_2 + k_{-1}) \quad \ldots \quad T.2.1.$$

for EP:-

$$(k_5 + k_1[G1P])(EP) = (ES)(k_{-1} + k_2) \quad \ldots\ldots \text{T.2.2.}$$

and for E:-

$$k_4[G16P](E) = k_{-4}(ES) + k_5(EP) \quad \ldots\ldots \text{T.2.3.}$$

Also for total enzyme concentration $E_T$:-

$$(E_t) = (E) + (EP) + (ES) + (EG1P) + (EPG16P) \quad \ldots\ldots \text{T.2.4.}$$

The initial velocity of reaction for this system is $k_2(ES)_1$ hence solving equations T.2.1-4 for (ES) in terms of $(E_T)$, the following expression can be derived for the initial velocity.

$$\frac{1}{V} = \frac{1}{k_2(ES)} = \frac{1}{k_2(E_T)}\left(1 + \frac{(k_{-1}+k_2)}{(k_5+k_1[G1P])}\left(1 + \frac{[G16P]}{K_{iA}}\right)\right.$$

$$\left.+\left(\frac{k_{-4}}{k_4[G16P]} + \frac{k_5(k_{-1}+k_2)}{k_4(k_5+k_1[G1P])[G16P]}\right)\left(1 + \frac{[G1P]}{K_{iA}}\right)\right) \quad .. \text{T.2.5.}$$

If $k_5 \ll k_1[G1P]$, i.e. there is only a small amount of phosphatase activity then the $K_m$'s for G1P and G16P are seen to be:-

$$K_{G1P} = \left(\frac{k_{-1}+k_2}{k_1}\right)\left(\frac{\left(1 + \frac{[G16P]}{K_{iB}}\right) + \frac{k_5}{k_4[G16P]}\left(1 + \frac{[G1P]}{K_{iA}}\right)}{1 + \frac{k_{-4}}{k_4[G16P]}\left(1 + \frac{[G1P]}{K_{iA}}\right)}\right) \quad \ldots\ldots \text{T.2.6}$$

$$K_{G16P} = \left(\frac{\frac{k_{-4}}{k_4} + \frac{k_5(k_{-1}+k_2)}{k_4k_1[G1P]}\left(1 + \frac{[G1P]}{K_{iA}}\right)}{1 + \frac{(k_{-1}+k_2)}{k_1[G1P]}\left(1 + \frac{[G1P]}{K_{iA}}\right)}\right) \quad \ldots\ldots \text{T.2.7.}$$

Thus if $G1P \ll K_{iB}$ and $k_5 = k_{-4}$ $K_{G1P}$ is independent of [G16P]. Similarly if $k_5 = k_{-4}$ (i.e. rate of hydrolysis of EP = rate of dissociation of G16P from ES) then $K_{G16P}$ is independent of [G1P]. Under these circumstances an intersecting line initial velocity pattern will be observed even though the enzyme mechanism is essentially ping-pong. As $k_5 \longrightarrow 0$ both $K_m$'s become dependent on the other substrate and in the absence of inhibition by G1P and G16P a parallel line pattern will be obtained.

These conclusions are important for although little or no

phosphatase activity has been demonstrated for phosphoglucomutase, and it has been possible to isolate a stable EP complex (see Historical survey) the rate of dissociation of G16P from the central enzyme complex is also small[1] (see also induced transport tests section). In the case of phosphoglycerate-mutase though little phosphatase activity has been demonstrated it has proved difficult to isolate an EP complex which suggests that hydrolysis takes place (see Historical survey) and though G16P readily dissociate from ES at high ionic strength the rate of dissociation becomes much less as the ionic strength is lowered[57]. Consequently the initial velocity pattern will depend upon the precise balance of these two factors and it cannot be assumed that phosphatase activity can be ignored.

To consider the effect of including an EP isomerization step on the $K_m$'s it has been assumed that no inhibition by G16P or G1P occurs and that there is no phosphatase activity in order to simplify the kinetic expressions obtained. The initial velocity of reaction for this mechanism, in the absence of the product, G6P, can be derived from equations T.1.3 and T.1.5 in the preceding section. Thus:-

$$V_i = \frac{k_1k_2k_3(E)}{\frac{(k_3+k_{-3})(k_{-1}+k_2)}{[G1P]} + \frac{k_3k_4}{k_{-4}[G16P]} + k_1(k_2+k_3)} \quad \ldots \quad T.2.8.$$

where (E) is the amount of enzyme present.

From eq. T.2.8:-

$$V_{max} = \frac{k_2k_3(E)}{(k_2 + k_3)} \quad \ldots\ldots \quad T.2.9.$$

$$K_{G16P} = \frac{k_3k_4}{k_{-4}k_1(k_2 + k_3)} \quad \ldots\ldots \quad T.2.10.$$

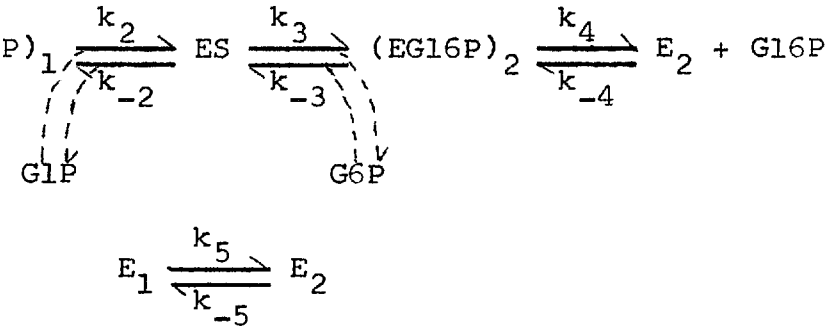
$$K_{G1P} = \frac{(k_3 + k_{-3})(k_{-1} + k_2)}{k_1(k_2 + k_3)} \quad \ldots\ldots \quad T.2.11.$$

If there is no isomerization of the enzyme then the form of the rate equation T.2.8 remains the same and may be obtained by setting $k_3 = k_{-3} = \infty$ . The expression for the $K_m$ of G1P (eq. T.2.5) will differ by a factor of 2 from that obtained by assuming that no isomerization occurs. A parallel line initial velocity pattern will be obtained. This equation is analagous to that derived by Cleland for an iso ping-pong mechanism by the King and Altman procedure[74].

Mechanism involving an enzyme-cofactor complex

A mechanism which involves a sequential and ordered addition of substrates and on isomerization of the free enzyme has been proposed for this reaction, see Scheme 2 below. Such a reaction in the terminology of Cleland[53] is called an iso ordered bi-bi mechanism. In this mechanism both G1P and G16P will be attached to the central enzyme complex as shown:-

Scheme 2



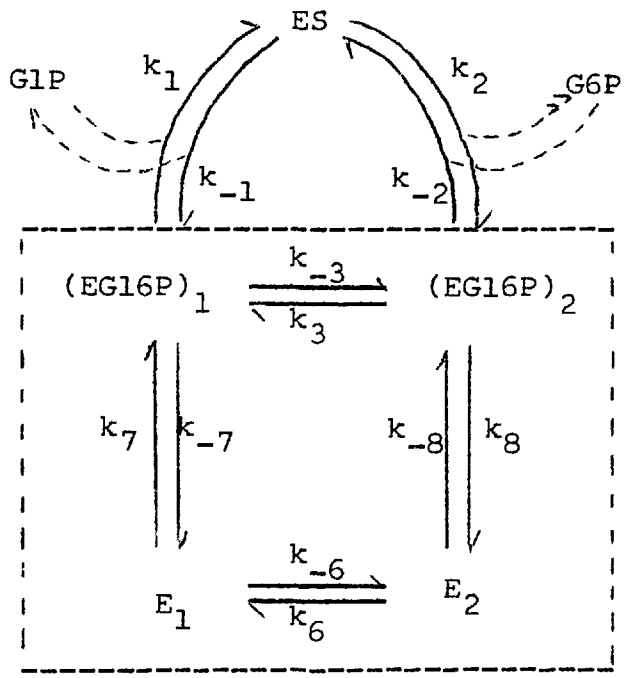

According to Cleland who used the King and Altman procedure[53, 74] the initial velocity of reaction (in his terminology) is given by:-

$$Vi = \frac{V_{max}}{1 + \frac{K_A}{A} + \frac{K_B}{B}\left(1 + \frac{K_{iA}}{A}\right)} \qquad \text{..... T.2.12}$$

where $K_{iA}$ is the inhibition constant of A.

This model, however, is not a realistic mechanism for Scheme 2 since $[EG16P]_2$ must isomerize to $[EG16P]_1$ at least occasionally to take into account the fact that exchange between monophosphates and diphosphate is slower than the overall rate of reaction. Furthermore Cleland's analysis appears not to take into account the fact that G16P will cause product inhibition since it is a product as well as a substrate. A more general scheme for this mechanism in which these points are considered is shown in Scheme 3 below.

Scheme 3



The first step in calculation of the initial velocity of reaction is to determine the values of $(E)_1$ and $(E)_2$ in terms of $(EG16P)_1$ and $(EG16P)_2$. This can be done using the method of Britton[64] to calculate the fluxes between different enzyme species, from which the overall fluxes can be calculated (see also section T.1 for determination of fluxes for mechanism 3, Scheme T.1.3). Thus for $(E)_1$, under steady state conditions:-

$$[G16P]\, k_7(E)_1 + \frac{k_{-6}k_{-8}[G16P](E)_1}{(k_6 + k_{-8}[G16P])} = k_{-7}(EG16P)_1 + \frac{k_8k_6(EG16P)_2}{(k_6+k_{-8}[G16P])}$$

..... T.2.13

and for $(E)_2$:-

$$k_{-8}[G16P](E)_2 + \frac{k_6k_7[G16P](E)_2}{(k_{-6}+k_7[G16P])} = k_8(EG16P)_2 + \frac{k_{-7}k_{-6}(EG16P)_1}{(k_{-6}+k_7[G16P])}$$

..... T.2.14.

Solving equations T.2.13 and T.2.14 an eq., T.2.15 was obtained where the constants $\alpha$ and $\beta$ are given by the expressions shown in equations T.2.16 and T.2.17 respectively.

$$(E)_1 + (E)_2 = \alpha(EG16P)_1 + \beta(EG16P)_2 \quad \text{..... T.2.15}$$

where;

$$\alpha = \frac{k_{-7}(k_6 + k_{-8}[G16P])}{k_7[G16P](k_6 + k_{-8}[G16P]) + k_{-6}k_{-8}[G16P]}$$

$$+ \frac{k_{-7}k_{-6}}{k_{-8}[G16P](k_7[G16P] + k_{-6}) + k_6k_7[G16P]} \quad \text{..... T.2.16}$$

$$\beta = \frac{k_8k_6}{k_7[G16P](k_6 + k_{-8}[G16P]) + k_{-6}k_{-8}[G16P]}$$

$$+ \frac{k_8(k_7[G16P] + k_{-6})}{k_{-8}[G16P](k_7[G16P] + k_{-6}) + k_6k_7[G16P]} \quad \text{..... T.2.17}$$

Calculating the flux from $(EG16P)_1$ to $(EG16P)_2$ and <u>vice versa</u> <u>via</u> $(E)_1$ and $(E)_2$ by the intermediate pool method of Britton[64] it follows that the total fluxes are given by the expressions below:-

$$\text{Flux } (EG16P)_1 \longrightarrow (EG16P)_2$$

$$= \left( \frac{k_{-7}k_{-6}k_{-8}}{k_{-8}(k_7[G16P] + k_{-6}) + k_6k_7} + k_{-3} \right) (EG16P)_1 = p(EG16P)_1$$

..... T.2.18

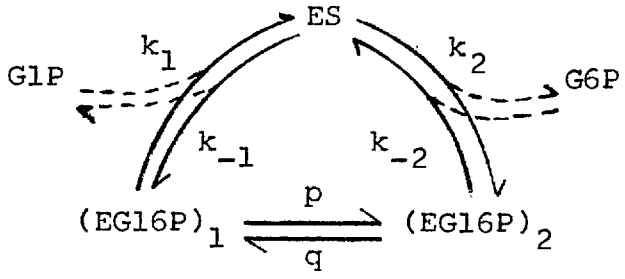
$$\text{Flux } (EG16P)_2 \longrightarrow (EG16P)_1$$

$$= \left( \frac{k_7k_6k_8}{k_{-8}(k_7[G16P] + k_{-6}) + k_6k_7} + k_3 \right) (EG16P)_2 = q(EG16P)_2$$

..... T.2.19

Thus it is possible to replace the steps in the dashed rectangle in Scheme 3 by a single reaction step with rate constants p and q. Thus Scheme 3 may now be represented as shown in Scheme 3A.

Scheme 3A



Under steady state conditions $(EG16P)_1$ and $(EG16P)_2$ can be expressed in terms of (ES). Thus using the intermediate pool technique of Britton[64] it can be shown that:-

for $(EG16P)_1$ in the absence of $[G6P]$

$$k_1(EG16P)_1[G1P] = (k_{-1} + k_2)(ES) \quad \text{..... T.2.20}$$

for $(EG16P)_2$

$$\frac{k_1 q[G1P](EG16P)_2}{([G1P]k_1 + p)} = \left( k_2 + \frac{k_{-1}p}{(p + k_1[G1P])} \right) (ES) \quad \text{..... T.2.21.}$$

For total enzyme concentration $(E_T)$:-

$$(E_T) = (ES) + (EG16P)_1(1 + \alpha) + (EG16P)_2(1 + \beta) \quad \text{... T.2.22.}$$

Hence ES can be expressed in terms of $E_T$ by solving eqs. T.2.20-22. Now as the initial velocity of reaction is $k_2(ES)$ the initial velocity expression may be written:-

$$\frac{1}{V} = \frac{1}{k_2(ES)} = \frac{1}{k_2(E_T)} \left( 1 + \frac{(k_{-1} + k_2)}{k_1[G1P]}(1 + \alpha) \right.$$

$$\left. \left( \frac{k_2(p + k_1[G1P]) + k_{-1}p}{k_1 q[G1P]} \right) (1 + \beta) \right) \quad \text{... T.2.23.}$$

This equation in its general form does not give Michaelis-

Menten kinetics.

Eq. T.2.23 derived above for the general mechanism for a sequential mechanism (Scheme 3) may now be simplified for various specific conditions so that the form of the initial velocity plots may be deduced. For example if $k_3 = k_{-3} = 0$ (i.e. if (EG16P) does not isomerize into $(EG16P)_2$) then the mechanism becomes the same as that proposed in Scheme 2 for an iso ordered bi-bi mechanism. The form of the rate equation is shown below in eq. T.2.24. p and q have been expressed in terms of the original rate constants.

$$\frac{1}{V} = \frac{1}{k_2(E_T)}\left( 1 + \frac{(k_{-1} + k_2)}{k_1[G1P]}(1 + \alpha) \right.$$

$$\left. + \left( \frac{k_2(k_7k_6 + k_{-6}k_{-8} + k_7k_{-8}[G16P])}{k_6k_7k_8} + \frac{k_{-6}k_{-7}k_{-8}(k_{-1}+k_2)}{k_1k_6k_7k_8[G1P]} \right)(1+\beta) \right)$$

..... T.2.24

where $\alpha$ and $\beta$ have the values given in eqs. T.2.16 and T.2.17 respectively. By inspection of the equations it can be shown that for G16P non-linear initial velocity plots are obtained, though linear plots will be obtained for G1P. The form of this equation is thus different from Cleland's equation (T.2.12). The difference probably arises due to

G16P inhibition, which will be present even when [G6P] = 0.

The form of the equation can be further simplified by setting $k_6 = k_{-6} = \infty$ (i.e. there is only one form of the enzyme E. Under these circumstances the following equation is obtained:-

$$\frac{1}{V} = \frac{1}{k_2(E_T)}\left( 1 + \frac{(k_{-1} + k_2)}{k_1[G1P]}\left(1 + \frac{k_{-7}k_{-8}}{k_7k_8}\right) + \frac{k_2(k_7 + k_{-8})}{k_7k_8} \right.$$

$$+ \frac{2(k_{-1} + k_2)k_{-7}}{(k_7 + k_{-8})k_1}\left(1 + \frac{k_{-8}}{k_7}\right)\frac{1}{[G16P]\,[G1P]}$$

$$\left. \frac{k_{-7}(k_{-1} + k_2)}{k_1k_7k_8} + \frac{2k_2}{k_7[G16P]} \right) \qquad \text{.....} \quad \text{T.2.25}$$

Straight line initial velocity plots are obtained for G1P and G16P. In general the lines will converge but parallel lines will be obtained if $k_{-7} \ll k_1$.

One further alternative is to put $k_6 = k_{-6} = 0$ (i.e. $(E)_1$ and $(E)_2$ cannot isomerize). The initial velocity equation then takes the form shown in eq. T.2.26 below.

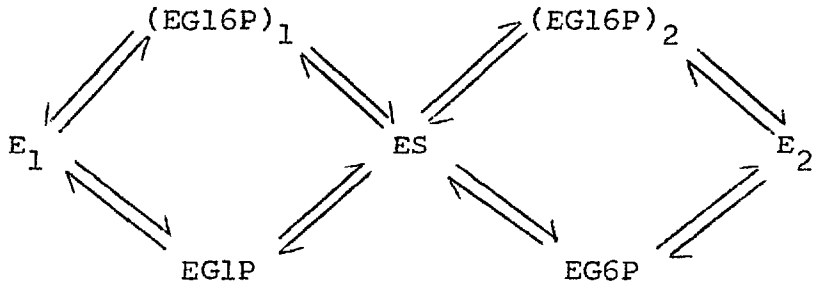
$$\frac{1}{V} = \frac{1}{k_2(E_T)}\left(1 + \frac{k_2}{k_3} + \frac{(k_{-1} + k_2)}{k_1[G1P]}\left(1 + \frac{k_{-3}}{k_3}\right) + \frac{k_2 k_8}{k_{-3}k_{-8}[G16P]}\right.$$

$$\left. + \frac{(k_{-1} + k_2)}{k_1[G1P][G16P]}\left(\frac{k_{-7}}{k_7} + \frac{k_8 k_{-3}}{k_{-8}k_3}\right)\right) \quad \ldots\ldots \text{T.2.26}$$

Straight line initial velocity plots are obtained for G1P and G16P with an intersecting line pattern.

This treatment shows that an iso ordered bi-bi mechanism does not necessarily lead to intersecting Lineweaver-Burke plots that under certain circumstances both non-linear and parallel-line plots may be obtained. Consequently it becomes difficult to distinguish between a sequential and a EP mechanism on the basis of the pattern of Lineweaver-Burke plots. Furthermore the possibility must be considered that the cofactor may dissociate from the central, ES, complex and that the reaction is iso-random bi-bi sequential, as shown in Scheme 4.

Scheme 4



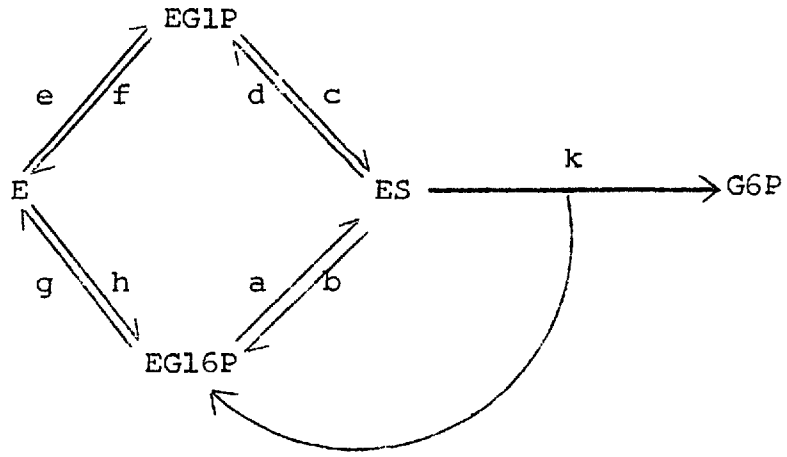

$$(EG16P)_1 \rightleftharpoons (EG16P)_2$$

$$E_1 \rightleftharpoons E_2$$

The initial velocity pattern for Scheme 4 was calculated by Chiba et al.[122] in the case where there is rapid equilibration of all steps (except the interconversion of the ES complex. In the concentration range where only substrate (G1P) inhibition occurs they concluded that $\frac{1}{G16P}$ plots are always linear, and convergent, whereas $\frac{1}{G1P}$ plots are linear and convergent only at low substrate concentrations. At high concentrations the plots become curved. They concluded also that a random sequential mechanism can always be distinguished kinetically from an EP (ping-pong) mechanism in the non-inhibitory concentration range as the latter has

parallel straight line plots for $\frac{1}{G1P}$ and $\frac{1}{G16P}$ (though intersecting line-plots are obtained at high concs of G1P and G16P). However, for the random sequential mechanism there is an intermediate concentration range in which $\frac{1}{G16P}$ plots become almost parallel especially if $K_{iG1P} \simeq K_{G1P}$ though it is still possible to distinguish this mechanism from the EP mechanism by drawing appropriate secondary plots[122]. These conclusions are only valid if the addition of substrates is fast. In the following treatment this assumption is not made. A general rate expression for Scheme 4 would be very complicated and would involve terms with $[G1P]^2$ and $[G16P]^2$ factors giving non-linear Lineweaver-Burke plots. It is possible, however, to preserve the important features of the reaction in a simplified scheme, Scheme 5, which is much more amenable to analysis. Only the forward reaction (G1P to G6P) is represented and the major pathway of reaction is assumed to be the addition of G1P to EG16P. The rate constants c, d, e, f, g and h, are assumed to be slow in comparison to a and b as the rate of exchange between monophosphates and diphosphates is small compared with the rate of conversion of G1P to G6P (or 2PGA to 3PGA at low $\mu$). An isomerization of EG16P is not considered.

Scheme 5




The concentrations of ES in terms of $E_T$, the total enzyme concentration may be calculated by the method of Britton[61] assuming steady state conditions as follows:

for (ES):-

$$(ES)(b + k) = (EG16P)a[G1P] \qquad \text{.....} \quad T.2.26$$

for (E):-

$$g(EG16P) + \frac{df(ES)}{(f + c[G16P])} = h[G16P](E) + \frac{ce[G1P][G16P](E)}{(c[G16P] + f)}$$

..... T.2.27

for (EG1P):-

$$d[G16P](EG1P) + \frac{(EG1P)fh[G16P]}{(e[G1P] + h[G16P])} = (ES)d + \frac{(EG16P)ge[G1P]}{(e[G1P] + h[G16P])}$$

..... T.2.28

and also $(E)_T = (ES) + (EG1P) + (E) + (EG16P)$ ..... T.2.29.

From equations T.2.26-29 initial velocity of reaction $V_i$ can be calculated

$$V_i = k(ES) = \frac{k(E)_T}{(1 + A + B + C)} \quad \text{..... T.2.30}$$

where:-

$$A = \frac{[G1P]dea + [G16P]dha + (b + k)ge}{a[G16P](ce[G1P] + ch[G16P] + fh)} \quad \text{..... T.2.31}$$

$$B = \frac{[G16P]gc(b + k) + dfa[G1P] + fg(b + k)}{a[G1P][G16P](ce[G1P] + ch[G16P] + fh)} \quad \text{..... T.2.32}$$

and:-

$$C = \frac{b + k}{a[G1P]} \quad \text{..... T.2.33.}$$

Under certain conditions equations T.2.30-33 can be further simplified, for example, by imposing restrictions upon individual rate constants. Suppose the rate of breakdown of EG16P was slow, i.e. the constant g is small. Then equation T.2.30 becomes:-

$$Vi = \frac{k(E)_T}{1 + \frac{(b+k)}{a[G1P]} + \frac{1}{[G16P]}\left(\frac{de[G1P] + dh[G16P] + df}{ce[G1P] + ch[G16P] + fh}\right)} \quad \ldots \quad T.2.34$$

If the further condition is added that the rate of breakdown of EG1P, f, is also small, the equation T.2.34 becomes:-

$$Vi = \frac{k(E)_T}{1 + \frac{(b+k)}{a[G1P]} + \frac{d}{c[G16P]}} \quad \ldots\ldots \quad T.2.35.$$

This equation has the same form as that of equation T.2.8, for the ping-pong mechanism of reaction, and therefore will give a parallel line pattern when the initial velocities are represented upon a Lineweaver-Burke plot. Equation T.2.34 is the result of making a particular pair of rate constants (one in each direction) rate limiting. This treatment can be extended to cover all the possible pairs of rate constants and the type of kinetic pattern obtained in each case has been

Table T.2.1.

Scheme showing kinetic initial velocity pattern of Lineweaver-Burke plots from possible combinations of rate limiting reaction steps making the assumption that the constants a and b represent the major reaction pathway at all times. Changes in the expected pattern for extra boundary conditions are also listed.

| Limiting Rate Constants | d | e | f | g | h |
|---|---|---|---|---|---|
| c | sequential<br>ping-pong if $\frac{g}{h}$ small | sequential<br>ping-pong if $\frac{g}{h}$ small | no reaction | non-linear plots<br>Simple Michaelis if $\frac{de}{fh}$ small | no reaction |
| d | - | non-linear plots<br>simple Michaelis if g small | non-linear plots<br>simple Michaelis if g small | simple Michaelis | non-linear plots<br>simple Michaelis if g small |
| e | | - | sequential<br>ping-pong if $\frac{g}{h}$ small | non-linear plots<br>ping-pong if c or f small | no reaction |
| f | | | - | ping-pong | non-linear plots<br>ping-pong if g small |
| g | | | | - | sequential<br>ping-pong if $\frac{df}{ce}$ small |

listed in Table T.2.1.

Thus it can be seen that there is a wide variety of kinetic behaviour possible for this reaction and consequently the use of initial velocity patterns on Lineweaver-Burke plots as a test of reaction mechanism does not necessarily give any useful information. It is interesting to notice that in the case in which e and f are the limiting rate constants the reaction will appear to be either sequential or ping-pong deoending upon the relative affinities of the enzyme E, and the enzyme-substrate complex EG1P, for the cofactor EG16P.

It should be pointed out that most of the possible combinations of rate limiting steps postulated in Table T.2.1. are quite feasible in terms of the mechanism of reaction. One particularly likely combination is for g and f to be slow. Thus a ping-pong kinetic pattern will arise if the enzyme has a very high affinity for the cofactor and if the E-substrate complex rarely breaks down. This would offer an attractive explanation for the fact that the rabbit-muscle enzyme has been isolated as a 23PGA-enzyme complex[75].

## SECTION 3. CALCULATION OF MAGNESIUM BINDING CONSTANTS WITH 8-HYDROXYQUINOLINE AND 2- AND 3-PHOSPHOGLYCERATE

The apparent binding constant $K_{1app}$, between magnesium ions and 8-hydroxyquinoline can be calculated from the initial gradients of optical density titrations of magnesium chloride against 8-hydroxyquinoline at 360 nm as described in the methods section M.2. If no account was taken of the effects of pH, and the formation of species other than $MgOx^+$, where Ox is the undissociated form of 8-hydroxyquinoline, the equilibria in solution can be written as:-

$$Mg^{2+} + Ox \rightleftharpoons MgOx^+ + H^+.$$

If $E_1$ is the extinction coefficient of $MgOx^+$, and $E_o$ the extinction coefficient of Ox, at 360 nm, then the increase in optical density at 360 nm, $\Delta$ OD, upon the addition of $Mg^{2+}$ ions to the solution can be written as in equation T.3.1.

$$\Delta \text{ OD} = (E_1 - E_o)[MgOx^+] \quad \ldots\ldots \quad \text{T.3.1}$$

where:-

$$K_{1app} = \frac{[MgOx^+][H^+]}{[Mg^{2+}][Ox]} \quad \ldots\ldots \quad \text{T.3.2}$$

and

$$[Mg^{2+}]_{Total} = [Mg^{2+}]_{free} + [MgOx^+] \quad \text{T.3.3.}$$

Substituting equations T.3.2 and T.3.3 in T.3.1:

$$\frac{\Delta\ OD}{[Mg^{2+}]_{Tot}} = \frac{K_{1app}(E_1 - E_0)[Ox]}{([H^+] + K_{1app}[Ox])} \qquad \text{..... T.3.4.}$$

At low concentrations of added magnesium, $[Ox] \simeq [Ox]_{Total}$ and let the initial optical density gradient $\frac{\Delta\ OD}{[Mg^{2+}]_{Tot}} = A.$

Then:-

$$A = \frac{K_{1app}(E_1 - E_0)[Ox]_{Tot}}{([H^+] + K_{1app}[Ox]_{Tot})} \qquad \text{..... T.3.4.}$$

Rearranging and putting $(E_1 - E_0)[Ox]_{Tot} = OD_{max}$ (from equation T.3.1 we have:-

$$K_{1app} = \frac{A[H^+]}{(OD_{max} - [Ox]_{Tot}.A)} \qquad \text{..... T.3.5.}$$

This equation did not give a constant value of $K_{1app}$ over the range of pH that optical density titrations were carried out, and consequently a more complete replication of the system was considered. Contributions from the ternary

complexes were taken into account, particularly the $Mg(Ox)_2$ complex, which had been observed by Burton (see results section R.4). It was also decided that there was the possibility of interaction of magnesium and its 8-hydroxyquinoline complexes with the Tris buffer in which the optical density determinations were carried out. Consequently the occurrence of the following equilibria in solution were considered:-

$$Mg^{2+} + Ox \overset{K_1}{\rightleftharpoons} MgOx^{+} + H^{+}$$

$$MgOx^{+} + Ox \overset{K^1}{\rightleftharpoons} Mg(Ox)_2 + H^{+}$$

$$Mg^{2+} + Tris \overset{K_3}{\rightleftharpoons} MgTris^{2+}$$

$$MgTris^{2+} + Ox \overset{K_4}{\rightleftharpoons} MgTrisOx^{+} + H^{+}$$

$$Tris + H^{+} \overset{K_5}{\rightleftharpoons} Tris\ H^{+}$$

$$Ox \overset{K_6}{\rightleftharpoons} Ox^{-} + H^{+}$$

where the K's represent the appropriate binding constants.

In order to simplify calculation the extinction coefficient of Mg- 8-hydroxyquinoline complex was assumed to be unaffected by the addition of further ligands to magnesium ion. Hence by a calculation identical to the one above, the

expression, equation T.3.6, was derived for the initial gradient of the optical density titration.

$$A = \frac{(E_1 - E_0)\left[\frac{K_1[Ox]}{[H^+]}\left(1 + \frac{2K^1[Ox]}{[H^+]} + \frac{K_3K_4K_5[Tris]}{K_1([H^+] + K_5)}\right)\right]}{\left(1 + \frac{K_1[Ox]}{[H^+]} + \frac{K_1K^1[Ox]^2}{[H^+]^2} + \frac{K_3K_5[Tris]}{([H^+]+K_5)}\left(1 + \frac{[Ox]K_4}{[H^+]}\right)\right)} \quad \ldots\ldots \text{T.3.6.}$$

Where A is the initial gradient (optical density x total magnesium conc$^{-1}$) and [Ox] and [tris] represent the total concentrations of 8-hydroxyquinoline and tris buffer respectively.

As tris binding of the various species containing magnesium can be shown to be negligible except at very high pH, equation T.3.6 can be simplified to the equation shown below:-

$$A = \frac{aK_1}{[H^+]} \frac{(1 + 2K^1[Ox][H^+]^{-1})}{(1 + K_1[Ox][H^+]^{-1} + K_1K^1[Ox]^2[H^+]^{-2})} \quad .. \text{T.3.7.}$$

Where a represents the maximum optical density at 360 nm when all the 8-hydroxyquinoline is complexed by

magnesium and the other quantities have the same definitions as before. If the titration is carried out in the presence of added phosphoglycerate then equation T.3.7 can be modified to account for this by the inclusion of a suitable term in the denominator. Such an equation has been derived by the method outlined above and it can be found in results section 4 as equation R.4.3. The difference between the initial optical density gradient, A, in the absence of added phosphoglycerate, and the initial gradient B, in the presence of phosphoglycerate is described by equation T.3.8, which was obtained by subtracting equation T.3.7 from R.4.3.

$$\frac{1}{B} - \frac{1}{A} = \frac{K_2 K_i (K_i + [H^+])^{-1} [PGA]_{Total}}{K_1 a( \quad + 2K^1 [Ox] [H^+]^{-1})} \qquad \ldots\ldots \quad T.3.8$$

where $K_2$ is the binding constant between magnesium and 2- or 3-phosphoglycerate, and $K_i$ is the appropriate 3rd acid dissociation constant of the phosphoglycerate.

Equilibration constant of the phosphoglyceratemutase reaction

The apparent equilibrium constant of the phosphoglycerate mutase reaction will be a measure of ratio of the total 2- and 3-phosphoglycerate concentrations when determined by the NADH coupled assay described in the methods section M.2.

Thus:-

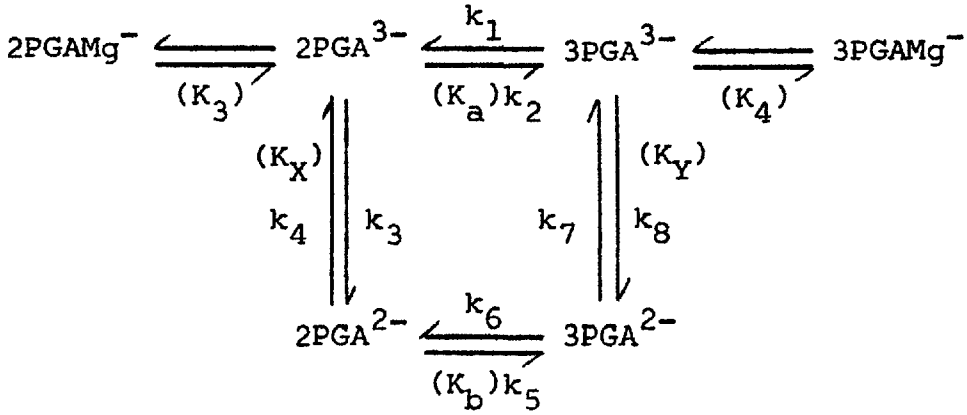
$$Keq_{app} = \frac{[3PGA]_{Total}}{[2PGA]_{Total}} \qquad \text{..... T.3.9.}$$

Under the conditions of the experiment, approximately neutral pH, and in the presence of $[Mg^{2+}]$ ions, equation T.3.9 becomes:-

$$Keq_{app} = \frac{[3PGA^{3-}] + [3PGA^{2-}] + [3PGAMg^{-}]}{[2PGA^{3-}] + [2PGA^{2-}] + [2PGAMg^{-}]} \qquad \text{..... T.3.10.}$$

Now as there is no evidence to suggest that rabbit-muscle phosphoglycerate mutase catalyses only the interconversion of the fully ionized species; $2PGA^{3-}$, and $3PGA^{3-}$ (48), the system at equilibrium will be represented by Scheme 1 below.

Scheme 1



As discussed in the results section, R.5, the change in $K_{eq\ app}$ with pH and $[Mg^{2+}]_{Free}$ is unaffected by the pathway of conversion. Thus $K_a$ and $K_b$ are not independent and are related as shown below:

$$k_1k_3k_5k_7 = k_2k_4k_6k_8 \quad \text{..... T.3.11.}$$

Hence:

$$\frac{K_a}{K_b} = \frac{K_y}{K_x} \quad \text{..... T.3.12.}$$

The expression for the expected variations in $K_{eq\ app}$ in terms of pH, $[Mg^{2+}]_{Free}$, and $K_a$ can be obtained from equation T.3.10 and is shown below:-

$$K_{eq\ app} = \frac{[3PGA^{3-}](1 + K_4[Mg^{2+}] + \frac{[H^+]}{K_y})}{[2PGA^{3-}](1 + K_3[Mg^{2+}] + \frac{[H^+]}{K_x})} \quad \text{.. T.3.13}$$

Where: $$K_a = \frac{[3PGA^{3-}]}{[2PGA^{3-}]}$$

# CHAPTER 5

# DISCUSSION

## INTRODUCTION

The investigations carried out in this thesis have been designed to elucidate and compare the mechanisms of action of rabbit-muscle phosphoglucomutase and rabbit-muscle phosphoglyceratemutase. This has been done by comparing the results of Induced transport tests, carried out on each enzyme as described in the previous sections. Unlike investigations of enzyme mechanism by isolation of reaction intermediates or kinetic studies these tests are less subject to the ambiguities to which more traditional methods are prone (see historical section and below). However, in order that an unambiguous assignment of mechanism could be made for phosphoglycerate-mutase it was necessary to carry out a series of preliminary experiments to establish the values of the equilibrium constant, the ratio of the maximum velocities of reaction in the forwards and reverse direction, and the $K_m$'s of 2PGA and 3PGA. These experiments were carried out at constant free magnesium ion concentration and consequently it was also necessary to establish the binding constants of magnesium with 2PGA and 3PGA under the appropriate

experimental conditions. The results obtained in these preliminary experiments are considered below.

## SECTION 1. PRELIMINARY INVESTIGATIONS OF PHOSPHOGLYCERATE MUTASE SYSTEM. BINDING CONSTANTS OF MAGNESIUM WITH 2PGA AND 3PGA

A preliminary study of magnesium-8-hydroxyquinoline binding was carried out under appropriate experimental conditions as described previously, which served partly as a test of the method employed. The binding constant obtained ($4.425 \times 10^{-6}$) and that of Näseänen[29], interpolated for $\mu = 0.02$ ($4.613 \times 10^{-6}$) were very similar and as $Mg(Ox)_2$ binding is not very important under the conditions of experiment this indicated that the use of changes in optical adsorption at 360 nm was a good way of following changes in magnesium-8-hydroxyquinoline binding at least in the region of pH 7.4. Unfortunately at both lower and higher pH's the method becomes unreliable (see results section) and consequently very little information could be obtained about the extent of formation of $Mg(Ox)_2$ and $Mgtris^+$. However the contribution of both of these complexes to magnesium binding is very small below pH 8 so that their contributions can be largely ignored.

The method in which the magnesium binding constants of 2PGA and 3PGA are measured by allowing them to compete for free magnesium ions with 8-hydroxyquinoline is not very sensitive even in the region of pH 7.4. The magnesium binding

constants of the phosphoglycerates are not very large so that the difference between the initial optical density gradients in the presence and absence of added phosphoglycerate is comparatively small. Consequently as the method relies on measuring this difference to evaluate the magnesium-phosphoglycerate binding constants experimental error is bound to be large. Though the standard deviation is approximately $\pm$ 7% this compares very favourably with that of other methods. Unfortunately, however, it was not possible to study the effect of pH upon the magnesium-phosphoglycerate binding constants due to the limitations of the method outlined previously. Thus it was not possible to check the statement by Wold and Ballou[38] that magnesium binds only with the triply ionized form of the phosphoglycerates. In the absence of other evidence their contention was accepted (see later).

The possibility that teriary complexes, for example $[Mg\ PGA_2]^{4-}$, $[Mg\ PGA\ Ox]^{2-}$, and $[Mg_2\ PGA]^{+}$ were formed in solution was considered but no evidence has been found for their formation. It was expected that if they were formed at all their formation constants would be small compared to the binary complexes and that therefore negligible amounts would be formed in solution under experimental conditions. Consequently their contribution to magnesium binding could be neglected in all calculations.

It proved to be difficult to compare the values of the magnesium binding constants obtained in this work and values reported by other authors. In the case of 3PGA though Pizer in a private communication reported a value of 480 $M^{-1}$ compared with the present value of 302 $M^{-1}$ no comparison was possible as Pizer did not give the experimental conditions under which the binding constant was measured. Wold and Ballou[38] measured the 2PGA binding constant at 25°C with a potentiometric titration technique and found values of 280 $M^{-1}$ at $\mu$ = 0.1 and 180 $M^{-1}$ at $\mu$ = 0.4. In the present investigation a somewhat larger value, 582 $M^{-1}$ was obtained at $\mu$ = 0.02 and at 30°C. Though the effect of temperature upon the binding constant is uncertain it should not be large. However, the difference in ionic strength may affect the activity coefficients of the ionic species sufficiently to account for much of the difference in the binding constant.

It is of interest to compare the magnesium binding constants of the glucose phosphates from the phosphoglucomutase system with the phosphoglycerates. These are shown in Table D.1.1 below.

Table D.1.1.

| hexose phosphates | $Mg^{2+}$ binding constant (Ref) | triose phosphates | $Mg^{2+}$ binding constant (Ref) |
|---|---|---|---|
| $G1P^{2-}$ | 91 $M^{-1}$ ) | $2PGA^{2-}$ | - $M^{-1}$ |
| $G6P^{2-}$ | 63 $M^{-1}$ ) | $3PGA^{2-}$ | - $M^{-1}$ |
| $G16P^{3-}$ | 380 $M^{-1}$ ) (6, 28, 35) | $2PGA^{3-}$ | 582 $M^{-1}$ ) this |
| | | $3PGA^{3-}$ | 302 $M^{-1}$ ) thesis |
| $G16P^{4-}$ | 1500 $M^{-1}$ ) | $23PGA^{(4-,\ 5-)}$ | 1111 $M^{-1}$ (108) |

As can be seen from this table the main factor influencing the binding constant appears to be the charge of the ligand. There is only a four fold increase in binding constant between the G1P dianion and the G16P trianion, just as there is only a four fold increase between the G16P trianion and tetra-anion. Consequently bidentate effects involving both phosphate groups would appear to be unimportant for these hexose phosphates. The effect of charge on the binding constant for the phosphoglycerates parallels that for the glucose phosphates, and for both glucose and glycerate phosphates similarly charged species have binding constants of the same order of magnitude. Consequently it appears that

bidentate effects are unimportant for the diphosphoglycerate and that the main determining factor for binding constant is charge. The similarity between the binding constants of similarly charged species suggests that $2PGA^{2-}$ and $3PGA^{2-}$ will possess magnesium binding constants of the order of 90 $M^{-1}$ though Wold and Ballou[38] suggested that no binding occurs. Unfortunately the pK's of 2PGA and 3PGA are in the region of pH 7.0 so that in order to study the magnesium binding of the dianions the pH of solution would have to be lowered below pH 6.50. Although this was done the method proved to be too insensitive in this pH region to give meaningful results. However, it can be shown with models that both $2PGA^{2-}$ and $3PGA^{2-}$ can be arranged without distortion so that the two charged oxygen atoms of the dianion are very close to hydroxyl hydrogen atoms. Hydrogen bonding would then lead to the formation of stable 8 membered ring structures. In contrast with $G1P^{2-}$ or $G6P^{2-}$ ring structures with only one hydrogen bond can be formed. Consequently $2PGA^{2-}$ and $3PGA^{2-}$ may lose the ability to complex magnesium though $G1P^{2-}$ and $G6P^{2-}$ clearly retain this power. Further studies under the conditions suggested above will be necessary to resolve this point satisfactorily.

Equilibrium constant of the phosphoglycerate mutase reaction

In the thermodynamic sense the pathway of conversion of 3PGA to 2PGA is unimportant and thus the equilibrium constant will be independent of the actual mechanism of reaction. Consequently it is not necessary to show whether each of $3PGA^{3-}$, $3PGA^{2-}$ and $3PGAMg^{-}$ (and each of the corresponding 2PGA species) may act as substrates for the enzyme. (Though this will be important in considering the changes of $V_{Fmax}$ and $V_{Rmax}$ with pH and $[Mg^{2+}]_{free}$.)

The equation for the equilibrium constant R.5.1 contains terms involving the dissociation constant of magnesium and the third ionic dissociation constant of 2PGA and 3PGA. Thus as each of these constants depends upon ionic strength it would appear that the apparent equilibrium constant, $K_{eq}^{app}$ will also be ionic strength dependent. However, as the di- and trianions of 2PGA and 3PGA, and their magnesium complexes are of the same size and have very similar charge distributions their activity coefficients will be very similar over a wide range of ionic strength. Consequently it is expected that $K_{eq}^{app}$ will become comparatively independent of ionic strength. This system should behave very similarly to the phosphoglucomutase system for which the actively coefficients of G1P and G6P have been shown to be the same over a range of ionic strength and for which the equilibrium constant is ionic

strength independent[33].

In the absence of $Mg^{2+}$ the apparent $K_{eq}$ will have a limiting value of 3.72 at low pH and a limiting value of 20.45 at high pH. The most rapid change in $K_{eq\ app}$ with pH will occur in the region of the pK's of $2PGA^{3-}$ and $3PGA^{3-}$ (i.e. pH 6.7-7.4). Though some measurements of $K_{eq\ app}$ with pH have been made over the range pH 5-7 it was reported that no change occurred in the value of $K_{eq\ app}$[44]. However, it seems most unlikely that there would be no change in this region. The limiting value of $K_{eq\ app}$ for low pH, 3.72, agrees fairly well with measurements made by Ballou and Fischer[41] (made at pH 0), who obtained values of 4.1 and 4.0 (see results section R.5, Table R.5.1).

Kinetic investigation of phosphoglycerate-mutase reaction

The previous kinetic investigation carried out by Grisolia and Cleland[54] was conducted at substrate concentrations such that 23PGA (0.15-6000 μM) sometimes $<$ $K_{i23PGA}$ and 3PGA (1-40 mM) always $>$ $K_{i3PGA}$ (constants measured in this thesis). Under these conditions the initial rate expressions for the ping-pong and sequential mechanisms of reaction become indistinguishable (see Historical Survey). Consequently it was impossible to see the basic kinetic

pattern (on Lineweaver-Burke plots) as this was overlaid by 3PGA inhibition. Moreover, at the high substrate concentrations employed potentiation of the enzyme could occur and this would also obscure the basic kinetic pattern. Due to the limitations of the enolase coupled assay system at low 3PGA concentrations it was not possible to carry out kinetic investigations at sufficiently low concentration of it to reduce 3PGA inhibition to an acceptable level (i.e. 3PGA $< \frac{1}{2}K_{i3PGA}$). However, the $NAD^+$/NADH coupled assay system developed in this thesis was capable of extending the range of substrate concentrations to the required levels. Thus a series of initial rate measurements were made in the range of substrate concentration; 3PGA 0.5-0.02 mM; 23PGA, 50-0.2 μM i.e. for 3PGA $< K_{i3PGA}$ and 23PGA $< K_{i23PGA}$. Under these circumstances an ordered sequential mechanism should give rise to an intersecting line initial velocity plot, and a ping-pong mechanism to a parallel line initial velocity plot. Although a parallel line plot was observed under these conditions a sequential mechanism of reaction could not be excluded as it has been shown in the Theory section that a random sequential mechanism can give rise to either type of plot depending only upon the values of the individual rate constants.

In the present investigation unlike that of Grisolia and

Cleland both the ionic strength and the free magnesium ion concentrations were kept constant. It is important to maintain the ionic strength constant firstly to keep activity coefficients constant and secondly because one of the features of the phosphoglycerate-mutase reaction is the marked labilizing of the 23PGA bound to the enzyme with increasing ionic strength. Since the rate constant for dissociation of 23PGA from the enzyme, $k_4$ in Scheme 1 of Theory section 2 increases more than the rate constant for reassociation $k_{-4}$, the $K_m$ for 23PGA also increases. Consequently a meaningful value of the $K_m$ will only be obtained at constant ionic strength. It is also important to maintain the $[Mg^{2+}]_{Free}$ concentration constant so that a constant proportion of both substrates remain uncomplexed by magnesium and can combine with the enzyme (see later). If this is not done a complicated kinetic pattern results (assumptions have to be made as to whether $Mg^{2+}$ complexed substrates combine with the enzyme and it is very difficult to calculate the true value of the constants). Although the concentration of chloride ion, which is also an inhibitor, was changed as a result of adding various amounts of magnesium chloride to substrate solutions (to keep the free magnesium ion concentration constant) the overall changes in chloride ion concentrations

were not large. Increases in chloride ion from this source were partly offset by a decrease in the quantity of KCl added to the solution to keep the ionic strength constant. Moreover the tris buffer was adjusted to pH 7.40 by the addition of hydrochloric acid and as this constituted a major source of chloride ion the percentage change in chloride concentration was very small. Consequently inhibition by chloride ion was effectively constant and was therefore ignored.

The strong sulphate inhibition of the enzyme reaction was investigated by measuring the $K_m$'s of 23PGA and 3PGA at two sulphate ion concentrations, one of which was very low, under conditions which were otherwise identical. If the finding of Grisolia and Cleland that the inhibition can be almost entirely relieved by increasing the 23PGA concentration[54] is taken to indicate that the inhibition is exclusively competitive then the inhibition constants and the true $K_m$'s for both substrate could be calculated as shown in Results section R.6.

These measurements of the inhibition constants of sulphate, competitive with 3PGA and 23PGA indicated that the inhibition constant with 23PGA was about 8-9 times less than the corresponding 3PGA inhibition constant, thus showing that

sulphate mainly competes with 23PGA.

A brief investigation has been carried out into the nature of the magnesium inhibition of the enzyme reaction. Changes observed in the apparent Michaelis constant of 23PGA with magnesium ion concentration indicated that the magnesium-3PGA complex is not a substrate. There was also some evidence that the magnesium-3PGA complex is not a substrate though no firm conclusion could be drawn from the available data as experimental variability made interpretation of the inhibition pattern difficult. It seems probable that this system is analogous to the phosphoglucomutase system for which it has been clearly demonstrated that magnesium competitively inhibits both G1P and G16P by the formation of inactive magnesium complexes[6].

From the ratio of the maximum velocities of reaction in forwards and reverse direction it was possible to calculate the $K_m$ for 2PGA from the $K_m$ for 3PGH by the Haldane relationship. Previously no value for this constant appears to have been reported.

The constants quoted are all apparent Michaelis constants as inhibition by magnesium and chloride ions under experimental conditions are included in the value given. It was considered to be more appropriate to quote the constants

in this way as chloride inhibition has not been systematically investigated and because the magnesium inhibition can easily be accounted for if the free magnesium ion concentration is known by multiplying the $K_m$ by the fraction of the substrate which is uncomplexed by magnesium. The activity coefficients of the substrates have not been taken into account in the calculation of the constants of reaction. However, as the investigation was carried out at a single known ionic strength it would be possible to include such terms in the calculations.

Comparison of inhibition and Michaelis constants for 3PGA and 23PGA obtained in 7.9 mM $SO_4^{2-}$ with those reported for the rabbit muscle and chicken breast enzymes[51] in the absence of sulphate ion (see Grisolia and Cleland[54] and Pizer and Ballou[102]) shows that although the Michaelis constants are about the same the inhibition constants obtained in the present investigation are smaller. Calculation of the $K_m$'s of 3PGA and 23PGA at zero sulphate concentration gave values which were less than the values quoted by other authors (see Table D.1.2) by a factor of 3-4 for 3PGA and by a factor of 5-6 for 23PGA.

Table D.1.2.

| | Present investigation | | Grisolia and Cleland[54] Low ionic strength |
|---|---|---|---|
| | $[SO_4^{2-}]$ = 7.9 mM | $[SO_4^{2-}]$ = 0.0 mM | |
| $K_{23PGA}$ | 3.27 μM | 0.275 μM | 1.4 μM |
| $K_{i23PGA}$ | 19 μM | - | 2.5 mM |
| $K_{3PGA}$ | 0.36 mM | 0.166 mM | 0.6 mM |
| $K_i$3PGA | 0.83 mM | - | 2.9 mM |

The difference in the inhibition constants illustrates the necessity for keeping the free magnesium ion concentration constant so that the extent of formation of the inactive magnesium-substrate complexes does not vary. A particularly large fall in the inhibition constant for 23PGA occurs when this is done. This arises because at high 23PGA concentrations (i.e. in the inhibition region) if $[Mg^{2+}]_{Free}$ is unbuffered an increasing and significant proportion of $Mg^{2+}$ becomes bound by 23PGA and hence since the $Mg^{2+}$ binding constant for 23PGA is greater than that for 3PGA less 3PGA

will be complexed by $Mg^{2+}$ as the 23PGA concentration is raised. As a result it will be necessary to add more 23PGA to cause a given degree of inhibition of 3PGA binding than would have been necessary if $[Mg^{2+}]_{Free}$ were constant onsequently $K_{i23PGA}$ will be greater than the true value.

The inhibition constants of the substrates measured with $[Mg^{2+}]_{Free}$ = constant are only slightly larger than their Michaelis constants. This suggests that there are two sites for substrate addition on the enzyme surface and that the substrates can attach at both sites though 23PGA shows a preference for one particular site. Such a description of the enzyme is consistent with the finding that it is possible to isolate E3PGA and E.23PGA complexes[75] though no firm conclusions can be drawn about the nature of phosphate transfer from this evidence (see later).

In the case of a phosphoenzyme mechanism the $K_m$ for the substrate relates to binding with the phosphoenzyme whereas the inhibition constant relates to binding with the dephosphoenzyme. As the presence of a phosphate group is presumably important for substrate binding it might be expected that the $K_i$ should be greater than the $K_m$. Similarly for the cofactor the presence or absence of a phosphate group would be expected to influence binding to a

considerable extent so that again $K_i$ should be greater than the $K_m$. Indeed for rabbit muscle phosphoglucomutase the $K_i$'s of both G1P and G16P are greater by several orders of magnitude than their respective $K_m$'s[1].

It was not expected that the constants quoted above would be affected by the potentiation of the phosphoglycerate-mutase reaction observed at high concentrations of both 3PGA ($\gtrsim$ 5 mM) and 23PGA ($\gtrsim$ 10 μM)[51] as all initial velocity measurements were made at concentrations below these. Evidence of a slow initial increase in the velocity of reaction was only found at the highest concentrations of both reagents which were similar to these limits. Though this phenomenon was briefly investigated to find out whether the order of addition of substrate affected the length of the period of enzyme induction, it was not possible to draw any firm conclusions. If potentiation occurs as described by Mantle and Garfinkel[51] by a "black box modifier" (see Fig. R.6.9) then the present results suggest that the order of addition of 23PGA and 3PGA must be random rather than obligatory and that 23PGA binds more slowly to the enzyme than 3PGA. If this induction of enzyme were to be systematically studied it would be useful to have apparatus capable of studying fast reactions.

## SECTION 2. DISCUSSION

### Introduction

Having completed the preliminary investigations of the phosphoglycerate-mutase system it is possible to attempt the interpretation of the induced transport and flux ratio tests and to compare the results with those obtained for phosphoglucomutase. However, before the general significance of the results can be discussed consideration must be given to the non-ideal behaviour of solution and to isotope effects. In the results section these were thought not to influence the interpretation and the justification for this assumption is now given.

### Induced-transport tests, non-ideal behaviour of solution and isotope effects

In non-ideal solutions it becomes necessary to redefine the conditions for the absence of induced transport in order to take account of activity coefficients and isotope factors. Thus it is shown in the Theory section that for no induced transport in an A ⟷ B reaction eq. T.1.63 must hold. This equation is reproduced below:

$$n\,\frac{m_{A\longrightarrow B}}{m_{B\longrightarrow A}} = \frac{f_A[A]f_{B^*}f^{\circ}_{A^*}}{f_B[B]_{\circ}Kf_{A^*}f^{\circ}_{B^*}} \qquad \text{..... D.2.1.}$$

The significance of the symbols is given in the theory section T.1.

From this equation it follows that lack of induced transport in a chemical reaction must be evidence for the reaction obeying the Independence relationship even if isotope effects are present and the solutions behave non-ideally, provided that the reaction has only one pathway and provided that the ratio of the activity coefficients of the labelled substrates is unaffected by the addition of unlabelled substrate. In contrast, the magnitude of any induced transport, will be influenced by isotope effects and may also be affected by the activity coefficients. Thus for ideal solutions and in the absence of isotope effects the magnitude of the induced transport is given by the integral of equation T.1.28 but when these factors are taken into account the magnitude is given by equation T.1. In this discussion alternative pathways are the different routes by which an enzyme may transfer labelled groups from substrate to product. Thus isoenzymes with similar reaction pathways, and minor reaction pathways such as occur in the phosphoglucomutase reaction when the substrate add to the enzyme before the addition of magnesium[6, 17] do not constitute alternative pathways for the present purposes, unless one isoenzyme discriminates against the labelled

material. When there are alternative pathways for the reaction lack of induced transport will not necessarily be evidence for the reaction obeying the Independence relationship, since the isotope may preferentially follow one pathway. However, induced transport tests may still provide evidence for steps in the reaction (see theory section).

Thus, if activity coefficients of the substrates can be ignored the lack of induced transport for $^{14}C$-labelled substrates at low substrate concentration for both rabbit-muscle phosphoglucomutase, and phosphoglycerate mutase must be good evidence for both reactions obeying the Independence relationship even if isotope effects are present. The counter-transport observed at high substrate concentrations with phosphoglucomutase is sensitive to isotope effects but such effects should be unimportant in relationship to the other uncertainties of measurement. In the case of induced-transport experiments with $^{32}P$-labelled substrates, isotope effects will be important as the magnitude of the induced transport is used to distinguish between various mechanisms of reaction. However, in the case of $^{32}P$-labelled substrates isotope effects should be very small, and for both enzymes the experiments themselves provided some evidence that isotope could not be very large. Thus the initial rates of fall of the substrate concentrations (G1P or 2PGA) and the $[^{32}P]$ G1P

or [$^{32}$P] 2PGA radioactivity, after the addition of inactive substrate were equal. Had there been a significant isotope effect present the rate of fall of the $^{32}$P-radioactivity would probably have been less rapid than the fall in substrate concentration.

As mentioned previously, lack of induced transport can only be used as a criterion for the reaction obeying the Independence relationship if the ratio of the activity coefficients of the labelled substrates is unaffected by the addition of unlabelled substrate. The ratio of the activity coefficients for the labelled species should be similar to the ratio for the unlabelled substrates; the fact that the equilibrium constants for the phosphoglucomutase and phosphoglycerate mutase reaction were the same for both $^{14}$C-labelled and unlabelled substrates within experimental error, provides support for this assumption (see Theory section). Thus it should be sufficient to show that the ratio for the unlabelled substrates is unchanged when additional substrate is added in order that the above criterion should be satisfied. In the case of the phosphoglucomutase reaction there is experimental evidence that the ratio for the unlabelled substrates is constant and close to unity (see below) and it is argued that the same is probably true for the substrates of the phosphoglycerate mutase reaction in view of the similarity of the two systems. Consequently lack of

induced transport will be evidence that the reaction obeys the Independence relationship. Whether or not the magnitude of any induced transport is also independent of activity coefficients under these conditions depends upon the nature of the interaction between the fluxes. The magnitude will be independent of the activity coefficients if the interaction between the fluxes is due to an indirect transfer of groups (as for example, is the case for phosphate transfer by mechanism 1 and 3 for phosphoglucomutase) but not if the induced transport is caused by an interaction due to an isomerizing step (since in the latter case the amount of induced-transport depends upon $\alpha$ which is influenced by activity coefficients).

For the phosphoglucomutase system the ratio of the activity coefficients of the substrates G1P and G6P (present mainly as dianions) may be expected to be close to unity as their properties are very similar. Thus G1P and G6P have very nearly the same ionic dissociation constants[109] and electrostatic effects should influence both equally; binding by magnesium should be small under experimental conditions[17] and may be expected to be similar for both; furthermore the similarity between the molecules suggests that non-ionic factors will influence both in the same way. Evidence that the ratio of the activity coefficients is 1:1 is given by the

fact that the equilibrium constant of reaction was independent of pH[34] and by the fact that the equilibrium constant was independent of the concentration of the reactants, within 2% in the present work. In addition experiments on depression of freezing point showed that both G1P and G6P had virtually the same activity coefficients over a wide range of concentration (0-200 mM) (see results section R.2).

For the phosphoglycerate mutase reaction the ratio of the activity coefficients of the same ionic forms of the substrates 2PGA and 3PGA was expected to be close to unity as their properties were very similar. The dianions and trianions of 2PGA and 3PGA have very nearly the same size, shape and charge distribution suggesting that both electrostatic and non-ionic factors should affect each equally. As their ionic dissociation constants differ by a factor of about 0.7 of a pH unit (pH 7.41 and 6.67 respectively) in the vicinity of pH 7.4 at which the induced transport experiments were carried out the dianions and trianions of each substrate will be present in different proportion. However, the ionic dissociation constants are equally affected by ionic strength and measurements of the equilibrium constant of the reaction over the range 1·5-25 mM of total monophosphoglycerate showed that it was constant within the limits of experimental

error. Thus suggesting that the ratio of the activity coefficients of the total 2PGA and 3PGA concentration were unaffected by addition of further substrate.

If the counter-transport of $^{14}C$-labelled substrate observed at high concentrations of substrates for the phosphoglucomutase reaction had been due to the ratio of the activity coefficients departing from unity, the departure would have to be relatively large, since the theoretical curve (for Fig. R.7) requires a 30% deviation of the flux ratio from equation D.2.1 immediately after the addition of non-radioactive G1P. Perhaps the only way in which this could occur would be if the G1P molecules interacted with each other, for example with the formation of dimers. This possibility appears to be excluded by the freezing-point data, especially since shortly after the addition of non-radioactive G1P the concentrations of the G1P and G6P become the same, and a direct comparison of the freezing point data over the same range of concentrations becomes possible. Further, dimer formation of the required order of magnitude should alter the equilibrium constant by about 7.5% at the end of the reaction, whereas no change was in fact observed. Indeed the constancy

of the equilibrium constant and the freezing-point data taken together suggest that neither G1P nor G6P associates in solution.

Significance of Induced transport test with $^{14}C$-labelled substrates

In the light of the above discussion it is seen that the virtual absence of induced transport of $^{14}C$-labelled substrate over a nearly 30 fold range of concentration for phosphoglucomutase and a hundred fold range for phosphoglycerate-mutase constitutes very good evidence for the glucose and glycerate fluxes obeying the Independence relationship over these ranges of concentration. A reaction which obeys the Independence relationship must either possess no steps that bring about an interaction between the fluxes, or if such steps exist the interactions they bring about must effectively cancel each other. The latter possibility would be excluded if it could be demonstrated that the Independence relationship was obeyed over a wide range of concentrations, since cancellation would only be expected over a limited range. Such an observation would be good evidence for a direct transfer of substrate from reactant to product (since an indirect transfer leads to interaction), for an absence of a rate-limiting isomerization of the enzyme, and

for an absence of certain types of interaction between active centres[59, 61, 63]. It might have been desirable to make observations at sufficiently low substrate concentrations so that the enzyme was only partially saturated but in the case of phosphoglucomutase the lack of non-competitive product inhibition and the fact that the enzyme has only one active centre[32], must provide strong evidence for the direct transfer of glucose residues whatever mechanism may be involved. Experiments carried out under these conditions for phosphoglycerate mutase showed no measurable deviation from the Independence relationship (at 3PGA = 0.1 mM; $K_m$ = 0.17 mM). The inability of a rate limiting interconversion of two forms of the enzyme to cause a mechanism involving indirect transfer of $^{14}C$-labelled substrates to simulate direct transfer at low substrate concentrations and the fact that no induced-transport occurs over a wide range of substrate concentrations provides strong evidence that the transfer of glycerate residues must be direct whatever the mechanism of reaction even though the enzyme appears to have two active centres[123].

In the phosphoglucomutase reaction at the highest substrate concentrations counter-transport of the $^{14}C$-labelled glucose moiety occurred. As discussed above, the result constitutes strong evidence that the flux ratio departs from the Independence relationship in the direction

of exchange diffusion[59, 61]. It follows that there must be a method of thermodynamically transferring the energy that is made available when the non-radioactive G1P is converted into G6P to the conversion of radioactive G6P and G1P, since this latter conversion occurs against the energy gradient. Two possible mechanisms have been proposed to account for this phenomenon[59, 61, 63]. In one there is a rate-limiting isomerization of the free-enzyme. During the reaction the two forms of the enzyme are not at equilibrium and the displacement of this step from equilibrium mediates the transfer of energy (cf. mobile-carrier mechanism used to explain exchange diffusion in the permeability field). The second mechanism involves an interaction between active centres in a multivalent enzyme. This was discussed by Britton[63], but the interaction must be of a very unlikely type since the conversion of G1P to G6P at one centre must facilitate the conversion of a molecule of G6P to G1P at the other site and further to explain the interaction some form of isomeric change of the enzyme would again seem to be required. Since rabbit muscle phosphoglucomutase has only one active centre per molecule[32] it would appear that the latter possibility can be excluded and counter-transport must constitute good evidence for an isomerization step involving the phospho-

enzyme. The assumption involved in deriving a numerical value for the rate constant are discussed in the Theory section. The true value of the rate constant (assuming $k_3 = k_{-3}$) can be calculated by taking into account the activity coefficients of G1P and G6P and assuming them to be equal. Thus from eq. T.1.48:-

$$k_3 = k_{-3} = 4.5 \times 10^7 \times \frac{f^1_{G1P}}{f^{11}_{G1P}} \qquad \text{.....} \quad D.2.2.$$

where $f^1_{G1P}$ is the activity coefficient of G1P under the conditions of the induced-transport test and $f^{11}$ is the activity coefficient of G1P under the conditions for which $K_{G1P}$ was determined. The ionic strength for expt. 3 (Table R.7.2) was 0.517 and from the investigation in Section R.2 the mean ionic activity coefficient was calculated to be 0.417. The ionic strength at which $K_{G1P}$ was determined was 0.1145[6] and the corresponding mean activity coefficient was found to be 0.792. If the activity coefficients for $Na^+$ are assumed to be 0.79 and 0.84 at the higher and lower ionic strengths it follows that

$$\frac{f^1_{G1P}}{f^{11}_{G1P}} \simeq \frac{1}{6}$$

Thus the true value of the rate constant may be expected to be about one-sixth of the apparent rate constant ($4.5 \times 10^7$ $sec^{-1}$) because of the low activity coefficients of the substrates at high concentrations.

There is bound to be a good deal of uncertainty about this value as the ratio of activity coefficients will be very sensitive to the calculated mean activity coefficient of GlP and also because of the assumptions made in evaluating $f_{Na^+}$. There is also an uncertainty because of the need to assume that the activity coefficients of the different enzyme species are equal.

Mantle and Garfinkel pointed out that at high concentrations of both substrates potentiation of phosphoglycerate-mutase occurred and they interpreted this to be the result of the addition of further molecules of 23PGA and 3PGA causing a conformational change in the enzyme[51]. This phenomenon could also be explained by the enzyme having two active centres which could act co-operatively[63] the second active centre having higher $K_m$'s for 23PGA and 3PGA than the first. However the co-operation between the centres is probably no more than an allosteric activation process as the change produced in enzyme activity is slow (see section R.6). As already

discussed the absence of induced transport over the whole range of substrate concentration very strongly suggests that though the enzyme appears to have two active centres (one on each of two subunits)[123] they do not act co-operatively in the sense that conversion of substrate to product at one site does not influence the direction of reaction at the other (i.e. only the rate of conversion may be affected). The lack of counter-transport at high substrate concentrations indicates that any isomerization of the free enzyme is not rate limiting. Lower limit to the rate constant for interconversion of the two forms of the enzyme of $2.7 \times 10^6$ $sec^{-1}$ was calculated as described in the results section R.8. The assumptions made in this calculation are the same as for phosphoglucomutase.

Significance of Induced transport tests with $^{32}P$-labelled substrates

As discussed previously, the induced transport of $^{32}P$-labelled substrates constitutes strong evidence, in the case of both enzymes, that the flux ratio for phosphates deviates from the Independence relationship in the direction of the long pore effect. It follows that in the induced transport test radioactive phosphate from the G1P or 2PGA is transferred to the G6P or 3PGA respectively against the

energy gradient[59], and it is necessary for the reaction to contain a step that will transfer the energy made available from the conversion of non-radioactive G1P or 2PGA to G6P or 3PGA respectively to the radioactive species. The indirect transfer of phosphate provides such a step, since when the $^{32}$P-labell . is attached to the enzyme (or to an enzyme cofactor complex) the subsequent fate of the $^{32}$P-labelled phosphate depends upon whether a molecule of the substrate, G1P or 2PGA, or product, G6P or 3PGA, reacts next with the enzyme, and it is immaterial whether this molecule is labelled or not. Interaction of this type is very similar to that proposed by Hodgkin and Keynes[65] in their long-pore model to explain $K^+$ permeability of the squid axon. Alternatively, interaction could occur if the enzyme were multivalent and there were interactions between active centres[59, 61, 63]. The type of interaction that would be required would be similar to that suggested to occur in multivalent carriers[65, 110]. However, phosphoglucomutase has only one active centre per molecule[32] and though phosphoglycerate mutase has two[123] it seems unlikely that there is a co-operative mechanism for transfer of $^{32}$P-label. Moreover the results obtained with $^{14}$C-labelled substrates for both enzymes are incomparible with such interactions since

the glucose and glycerate flux ratios should be similarly influenced. Thus indirect transfer of phosphate appears to provide the only possible explanation of the experimental results.

Since for both phosphoglucomutase and phosphoglycerate mutase the flux ratio was equal to the square of the right-hand side of the Independence relationship (eq. T.1.21) over a 2-2½ range of substrate concentrations, it can further be argued that there must be only one intermediate phosphate in the reaction whatever the mechanism. Thermodynamic considerations[60] indicate an average of at least one intermediate phosphate, but a larger number of intermediate phosphates would give rise to a greater degree of interaction, as is seen when the enzyme is considered as a molecular shuttle[61]. Although it would be possible for different steps in the reaction to cancel each other, the degree of cancellation would be expected to depend upon substrate concentrations, and the fact that the $^{32}P$ flux ratio was independent of these concentrations constitutes strong evidence for a single reaction intermediate. Moreover the limitations imposed upon the rate of isomerization of the free enzyme by the $^{14}C$-induced transport experiments prevents a mechanism in which there are two phosphate intermediate simulating a mechanism in which there was only one, as αKB

(where B = G6P or 3PGA, see eq. T.1.18) cannot be very much greater than unity.

Ratio of $^{14}C$- to $^{32}P$-fluxes at equilibrium

The ratio of the fluxes at chemical equilibrium will not be influenced by non-ideal behaviour of the substrates except insofar as there be a rate-limiting isomerization of the enzyme, when the numerical value of $\alpha$ (see equations T.1.10, T.1.20) will be affected. On the other hand the flux ratio will be sensitive to any isotope effects. The fact that for phosphoglucomutase the ratio of the fluxes was 2:1 over a $2\frac{1}{2}$ fold concentration range is compatible with the direct transfer of glucose residues and the indirect transfer of phosphate with a single intermediate phosphate, and with an isomerization of the free enzyme moiety that is not substantially rate-limiting at the substrate concentrations used. In the case of phosphoglycerate mutase the flux ratio was only measured at one substrate concentration and consequently the result is not quite as unambiguous as in the case of phosphoglucomutase. However, a flux ratio of 2:1 was measured which was compatible with the direct transfer of glycerate residues and the indirect transfer of phosphate with a single intermediate phosphate, and with a non-rate

limiting isomerization of the free enzyme moiety. Although they may appear unlikely there are other mechanisms that would yield the same result as the ones postulated above. Thus the glucose or glycerate residue might be transferred indirectly with one intermediate, and the phosphate might be transferred via three intermediate phosphates. Correspondingly, there might be two intermediate glucose or glycerate residues and five intermediate phosphate groups. Unlike the induced-transport tests, interaction between active centres will not influence the flux ratio at equilibrium.

It can be seen from equation T.1.10 that the flux ratio should increase at high substrate concentration if there is a slow isomerization of the free enzyme. However, calculation shows that this is a far less sensitive test for such a step than the induced-transport test with $^{14}C$-labelled substrates. Thus a value of $\alpha$ that provide a 10% rise in the radioactivity in the GlP in the induced-transport test will produce only a 0.8% change in the ratio of the $^{14}C$- to $^{32}P$-flux when equilibrium has been reached.

## Summary

The induced transport tests and the flux ratios at

equilibrium provide very strong evidence for the phosphoglucomutase and phosphoglycerate mutase reaction proceeding by direct transfer of glucose or glyceric acid respectively from reactant to product and the indirect transfer of phosphate with one intermediate phosphate. These conclusions are independent of the detailed reaction mechanism and require no assumption about the constancy of rate constants. Further, for phosphoglucomutase, strong evidence is obtained for an isomerization of the free "phosphoenzyme" with an apparent rate constant of about $4.5 \times 10^7$ $sec^{-1}$. The true rate constant would appear to be about one sixth of this value. In the case of phosphoglycerate mutase no evidence could be obtained for an isomerization step. However, assuming such a step to occur it was possible to put a lower limit of $2.7 \times 10^6$ $sec^{-1}$ to the rate constant of interconversion. Of the three models proposed in the theory section it is clear that mechanism 1, or E23PGA (or EG16P) acting as a phosphoenzyme represent the only possible mechanism for both enzymes. It is also clear in both cases that the isomerization of the "phosphoenzyme" is so rapid that it can only involve a small chemical change (see Historical survey). This might, in the case of phosphoglucomutase involve a rearrangement of hydrogen-bonding of the phosphate by histidine.

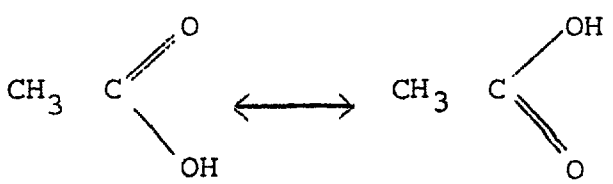
It has been reported that rabbit muscle phosphoglucomutase contains four isoenzymes[82] and that rabbit muscle phosphoglycerate mutase contains two isoenzymes[98]. However, the conclusions drawn about the mechanism of reaction are unaffected, since these do not depend on the rate constants. Some uncertainty must be introduced about the rate constants for isomerization, since the isoenzymes may possess different rate constants, but conclusions about the order of magnitude of these will still be valid.

Interpretation of results in terms of reaction mechanism

In the following an attempt is made to describe possible reaction pathways for the phosphomutases in the light of the preceding results. It has been shown for phosphoglucomutase and for phosphoglycerate-mutase at low and high ionic strength that reaction appears to take place by means of the phosphoenzyme mechanism shown in Scheme 1, Fig. T.1.2 (Theory section). Thus the mechanism proposed for rabbit muscle phosphoglucomutase by Ray and Roscelli[1] appears to be upheld whereas the sequential mechanism proposed for muscle phosphoglycerate-mutase by Jacobs and Grisolia[75] in Scheme H.2.1 is rejected in favour of the phosphoenzyme mechanism proposed by Grisolis and Cleland[54] in Scheme H.2.2.

Nevertheless there is still conflicting evidence about whether rabbit muscle phosphoglucomutase is isolable as an EP. or EG16P complex from solutions containing its substrates. Muscle phosphoglycerate-mutase seems only to have been isolated as the E23PGA complex, though the bound 23PGA is in a highly distorted state as it is readily hydrolysable in acid solution. A recent investigation by Rose[123], however, suggests that rabbit-muscle phosphoglyceratemutase has two subunits, and that each binds one molecule of phosphate. $EP_2$ was proposed to be the active form of the enzyme though there was insufficient evidence to eliminate E(23PGA) with 23PGA distorted as a possible alternative (see Historical section).

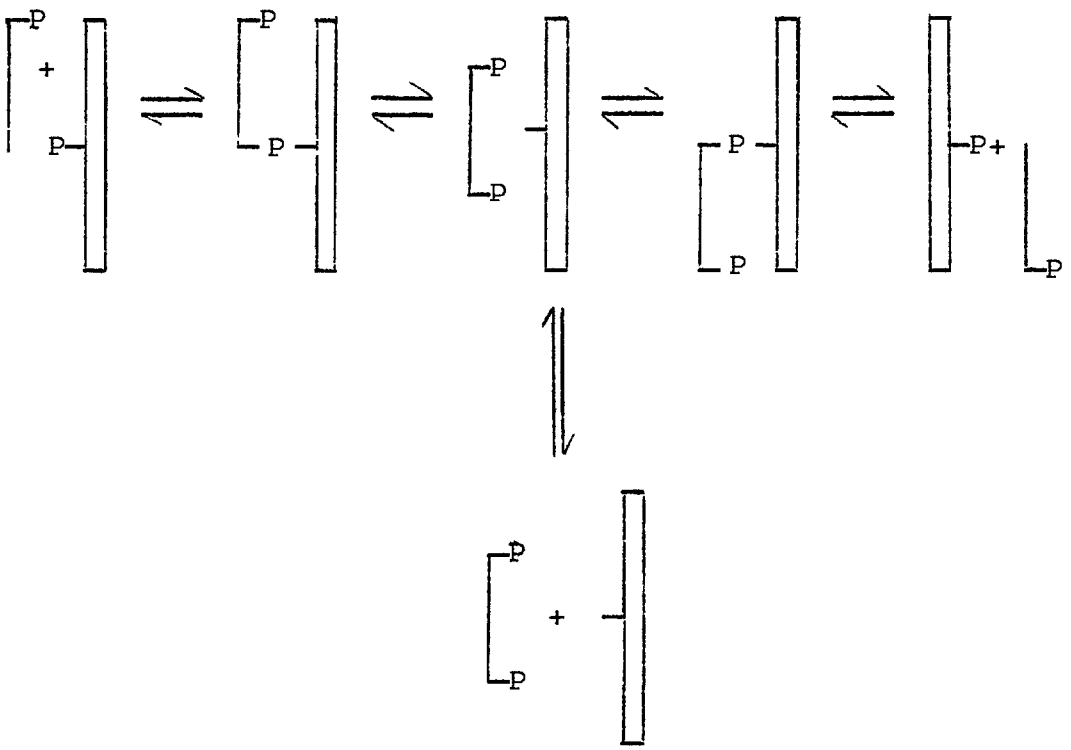
For both enzymes the rate of interconversion of the two forms of the phosphoenzyme (or E cofactor complex) are very fast ( $10^7$ $sec^{-1}$). Indeed this is much faster than any known group transfer enzymes $V_{max}$[87] and the rates of conversion are approximately equal to the intramolecular rate constants for transfer of $H^+$ <u>via</u> a water bridge between two groups in solution, for example:-



Thus provided phosphate and glucose (or glyceric acid) transfer occur *via* the pathway described the data excludes the possibility of isomeric phosphoenzymes. There appears to be structural evidence, however, for two phosphoenzymes in the case of rabbit-muscle phosphoglucomutase. Bocchini *et al.*[124] have reported two active site sequences each with a phospho-seryl residue attached to different parts of similar amino-acid sequences. Though they concluded that this might be evidence for two phosphoenzymes the alternative explanation that two isoenzymes are involved cannot be excluded on the basis of the available evidence.

If it should be true that there is only one form of the phosphoenzyme an alternative mechanism must be sought to account for the properties of the reaction. Such a mechanism is shown in Scheme D.2.1 below for phosphoglucomutase, in which only a single site of phosphate attachment to the enzyme is proposed.

Scheme D.2.1.



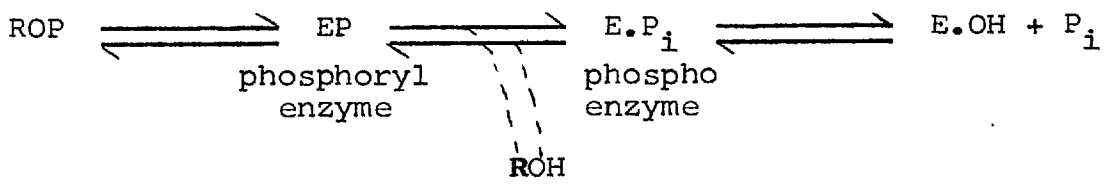
This offers an attractive alternative which explains why Ray and Roscelli[32] have obtained different $K_m$'s for G16P when the one obtained from studies of the forwards reaction (G1P $\longrightarrow$ G16P) was compared with that obtained from studies of the reverse reaction (G6P $\longrightarrow$ G1P). This mechanism also shows a great similarity to that of alkaline phosphatase (see below), and would, if EP were unstable, exhibit diphosphatase activity. Several mutase show at least a small extent of

such diphosphatase activity. It thus seems possible that all diphosphate dependent mutases may have a common mechanism and that they may be evolutionarily related to the phosphatases. 23PGA diphosphatase from red cells is similar in properties to red cell phosphoglycerate mutase[127] and phosphoglycerate-mutase activity cannot be separated from it. Alkaline phosphatase and other phosphatases have transphosphorylase activity (which might be viewed as rudimentary mutase activity) and it has been shown that a phosphoenzyme is involved in this reaction (see Scheme D.2.2).

The amino acid sequence at the active site of alkaline phosphatase from E. coli has been shown to be thr-asp-ser(P)-ala-ala-ser-ala[114] with the phosphate group of the phosphorylated enzyme forming a covalent bond with one serine as shown. This sequence is very similar to that for rabbit-muscle[115], flounder muscle[116] and E. coli[117] phosphoglucomutase; thr-ala-ser(P)-his-asp, though an alternative sequence for rabbit-muscle phosphoglucomutase; asp-ser-gly-glu-ala-val has been proposed[118].

The mechanism of reaction of E. coli alkaline phosphatase, first proposed by Engstrom and Agren[119] and later confirmed by many others, is shown in Scheme D.2.2.

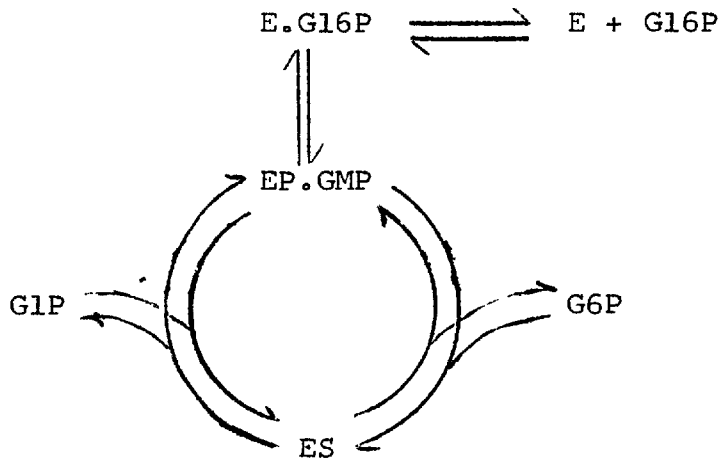
Scheme D.2.2.




This may be compared with postulated common mechanism of the phosphomutases shown in Scheme D.2.1 for phosphoglucomutase. It can be seen that only a minimal modification of a diphosphatase might be required to give mutase activity. However it is not possible by kinetic techniques to distinguish between an EP complex and an EG16P complex in which one of the phosphates has been made labile. Thus there is the alternative possibility, shown in Scheme D.2.3 that the phosphatase has developed a subsidiary binding site for this monophosphate, and that the EP.GMP complex formed by reaction of the enzyme with G16P acts as a phosphoenzyme.

Scheme D.2.3

(shown for phosphoglucomutase)




If this scheme represents the mechanism of reaction of the phosphomutases it may be that apparent discrepancies between enzymes from different sources are due to differences in the relative stabilities of the EG16P, EP.GMP, and EP complexes. In this context it may be significant that these enzymes fall into two classes; those with pH optima in the region of pH 7.4, and those with pH optima in the region of pH 8.5. This division could be important as

it is likely that the relative stability of the enzyme cofactor complexes will depend upon pH just as has been shown for alkaline phosphatase. In this case though the phosphoryl enzyme is stable relative to the free enzyme and inorganic phosphate at all pH's studied the addition complex between enzyme and Phosphate ($E.P_i$) is even more stable in the region of pH 7.0-8.0. Consequently in this pH region only the phosphoenzyme $E.P_i$ can be obtained. However, as the pH is lowered, the relationship changes so that at pH 5.5 the covalent phosphoryl-enzyme is much more stable than the enzyme-phosphate complex; hence in this pH region E.P will be the predominant form[113]. The environment of the active site of the mutases will probably be somewhat dissimilar to that of the E. coli alkaline phosphatase and consequently the relative stabilities of the phosphoenzyme and E. cofactor complexes may be somewhat different to the relative stabilities of the EP and $E.P_i$ complexes of the phosphatase. However, it seems possible that the broad outlines of the scheme, outlined above may remain the same. Thus the phosphoglucomutases from rabbit, flounder and shark muscle which all have pH optima at pH 7.4 could be isolated as phosphoenzymes whereas the phosphoglucomutases from M. lysodeiticus and B. cereus which have pH optima at

about pH 8.5 could not be isolated as phosphoenzymes, and it seemed that the E.G16P complex was too labile, at least with M. lysodeikticus[80], to permit isolation.

Phosphoglucomutase from E. coli with a pH optimum in the region of pH 8.5 proved to be an exception to this rule as there seemed to be evidence for the isolation of a phosphoenzyme[117]. However, this enzyme was different from the others in that its one active SH group appeared to be essential for enzyme activity[56] whereas this was not generally the rule for the other phosphoglucomutases. The difference in the stabilities of E.G16P and EP do not seem to be very large as rabbit-muscle phosphoglucomutase could be isolated as either an E.G16P complex or as a phosphoenzyme depending upon the experimental conditions. Thus if "gentle" experimental procedures are used to separate the enzyme from its substrates, such as column chromatography an EG16P complex is isolated, whereas more rigorous procedures, such as prolonged dialysis yield the phosphoenzyme. Consequently much intermediate behaviour might be expected of these enzymes and thus only with the rabbit muscle enzyme is there a phosphoenzyme with 1 mole phosphate per mole enzyme. The flounder muscle and shark muscle enzyme-phosphate complexes are isolated with about 0.5 moles phosphate per mole enzyme.

For the phosphoglycerate mutases a direct comparison with alkaline phosphatase may not be as useful as in the case of the phosphoglucomutases since the phosphoglycerate mutases do not require added metal ions for activity. Consequently the nature of their active sites may be expected to be different. Indeed recent studies suggest that the phosphate group is bound to histidine rather than serine[123]. If this were so it would explain the lability of the enzyme-phosphate bond at low pH. However, Scheme D.2.3 would seem to be applicable to the muscle enzymes at least as the induced transport tests favour as a kinetic intermediate a phosphoenzyme or EP.PGA complex, whereas attempts to isolate the phosphoenzyme have all yielded E.23PGA complexes. It seems that the 23PGA molecule in this complex is highly distorted (see earlier) so that the nature of this complex may be closer to that of EP.PGA than E.23PGA. The induced transport experiments also show that for rabbit muscle phosphoglycerate mutase there is no change in mechanism with ionic strength. This observation confirms the belief of other investigators that the prime effect of ionic strength is to change the rate constants for the formation and dissociation of the E.23PGA complex. Thus the basic mechanism remains the same as for the

phosphoglucomutases. Indeed as with the phosphoglucomutases it is possible to split up the phosphoglyceratemutases on the basis of their pH optima into two groups with apparently different mechanisms. Thus the muscle (rabbit and chicken breast) and yeast enzymes have pH optimas at approximately pH 7, and require 23PGA cofactor for activity whereas the plant enzymes with pH optimas at pH 9 (approx.) do not have this requirement. Comparison with the mechanism of alkaline phosphatase is not helpful except to suggest that the alteration in mechanism may only be in detail and not in kind. In support of this argument it may be noted that the rabbit muscle enzyme can be activated by arsenate ions[97] and it would seem more likely under these circumstances that transfer of phosphate from 2PGA to 3PGA occurred by a mechanism similar to that of the plant enzymes. Furthermore, to a certain extent, the yeast enzyme exhibits the characteristics of the plant enzymes as the enzyme retains about 10% of activity in the absence of 23PGA (see historical survey). Thus it is possible to propose two mechanisms for the plant enzymes which are based upon the mechanism of action of the rabbit muscle enzyme. In the first it is proposed that cooperation between active groups at the site of substrate binding facilitate the intra-molecular shift of phosphate between the two and three

position of the glyceric acid. Transfer occurs via a five-members heterocyclic ring in which phosphate bridges the two and three positions of the glyceric acid. In the second mechanism it is proposed that transfer involves a molecule of 2PGA or 3PGA acting as a substitute for 23PGA, so that the phosphate from 2PGA or 3PGA can act in the same way as the phosphate of a phosphoenzyme. Thus this mechanism will lead to be indirect transfer of phosphate.

Both these mechanisms seem equally likely in the light of the available evidence. The small mono- and diphosphatase activities associated with the wheat germ enzyme are compatible with either mechanism. However, they can be distinguishable by means of induced transport tests. The first will give no induced transport with either $^{32}P$- or $^{14}C$-labelled substrates but the second will give co-transport with $^{32}P$-labelled substrates (though not with $^{14}C$-labelled substrates).

In the light of the above discussion it would be interesting to study the effect of pH upon the mechanism of reaction. For the yeast and muscle phosphoglycerate-mutases as the pH is increased EP or E.23PGA should become more stable[123] due to the increasing stability of the phosphoryl-histidine bond. At pH's greater than the pH optimum of the enzyme the phosphate bound by histidine may not

be able to participate in exchange. For the yeast enzyme in particular it would be interesting to see if the partial requirement of 23PGA for activity could be abolished completely at high pH. Under these circumstances kinetic behaviour similar to that expected for the plant enzymes (which have a high pH optimum) may be observed.

If there is a common mechanism for all phosphoglucomutases and for phosphoglycerate mutases requiring cofactor 23PGA, and if this common mechanism involves a phosphoenzyme EP then a parallel line initial velocity pattern would be expected for Lineweaver-Burke plots. However, both the M. lysodeikticus and B. cereus phosphoglucomutases give converging line plots. Such a plot can only be obtained from an EP mechanism if there is some phosphatase activity (see Theory section T.2), but as these enzymes have a very low phosphatase activity it is more likely that the kinetic mechanism is sequential or random sequential (i.e. if rate of hydrolysis of EP rate of dissociation of G16P from ES). On the basis of the induced transport experiments, EP.GMP or EP would be preferred as reaction intermediates, whereas the high pH optimum of these enzymes and the kinetics of reaction suggest EG16P or EP.GMP rather than EP. Induced transport experiments, not reported in this thesis, carried out on phosphoglucomutase from

M. lysodeikticus have indicated that there is one intermediate for phosphate transfer and that glucose transfer from substrate to product is direct[120]. Consequently EP.GMP seems to be the most likely intermediate for the two bacterial phosphoglucomutases. Indeed it would be possible for all the mutases to have EP.GMP or EP.PGA as their kinetic intermediates. The common kinetic mechanism would then be a random sequential mechanism which would give parallel line or intersecting line Lineweaver-Burke plots depending on the relative values of the individual rate constants as proposed in the Theory section T.2. On the basis of this mechanism the phosphoenzyme of the muscle phosphoglucomutases would represent an artifact of the separation procedure. However, it is not really possible to distinguish between EP and EP.GMP or EP.PGA as the kinetic intermediates on the basis of the available evidence and it may be unrealistic to insist upon a common intermediate for enzymes which show such disparate properties. Nevertheless it seems clear that a random sequential mechanism with the basic pathway proposed in Scheme D.2.3 is a possible alternative to the phosphoenzyme Scheme D.2.1.

## Conclusion

The current work based upon the flux approach of Britton has examined the mechanism of reaction of rabbit-muscle phosphoglucomutase and phosphoglycerate-mutase. By means of Induced transport tests carried out in the presence of radioactively labelled substrates it has been possible to distinguish unambiguously between three mechanisms of reaction proposed for these enzymes on the basis of conventional techniques and to determine the rate constant of isomerization of the phosphoenzyme for rabbit-muscle phosphoglucomutase. Unlike conventional kinetic methods the results obtained by this technique are unequivocal, and do not rely on either a detailed knowledge of the reaction mechanism or upon the assumption that individual rate constants do not change.

In view of the proposed mechanisms of reaction of the mutases it would be of great interest to extend the present studies to include phosphoglucomutase from M. lysodeikticus, and phosphoglyceratemutases from plants as these enzymes apparently have different kinetic mechanisms from the others. Studies of both systems are now in progress, and preliminary results from the bacterial phosphoglucomutase suggest that the mechanism is the same as for the rabbit-muscle enzyme. The

flux approach outlined by Britton and in this thesis should also be of particular use in the study of bimolecular enzyme reactions especially where conventional kinetic studies have yielded conflicting conclusions. It should be possible to obtain unequivocal information about: the order of addition of substrates and release of products, the existence of any isomerization of the free enzyme, the isomerization of EX complexes where X if the group transferred, the number of intermediates in the transfer of the groups, and the nature of any co-operative phenomenon between active centres. In the future it is hoped to use this technique to investigate the order of addition of substrates etc. for hexokinase (from yeast and mammalian sources), glucokinase, glycerolkinase, and fructokinase.

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# The Mechanism of the Phosphoglucomutase Reaction

## STUDIES ON RABBIT MUSCLE PHOSPHOGLUCOMUTASE WITH FLUX TECHNIQUES

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1. The kinetics of phosphoglucomutases from different sources are discussed and it is concluded that on the available evidence there are in all cases three possible mechanisms for the reaction. These are an indirect transfer of phosphate involving the phosphoenzyme (mechanism 1), a direct transfer of phosphate (mechanism 2), and an intermolecular transfer of phosphate from glucose 1,6-diphosphate to the substrate (mechanism 3). Conventional net flux measurements are shown not to differentiate between these mechanisms. 2. Flux equations are developed and it is shown that there are three flux ratios that characterize and distinguish between the mechanisms. 3. To examine these flux ratios induced-transport tests are described with $^{14}C$- and $^{32}P$-labelled substrates. The fluxes determined with $^{14}C$- and $^{32}P$-labelled substrates are also compared at chemical equilibrium. 4. With rabbit muscle phosphoglucomutase the results of these tests were completely consistent with mechanism 1 and unequivocally excluded any substantial part of the reaction proceeding by mechanism 2 or mechanism 3. Evidence was also obtained for an isomerization of the phosphoenzyme with an apparent rate constant about $4{\cdot}5 \times 10^7$ sec.$^{-1}$. Taking into account the activity coefficients of the substrates the true rate constant appears to be about one-sixth of this value. 5. Isotope effects and non-ideal behaviour of the solutions are discussed and the activity coefficients of the substrates are shown to be equal by measurement of the depression of freezing point. It is concluded that these factors do not influence the tests significantly. 6. Alternative mechanisms are considered and it is concluded that the tests show that the glucose residue is transferred directly, that the phosphate is transferred indirectly with one intermediate phosphate, and that there is an isomerization of the free phosphoenzyme without reference to any other details of the reaction. Further, no assumptions are required about the constancy of rate constants. 7. The relative merits of induced transport and product inhibition for detecting isomerization of the enzyme are discussed. It is concluded that the induced-transport test is more sensitive and that its interpretation is less equivocal. 8. The application of the tests to other enzyme systems is briefly considered.

Phosphoglucomutase (EC 2.7.5.1) from rabbit muscle is believed to catalyse the conversion of G1P* into G6P by the mechanism (mechanism 1) shown in Scheme 1 (Ray & Roscelli, 1964*a*,*b*, 1966*a*,*b*; Ray, Roscelli & Kirkpatrick, 1966; Najjar & Pullman, 1954). When a molecule of G1P is converted into G6P by this mechanism the phosphate from the G1P is transferred to the enzyme and the phosphate attached to the enzyme is transferred to the 6-position of the substrate. There is thus an intermediate phosphate involved in phosphate transfer. This mechanism was proposed because of the requirement of the enzyme for G1P6P as a cofactor, because of the existence of a phosphorylated form of the enzyme and because the reciprocal of the initial reaction velocity plotted against the reciprocal of the G1P concentration, at different G1P6P concentrations, gave a series of parallel lines. A series of parallel lines was also obtained when the reciprocal of the initial reaction velocity was plotted against the reciprocal of the G1P6P concentration at different G1P concentrations. Free G1P6P in solution, as is indicated in Scheme 1, did not appear to be an obligatory intermediate in the reaction since $^{32}P$-labelled enzyme incubated for a short period with a large excess of G1P and G1P6P transferred most of its label to the monophosphates and

*Abbreviations: G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; G1P6P, glucose 1,6-diphosphate.

$$G^*1P^* + EP_I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EP_{II}^* + G^*6P$$

$$EP_{II}^* \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} EP_I^*$$

$$G1P + EP_I^* \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EP_{II} + G6P^*$$

$$EP_{II} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} EP_I$$

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

Scheme 1. Scheme (mechanism 1) to show transfer of labelled glucose and labelled phosphate from G1P to G6P by a mechanism involving the transfer of the phosphate from the substrate to the phosphoenzyme. ES represents the enzyme–substrate complex; $EP_I$ and $EP_{II}$ represent different forms of the phosphoenzyme, and E represents the dephosphoenzyme. $k_{+1}$, $k_{+2}$ etc. are rate constants. G* and P* represent labelled molecules. The labelled glucose is transferred directly whereas two G1P molecules have to react in succession with the enzyme to transfer the phosphate.

$$G^*1P^* + EG1P6P_I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EG^*1P^*6P_{II} + G6P$$

$$EG^*1P^*6P_{II} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} EG^*1P^*6P_I$$

$$G1P + EG^*1P^*6P_I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EG1P6P_{II}^* + G^*6P$$

$$EG1P6P_{II}^* \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} EG1P6P_I^*$$

$$G1P + EG1P6P_I^* \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EG1P6P_{II} + G6P^*$$

$$EG1P6P_{II} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} EG1P6P_I$$

Scheme 2. Scheme (mechanism 3) to show transfer of labelled glucose and labelled phosphate from G1P to G6P by a mechanism involving the intermolecular transfer of phosphate from G1P6P to G1P. $EG1P6P_I$ and $EG1P6P_{II}$ represent different forms of the enzyme–G1P6P complex. G* and P* represent labelled molecules. $k_{+1}$, $k_{+2}$ etc. are rate constants. The reaction is supposed to take place in the presence of excess of G1P6P, so that dissociation of the enzyme–G1P6P complex can be ignored. Two molecules of G1P have to react in succession with the enzyme to transfer the labelled glucose. At least three molecules of G1P have to react to transfer the phosphate.

particularly to G6P (Ray & Roscelli, 1964*a*). Two forms of the phosphorylated enzyme, designated $EP_I$ and $EP_{II}$, are shown since the mechanism would seem to demand their existence. However, Ray & Roscelli (1964*b*) were unable to find any evidence for phosphoenzyme isomerization from product inhibition and they concluded that the apparent rate constant for the interconversion of the species, if they should exist, must be in excess of $10^7 sec.^{-1}$. The corresponding limit for the true rate constant will be somewhat lower than this since these authors did not take into account the non-ideal behaviour of the solutions.

Although the transfer of $^{32}P$-labelled phosphate from the phosphoenzyme to the substrate strongly suggests that the phosphate attached to the enzyme takes part in the reaction in the way shown in Scheme 1 (mechanism 1), reaction times of the order of seconds were used and it is conceivable that over these relatively long periods the phosphate may have been transferred from the enzyme by side reactions. Thus in the normal enzyme reaction there could be a direct transfer of phosphate from the 1-position to the 6-position with the phosphate attached to the enzyme performing some indirect role such as the formation of a pyrophosphate bond. This mechanism is referred to below as mechanism 2. Such a direct transfer of phosphate might explain why the enzyme still shows some activity in the absence of G1P6P (Ray & Roscelli, 1964*a*).

Unlike the enzyme from rabbit muscle, the phosphoglucomutases obtained by Hanabusa, Dougherty, del Río, Hashimoto & Handler (1966) from *Micrococcus lysodeikticus* and *Bacillus cereus* could not be isolated in phosphorylated forms although G1P6P was still required as a cofactor. These authors also found that the kinetics of these enzymes differed from that of rabbit muscle, in that the reciprocal of the initial velocities plotted against the reciprocal of the G1P concentration at different G1P6P concentrations gave a series of intersecting lines, indicating that the apparent $K_m$ for G1P was independent of G1P6P concentration; and a similar series of intersecting lines was obtained when the reciprocal of the initial velocity was plotted against the reciprocal of the G1P6P concentration at different G1P concentrations. They therefore proposed that these enzymes catalyse an intermolecular transfer of phosphate from G1P6P to the substrates as is shown in Scheme 2 (mechanism 3). In this mechanism there is not only an indirect transfer of phosphate, which involves two intermediate phosphate groups, but an indirect transfer of the glucose with a single intermediate glucose molecule.

It is surprising that the mechanisms for phosphoglucomutases from different sources should be so different. Mechanism 1 appears not to be a possible mechanism for the phosphoglucomutases from *M. lysodeikticus* and *B. cereus* because these enzymes do not exist in phosphorylated forms, and because

the $K_m$ for G1P is unaffected by G1P6P concentration. However, if the phosphoenzyme spontaneously dephosphorylated with the same rate constant as the dissociation of G1P6P from the enzyme–substrate complex then appropriate kinetics would be obtained and the absence of a phosphorylated form of the enzyme would also be explained. Alternatively, one of the phosphates of the enzyme–G1P6P complex shown in Scheme 2 (mechanism 3) might play the same role as the phosphate in the phosphoenzyme. The direct transfer of phosphate would clearly be a possible mechanism for all types of phosphoglucomutases. Mechanism 3 has been rejected as a possible mechanism for rabbit muscle phosphoglucomutase because parallel lines were obtained when the reciprocals of the initial velocities were plotted against the reciprocals of the G1P concentration at different G1P6P concentrations. However, if the free enzyme had a much greater affinity for G1P6P than had the enzyme combined with substrate then it can be shown that the kinetics of the rabbit muscle enzyme may be obtained. If mechanism 3 describes the mechanism of rabbit muscle phosphoglucomutase, the phosphoenzyme must represent an artifact of the separation procedure, since the effective agent will be the enzyme–G1P6P complex, and it may be noted that Hanabusa *et al.* (1966) have reported the isolation of the enzyme with a rather tightly bound molecule of G1P6P.

It may also be relevant that Brody & Tatum (1967) found that phosphoglucomutase from the wild forms of *Neurospora crassa* gave kinetics resembling those of the *M. lysodeikticus* and *B. cereus* enzymes, whereas the enzyme derived from a mutated strain gave kinetics resembling those of the rabbit enzyme. Unless two enzymes are involved it would seem unlikely that a mutation would alter the reaction mechanism.

Thus all three mechanisms would seem to be possible for phosphoglucomutase. The flux approach of Britton (1965*a*, 1966*a*,*b*, 1967*a*,*b*) offers a method of distinguishing between these various possibilities and also provides a very sensitive method of detecting any isomerization of the free enzyme. It is used in the present paper to show that in rabbit muscle phosphoglucomutase the glucose moiety is directly transferred between the substrates and that phosphate is transferred indirectly with one intermediate phosphate. Mechanism 1 thus represents the only possible mechanism. Evidence is also obtained for the existence of two forms of the free phosphorylated enzyme. The apparent rate constant for the interconversion of the species is about $4{\cdot}5 \times 10^7$ sec.$^{-1}$, but when the activity coefficients of the substrates are taken into account the true rate constant would appear to be about one-sixth of this value.

The role of $Mg^{2+}$, an essential cofactor, has not been considered in the above discussion. Ray & Roscelli (1966*a*,*b*) and Ray *et al.* (1966) have extensively investigated this aspect of the reaction and have obtained very strong evidence that the $Mg^{2+}$ remains attached to the enzyme during the whole of the enzymic process. All the experimental work described below was carried out in the presence of excess of $Mg^{2+}$.

## THEORY

In the following, the term flux is used in the way defined by Britton (1966*b*). The flux of phosphate from G1P to G6P is the actual number of phosphate groups transferred from the 1-position to the 6-position of the glucose in unit time under the particular conditions of concentrations, temperature etc. that are employed, and the flux of glucose from G1P to G6P is the rate of transfer of glucose from G1P to G6P.

*Derivation of flux equations.* (*a*) Flux equations for mechanism 1. In a previous publication (Britton, 1966*b*) the fluxes for a reaction of the type:

$$RX + R' \rightleftharpoons R + R'X$$

were calculated for a mechanism in which the enzyme forms a stable intermediate with the group transferred. Thus the initial reaction is the transference of X from RX to the enzyme to form an EX complex. The EX complex then isomerizes to a form that can transfer X to R′ to yield R′X. Finally the free enzyme isomerizes to its original form. In the previous publication (Britton, 1966*b*) RX=A, R′=B, R=C and R′X=D. Considering mechanism 1 (Scheme 1), and if A=B=G1P and C=D=G6P, then at any one instant in time one-half of the EP molecules may be regarded as transferring phosphate from A to D and the other half as transferring phosphate from B to C. Thus by comparison with eqns. 24–29 in Britton (1966*b*): flux of phosphate from G1P to G6P=2×flux of A to D; flux of phosphate from G6P to G1P=2×flux of D to A; flux of glucose residues from G1P to G6P=2×flux of A to C; flux of glucose residues from G6P to G1P=2×flux of C to A; $(EP_I)=2E_A=2E_B$ and $(EP_{II})=2E_C=2E_D$, where $(EP_I)$, $(EP_{II})$, $E_A$, $E_B$, $E_C$ and $E_D$ represent the quantities of the enzyme species; [G1P]=A=B and [G6P]=C=D, where the symbols represent concentrations; and $k_{+1}=a=g$, $k_{-1}=b=h$, $k_{+2}=c=k$, $k_{-2}=d=l$, $k_{+3}=e=m$ and $k_{-3}=f=n$.

Substituting:

$$\text{Flux of phosphate from G1P to G6P} = \frac{k_{+1}^2 k_{+2}^2 k_{+3}[\text{G1P}]^2(\text{EP}_\text{I})}{k_{+1}k_{+2}k_{+3}(k_{-1}+k_{+2})[\text{G1P}]+k_{-1}k_{-2}k_{-3}(k_{-1}+k_{+2})[\text{G6P}]+k_{+1}k_{+2}k_{-1}k_{-2}[\text{G1P}][\text{G6P}]} \quad (1)$$

$$\text{Flux of phosphate from G6P to G1P} = \frac{k_{-1}^2k_{-2}^2k_{-3}[\text{G6P}]^2(\text{EP}_{\text{II}})}{k_{+1}k_{+2}k_{+3}(k_{-1}+k_{+2})[\text{G1P}]+k_{-1}k_{-2}k_{-3}(k_{-1}+k_{+2})[\text{G6P}]+k_{+1}k_{+2}k_{-1}k_{-2}[\text{G1P}][\text{G6P}]} \quad (2)$$

Flux of glucose residues from G1P to G6P

$$= \frac{k_{+1}k_{+2}[\text{G1P}](\text{EP}_{\text{I}})}{k_{-1}+k_{+2}} \quad (3)$$

and

Flux of glucose residues from G6P to G1P

$$= \frac{k_{-1}k_{-2}[\text{G6P}][\text{EP}_{\text{II}}]}{k_{-1}+k_{+2}} \quad (4)$$

Further, in the steady state:

$$(\text{EP}_{\text{I}}) = \frac{(\text{E})\{k_{+3}(k_{-1}+k_{+2})+k_{-1}k_{-2}[\text{G6P}]\}}{D_1} \quad (5)$$

and

$$(\text{EP}_{\text{II}}) = \frac{(\text{E})\{k_{-3}(k_{-1}+k_{+2})+k_{+1}k_{+2}[\text{G1P}]\}}{D_1} \quad (6)$$

where:

$$\begin{aligned} D_1 = {} & k_{+1}(k_{+2}+k_{+3})[\text{G1P}]+k_{-2}(k_{-1}+k_{-3})[\text{G6P}] \\ & +k_{+1}k_{-2}[\text{G1P}][\text{G6P}]+(k_{+3}+k_{-3})(k_{-1}+k_{+2}) \\ & +\frac{k_{+4}}{k_{-4}[\text{G1P6P}]}(k_{+3}[\text{G1P}]+k_{-3}[\text{G6P}] \\ & +k_{+1}k_{-2}[\text{G1P}][\text{G6P}]) \end{aligned}$$

and (E) is the total quantity of enzyme.

The rate constants must also be related by the expression:

$$Kk_{+1}k_{+2}k_{+3} = k_{-1}k_{-2}k_{-3} \quad (7)$$

where $K$ is the equilibrium constant.

From eqns. (1), (2), (5), (6) and (7):

$$\frac{\text{Flux of phosphate from G1P to G6P}}{\text{Flux of phosphate from G6P to G1P}} = \frac{[\text{G1P}]^2(\text{EP}_{\text{I}})k_{-3}}{[\text{G6P}]^2K^2(\text{EP}_{\text{II}})k_{+3}} = \frac{[\text{G1P}]^2(1+\alpha K[\text{G6P}])}{[\text{G6P}]^2K^2(1+\alpha[\text{G1P}])} \quad (8)$$

From eqns. (3), (4), (5), (6) and (7):

$$\frac{\text{Flux of glucose residues from G1P to G6P}}{\text{Flux of glucose residues from G6P to G1P}} = \frac{[\text{G1P}](\text{EP}_{\text{I}})k_{-3}}{[\text{G6P}]K(\text{EP}_{\text{II}})k_{+3}} = \frac{[\text{G1P}](1+\alpha K[\text{G6P}])}{[\text{G6P}]K(1+\alpha[\text{G1P}])} \quad (9)$$

And at chemical equilibrium from eqns. (1), (3) and (7):

$$\frac{\text{Flux of glucose residues from G1P to G6P}}{\text{Flux of phosphate from G1P to G6P}} = 2+\alpha[\text{G1P}] \quad (10)$$

where:

$$\alpha = k_{+1}k_{+2}/k_{-3}(k_{-1}+k_{+2}) \quad (11)$$

Since $\alpha$ is inversely related to $k_{-3}$, $\alpha$ will be zero when the isomerization of the phosphoenzyme is not rate-limiting.

(*b*) Flux equations when there is direct transfer of phosphate (mechanism 2). For a mechanism similar to mechanism 1 but in which there is a direct transfer of phosphate between the 1-position and the 6-position (i.e. mechanism 2) the expressions for both the glucose and phosphate fluxes will be those given for the glucose fluxes in Scheme 1. The ratio of the glucose flux to the phosphate flux will be unity. Similar flux-ratio equations would be obtained if the effective agent were an enzyme–G1P6P complex as in mechanism 3 (Scheme 2).

(*c*) Flux equations for mechanism 3. For mechanism 3 an entirely analogous method of calculation as for mechanism 1 may be used and it is possible to write:

Flux of phosphate from G1P to G6P

$$= \frac{k_{+1}^3k_{+2}^3k_{+3}^2[\text{G1P}]^3(\text{EG1P6P}_{\text{I}})}{D_2} \quad (12)$$

Flux of phosphate from G6P to G1P

$$= \frac{k_{-1}^3k_{-2}^3k_{-3}^2[\text{G6P}]^3(\text{EG1P6P}_{\text{II}})}{D_2} \quad (13)$$

where:

$$\begin{aligned} D_2 = {} & k_{+1}^2k_{+2}^2k_{+3}^2(k_{-1}+k_{+2})[\text{G1P}]^2+k_{-1}^2k_{-2}^2k_{-3}^2(k_{-1}+k_{+2})[\text{G6P}]^2+k_{+1}k_{+2}k_{+3}k_{-1}k_{-2}k_{-3}(k_{-1}+k_{+2})[\text{G1P}][\text{G6P}] \\ & +k_{+1}k_{+2}k_{-1}^2k_{-2}^2k_{-3}[\text{G1P}][\text{G6P}]^2+k_{+1}^2k_{+2}^2k_{+3}k_{-1}k_{-2}[\text{G1P}]^2[\text{G6P}] \end{aligned}$$

$$\text{Flux of glucose from G1P to G6P} = \frac{k_{+1}^2k_{+2}^2k_{+3}[\text{G1P}]^2(\text{EG1P6P}_{\text{I}})}{k_{+1}k_{+2}k_{+3}(k_{-1}+k_{+2})[\text{G1P}]+k_{-1}k_{-2}k_{-3}(k_{-1}+k_{+2})[\text{G6P}]+k_{+1}k_{+2}k_{-1}k_{-2}[\text{G1P}][\text{G6P}]} \quad (14)$$

and

$$\text{Flux of glucose from G6P to G1P} = \frac{k_{-1}^2k_{-2}^2k_{-3}[\text{G6P}]^2(\text{EG1P6P}_{\text{II}})}{k_{+1}k_{+2}k_{+3}(k_{-1}+k_{+2})[\text{G1P}]+k_{-1}k_{-2}k_{-3}(k_{-1}+k_{+2})[\text{G6P}]+k_{+1}k_{+2}k_{-1}k_{-2}[\text{G1P}][\text{G6P}]} \quad (15)$$

In addition in the presence of excess of G1P6P:

$$(\mathrm{EG1P6P_I}) = \frac{(\mathrm{E})\{k_{+3}(k_{-1}+k_{+2})+k_{-1}k_{-2}[\mathrm{G6P}]\}}{k_{+1}(k_{+2}+k_{+3})[\mathrm{G1P}]+k_{-2}(k_{-1}+k_{-3})[\mathrm{G6P}]+k_{+1}k_{-2}[\mathrm{G1P}][\mathrm{G6P}]+(k_{+3}+k_{-3})(k_{-1}+k_{+2})} \quad (16)$$

and

$$(\mathrm{EG1P6P_{II}}) = \frac{(\mathrm{E})\{k_{-3}(k_{-1}+k_{+2})+k_{+1}k_{+2}[\mathrm{G1P}]\}}{k_{+1}(k_{+2}+k_{+3})[\mathrm{G1P}]+k_{-2}(k_{-1}+k_{-3})[\mathrm{G6P}]+k_{+1}k_{-2}[\mathrm{G1P}][\mathrm{G6P}]+(k_{+3}+k_{-3})(k_{-1}+k_{+2})} \quad (17)$$

where (E) is the total quantity of enzyme.

The rate constants must also be related as in eqn. (7).

From eqns. (12), (13), (16), (17) and (7):

$$\frac{\text{Flux of phosphate from G1P to G6P}}{\text{Flux of phosphate from G6P to G1P}} = \frac{[\mathrm{G1P}]^3(\mathrm{EG1P6P_I})k_{-3}}{[\mathrm{G6P}]^3K^3(\mathrm{EG1P6P_{II}})k_{+3}} = \frac{[\mathrm{G1P}]^3(1+\alpha K[\mathrm{G6P}])}{[\mathrm{G6P}]^3K^3(1+\alpha[\mathrm{G1P}])} \quad (18)$$

From eqns. (14), (15), (16), (17) and (7):

$$\frac{\text{Flux of glucose from G1P to G6P}}{\text{Flux of glucose from G6P to G1P}} = \frac{[\mathrm{G1P}]^2(\mathrm{EG1P6P_I})k_{-3}}{[\mathrm{G6P}]^2K^2(\mathrm{EG1P6P_{II}})k_{+3}} = \frac{[\mathrm{G1P}]^2(1+\alpha K[\mathrm{G6P}])}{[\mathrm{G6P}]^2K^2(1+\alpha[\mathrm{G1P}])} \quad (19)$$

And at chemical equilibrium from eqns. (12), (14) and (7):

$$\frac{\text{Flux of glucose from G1P to G6P}}{\text{Flux of phosphate from G1P to G6P}} = \frac{3+2\alpha[\mathrm{G1P}]}{2+\alpha[\mathrm{G1P}]} \quad (20)$$

where $\alpha$ is defined as in eqn. (11) but with the rate constants referring to mechanism 3 (Scheme 2).

At high concentrations of substrates or in solutions of high electrolyte strength the activity coefficients of G1P and G6P should be introduced into the flux equations. The activity coefficients of the different enzymic species should also be included in relation to the appropriate rate constants, but if the activity coefficients of all the enzymic species are the same these coefficients cancel. The latter assumption may not necessarily be true if the electrical charge on the enzyme changes on the addition of substrate, and as a consequence the apparent rate constants may change in solutions of high electrolyte strength. However, except for the numerical value of $\alpha$, the flux-ratio equations will be unaltered.

The flux-ratio equations for mechanisms 1 and 2 are independent of the G1P6P concentration. For mechanism 3 the flux-ratio equations will remain of the same form at low G1P6P concentrations, but the significance of the rate constants may change somewhat if an appreciable part of the reaction takes place by the substrates combining with the free enzyme and with the G1P6P subsequently adding to the enzyme–substrate complex. The latter is unlikely to be the case if G1P6P dissociates only occasionally from the enzyme except at very low substrate concentrations.

In deriving the flux equations it has been assumed either that the free G1P6P in solution exchanges rapidly and comes into a steady state or that the rate of exchange of G1P6P is negligible. The experimental result lies between the two extremes, but as the quantity of G1P6P present is small the effect is relatively unimportant. It is discussed in the Results section.

When the isomerization of the enzyme is not rate-limiting an alternative way of deriving the flux-ratio equations is by the probability method of Hodgkin & Keynes (1955).

*Significance of the flux ratios.* If G1P molecules are converted into G6P molecules independently then the flux ratio should obey the Independence relationship (Britton, 1965*a*).

Thus:

$$\frac{\text{Flux of G1P to G6P}}{\text{Flux of G6P to G1P}} = \frac{f_{\mathrm{G1P}}[\mathrm{G1P}]}{f_{\mathrm{G6P}}[\mathrm{G6P}]K} \quad (21)$$

where $f_{\mathrm{G1P}}$ and $f_{\mathrm{G6P}}$ represent the activity coefficients of G1P and G6P respectively and the other symbols are as defined above.

Consider a net conversion of G1P into G6P. For mechanism 1 the glucose flux ratio (eqn. 9) should be less than that expected from the Independence relationship, and the chemical equivalent of exchange diffusion should occur unless $\alpha$[G1P] and $\alpha K$[G6P] are very small, when the Independence relationship should be obeyed. The same will be true for the phosphate flux ratio if there is a direct transfer of phosphate as in mechanism 2. However, for mechanism 1 the phosphate flux ratio (eqn. 8) should exceed that to be expected from the Independence relationship, so that the chemical analogue of the long-pore effect should be found. For mechanism 3 both the glucose flux ratio (eqn. 19) and the phosphate flux ratio (eqn. 18) should show the long-pore effect. Examination of the reaction for the chemical analogues of exchange diffusion and the long-pore effect should thus provide a method of distinguishing between the models. The ratio of the glucose flux to the phosphate flux at chemical equilibrium will provide a further method of making a distinction, since the ratio will be 1:1 if there is a direct transfer of phosphate (mechanism 2), but will be greater than 1:1 for mechanisms 1 and 3 (eqns. 10 and 20). In contrast, the expressions for the net fluxes for all three models are identical in the presence of excess of G1P6P. At lower concentrations of G1P6P the expressions for the net fluxes for mechanisms 1 and 2 remain identical but for mechanism 3 the effect of a low G1P6P concentration will depend on the relative values of the rate constants, as has been discussed in the introduction.

To examine an A$\rightleftharpoons$B reaction of the present type for the chemical analogue of exchange diffusion or the long-pore effect, it is convenient to use tests based on induced transport (Britton, 1963, 1965*a*, 1966*b*; and see the Appendix). To carry out these tests appropriately labelled substrate (G1P or G6P) is allowed to come into equilibrium in the presence of the enzyme and then unlabelled substrate (G1P) is added. If activity coefficients and isotope effects are ignored, then, if the flux ratio obeys the Independence

relationship, the distribution of radioactivity between the two substrates will remain unchanged during the reaction. If the reaction shows the chemical analogue of exchange diffusion there will be a transient flow of labelled material in a direction opposite to the net flow; by analogy with the permeability studies this may be called counter-transport. Finally, if the reaction shows the chemical analogue of the long-pore effect there will be a transient flow of labelled material in the same direction as the net flow; this may be termed co-transport. The influence of isotope effects and non-ideal behaviour of solutions on these tests is considered in the Discussion section.

## MATERIALS AND METHODS

*Reagents and materials.* G1P, G6P and G1P6P, the monophosphates as the sodium salts and the diphosphate as the cyclohexylammonium salt, were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). Rabbit skeletal-muscle phosphoglucomutase was purchased from the same source as a crystalline suspension in 0·25 M-$(NH_4)_2SO_4$. The enzyme was separated from $(NH_4)_2SO_4$ by placing it on a short column of Sephadex G-25 that had been equilibrated with 9·6 mM-tris–chloride buffer, pH 7·4, and by eluting with the same buffer. The enzyme was used without further purification, but was activated immediately before use by adding a histidine–tris buffer, pH 7·4, to give final concentrations of histidine (40 mM), tris (12 mM), $MgCl_2$ (1·1 mM) and gelatin (50 $\mu$g./ml.) and incubating for 10 min. at 30° as described by Ray & Roscelli (1964*a*, 1966*a*). When assayed according to the method of Ray & Roscelli (1964*a*) an activity of about 50–100 units/$\mu$g. was obtained, which may be compared with the value of 105 units/$\mu$g. obtained by Ray & Roscelli (1964*a*) for their most active preparations.

Solutions of G1P and G6P were adjusted to pH 7·4 with HCl at a glass electrode.

[G-$^{14}$C]G6P was obtained from The Radiochemical Centre (Amersham, Bucks.) and was used without further purification.

[$^{32}$P]G1P was prepared by the following procedure. A 1 mC sample of sodium [$^{32}$P]phosphate, supplied as a sterilized solution, pH 3, by The Radiochemical Centre, was evaporated to dryness in a vacuum desiccator over NaOH. Then 0·2 ml. of 10 mM-sodium phosphate buffer, pH 7, was added, followed by 0·1 ml. of aq. 4% (w/v) glycogen solution and 0·1 ml. of rabbit muscle phosphorylase solution [supplied by Sigma Chemical Co. (St Louis, Mo., U.S.A.) as a 2 mg./ml. suspension in 1·5 mM-EDTA, 1·92 mM-sodium glycerophosphate adjusted to pH 6·6 with acetic acid and 0·1 M-NaF; the activity was 1434 units/mg. (Cori, Cori & Green, 1943)]. After incubation at 30° for 90 min. the mixture was evaporated to dryness in a vacuum desiccator. It was then dissolved in a small quantity of water and chromatographed successively once with butan-1-ol–acetic acid–water (12:3:5, by vol.) (as a 2 in.-wide strip) and twice with 2-methoxyethanol–ethyl methyl ketone–aq. 3 N-$NH_3$ (7:2:3, by vol.) (Mortimer, 1952) on Whatman no. 4 paper.

All glassware was cleaned with Decon 75 (A. Gallenkamp and Co. Ltd., London, E.C. 2) before use.

*Analytical methods.* Total enzyme protein was determined from the extinction at 278 m$\mu$ by using $E^{1\%}_{1\,cm.}$ 7·70 (Najjar, 1948).

G1P was determined by a slight modification of Bartlett's (1959) phosphate method as described by Ray & Roscelli (1964*a*). Samples (0·1 ml.) were taken from the reaction mixture and added to 4 ml. of 0·18% (w/v) ammonium molybdate in N-$H_2SO_4$ in stoppered test tubes. Then 0·2 ml. of reducing solution (2·4 g. of anhydrous sodium sulphite, 12 g. of sodium metabisulphite and 0·2 g. of 1-amino-2-naphthol-4-sulphonic acid in 100 ml. of water) was added and the test tubes were immersed in a boiling-water bath for 10 min. The extinctions were measured at 830 m$\mu$. Hydrolysis of the G1P and colour development take place at the same time in this procedure.

[$^{32}$P]G1P was determined by a variation of the method of Ray *et al.* (1966) based on the phosphate precipitation technique of Sugino & Miyoshi (1964). A mixture of 57 mM-ammonium molybdate, 74 mM-triethylamine and 0·9 N-$HClO_4$ (2:1:1, by vol.) was prepared shortly before use and 0·4 ml. portions were placed in 15 ml. conical centrifuge tubes. Samples (0·1 ml.) taken from the reaction mixture were added to the centrifuge tubes followed by 0·5 ml. of a solution containing G1P (0·4 mM) and G6P (0·4 mM). The centrifuge tubes were placed in a boiling-water bath for 10 min., cooled thoroughly in cold running water and kept for at least 1 hr. at 4°. After centrifuging at 2000*g* for 20 min., the supernatants were removed and the precipitates washed three times, without disturbing the compacted material, with a mixture consisting of 57 mM-ammonium molybdate, 74 mM-triethylamine, 0·9 N-$HClO_4$ and water (2:1:1:6, by vol.) to which a trace of phosphate had been added. The precipitates were then dissolved with gentle warming in 0·2 ml. of N-NaOH and each solution was transferred quantitatively with three washings of 0·2 ml. of water to a scintillation vial containing 15 ml. of a scintillation mixture [60 g. of naphthalene, 4 g. of 2,5-diphenyloxazole, 0·2 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 100 ml. of methanol, 20 ml. of ethylene glycol and dioxan to 1 l.] to which Cab-O-Sil (Packard Instrument Co., Wembley, Middx.) had been added as a 5% suspension to prevent loss of radioactivity as a result of adsorption on the wall of the glass vial. The vials were counted in a Packard Tri-Carb liquid-scintillation counter (Packard Instrument Co.). No correction was applied for quenching.

[$^{32}$P]G6P was counted by using the phosphate precipitation technique described for [$^{32}$P]G1P. After centrifugation, portions (0·4 ml.) of the supernatants were added to scintillation vials containing scintillation mixture and Cab-O-Sil as in the determination of [$^{32}$P]G1P. Approx. 0·15 ml. of N-NaOH was added to discharge the yellow colour and the vials were counted as described above.

[$^{14}$C]G1P was determined as glucose after hydrolysis and removal of G6P and G1P6P with $ZnSO_4$ and $Ba(OH)_2$. In experiments at low concentrations of substrates 0·1 ml. samples from the reaction mixture were each added to 0·1 ml. of N-$H_2SO_4$ in glass-stoppered test tubes. The tubes were heated in a boiling-water bath for 10 min. and, after cooling, 0·5 ml. of a solution containing glucose (0·1 mg./ml.) was added to each followed by 0·65 ml. of $Ba(OH)_2$ solution (approx. 0·15 M). The tubes were agitated thoroughly, 0·2 ml. of $ZnSO_4$ solution (approx. 5%, w/v) was added to each and the tubes were again thoroughly agitated before the precipitate was compacted by centrifugation. Finally, 0·1 ml. of the $Ba(OH)_2$ solution followed by 0·1 ml. of the $ZnSO_4$ solution were added with thorough agitation. After centrifugation 0·1–0·2 ml. (0·7–1·0 ml. in

equilibrium-flux experiments) of each supernatant was pipetted into 15ml. of dioxan scintillation medium in scintillation vials as in the determination of [$^{32}$P]G1P, but Cab-O-Sil was omitted. The vials were counted and corrected for quenching by the channels-ratio method.

The concentrations of $Ba(OH)_2$ and $ZnSO_4$ solutions were adjusted until 9·8–9·9ml. of $Ba(OH)_2$ solution added to 10ml. of $ZnSO_4$ solution (plus 5ml. of water) produced a faint pink colour with phenolphthalein (Somogyi, 1945; Nelson, 1944). It was found that 6·4ml. of this $Ba(OH)_2$ solution also gave a faint pink colour with phenolphthalein when added to 2ml. of $ZnSO_4$ solution and 1ml. of N-$H_2SO_4$. There was thus a very slight excess of $Ba^{2+}$ at the end of each of the precipitation steps.

The procedure outlined above was suitable for experiments in which the total substrate concentration did not exceed approx. 5mM. For higher concentrations the volumes were increased. At 30·4mM total concentration (Fig. 2) the volumes of reagents were doubled and the sample volume was decreased to 0·05ml. At 86·4mM total concentration (Fig. 3) the reagent volumes were increased 20-fold, apart from the final addition of $ZnSO_4$ and $Ba(OH)_2$, where 1ml. of each solution was added; 2ml. of the supernatant was taken for counting.

Total radioactivity in the reaction mixtures was determined by taking 0·1ml. samples and adding to 15ml. of scintillation mixture containing Cab-O-Sil.

[$^{14}$C]G1P6P was determined as follows. Samples (0·1ml.) taken from the reaction mixture were heated very briefly at 100° and applied to DEAE-cellulose chromatography paper (Whatman DE81). Descending chromatography was then carried out for 6hr. with 0·18M-ammonium formate and the chromatogram was scanned with a Packard scanner (Packard Instrument Co.).

*Induced-transport tests.* The enzyme was activated in histidine–tris buffer as described under 'Reagents and materials' above and suitably diluted with the same buffer. G1P and [$^{32}$P]G1P, or G6P and [$^{14}$C]G6P, and G1P6P were then added and the system was incubated for 60–90min. at 30° to allow it to come into equilibrium. Non-radioactive G1P was then added in small volume to the reaction mixture. Samples (0·1ml.) were taken for chemical and radioactivity determinations before and after the addition of non-radioactive G1P. In one series of experiments (*A* and *C* in Fig. 1 and Figs. 4 and 5), 4ml. of the enzyme solution was taken and 1ml. of a solution containing G1P, or G6P, and G1P6P was added; the non-radioactive G1P was added in a volume of 0·4ml. to 4·6ml. of this solution. The concentrations of histidine, tris and $MgCl_2$ were 29·4, 8·8 and 0·8mM respectively after the addition of the non-radioactive G1P. In the other series of experiments (*B* in Fig. 1 and Figs. 2 and 3), 1·35ml. of a solution containing G6P and G1P6P was added to 4ml. of the enzyme solution; the non-radioactive G1P was added in a volume of 0·4ml. to 4·95ml. of this solution. In this case the final concentrations of histidine, tris and $MgCl_2$ were 27·7, 8·3 and 0·76mM respectively.

When [$^{14}$C]G1P was determined it was necessary to allow for radioactivity due to incomplete precipitation of G6P by $ZnSO_4$ and $Ba(OH)_2$, partial hydrolysis of G6P and radioactive impurities in the [$^{14}$C]G6P. Of these the last appeared to be the most important. Radioactivity from these sources, which amounted to about 10% of the total, was determined by carrying out a blank experiment in which no enzyme was present. When allowance was made for this the radioactivity in the G1P agreed with that calculated to be present from the equilibrium constant.

When [$^{32}$P]G1P was determined allowance had to be made, not only for partial hydrolysis of G6P and radioactive impurities of the G1P, but for the contribution from the phosphate derived from the 1-position of G1P6P. A correction factor was derived by subtracting the radioactivity, calculated to be present in the G1P from the total radioactivity added and the equilibrium constant, from the radioactivity found in the G1P during the equilibrium period. The correction factor usually amounted to about 15–20% of the total radioactivity found in the G1P. An equilibrium constant of 1/17·2 was assumed (Colowick & Sutherland, 1942; Ray & Roscelli, 1964*b*); the chemical determinations of G1P in the present paper yielded equilibrium constants within 2% of this value at all concentrations.

*Comparison of glucose and phosphate fluxes at chemical equilibrium.* A solution of the enzyme in histidine–tris buffer was prepared and activated as described under 'Reagents and materials' above. G1P and G1P6P were added as in the induced-transport experiments (0·42ml. to 7·78ml. of enzyme–buffer solution) and the system was allowed to come into equilibrium at 30°. The solution was divided into two equal 4ml. portions and [$^{32}$P]G1P and [$^{14}$C]G6P were added respectively to the two portions in small volume (0·01–0·025ml.). Samples (0·1ml.) were then taken at intervals for measurement of radioactivity. The concentrations of histidine, tris and $MgCl_2$ were 37·5, 11·2 and 1·03mM in the final reaction mixture.

To calculate the fluxes the logarithms to base 10 of the differences between the actual counts in individual samples and the final equilibrium value were plotted against time and the best straight line was drawn. The fluxes were derived from the slope of the line by using the expression:

$$\text{Flux} = \frac{2{\cdot}303\,V}{\left(\frac{1}{[\text{G1P}]}+\frac{1}{[\text{G6P}]}\right)(\text{E})}(\text{Slope})$$

where $V$ is the volume of solution, (E) is the total quantity of enzyme and [G1P] and [G6P] represent concentrations. No allowance was made for radioactivity appearing in the G1P6P, since Ray *et al.* (1966) found a negligible transfer of radioactivity into this material. Further, the concentration of the G1P6P was only about 5% of the G1P concentration.

*Depression of freezing point.* Freezing-point determinations were carried out on 0·4ml. samples with an Advanced osmometer (Advanced Instruments Inc., Newton Highlands, Mass., U.S.A.).

## RESULTS

In all the experiments the reaction was carried out in a histidine–tris buffer, pH 7·4, at 30° with activated enzyme (Ray & Roscelli, 1964*a*) and in the presence of excess of G1P6P. In considering the concentration of G1P6P competition from G1P and G6P had to be taken into account, but calculations from the data of Ray & Roscelli (1964*a*,*b*) indicated that concentration of G1P6P exceeded the effective $K_m$ for G1P6P by five- to ten-fold.

*Induced-transport tests with* $^{14}C$*-labelled substrates.* The results of a series of induced-transport tests at different substrate concentrations with $^{14}C$-labelled substrates are illustrated in Figs. 1–3. Where single experiments only are shown the results have been confirmed by duplicate experiments that are not illustrated. At low concentrations (Fig. 1) the radioactivity in the G1P remained substantially unchanged during the reaction, but at the concentrations used in Fig. 2 there was probably a small rise in the radioactivity, and at the high concentrations in Fig. 3 there was a rise of about 10% while the reaction was in progress. The increase in radioactivity in Fig. 3 appeared to be significant, since it occurred in all experiments, and no rise took place in control experiments in which the enzyme was inactivated by brief heating immediately before the addition of non-radioactive G1P. At the end of the reaction the radioactivity in the G1P always returned substantially to the value existing before the addition of non-radioactive G1P.

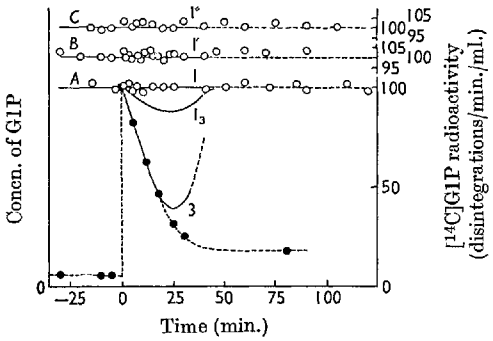



Fig. 1. Induced-transport tests at low substrate concentrations with $^{14}C$-labelled substrates showing absence of induced transport. [$^{14}C$]G6P was incubated at 30° with rabbit muscle phosphoglucomutase in a histidine–tris buffer, pH 7·4, containing G1P6P until equilibrium was reached. Samples were then taken from the reaction mixture and at zero time non-radioactive G1P was added. ●, Concn. of G1P; ○, [$^{14}C$]G1P radioactivity (disintegrations/min./ml.) corrected for dilution caused by addition of G1P. *A*, Non-radioactive G1P was added in a volume of 0·4ml. to 4·6ml. of solution. Concentrations before addition: G1P, 0·0404mM; G6P, 0·695mM; G1P6P, 2·13$\mu$M; concentrations immediately after addition: G1P, 1·044mM; G6P, 0·630mM; G1P6P, 2·0$\mu$M. *B*, Non-radioactive G1P was added in a volume of 0·4ml. to 4·95ml. of solution. Concentrations before addition: G1P, 0·087mM; G6P, 1·51mM; G1P6P, 4·6$\mu$M; concentrations immediately after addition: G1P, 1·58mM; G6P, 1·39mM; G1P6P, 4·2$\mu$M. The points shown represent means of duplicate or triplicate observations. *C*, Non-radioactive G1P was added in a volume of 0·4ml. to 4·6ml. of solution. Concentrations before addition: G1P, 0·0808mM; G6P, 1·39mM; G1P6P, 4·26$\mu$M; concentrations immediately after addition: G1P, 2·088mM; G6P, 1·26mM; G1P6P, 4·0$\mu$M. In individual experiments the time-course of the reaction varied slightly. The time-scale of each experiment was therefore adjusted so that the course of the reaction was represented by the curve shown. The continuous lines are theoretical curves: 1, 1′ and 1″ for mechanism 1 or mechanism 2, assuming $\alpha = 0{\cdot}637 \times 10^{-2}$ mM$^{-1}$; 3, for mechanism 3; $1_3$, for 80% of the glucose flux from G1P to G6P proceeding by mechanism 1 or mechanism 2 and 20% proceeding by mechanism 3.

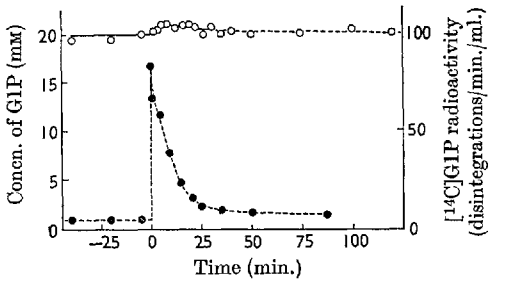



Fig. 2. Induced-transport test with $^{14}C$-labelled substrates at intermediate substrate concentrations. For symbols and experimental procedure see the legend to Fig. 1. At zero time non-radioactive G1P in 0·4ml. was added to 4·95ml. of reaction mixture. Concentrations before addition of non-radioactive G1P: G1P, 0·869mM; G6P, 14·92mM; G1P6P, 0·0489mM; concentrations immediately after addition: G1P, 16·53mM; G6P, 13·74mM; G1P6P, 0·0450mM. The continuous line is the theoretical curve for mechanism 1 or mechanism 2 assuming $\alpha = 0{\cdot}637 \times 10^{-2}$ mM$^{-1}$.

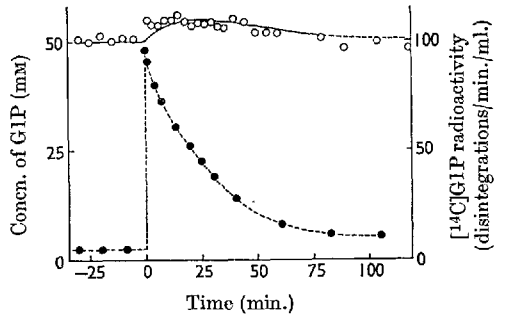



Fig. 3. Induced-transport test at high substrate concentrations with $^{14}C$-labelled substrates showing counter-transport. For symbols and experimental procedure see the legend to Fig. 1. The points shown represent means of duplicate or triplicate determinations. At zero time non-radioactive G1P in 0·4ml. was added to 4·95ml. of reaction mixture. Concentrations before addition: G1P, 2·465mM; G6P, 42·4mM; G1P6P, 0·1155mM; concentrations immediately after addition: G1P, 47·1mM; G6P, 39·2mM; G1P6P, 0·1059mM. The continuous line is the theoretical curve for mechanism 1 or mechanism 2 assuming $\alpha = 0{\cdot}637 \times 10^{-2}$ mM$^{-1}$.

It is possible that after the addition of non-radioactive G1P (Figs. 1–3) some radioactivity was transferred from the G1P6P to the G1P, but (see below) any contribution from this source must have been very small. Consequently a change in the radioactivity in the G1P must very largely reflect a corresponding change in the radioactivity in the G6P. Thus the experiments shown in Figs. 1–3 provide strong evidence for a lack of induced transport of $^{14}C$-labelled substrates at low substrate concentrations and counter-transport at high concentrations. If isotope effects and non-ideal behaviour of solutions, which are probably relatively unimportant (see below), are ignored, it follows that the glucose fluxes must obey the Independence relationship at low substrate concentrations, but that at high concentrations the flux ratio must deviate from this relationship in the direction of exchange diffusion. Such a result is clearly compatible with either mechanism 1 or mechanism 2, for which the equations are identical, and the continuous lines drawn through the experimental points in Figs. 1–3 are the theoretical lines for $\alpha = 0{\cdot}637 \times 10^{-2}$ mM$^{-1}$ (eqn. 9). Exchange diffusion provides evidence that there is a rate-limiting isomerization of the phosphoenzyme, and if non-ideal behaviour of the solution is ignored the apparent rate constant for the isomerization step (if $k_{+3} = k_{-3}$) will be about $4{\cdot}5 \times 10^7$ sec.$^{-1}$, as is shown in the Appendix.

The results in Figs. 1–3 exclude mechanism 3, since for this mechanism the glucose fluxes should show the chemical analogue of the long-pore effect at low concentrations of substrate and co-transport should have occurred. Thus the theoretical curves 3 and $1_3$ in Fig. 1 show the calculated results if the whole reaction had proceeded via mechanism 3 or if 20% of the flux from G1P to G6P had proceeded via mechanism 3, assuming that $\alpha$ (eqn. 19) is small, and the theoretical curves clearly diverge from the experimental results. If the value of $\alpha$ (eqn. 19) were large the predicted amount of co-transport would be less and the theoretical lines would be closer to the experimental results. However, if this were the case, the net reaction should have shown product inhibition of a partly non-competitive nature, and Ray & Roscelli (1964*b*) were unable to detect any inhibition of this kind. Thus it may be concluded that the experimental results exclude even a small contribution of mechanism 3 to the reaction.

As mentioned above, the possibility that radioactivity was transferred transiently from the G1P6P to the G1P when the non-radioactive G1P was added has to be considered, because of the low specific radioactivity of the G1P immediately after the addition. To test for this possibility samples were taken and chromatographed on DEAE-cellulose paper with ammonium formate to separate the G1P6P from the monophosphates. The chromatograms were then scanned for radioactivity. The G1P6P had the same specific radioactivity as the substrates at the end of the equilibration period, and when the non-radioactive G1P was added the specific radioactivity fell progressively to reach the expected value of about one-half the initial radioactivity. However, at the time when the radioactivity in the G1P was elevated in Fig. 3, the radioactivity in the G1P6P had fallen by less than 20%. The total radioactivity in the G1P6P at the end of the equilibration period was only about 5% at the lower concentrations, and 2·5% at the highest concentrations of the radioactivity in the G1P. Thus the G1P6P could have contributed at most only 0·5–1% to the radioactivity in the G1P. Further, it is extremely unlikely that all the radioactivity disappearing from the G1P6P would be transferred to the G1P. For example, by using the rate constants obtained by Ray & Roscelli (1964*a*,*b*, 1966*a*) it may be calculated that only about one-quarter of the radioactivity would be so transferred. However, the latter calculation may not be valid, since Ray & Roscelli (1964*a*,*b*, 1966*a*,*b*) in their analysis assumed only one intermediate enzyme–substrate complex, and there is evidence suggesting that there may be at least two complexes and that G1P6P can dissociate from both of them. Thus from initial-rate studies with G1P Ray & Roscelli (1964*a*,*b*) obtained an apparent $K_m$ for G1P6P of $6{\cdot}2 \times 10^{-8}$ M, whereas they obtained a value of $1{\cdot}1\ (\pm 0{\cdot}5) \times 10^{-8}$ M from initial-rate studies with G6P under similar experimental conditions. Such a difference could be most simply explained by assuming two intermediate enzyme–substrate complexes. However, it seems that the G1P6P must have contributed very little radioactivity to the G1P during the reaction and that any radioactivity appearing in the G1P must have been largely derived from the G6P. At the end of the reaction there will be a small contribution from the G1P6P to the G1P, but this will be less than 0·1% of the initial radioactivity.

Ray & Roscelli (1964*a*), using $^{32}P$-labelled phosphoenzyme, concluded that the enzyme–substrate complex dissociated into G1P6P on about 5% of occasions, but the present observations suggest that the frequency of dissociation must be rather smaller. Hashimoto & Handler (1966) studied the rate of incorporation of $^{32}P$-labelled phosphate into G1P6P with [$^{32}P$]G1P as substrate and also obtained results that indicated a low rate of dissociation of the enzyme–substrate complex. Further, in later experiments Ray *et al.* (1966) found negligible incorporation of radioactivity into G1P6P in equilibrium flux measurements with $^{32}P$-labelled substrates. It is therefore

possible that in the original experiments of Ray & Roscelli (1964*a*) the frequency of dissociation was overestimated. This could have arisen if there were some [$^{32}$P]G1P6P rather tightly bound to the phosphoenzyme; alternatively, there may have been some alteration of the enzyme during the mixing procedure, perhaps as a result of transient electrolyte changes. The latter might also explain the relatively rapid incorporation of $^{32}$P-labelled phosphate into G1P6P that occurred in the initial stages of the experiments of Hashimoto & Handler (1966). That such enzyme changes may occur is suggested by the work of Cascales & Grisolia (1966) on glycerophosphomutase in which the rate of incorporation of $^{32}$P-labelled phosphate into diphosphoglyceric acid from the substrates was greatly increased when the ionic strength of the solution was raised.

*Induced-transport tests with $^{32}$P-labelled substrates.* Induced-transport tests with $^{32}$P-labelled substrates are illustrated in Figs. 4 and 5. In Fig. 4 the results of a single experiment are shown in which the radioactivity in the G1P fell after the addition of non-radioactive G1P and the initial rate of fall was similar to the rate of chemical reaction. Therefore under these conditions the net flux must have been virtually equal to the flux of [$^{32}$P]G1P to G6P, and the flux of [$^{32}$P]G6P to G1P must have been very small. As the reaction progressed, however, the two curves diverged, and the radioactivity in the G1P reached a minimum of 39% and then returned to its initial value. Fig. 4 also shows for comparison the results of the comparable experiment with $^{14}$C-labelled substrates. A second experiment under the conditions shown in Fig. 4 yielded a very similar result with the same minimum of 39% for the radioactivity in the G1P. In Fig. 5 the experimental conditions were similar to those in Fig. 4, but the concentrations of substrates were doubled. Again a very similar result was obtained. The minima for the radioactivity in the G1P for the two experiments from which the data shown in Fig. 5 were obtained were 37 and 40%.

Clearly in these experiments considerable co-transport of the $^{32}$P-labelled substrates occurred, but, as in the experiments with $^{14}$C-labelled substrates, the contribution from the G1P6P to the radioactivity measured in the G1P has to be considered. This is discussed below, but the effect is slight and amounts to a probable overestimate of the amount of co-transport by rather less than 1% of the initial radioactivity in the G1P. The fact that co-transport occurred indicates that the phosphate flux ratio must show the chemical analogue of the long-pore effect. This observation therefore qualitatively supports either mechanism 1 or mechanism 3 and excludes mechanism 2. It is possible to calculate quantitatively the amount of co-transport

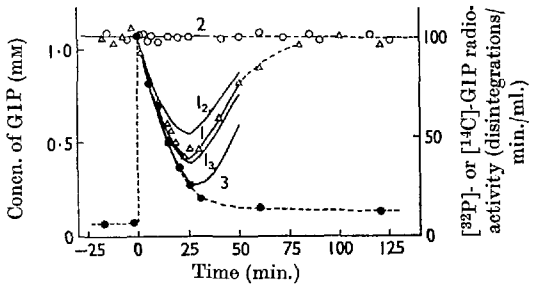



Fig. 4. Induced-transport test with $^{32}$P-labelled substrates showing co-transport. [$^{32}$P]G1P was incubated at 30° with rabbit muscle phosphoglucomutase in a histidine–tris buffer, pH 7·4, until equilibrium was reached. Samples were then taken from the reaction mixture and at zero time non-radioactive G1P was added in a volume of 0·4 ml. to 4·6 ml. of the mixture. ●, Concn. of G1P; △, [$^{32}$P]G1P radioactivity (disintegrations/min./ml.) corrected for dilution caused by addition of non-radioactive G1P; ○, [$^{14}$C]G1P radioactivity (disintegrations/min./ml.) of comparable experiment taken from Fig. 1. Concentrations before addition: G1P, 0·0404 mM; G6P, 0·695 mM; G1P6P, 2·13 $\mu$M. Concentrations immediately after addition: G1P, 1·044 mM; G6P, 0·630 mM; G1P6P, 2·0 $\mu$M. The continuous lines are theoretical curves for $^{32}$P-labelled substrates: 1, for mechanism 1; 2, for mechanism 2; 3, for mechanism 3; $1_2$, for 80% of flux of G1P to G6P proceeding by mechanism 1 and 20% proceeding by mechanism 2; $1_3$, for 80% of flux from G1P to G6P proceeding by mechanism 1 and 20% proceeding by mechanism 3.

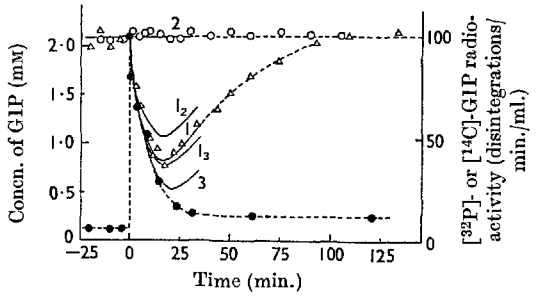



Fig. 5. Induced-transport tests with $^{32}$P-labelled substrates to show that the co-transport is quantitatively unaffected by a twofold increase in substrate concentration. For symbols and experimental procedure see the legend to Fig. 4. The points shown represent means of duplicate observations. Concentrations before addition: G1P, 0·0808 mM; G6P, 1·39 mM; G1P6P, 4·26 $\mu$M. Concentrations immediately after addition: G1P, 2·088 mM; G6P, 1·26 mM; G1P6P, 4·0 $\mu$M.

for the different mechanisms and for various combinations of them (see the Appendix). The theoretical curves, which are the same at both concentrations, are shown as continuous lines in Figs. 4 and 5.

The experimental results correspond closely to the curve for mechanism 1, and the theoretical minimum for the radioactivity in the G1P of 39·1% agrees well with the minima for the radioactivity in the individual experiments. The curves for mechanisms 2 and 3 differ widely from the experimental results, so that these mechanisms can be excluded as major reaction pathways. The curve for 20% of the flux from G1P to G6P proceeding via mechanism 2 and 80% proceeding via mechanism 1 also differs considerably from the experimental results, so that the possibility of any substantial but minor contribution from mechanism 2 is also excluded. The results do not exclude the possibility of a small part of the reaction proceeding via mechanism 3; however, this has already been excluded by the induced-transport tests with $^{14}$C-labelled substrates.

To calculate the induced-transport curves $\alpha$ (eqns. 8 and 18) has been assumed to be small and of the order of that found in the induced-transport tests with $^{14}$C-labelled substrates. The justification for this assumption has already been discussed above in relation to these experiments.

In the above, isotope effects and non-ideal behaviour of the solutions have been ignored, but these effects should be unimportant (see below).

To consider the contribution from the G1P6P to the radioactivity found in the G1P it should be remembered that the method of estimation of G1P also determined the phosphate in the 1-position of the G1P6P. An allowance was made for the radioactivity contributed by the G1P6P by comparing the radioactivity found in the G1P, at the end of the equilibration period, with the radioactivity calculated to be present in this substrate (see the Materials and Methods section). This correction will be valid after the addition of non-radioactive G1P only if the amount of radioactivity in the 1-position of the G1P6P remains constant. Immediately after the addition of non-radioactive G1P, the enzyme–substrate complex was most frequently formed by reaction of the phosphoenzyme with G1P, and since the specific radioactivity of the G1P fell by a large factor the enzyme–substrate complex and any G1P6P formed by dissociation of this complex subsequent to the addition must have had little radioactivity in the 1-position. However, from the chromatographic results obtained in the experiments with $^{14}$C-labelled substrates not more, and probably less, than 20% of the G1P6P present at the time of the minima in Figs. 4 and 5 had been formed after the addition of the non-radioactive G1P. Thus, as the phosphate in the 1-position of the G1P6P represented about 5% of the total acid-labile phosphate, it follows that the fall in the amount of labelling in the 1-position of the G1P6P must have represented not more than 1% of the initial radioactivity in the G1P. A proportion of the radioactivity lost from the 1-position of the G1P6P must have been transferred to the G1P, as has been discussed above in relation to the results obtained with $^{14}$C-labelled substrates, and this part would not have contributed to the error.

*Comparison of glucose and phosphate fluxes at chemical equilibrium.* The results of equilibrium flux-ratio measurements at two different substrate concentrations are shown in Table 1. The ratio of the glucose flux to the phosphate flux determined with $^{14}$C- and $^{32}$P-labelled substrates was close to 2:1 at both concentrations. This result excludes mechanism 2, which would have given a 1:1 ratio, but is in accord with mechanism 1 provided that $\alpha$ is small, as was indicated by the induced-transport experiments with $^{14}$C-labelled substrates. For mechanism 3 the flux ratio would be 3:2 if $\alpha$ were small, but would approach 2:1 if $\alpha$ were large. However, the latter possibility is excluded, since Ray & Roscelli (1964*b*) could not detect any product inhibition that was not strictly competitive. Thus the equilibrium flux ratios are only compatible with mechanism 1 as the major pathway. If a minor part of the reaction proceeded by mechanism 2 or mechanism 3 the effect would be to decrease the flux ratio, but the equilibrium flux ratio is not a very sensitive test for such pathways. Thus if 20% of the glucose flux proceeded by mechanism 2 or

Table 1. *Fluxes of glucose and phosphate at chemical equilibrium determined by the use of $^{14}$C- and $^{32}$P-labelled substrates*

The measurements were made in a histidine–tris buffer, pH 7·4, at 30°.

| Concn. of hexose phosphates (mM) | Concn. of enzyme (μg./ml.) | Glucose flux/ μg. of enzyme (μmoles/min./μg.) | Phosphate flux/ μg. of enzyme (μmoles/min./μg.) | Glucose flux / Phosphate flux ratio | |
|---|---|---|---|---|---|
| G1P 0·055<br>G6P 0·945<br>G1P6P 0·003 | 0·686 | 25·2 | 12·0 | 2·09 | Mean 1·99 |
| | | 32·7 | 17·3 | 1·89 | |
| G1P 0·138<br>G6P 2·362<br>G1P6P 0·0077 | 1·65 | 32·2 | 17·6 | 1·82 | Mean 1·95 |
| | | 48·7 | 23·6 | 2·07 | |

mechanism 3 the flux ratios would become 1·8:1 and 1·9:1 respectively, assuming $\alpha$ to be small as is required by the lack of non-competitive product inhibition.

*Summary of results.* The induced-transport tests with $^{14}C$-labelled substrates were compatible with mechanisms 1 and 2, but excluded even a minor contribution from mechanism 3. The induced-transport tests with $^{32}P$-labelled substrates, on the other hand, gave the expected result for mechanism 1 and excluded any contribution from mechanism 2; any large contribution from mechanism 3 was also excluded. The equilibrium flux-ratio measurements again indicated that mechanism 1 must be the only major pathway. Thus, of the three, mechanism 1 appears to be the only possible mechanism for the reaction. The occurrence of counter-transport with $^{14}C$-labelled substrates at high concentrations provides, in addition, evidence for a rate-limiting isomerization of the phosphoenzyme.

## DISCUSSION

In the foregoing, the influence of isotope effects and non-ideal behaviour of the solutions has been ignored and the results have been discussed in relation to particular models only. These factors and the more general significance of the results are now considered. Induced-transport tests and equilibrium flux-ratio measurements are discussed separately.

*Induced-transport tests.* (*a*) Isotope effects and non-ideal behaviour of solutions. In the present work the absence of induced transport has been used as evidence for the flux ratio obeying the Independence relationship (eqn. 21) and the magnitude of any induced transport has been used as a measure of the deviation of the flux ratio from this relationship. However, it is shown in the Appendix that, in fact, there will be an absence of induced transport when the flux ratio is given by Appendix eqn. (10) and the magnitude of any induced transport will be given by the integral of Appendix eqn. (21). The relationship between these equations and eqn. (21) is discussed in the Appendix and it is concluded that lack of induced transport must necessarily be evidence for the reaction obeying the Independence relationship even if isotope effects are present and the solutions behave non-ideally, provided that the reaction has only one pathway and provided that the ratio of the activity coefficients for the labelled substrates is unaffected by the addition of unlabelled substrate. In contrast, the magnitude of any induced transport will be influenced by isotope effects and may be also affected by the activity coefficients. When there are alternative pathways for the reaction lack of induced transport will not necessarily be evidence for the reaction obeying the Independence relationship, since the isotope may preferentially follow one pathway, but the induced-transport test may still provide evidence for steps in the reaction (see the Appendix).

Unless the isotopically labelled substrate followed one pathway to the exclusion of other pathways, the present results provide strong evidence that the reaction proceeds by only one pathway. Isoenzymes with similar reaction pathways (see below), and the very small part of the reaction that involves the substrates adding to the enzyme before the attachment of $Mg^{2+}$ (Ray & Roscelli, 1966*b*; Ray *et al.* 1966), do not constitute alternative pathways for present purposes. Thus, if activity coefficients are ignored, the lack of induced transport of $^{14}C$-labelled substrates at low substrate concentrations must be good evidence for the reaction obeying the Independence relationship even if isotope effects are present. The counter-transport that was seen at high substrate concentrations is sensitive to isotope effects, but such effects would seem to be unimportant in relation to the other uncertainties of measurement. In the induced-transport experiments with $^{32}P$-labelled substrates isotope effects would be important, since the magnitude of the co-transport was used to distinguish between the mechanisms. However, with $^{32}P$-labelled substrates isotope effects should be very small, and the experiments themselves provided some evidence that such effects were not significant. Thus the initial rates of fall of G1P concentration and [$^{32}P$]G1P radioactivity, after the addition of non-radioactive G1P, in Figs. 4 and 5, were equal. If an isotope effect had been present the rate of fall of [$^{32}P$]G1P radioactivity might have been expected to be less rapid than the fall in G1P concentration.

As mentioned above, lack of induced transport can only be used as a criterion for the reaction obeying the Independence relationship if the ratio of the activity coefficients for the labelled substrates is uninfluenced by the addition of unlabelled substrate. The ratio of the activity coefficients for the labelled species should be similar to the ratio for the unlabelled substrates; the fact that the equilibrium constant was the same for both $^{14}C$-labelled and unlabelled substrates provides experimental support for this assumption (see the Materials and Methods section). Thus it would seem sufficient to show that the ratio for the unlabelled substrates is unchanged when additional substrate is added in order to assume that the ratio for the labelled substrates remains constant. In the present case there is experimental evidence (see below) that the ratio for the unlabelled substrates is constant and close to unity. Lack of induced transport will thus be evidence for the reaction obeying the Independence relationship. Whether the

magnitude of any induced transport is also independent of the activity coefficients under these conditions depends on the nature of the interaction between the fluxes. The magnitude will be independent of the activity coefficients if the interaction between the fluxes is due to an indirect transfer of groups (as, for example, is the case with phosphate in mechanisms 1 and 3), but not if the induced transport is caused by an interaction due to an isomerizing step (see Appendix eqn. 21).

The properties of G1P and G6P are very similar and the ratio of their activity coefficients may be expected to be close to 1:1. Thus G1P and G6P have very nearly the same ionic dissociation constants (Cori, Colowick & Cori, 1937) and electrostatic effects should influence both equally; binding by $Mg^{2+}$ should be small (Ray *et al.* 1966) and may be expected to be similar for both; and the similarity between the molecules suggests that non-ionic factors will influence both in the same way. Evidence that the ratio of the activity coefficients is 1:1 is given by the fact that the equilibrium constant was independent of pH (Colowick & Sutherland, 1942) and by the fact that the equilibrium constant was independent of the concentration of the reactants, within 2%, in the present work (see the Materials and Method section). In addition, experiments on depression of freezing point, which are illustrated in Fig. 6, showed that G1P and G6P gave virtually the same freezing-point depression despite the fact that the activity coefficients fell substantially at the higher concentrations.

If the counter-transport of $^{14}C$-labelled substrates at high concentrations (Fig.3) had been due to the ratio of the activity coefficients departing from 1:1 the departure would have had to be relatively large, since the theoretical curve in Fig. 3 requires a 30% deviation of the flux ratio from Appendix eqn. (10) immediately after the addition of non-radioactive G1P. Perhaps the only way in which this could occur would be if the G1P molecules interacted with each other, for example with the formation of dimers. This possibility appears to be excluded by the freezing-point data, especially since shortly after the addition of the non-radioactive G1P the concentrations of G1P and G6P became the same, and a direct comparison of the freezing-point data over the same range of concentrations becomes possible. Further, dimer formation of the required order of magnitude should alter the equilibrium constant by about 7·5% at the end of the reaction, whereas in fact no change was observed. Indeed, the constancy of the equilibrium constant and the freezing-point data taken together suggest that neither G1P or G6P associates in solution.

(*b*) Significance of induced-transport tests with $^{14}C$-labelled substrates. From the above it is seen that the virtual absence of induced transport of

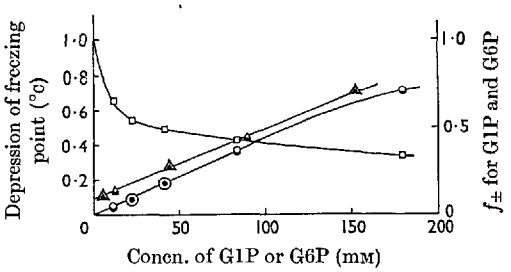



Fig. 6. Depression of freezing point of solutions of G1P and G6P. ○ and ●, $Na_2G1P$ and $Na_2G6P$ in water; △ and ▲, $Na_2G1P$ and $Na_2G6P$ in a histidine–tris buffer, pH 7·4 (histidine, 32mM; tris, 9·6mM; $MgCl_2$, 0·88mM). □, Mean ionic activity coefficients of G1P and G6P at 0° calculated from the data for $Na_2G1P$ and $Na_2G6P$ in water (for details of the calculation see the Appendix). Equimolar mixtures of G1P and G6P gave freezing-point depressions that were the means of the results for the corresponding solutions of G1P and G6P of the same total molarity.

$^{14}C$-labelled substrates over a nearly 30-fold concentration range must constitute very good evidence for the glucose fluxes obeying the Independence relationship (eqn. 21) over this range of concentrations. A reaction obeying the Independence relationship must either possess no steps that bring about an interaction between the fluxes, or if such steps exist the interactions they bring about must effectively cancel each other. The latter possibility would be excluded if it could be demonstrated that the Independence relationship was obeyed over a wide range of concentrations, since cancellation would be expected only over a limited range. Such an observation would be good evidence for a direct transfer of substrate (since an indirect transfer leads to interaction), for an absence of a rate-limiting isomerization of the enzyme and for an absence of certain types of interaction between active centres (Britton, 1965*a*, 1966*b*, 1967*b*). It might have been desirable to make observations at lower substrate concentrations where the enzyme was only partially saturated. Nevertheless, the present results, especially when account is taken of the lack of non-competitive product inhibition and the fact that the enzyme has only one active centre (Ray & Roscelli, 1964*b*), must provide strong evidence for the direct transfer of glucose residues whatever mechanism may be involved.

At the highest concentrations (Fig. 3) counter-transport of $^{14}C$-labelled substrates occurred. As discussed above, this result constitutes strong evidence that the flux ratio departs from the Independence relationship (eqn. 21) in the direction of exchange diffusion (Britton, 1965*a*, 1966*b*). It follows that there must be a method of thermodynamically coupling the energy that is made

available when the non-radioactive G1P is converted into G6P in the induced-transport test to the conversion of radioactive G6P into G1P, since this latter conversion occurs against the energy gradient. Two possible mechanisms have been proposed (Britton, 1965*a*, 1966*b*, 1967*b*). In one there is a rate-limiting isomerization of the free enzyme. During the reaction the two isomeric forms are not at equilibrium and the displacement of this step from equilibrium mediates the transfer of energy. This is comparable with the mobile-carrier mechanism that has been used to explain exchange diffusion in the permeability field. The other mechanism involves an interaction between active centres in a multivalent enzyme. This was discussed by Britton (1967*b*), but to explain the interaction some form of isomeric change in the enzyme again seems to be required. Since rabbit phosphoglucomutase has only one active centre/molecule (Ray & Roscelli, 1964*b*) it would appear that the latter possibility can be excluded, and counter-transport must constitute good evidence for an isomerizing step involving the free enzyme. The assumptions involved in deriving a numerical value for the rate constant are discussed in the Appendix. However, the true value of the rate constant may be expected to be about one-sixth of the apparent rate constant ($4{\cdot}5 \times 10^7$ sec.$^{-1}$) because of the low activity coefficients of the substrates at high concentrations. There is an additional uncertainty because of the need to assume that the activity coefficients of the different enzyme species are equal.

(*c*) Significance of the induced-transport tests with $^{32}$P-labelled substrates. As discussed above, the induced transport of $^{32}$P-labelled substrates constitutes strong evidence that the flux ratio for phosphate deviates from the Independence relationship (eqn. 21) in the direction of the long-pore effect. It follows that in the induced-transport test radioactive phosphate from the G1P is transferred to the G6P against the energy gradient (Britton, 1965*a*), and it is necessary for the reaction to contain a step that will transfer the energy made available from the conversion of non-radioactive G1P into G6P to the radioactive species. The indirect transfer of phosphate provides such a step, since when the $^{32}$P-labelled phosphate is attached to the enzyme (or to an enzyme–G1P6P complex) the subsequent fate of the $^{32}$P-labelled phosphate depends on whether a molecule of G1P or G6P reacts next with the enzyme, and it is immaterial whether this molecule is labelled or not. Interaction of this type is very similar to that proposed by Hodgkin & Keynes (1955) in their long-pore model to explain $K^+$ permeability of the squid axon. Alternatively, interaction could occur if the enzyme were multivalent and there were interaction between active centres (Britton, 1965*a*, 1966*b*, 1967*b*). The type of interaction that would be required would be similar to that suggested to occur in multivalent carriers (Hodgkin & Keynes, 1955; Britton, 1965*b*). However, in the present case the enzyme has only one active centre/molecule (Ray & Roscelli, 1964*b*), and the results obtained with $^{14}$C-labelled substrates are incompatible with such interactions since the glucose flux ratio should be similarly influenced. Thus the indirect transfer of phosphate appears to provide the only possible explanation of the experimental results.

Since the phosphate flux ratio was equal to the square of the right-hand side of the Independence relationship (eqn. 21) over a considerable concentration range, it can further be argued that there must be only one intermediate phosphate in the reaction whatever the mechanism. Thermodynamic considerations (Britton, 1966*a*) indicate an average of at least one intermediate phosphate, but a larger number of intermediate phosphates would give rise to a greater degree of interaction, as is seen when the enzyme is considered as a molecular shuttle (Britton, 1966*b*). Although it would be possible for interactions produced by different steps in the reaction to cancel each other, the degree of cancellation would be expected to depend on the substrate concentrations, and the fact that the phosphate flux ratio was independent of these concentrations constitutes strong evidence for a single intermediate phosphate.

*Ratio of glucose flux to phosphate flux at chemical equilibrium.* The ratio of the fluxes at chemical equilibrium will not be influenced by non-ideal behaviour of the substrates except insofar as there may be a rate-limiting isomerization of the enzyme, when the numerical value of $\alpha$ (see eqns. 10 and 20) will be affected. On the other hand the flux ratio will be sensitive to any isotope effects. The fact that the ratio of the fluxes was 2:1 over a $2\frac{1}{2}$-fold concentration range is compatible with the direct transfer of glucose residues and the indirect transfer of phosphate with a single intermediate phosphate, and with an isomerization of the free phosphoenzyme that is not substantially rate-limiting at the substrate concentrations used. There are, however, other mechanisms that would yield the same result, though they may appear to be unlikely. Thus the glucose residue might be transferred indirectly with one intermediate glucose and the phosphate might be transferred via three intermediate phosphates. Correspondingly, there might be two intermediate glucose residues and five intermediate phosphate groups. Unlike the induced-transport tests, interaction between active centres will not influence the flux ratio at equilibrium.

It is seen from eqn. (10) that the flux ratio should increase at high substrate concentrations if there is a slow isomerization of the free enzyme. However,

calculation shows that this is a far less sensitive test for such a step than the induced-transport test with $^{14}C$-labelled substrates. Thus a value of $\alpha$ that provides a 10% rise in the radioactivity in the G1P in the induced-transport test will produce only an 0·8% change in the ratio of the glucose flux to the phosphate flux when equilibrium has been reached.

*General conclusions.* To summarize, the induced-transport tests and the flux ratio at equilibrium provide very strong evidence for the reaction proceeding entirely by the direct transfer of glucose from product to reactant and the indirect transfer of phosphate with one intermediate phosphate. These conclusions are independent of the detailed reaction mechanism and require no assumptions about the constancy of the rate constants. Further, strong evidence is obtained for an isomerization of the free phosphoenzyme with an apparent rate constant of about $4{\cdot}5 \times 10^7 sec.^{-1}$. The true rate constant would appear to be about one-sixth of this value. Of the three models proposed in the introduction it is clear that mechanism 1 represents the only possible mechanism. It is also clear that the isomerization of the phosphoenzyme is so rapid that it can only involve a small chemical change. This might involve a rearrangement of hydrogen-bonding of the phosphate by histidine.

It has been reported that rabbit muscle phosphoglucomutase contains two isoenzymes (Joshi *et al.* 1967). However, the conclusions drawn about the mechanism of the reaction are unaffected, since these do not depend on the rate constants. Some uncertainty must be introduced about the value of the rate constant for the isomerization, since one isoenzyme might possess a higher rate of isomerization than the other, but conclusions about the order of magnitude of this rate constant will still be valid.

Ray & Roscelli (1964*b*) found no evidence for isomerization of the phosphoenzyme when they examined the reaction for product inhibition, and they concluded that the apparent rate constant for the isomerization step, if it should exist, must be greater than $10^7 sec.^{-1}$. Similar concentrations of substrates were used as in the induced-transport tests with $^{14}C$-labelled substrates, and it therefore appears that induced transport is a more sensitive test for isomerization of the enzyme. Further, the induced-transport test does not require the assumption that the rate constants are unaffected by the presence of substrate. Both tests, however, will be similarly influenced by non-ideal behaviour of the solutions.

Although in the above the induced-transport tests and the flux ratio at equilibrium have been discussed particularly in relation to the phosphoglucomutase reaction, it is clear that such tests should be generally applicable to enzyme studies. The relative freedom of induced-transport tests from isotope effects and the relatively unambiguous interpretation of the results should make them particularly useful as kinetic tools.

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## APPENDIX

*Part I: conditions for induced transport*

Consider a chemical reaction:

$$A \rightleftharpoons B$$

and suppose that both labelled and unlabelled reactants are present. Then at equilibrium:

$$\frac{f_A^0[A]_0}{f_B^0[B]_0} = K \quad (1)$$

and

$$\frac{f_{A^*}^0[A^*]_0}{f_{B^*}^0[B^*]_0} = K^* \quad (2)$$

where $[A]_0$ and $[B]_0$ are the concentrations, $f_A^0$ and $f_B^0$ are the activity coefficients and $K$ is the equilibrium constant for the unlabelled species, and $[A^*]_0$, $[B^*]_0$ etc. have the same significance for the labelled species.

Let:

$m_{A\to B}$ = flux of unlabelled A to B

$m_{B\to A}$ = flux of unlabelled B to A

Then:

$$\text{Flux of labelled A to B} = p_0 m_{A\to B}\frac{f_{A^*}^0[A^*]_0}{f_A^0[A]_0} \quad (3)$$

$$\text{Flux of labelled B to A} = q_0 m_{B\to A}\frac{f_{B^*}^0[B^*]_0}{f_B^0[B]_0} \quad (4)$$

where $p_0$ and $q_0$ are factors to allow for isotope effects at chemical equilibrium.

Since there is equilibrium:

$$p_0 m_{A\to B}\frac{f_{A^*}^0[A^*]_0}{f_A^0[A]_0} = q_0 m_{B\to A}\frac{f_{B^*}^0[B^*]_0}{f_B^0[B]_0} \quad (5)$$

From eqns. (1), (2) and (5):

$$\frac{p_0}{q_0} = \frac{K}{K^*} \quad (6)$$

Suppose that unlabelled A is added to the equilibrium mixture. Then:

$$\text{Flux of labelled A to B} = p m_{A\to B}\frac{f_{A^*}[A^*]_0}{f_A[A]} \quad (7)$$

$$\text{Flux of labelled B to A} = q m_{B\to A}\frac{f_{B^*}[B^*]_0}{f_B[B]_0} \quad (8)$$

where $f_A$, $f_B$ etc. are activity coefficients and $p$ and $q$ are isotope factors for the particular non-equilibrium conditions.

Let:

$$\frac{p/q}{p_0/q_0} = n \quad (9)$$

Co-transport will occur if the flux of labelled A to B exceeds the flux of labelled B to A, and counter-transport will take place if the converse should hold. If the two fluxes are equal no induced transport of the labelled species will occur. Thus from eqns. (2), (6), (7) and (8):

$$n\frac{m_{A\to B}}{m_{B\to A}} = \frac{f_A[A]f_{B^*}f_{A^*}^0}{f_B[B]_0 K f_{A^*}f_{B^*}^0} \quad (10)$$

for no net transport of labelled material;

$$n\frac{m_{A\to B}}{m_{B\to A}} > \frac{f_A[A]f_{B^*}f_{A^*}^0}{f_B[B]_0 K f_{A^*}f_{B^*}^0} \quad (11)$$

for co-transport of labelled material;

$$n\frac{m_{A\to B}}{m_{B\to A}} < \frac{f_A[A]f_{B^*}f_{A^*}^0}{f_B[B]_0 K f_{A^*}f_{B^*}^0} \quad (12)$$

for counter-transport of labelled material.

In eqns. (10), (11) and (12) $m_{A\to B}$ and $m_{B\to A}$ are the fluxes of unlabelled material. Clearly, however, when the isotope is present in trace quantities only these will approximate to the total fluxes.

*Isotope effects.* $K^*$ does not appear in eqns. (10), (11) and (12), and the extent by which $K^*$ differs from $K$ as a result of isotope effects will not influence the criteria for induced transport. The factor $n$ will also be unity even in the presence of isotope effects if the reaction has only one pathway, since the labelled and unlabelled molecules must take part in exactly the same reactions and $p$ and $q$ will always remain in constant proportion. However, $n$ will not necessarily be unity if the reaction has alternative pathways, since the labelled species may follow one pathway preferentially and the labelled species will be influenced by the flux ratio for this particular pathway rather than by the flux ratio for the overall reaction. Nevertheless the occurrence of induced transport may still give rise to the thermodynamic necessity of postulating a mechanism to account for the transfer of energy from the unlabelled to the labelled species (Britton, 1965, 1966), and may therefore provide evidence for or against certain steps in the reaction. The influence of isotope effects on the activity coefficients is discussed below.

*Non-ideal behaviour of solutions.* If the ratio $f_{A^*}/f_{B^*}$ is uninfluenced by the addition of non-radioactive A, then the activity coefficients for the labelled species cancel out in eqns. (10), (11) and (12), and assuming $n$ is unity the criteria for no induced transport, co-transport and counter-transport become that the flux ratio should obey the Independence relationship (eqn. 21 of the main paper), should show the long-pore effect and should

show exchange diffusion respectively. Isotope effects may be expected to have little influence on activity coefficients, and in most cases it would seem sufficient to demonstrate that the ratio $f_A/f_B$ is uninfluenced by the addition of A in order to assume that the activity coefficients for the labelled species cancel.

From the above it is seen that it should also be possible to use an alternative substrate rather than labelled material in the induced-transport test.

Although isotope effects may often have no influence on the criteria for the occurrence of induced transport, the magnitude of any induced transport is sensitive to isotope effects, as shown in part II of the Appendix.

Although from general considerations $n$ may be expected to be unity if there is a single pathway, the following argument, which may be employed when the isotope is present in trace quantities, may be useful.

Consider, for example, the transport of $^{32}$P-labelled phosphate groups from G1P to G6P as is shown for mechanism 1 in Scheme 1 of the main paper, and let the rate constants for the reactions involving $^{32}$P-labelled phosphate (as shown in Scheme 1 of the main paper) be $k'_{+1}$, $k'_{+2}$ etc. and the rate constants for the non-isotopic reactions be $k_{+1}$, $k_{+2}$ etc. Since $^{32}$P-labelled phosphate groups are present in trace quantities only, the possibility that two molecules of [$^{32}$P]G1P react successively with the enzyme can be ignored, so that the sequence shown will be the only one for the transfer of $^{32}$P-labelled phosphate from G1P to G6P.

The overall flux ratio is the product of the flux ratio for the individual steps (Britton, 1963) and it is possible to write:

$$\frac{\text{Flux of [}^{32}\text{P]phosphate from G1P to G6P}}{\text{Flux of [}^{32}\text{P]phosphate from G6P to G1P}} = \frac{f_{[^{32}\text{P]G1P}}[[^{32}\text{P]G1P}]f_{\text{G1P}}[\text{G1P}]k'_{+1}k'_{+2}k'_{+3}}{f_{[^{32}\text{P]G6P}}[[^{32}\text{P]G6P}]f_{\text{G6P}}[\text{G6P}]k'_{-1}k'_{-2}k'_{-3}} \quad (13)$$

and

$$\frac{\text{Flux of unlabelled phosphate from G1P to G6P}}{\text{Flux of unlabelled phosphate from G6P to G1P}} = \frac{f_{\text{G1P}}{}^2[\text{G1P}]^2 k_{+1}k_{+2}k_{+3}}{f_{\text{G6P}}{}^2[\text{G6P}]^2 k_{-1}k_{-2}k_{-3}} \quad (14)$$

where [G1P], [G6P] etc. represent concentrations and $f_{\text{G1P}}$ etc. activities.

Let G1P = A and G6P = B; then by comparisons with eqns. (7) and (8):

$$\frac{p}{q} = \frac{k'_{+1}k'_{+2}k'_{+3}k_{-1}k_{-2}k_{-3}}{k'_{-1}k'_{-2}k'_{-3}k_{+1}k_{+2}k_{+3}} = \frac{K}{K^*} \quad (15)$$

Thus by comparison with eqn. (6) $n$ will be unity.

*Part II*

Consider a chemical reaction:

$$A \rightleftharpoons B$$

Net rate of accumulation of isotopically labelled A is given by:

$$V\frac{d[A^*]}{dt} = q'\frac{[B^*]}{[B]}\,(\text{flux of B to A}) - p'\frac{[A^*]}{[A]}\,(\text{flux of A to B}) \quad (16)$$

where [A] and [B] represent the total concentrations and [A*] and [B*] the concentrations of labelled A and B, $t$ is time, $p'$ and $q'$ are factors to allow for isotope effects and $V$ is volume of the solution.

Let:

$$\frac{\text{Flux of A to B}}{\text{Flux of B to A}} = r \quad (17)$$

Then since:

$$\text{Net flux} = (\text{flux of A to B}) - (\text{flux of B to A})$$

$$\text{Flux of A to B} = \frac{r}{r-1}\,(\text{net flux of A to B}) \quad (18)$$

and

$$\text{Flux of B to A} = \frac{1}{r-1}\,(\text{net flux of A to B}) \quad (19)$$

Also

$$\text{Net flux of A to B} = -V\frac{d[A]}{dt} \quad (20)$$

Substituting eqns. (18), (19) and (20) into eqn. (17):

$$\frac{d[A^*]}{dt} = -\frac{d[A]}{dt}\left(q'\frac{[B^*]}{[B](r-1)} - p'\frac{[A^*]r}{[A](r-1)}\right) \quad (21)$$

If $p' = q' = 1$ (i.e. no isotopic effects are present):

$$\frac{d[A^*]}{d[A]} = \frac{[A^*]r}{[A](r-1)} - \frac{[B^*]}{[B](r-1)} \quad (22)$$

It is also possible to write:

$$[A] + [B] = T \quad (23)$$

and

$$[A^*] + [B^*] = N \quad (24)$$

where $T$ is total concentration of substrate and $N$ is the total concentration of labelled substrate. Eqn. (24) assumes that the amount of label associated with the cofactor is negligible or constant.

The following relationships for $r$ were introduced into eqn. (22) derived from the flux-ratio equations

given in the Theory section of the main paper ([A]=[G1P], [B]=[G6P]):

$$(a) \qquad r = \frac{[A](1+\alpha K[B])}{K[B](1+\alpha[A])} \qquad (25)$$

derived from eqn. (9) of the main paper.

$$(b) \qquad r = \frac{[A]^2}{K^2[B]^2} \qquad (26)$$

derived from eqn. (8) or eqn. (19) of the main paper assuming:

$$\frac{1+\alpha K[B]}{1+\alpha[A]} = 1$$

This simplification was adopted in view of the numerical value of $\alpha$ and the low concentrations of G1P and G6P employed in the experiments.

$$(c) \qquad r = \frac{[A]^3}{K^3[B]^3} \qquad (27)$$

derived from eqn. (18) of the main paper by making the same assumption as in (*b*) above.

$$(d) \qquad r = \frac{[A]^2}{(1-p)K^2[B]^2+pK[A][B]} \qquad (28)$$

derived by assuming that a fraction $(1-p)$ of the flux A to B was associated with a flux of B to A given by the value of $r$ in eqn. (26) and that a fraction $(p)$ of the flux of A to B was associated with a flux of B to A given by eqn. (25), assuming:

$$\frac{1+\alpha K[B]}{1+\alpha[A]} = 1$$

as in (*b*) above.

$$(e) \qquad r = \frac{[A]^3}{(1-p)K^2[A][B]^2+pK^3[B]^3} \qquad (29)$$

derived by assuming that a fraction $(1-p)$ of the flux of A to B was associated with a flux of B to A given by eqn. (26) and that a fraction $(p)$ of the flux of A to B was associated with a flux of B to A given by eqn. (27).

[B] and [B*] were eliminated from eqn. (22) by using eqns. (23) and (24) and the resulting equation was integrated numerically with an ICL 1900 series computer from the initial conditions given in the legend to Fig. 1. It is seen that $\alpha$ was closely proportional to the magnitude of the counter-transport over the range calculated.

If the solutions show non-ideal behaviour activity coefficients should be appropriately introduced into the expressions for $r$. However, when the activity coefficients for A and B are equal, as in the present case, the coefficients cancel in eqns. (26)–(29), but the numerical value of $\alpha$ in eqn. (25) is changed (see part IV of the Appendix).

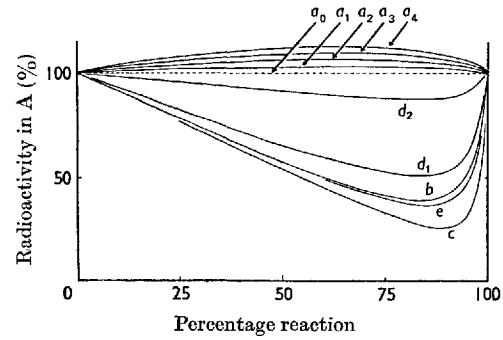



Fig. 1. Theoretical induced-transport curves obtained by integrating eqn. (22). The letters *a*, *b*, *c* etc. refer to the expressions for $r$ that were substituted into eqn. (22). For curve $a_0$, $\alpha=0$; for curves $a_1$, $a_2$, $a_3$ and $a_4$, $\alpha[A]_I$ was 0·1, 0·2, 0·3 and 0·4 respectively, where $[A]_I$ represents the initial value of A. For curve $d_1$, $p=0{\cdot}8$; for curve $d_2$, $p=0{\cdot}2$; for curve *e*, $p=0{\cdot}2$. Initial values: for curves $a_0$–$a_4$, $[A]_I=47{\cdot}14$, $[B]_I=39{\cdot}2$; for curves *b*, *c*, $d_1$, $d_2$ and *e*, $[A]_I=1{\cdot}044$, $[B]_I=0{\cdot}630$. $K=1/17{\cdot}2$. If $\alpha[A]_I=0{\cdot}3$, $\alpha=0{\cdot}637\times10^{-2}$.

In eqns. (28) and (29) for simplicity it has been arbitrarily assumed that the fluxes of A to B through the different pathways remain in the same proportion throughout the reaction. If two enzymes are involved, for example, it may be more realistic to assume that the net fluxes remain in constant proportion. A consideration of the relationship between the fluxes (eqn. 18), however, shows that the induced-transport curves will remain of the same form for the initial part of the reaction and that the differences in the later parts of the curves will be relatively small.

*Part III: calculation of mean ionic activity coefficients for G1P and G6P*

The mean ionic activity at the lowest concentration at which measurements were made (11·78 mM) in Fig. 6 of the main paper was calculated from the simplified Debye–Hückel relationship:

$$f_{\pm} = \frac{0{\cdot}580\times2\times\sqrt{s}}{1+\sqrt{s}}$$

where $f_{\pm}$ is the mean ionic activity at 0° and $s$ is the ionic strength. The change in activity coefficient with increasing concentration was then calculated from the freezing-point-depression curve by using standard techniques (see Moore, 1957).

*Part IV: calculation of the rate constants for the isomerization of the phosphoenzyme*

From eqns. (3) and (5) in the main paper in the presence of excess of G1P6P:

$$K_{G1P} = \frac{(k_{+3}+k_{-3})(k_{-1}+k_{+2})}{k_{+1}(k_{+2}+k_{+3})} \quad (30)$$

where $K_{G1P}$ is the apparent Michaelis constant for G1P in the presence of excess of G1P6P, and

$$\frac{V_{max.}}{[E]} = \frac{k_{+2}k_{+3}}{k_{+2}+k_{+3}} \quad (31)$$

where $V_{max.}$ is the maximum initial velocity with G1P as substrate in the presence of excess of G1P6P.

From the expression for $\alpha$ (eqn. 11 of the main paper) it follows that:

$$\frac{\alpha K_{G1P}[E]}{V_{max.}} = \frac{1}{k_{+3}} + \frac{1}{k_{-3}} \quad (32)$$

From the present work $\alpha = 0{\cdot}637 \times 10^{-2}$ mM$^{-1}$ and, from Ray & Roscelli (1966), $K_{G1P} = 5{\cdot}2\,\mu$M and $V_{max.}/[E] = 750$ sec.$^{-1}$. Thus:

$$\frac{1}{k_{+3}} + \frac{1}{k_{-3}} = \frac{0{\cdot}637 \times 10^{-2} \times 5{\cdot}2 \times 10^{-3}}{750}$$

and if $k_{+3} = k_{-3}$:

$$k_{+3} = k_{-3} = 4{\cdot}5 \times 10^{7}\,\text{sec.}^{-1}$$

This value of $k_{+3}$ represents an apparent value, since the activity coefficients for G1P and G6P and for the enzyme species have been assumed to be unity.

Taking into account the activity coefficients of G1P and G6P and assuming them to be equal (see the Discussion section of the main paper and part III of the Appendix):

$$k_{+3} = k_{-3} = 4{\cdot}5 \times 10^{7} \times \frac{f'_{G1P}}{f''_{G1P}}$$

where $f'_{G1P}$ is the activity coefficient of G1P under the conditions of the induced-transport test and $f''_{G1P}$ is the activity coefficient of G1P under the conditions under which $K_{G1P}$ was determined.

The ionic strength in the experiments shown in Fig. 3 of the main paper from which $\alpha$ was determined was calculated to be 0·517, and from Fig. 6 of the main paper the mean ionic activity coefficient for G1P was found to be 0·417. The ionic strength in the experiments of Ray & Roscelli (1966) in which $K_{G1P}$ was determined was calculated to be 0·1145 and the corresponding mean ionic activity of G1P was found to be 0·792. If the activity coefficients for $Na^+$ are assumed to be 0·79 and 0·84 at the higher and lower ionic strengths it follows that:

$$\frac{f'_{G1P}}{f''_{G1P}} \simeq \frac{1}{6}$$

Thus the true rate constant will be about one-sixth of the apparent value. Clearly, however, such a calculation can only be regarded as approximate and is subject to the uncertainty involved in the determination of individual ionic activities.

The activity coefficients for the different enzyme species should be introduced in relation to the appropriate rate constants in eqns. (30), (31) and (32), but these activity coefficients cancel if they are equal. The latter will not necessarily be true if the electrical charge on the enzyme molecule is altered by the addition of substrate and this introduces an additional uncertainty into the calculation of the true rate constant. However, it would seem unlikely that such factors would alter the order of magnitude of the rate constants.

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