Mechanism of Yeast Phosphoglycerate Mutase†

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ABSTRACT: Possible mechanisms for yeast phosphoglycerate mutase are reviewed and it is concluded from the available evidence that the reaction may proceed, possibly by (a) an intramolecular transfer of phosphate, or more probably by either (b) an intermolecular transfer of phosphate from diphosphoglycerate or (c) a phosphoenzyme mechanism. Induced transport tests at intermediate concentration (1.0 and 0.5 mM) showed cotransport with ^{32}P substrates and no induced transport with ^{14}C substrates. These results excluded (a) but were quantitatively compatible with (c) provided any isomerization of the phosphoenzyme were not rate limiting or with (b) if an isomerization of the enzyme–diphosphoglycerate complex were the rate-limiting step. Similar results at very low concentrations (9.5 μM) however were incompatible with (b) which would have required the K_m for 3-phosphoglycerate to have been less than 1.7 μM compared to a measured value of 200 μM. The results thus indicate a phosphoenzyme mechanism. Induced transport experiments with ^{14}C substrate at high concentration (20 mM) showed no induced transport and indicated that any isomerization of the phosphoenzyme must have a rate constant in excess of $\sim 10^6$ sec^{-1}. The lack of induced transport with ^{14}C substrate over a very wide concentration range also excluded certain types of interaction between active centers. In accord with a phosphoenzyme mechanism ^{14}C substrates exchanged twice as rapidly as ^{32}P substrates at chemical equilibrium. A mechanism is proposed that does not involve a formal isomerization of the phosphoenzyme.

With kinetic methods derived from the permeability field the DPGA[1]-dependent phosphoglycerate mutase from rabbit muscle has been shown to possess a Ping-Pong or phosphoenzyme mechanism (Scheme C or C′, Figure 1) (Britton and Clarke, 1969, and unpublished data), whereas the DPGA-independent enzyme of wheat germ catalyzes an intramolecular transfer of phosphate (Scheme A, Figure 1) (Britton *et al.*, 1971). In view of the difference in mechanism it seemed of interest to investigate the phosphoglycerate mutase of yeast since although this enzyme is DPGA dependent, the organism may be more closely related to the plant than to the animal kingdom.

The enzymic conversion of 2-PGA to 3-PGA may be represented by the steps

$$\text{2-PGA} + E_1 \underset{k_{-1}}{\overset{k_{-1}}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{-2}}{\rightleftharpoons}} E_2 + \text{3-PGA} \qquad (1)$$

$$E_1 \underset{k_{+3}}{\overset{k_{-3}}{\rightleftharpoons}} E_2 \qquad (2)$$

where ES represents the enzyme–substrate complex and $E_1 \rightleftharpoons E_2$ an isomerization of the free enzyme. k_{+1}, k_{-1}, etc., are the respective rate constants. Possible mechanisms are illustrated in Figure 1. Scheme A is the intramolecular transfer of phosphate (Meyerhof and Kiessling, 1935), Scheme B is the sequential mechanism involving an intermolecular transfer of phosphate from DPGA (Sutherland *et al.*, 1949), Scheme C is the Ping-Pong or phosphoenzyme mechanism originally proposed by Najjar and Pullman (1954) for phosphoglucomutase, and Scheme C′ is a variant of this mechanism in which an enzyme–DPGA complex performs the role of a phosphoenzyme.

Schemes B, C, and C′ explain the exchange of ^{32}P between DPGA and the monophosphoglycerates and therefore represent the most probable mechanisms.[2] The rate of exchange is much slower than the enzymic reaction except in the presence of salts (Grisolia and Cascales, 1966; Cascales and Grisolia, 1966). This is to be expected with all three schemes if DPGA normally dissociates only infrequently from the enzyme; but dissociates more rapidly when salt is present. Grisolia and Cleland (1968) obtained parallel lines when they plotted the reciprocal of the initial velocity at pH 7.4 against the reciprocal of the substrate concentration at different DPGA concentrations and interpreted this as evidence for Scheme C rather than Scheme B. However, if DPGA could dissociate from the enzyme–substrate complex, Scheme B could give parallel lines under some conditions (Britton and Clarke, 1968, and unpublished data) and the kinetics were complicated by Mg^{2+} binding by the substrates. More recently Chiba *et al.* (1970) at pH 5.9 reported a converging pattern. This was interpreted as evidence for Scheme B, but Scheme C may also give converging lines if the phosphoenzyme is unstable as is discussed later. Scheme C′ might give either a converging or a parallel line pattern. Initial velocity studies have thus given conflicting results and the interpretation of such data is equivocal. Britton, Carreras, and Grisolia (unpublished data) incubated the enzyme with [^{14}C]- and

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[1] Abbreviations used are: 3-PGA, D-3-phosphoglyceric acid; 2-PGA, D-2-phosphoglyceric acid; DPGA, D-2,3-diphosphoglyceric acid; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate.

[2] ^{14}C also exchanges between the substrates and DPGA. With Scheme C′ this requires that the DPGA attached to the enzyme should occasionally donate a phosphate group to the substrate. The molecule of monophosphoglycerate formed from the DPGA must then exchange with the substrates in solution. This will also lead to exchange of both phosphate groups of the DPGA.

[^{32}P]DPGA and isolated a phosphoenzyme virtually free of ^{14}C label by gel filtration. After denaturation with sodium dodecyl sulfate, the phosphoprotein was acid labile and alkali stable suggesting the involvement of a phosphohistidine as has recently also been reported by Rose (1972). Spectrophotometric evidence for the formation of an active unstable phosphoenzyme was also obtained. These latter observations strongly suggest a phosphoenzyme mechanism (Scheme C). The phosphoenzyme cannot be an intermediate in the transfer of phosphate as in Scheme B rather than Scheme C since this would require that the exchange between DPGA and substrates should be as rapid as the mutase reaction.

If a molecule of [^{32}P]2-PGA reacts with the enzyme according to mechanism C, the label will be transferred to the phosphoenzyme and to transfer the label to 3-PGA a second molecule of 2-PGA (which need not be labeled) must react with the enzyme. There is therefore an intermediate step, the phosphoenzyme in the phosphate transfer. In contrast if [^{14}C]2-PGA reacts with the enzyme, the label appears in the product without an intermediate step. Adopting the same arguments there are two intermediate steps in the transfer of ^{32}P from 2-PGA to 3-PGA by Scheme B and one intermediate step in the transfer of ^{14}C.

In the work to be described particular use has been made of the induced transport test. Radioactively labeled substrate is allowed to come into equilibrium with the product and then unlabeled substrate (2-PGA) is added. Since the labeled and unlabeled substrates are separate species, the equilibrium of the radioactive species should not be disturbed by the addition and subsequent reaction of the unlabeled material. The radioactivity in the substrate (*total* radioactivity) should therefore remain constant. However there may be an interaction between the flows of unlabeled and labeled material giving rise to an induced flow of the labeled species. If the interaction causes a flow of radioactive material in the same direction as the flow of unlabeled material, the label will be transiently carried from substrate to product. The radioactivity in the substrate will therefore fall during the chemical reaction. This is termed cotransport. Alternatively the interaction may be in the opposite sense giving rise to countertransport. Relatively few molecular mechanisms produce interaction between flows (Britton, 1966, 1967). The indirect transfer of label[3] discussed in relation to Scheme C, C′, and B, and certain types of interaction between active centers will produce cotransport. A rate limiting isomerization of the free enzyme[4] or an interaction between active centers will produce countertransport. To calculate the expected amount of induced transport for any mechanism it is convenient to regard the net chemical reaction as the algebraic sum of two flows in opposite directions. These unidirectional flows are termed fluxes by analogy with the use of the term in permeability studies (Britton, 1965, 1966). (At equilibrium the fluxes are equal and the term is synonymous with equilibrium reaction rate.) Induced transport was calculated from the ratio of the two fluxes as described in Britton and Clarke (1968). Mg^{2+} binding of the substrates was taken into account as outlined in Britton *et al.* (1971).

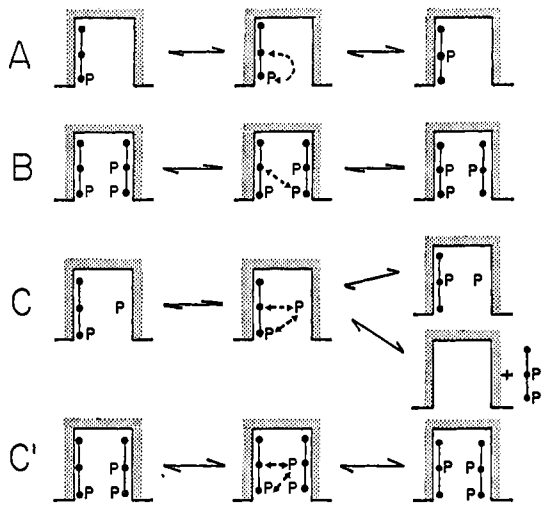

FIGURE 1: Schemes for the phosphoglycerate mutase reaction. Scheme A represents an intramolecular transfer of the phosphate group, Scheme B an intermolecular transfer of phosphate from DPGA to monophosphoglycerate and Scheme C a phosphoenzyme mechanism. Scheme C′ is a modified phosphoenzyme mechanism in which an enzyme–DPGA complex performs the role of a phosphoenzyme.

Materials

Yeast phosphoglycerate mutase was prepared by the method of De la Morena *et al.* (1968). Other materials were obtained as previously described (Britton *et al.*, 1971).

Methods

Determination of [^{32}P]*2-PGA*, [^{14}C]*2-PGA, and* [^{14}C]*DPGA.* AT INTERMEDIATE CONCENTRATIONS. [^{32}P]- and [^{14}C]2-PGA were estimated as described by Britton *et al.* (1971). The radioactivity in the DPGA was estimated by a modification of the procedure described to determine total [^{14}C]monophosphoglycerate. 3-PGA and 2-PGA were converted to lactate and DPGA was precipitated by Zn and Ba. After incubation of the samples with wheat-germ phosphoglycerate mutase, enolase, pyruvate kinase, and lactic dehydrogenase for 30 min at room temperature, 10 μl of 10 mM 3-PGA was added. After 30 min the addition of 3-PGA was repeated, 1.9 ml of the solution was mixed with 100 μl of 16 mM sodium lactate, and 8 μl of 0.25 mM DPGA, 0.1 ml of $ZnSO_4$, and 0.1 ml of $Ba(OH)_2$ were added. The mixture was thoroughly agitated, and allowed to stand for 20 min. The precipitate was washed three times with 0.8 mM sodium lactate, suspended in 0.3 ml of water, and transferred quantitatively to a scintillation vial with two washings of H_2O (0.3 and 0.4 ml). Scintillation fluid (10 ml) containing Cab-O-Sil was added and the solution was thoroughly shaken. The procedure depends upon the fact that the wheat-germ mutase does not catalyze exchange between DPGA and monophosphoglycerate (Britton *et al.*,

[3] The interaction, when there is an indirect transfer of label, arises in the following manner. Consider Scheme C and suppose that the substrate is labeled with ^{32}P. Reaction of the labelled substrate with the enzyme results in the formation of ^{32}P-labeled phosphoenzyme. Subsequent reaction of the phosphoenzyme with a molecule of 2-PGA (which need not be labeled) will transfer the label to 3-PGA whereas reaction with 3-PGA will transfer the label to 2-PGA. When excess unlabeled 2-PGA is added, in the induced transport test, reaction occurs most frequently with 2-PGA and therefore the label is preferentially transferred to the 3-PGA.

[4] A rate-limiting isomerization produces counter transport since a net flow of 2-PGA to 3-PGA will be associated with a corresponding flow of E_2 to E_1. Consequently the ratio $[E_2]:[E_1]$ will be greater than the value existing at chemical equilibrium. Radiaoctive 3-PGA molecules therefore have a greater chance of encountering molecules of E_2 than radioactive 2-PGA molecules have of finding molecules of E_1. A net flow of radioactivity from 3-PGA to 2-PGA results.

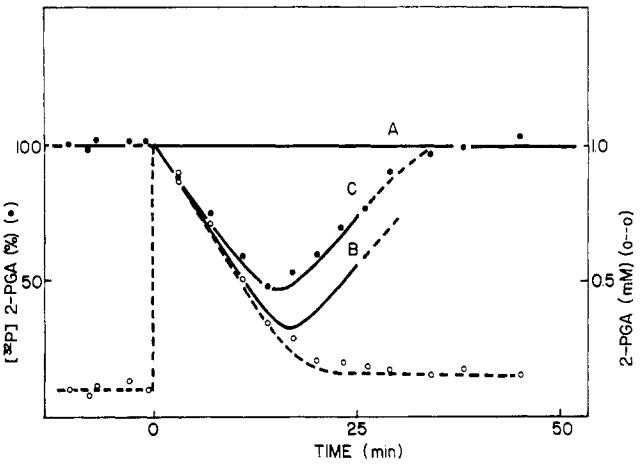

FIGURE 2: Induced transport test with ^{32}P-labeled substrate at intermediate substrate concentration. (○) Concentration of 2-PGA, mM; (●) [^{32}P]2-PGA expressed as per cent of the initial value before the addition of nonradioactive 2-PGA. The continuous lines are theoretical curves for the radioactive 2-PGA, for the different schemes (Figure 1). 100 μl of a mixture of 3-PGA and 2-PGA (10:1 total concentration, 31.2 mM), 30 μl of DPGA (0.25 mM), 3.37 ml of buffer (Tris-Cl$^-$ (16.7 mM, pH 7.4), $MgCl_2$ (3.33 mM), and bovine serum albumin (0.02%)), and 10 μl of yeast phosphoglycerate mutase (4.8 units) were incubated at 30° for 30 min. 100 μl of [^{32}P]-3-PGA (0.6 μCi) was then added. After a further 30-min incubation, seven samples of 100 μl were taken and at zero time 100 μl of 2-PGA (29.2 mM) was added to the remaining solution (2.9 ml). 100-μl samples were assayed for 2-PGA and [^{32}P]2-PGA. All the reagents, with the exception of the DPGA, were dissolved in the Tris buffer. The concentrations immediately after the addition of 2-PGA were 2-PGA (1.04 mM), 3-PGA, (0.804 mM), and DPGA (2.01 μM).

1971). Although about 0.7% of lactate was precipitated the method was reproducible and suitable to determine whether changes in the radioactivity in the DPGA contributed appreciably to the 2-PGA.

AT VERY LOW CONCENTRATIONS. Fluorimetric modifications of methods previously described (Britton *et al.*, 1972) were used. Samples (100 μl) from the reaction mixture were added to 100 μl of 0.6 M $HClO_4$ in small Pyrex test tubes (75 × 10 mm). One-hundred microliters of 0.6 N NaOH was added to neutralize the solution and then 0.7 ml of buffer was added (triethanolamine-Cl$^-$ (pH 7.6, 0.04 M), KCl (6 mM), $MgCl_2$ (6.4 mM), ADP (0.2 mM), NADH (2 μM), lactate dehydrogenase (5 μg/ml), and pyruvate kinase (10 μg/ml)). The fluorescence of the solution was measured against a quinine standard in a fluorimeter (Model A-3 Farrand Optical Co. Inc., New York) with a 7-60 primary filter and 4-72 and 3-72 secondary filters (Corning Glass Works, Corning, N. Y.). Enolase (10 μl) (Boehringer, 10 mg/ml diluted 1 : 10 with H_2O) was then added and the fluorescence was redetermined 40 min later when the reaction had gone to completion. To determine [^{14}C]2-PGA 50 μl of 16 mM sodium lactate was added followed by 0.25 ml of $ZnSO_4$ and 0.25 ml of $Ba(OH)_2$. After thorough agitation, the solution was allowed to stand overnight and centrifuged and 1 ml of the supernatant was removed for scintillation counting. To determine [^{14}C]DPGA the same procedure was followed but 10 μl of 36 μM DPGA was added after addition of the sample to the perchloric acid and 5 μl (0.7 unit) of wheat-germ mutase was added with the enolase. The reaction was allowed 60 min to ensure its completion. Then 50 μl of 16 mM sodium lactate, 50 μl of 0.15 M $ZnSO_4$, and 50 μl of 0.15 M $Ba(OH)_2$ were added. The precipitate was washed three times with 0.8 mM sodium lactate, suspended and counted as described for the estimation at intermediate concentrations. To determine [^{32}P]2-PGA, 100 μl of 2.55 M $HClO_4$ was added to each tube after the enzymic reaction had reached completion and the solutions were heated on a boiling-water bath for 30 min. After cooling 0.35 ml of phosphate precipitating mixture was added (two volumes of 0.8 M ammonium molybdate, one volume of 0.2 M triethylamine hydrochloride, and four volumes of H_2O) and the mixture allowed to stand overnight at 4°. The precipitate was washed, dissolved, and counted as described by Britton *et al.* (1971). To determine [^{32}P]DPGA, 5 μl of 3 mM DPGA was added after addition of the sample to the perchloric acid and 5 μl (0.7 unit) of wheat-germ mutase was added with the enolase. After 60 min, 100 μl of 2.55 M $HClO_4$ was added and the tubes were heated in a water bath for 60 min. Phosphate precipitating mixture (0.35 ml) was then added and after standing overnight at 4° the tubes were centrifuged and 0.8 ml of the supernatant was transferred to a scintillation vial. Two-tenths milliliter of 1 N NaOH and 10 ml of scintillation solution containing Cab-O-Sil were added.

Results

Induced Transport Tests at Intermediate Concentrations. WITH ^{32}P-LABELED SUBSTRATE. Figure 2 shows an induced transport experiment with ^{32}P-labeled substrates. The ^{32}P-labeled substrates were incubated with the enzyme until chemical equilibrium had been reached and then unlabeled 2-PGA was added. During the reaction that followed the addition of the unlabeled 2-PGA, the radioactive 2-PGA fell to a minimum of about 47% and returned to its original value. A duplicate experiment gave a similar result with a minimum of about 45%. Since the fall in radioactive 2-PGA must have been associated with a corresponding rise in radioactive 3-PGA cotransport of the ^{32}P label must have occurred. When the nonradioactive 2-PGA was added some radioactivity may also have been transferred from DPGA to the 2-PGA since the specific activity of the 2-PGA was reduced by a large factor when the unlabeled 2-PGA was added, and such a transfer would reduce the apparent amount of cotransport. However the quantity of DPGA represented only 2% of the 2-PGA before the unlabeled 2-PGA was added, and as discussed in relation to phosphoglucomutase (Britton and Clarke, 1968), only one of the phosphates of the DPGA would be expected to be transferred to the 2-PGA. The maximum contribution of radioactivity to the 2-PGA would therefore be only 2% and it is unlikely to have been as large as this since some of the radioactivity leaving the DPGA must have been transferred to the 3-PGA. If it is assumed that any isomerization of the free enzyme (eq 2) is not rate limiting, lines A, B, and C represent the theoretical curves for the radioactivity in the 2-PGA for Schemes A, B, C, and C′, respectively (Figure 1). These curves, the derivation of which is outlined in the introduction, do not depend upon rate constants and are determined only by the substrate concentrations and the equilibrium constant. They therefore apply generally. The experimental result thus corresponds closely with that expected for Schemes C and C′. Two further experiments in which the concentration of substrate and cofactor were one-half of those shown in Figure 2 gave minima of 46 and 43% compared to the theoretical minimum of 47% for Schemes C and C′ in the absence of a rate-limiting isomerization of the phosphoenzyme. The effect of rate-limit-

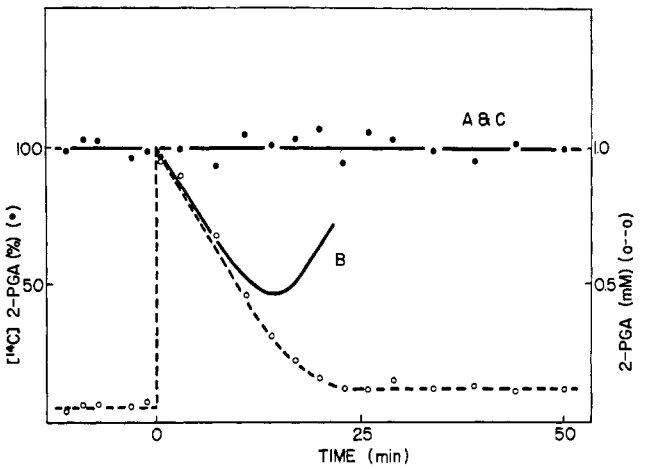

FIGURE 3: Induced transport test with ^{14}C-labeled substrate at intermediate substrate concentration. (○) Concentration of 2-PGA, mM; (●) [^{14}C]2-PGA expressed as per cent of the initial value before addition of nonradioactive 2-PGA. The continuous lines are theoretical curves for the different schemes in Figure 1 (see text). The details of the procedure and the concentrations were those described in Figure 2, except that [^{14}C]3-PGA (100 μl, 2 μCi) was added and the samples were assayed for the ^{14}C isotope.

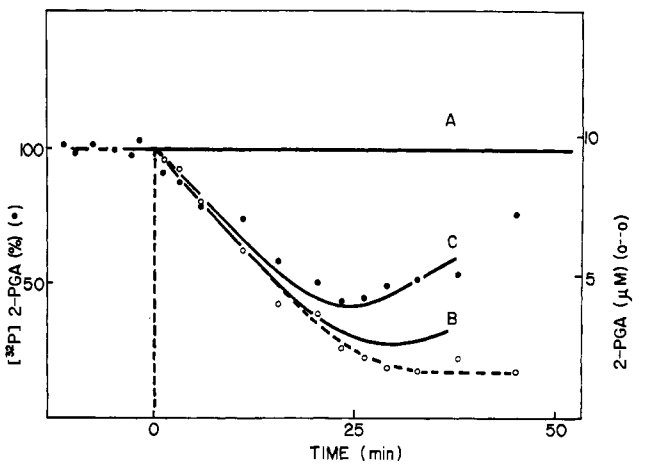

FIGURE 4: Induced transport test with ^{32}P-labeled substrate at low substrate concentration. (○) Concentration of 2-PGA, μM; (●) [^{32}P]2-PGA expressed as per cent of initial value before the addition of nonradioactive 2-PGA. The continuous lines are the theoretical curves (see text). 10 μl of [^{32}P]3-PGA (0.95 mM), 10 μl of DPGA (2.14 μM), 3.58 ml of buffer (Tris-Cl$^-$ (pH 7.4, 16.7 mM), $MgCl_2$ (3.33 mM), and bovine serum albumin (0.02%)), and yeast phosphoglycerate mutase (0.08 unit in 10 μl) were incubated at 30° for 50 min. Seven samples of 100 μl were taken and at zero time 10 μl of 2-PGA (2.68 mM containing DPGA, 5 μM) was added to the solution. 100-μl samples were taken during the ensuing chemical reaction. The samples were assayed for 2-PGA and [^{32}P]2-PGA as described in the text. The concentrations immediately after the addition of 2-PGA were 2-PGA (9.51 μM), 3-PGA (2.42 μM), and DPGA (23 nM).

ing isomerization of the free enzyme upon the theoretical curves will be discussed later.

WITH ^{14}C-LABELED SUBSTRATES. Figure 3 shows an experiment similar to that shown in Figure 2 but with ^{14}C-labeled substrates. In contrast to the ^{32}P experiments the radioactivity in the 2-PGA remained substantially constant throughout the reaction, indicating no induced transport. Transfer of radioactivity from DPGA to 2-PGA must be considered as discussed with the ^{32}P-induced transport experiments. However the contribution of radioactivity to the 2-PGA could not have exceeded 2% at the very most and further estimates of the radioactivity in the DPGA showed a negligible change. Essentially similar results were obtained in a duplicate experiment and in two other experiments where the substrate and cofactor concentrations were halved. A lack of induced transport is the expected result for Schemes A, C, and C′, as is shown by line A and C (Figure 3) provided that any isomerization of the free enzyme is rapid (eq 2). As discussed with the ^{32}P experiments, these theoretical lines are independent of rate constants.

The data from the above experiments with ^{32}P- and ^{14}C-labeled substrates were entirely compatible with Schemes C or C′ and appeared to exclude the other schemes. The assumption was made however that any isomerization of the free enzyme (eq 2), if such a step should exist, was rapid. With Scheme C or C′ a rate-limiting isomerization would give countertransport with ^{14}C substrates and a reduced amount of cotransport with ^{32}P-labeled substrates. With Scheme A countertransport would occur with both ^{14}C- and ^{32}P-labeled substrate. With Scheme B the amount of cotransport with ^{14}C and ^{32}P substrates would be reduced so that, in the limit, there would be no induced transport of the ^{14}C label and the amount of cotransport with ^{32}P-labeled substrates would be that expected of Scheme C or C′. The data could thus be compatible with Scheme B if an isomerization of the free enzyme were the major rate-limiting step. To resolve the ambiguity, experiments were carried out at very low concentrations of substrates.

Induced Transport Test at Very Low Concentrations. WITH ^{32}P-LABELED COMPOUNDS. The results of one experiment with ^{32}P-labeled substrates are shown in Figure 4. As in the experiments at intermediate concentrations, the induced transport curve followed line C, the curve for Schemes C and C′ in the absence of a rate-limiting isomerization of the free enzyme. Transfer of radioactivity from DPGA to 2-PGA as discussed in relation to the experiments at intermediate concentration must have been very small since the radioactivity in DPGA showed no significant change and the DPGA represented only 2% of the 2-PGA before the addition of nonradioactive substrate. A second experiment with a slightly different initial concentration of 3-PGA (4 μM) gave a minimum for the radioactivity in the DPGA of about 47% in tolerable agreement with the theoretical curve for Schemes C and C′ which indicated a minimum of 43%.

WITH ^{14}C-LABELED SUBSTRATES. In the experiment illustrated in Figure 5 the radioactivity in the 2-PGA remained constant indicating a lack of induced transport. As in the previous experiments the DPGA represented only 2% of the 2-PGA present and estimates of the radioactivity in the DPGA were in fact slightly higher during the chemical reaction. Thus transfer of radioactivity from DPGA to 2-PGA appeared to be negligible. Similar results were obtained in two further experiments. An essential lack of induced transport is the expected result for Schemes A, C, and C′ provided that any isomerization of the enzyme is not rate limiting (see line A and C, Figure 5). Line B (Figure 5) is the expected result for Scheme B if the isomerization of the enzyme is rapid and line B* represents one of the family of curves that is obtained as the isomerization is made rate limiting. However, line B* requires that the K_m for 3-PGA should be less than 1.7 μM (see Appendix) and to obtain curves showing a smaller amount of cotransport and a closer fit to the experimental result the K_m would have to be lower. Since this value for the K_m is

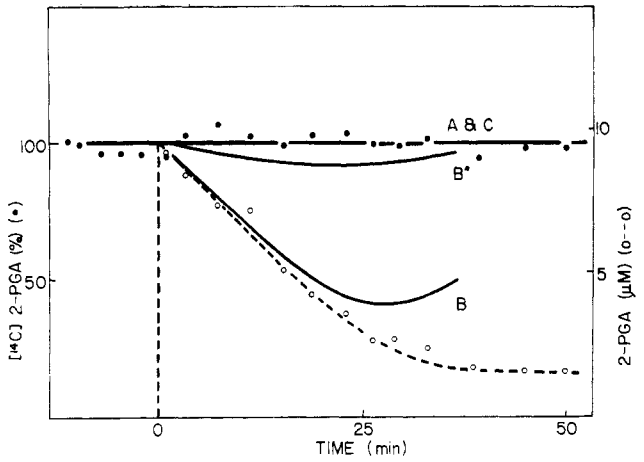

FIGURE 5: Induced transport test with ^{14}C-labeled substrate at low substrate concentration. (○) Concentration of 2-PGA, μM; (●) [^{14}C]2-PGA expressed as per cent of the initial value before the addition of nonradioactive 2-PGA. The continuous lines are the theoretical curves (see text). The details of the procedure and the concentrations were those described in Figure 4 except that 0.6 μCi of [^{14}C]3-PGA (15 μl, 0.63 mM) was added and the samples were assayed for the ^{14}C isotope.

TABLE I: Fluxes of Glycerate and Phosphate at Chemical Equilibrium Determined by the Use of ^{14}C- and ^{32}P-Labeled Substrate.[a]

Quantity of Enzymes Added (Unit)	^{14}C Flux (μmole/min per Unit)	^{32}P Flux (μmole/min per Unit)	^{14}C Flux : ^{32}P Flux
0.57	0.0365	0.0171	2.14
0.15	0.033	0.0175	1.88

[a] A 1.5-ml buffer (pH 7.4) (Tris-HCl (16.7 mM), $MgCl_2$ (3.33 mM), and bovine serum albumin (0.02%)), 25 μl of a solution of 26.4 mM 3-PGA and 2.64 mM 2-PGA dissolved in the same buffer, and 5.5 or 25 μl of enzyme were incubated at 30° for 30 min. A solution (30 μl) of [^{32}P]3-PGA and [^{14}C]3-PGA were then added (representing less than 2% of the 3-PGA already present). The radioactivity in the 2-PGA and the fluxes were determined as described in Britton *et al.* (1971). The concentrations of 2-PGA and 3-PGA were 0.0379 and 0.428 mM in the final solution.

very much less than the experimental value of 200 μM (see below), it would seem that Scheme B must be excluded.

Induced Transport Tests at High Concentrations of Substrates. The experiments described so far indicate that the reaction proceeds by Scheme C or C′ and that isomerization of the enzyme cannot be substantially rate limiting. A rate-limiting isomerization would produce countertransport with ^{14}C substrates and this effect would be more prominent at high substrate concentrations. An experiment at 20 mM substrate concentration was therefore carried out (Figure 6). Since the equilibrium constant should not alter during the reaction, a pH of 5.9 was used to minimize pH changes due to the different ionization constants of 2-PGA and 3-PGA, and Mg^{2+} was omitted to avoid Mg^{2+} binding. The radioactivity in the 2-PGA remained constant throughout the reaction indicating a lack of induced transport. As in the previous experiments the DPGA represented only 2% of the 2-PGA before the addition of nonradioactive 2-PGA and the contribution from DPGA to 2-PGA must have been very small. The equilibrium constant [2-PGA]:[3-PGA] was 1:8.3. Chiba *et al.* (1970) obtained a K_m for 3-PGA of 0.6 mM at pH 5.9, and from their data and a molecular weight of 112,000 (Edelhoch *et al.*, 1957; Pizer, 1960), a V_{max} of 1.56×10^3 sec^{-1} may be derived. From these figures it may calculated (Britton and Clarke, 1968) that if $k_{+3} = k_{-3} = 3.2 \times 10^6$ sec^{-1} a rise of 10% would have occurred in the radioactivity in the 2-PGA during the reaction. The rate constant for isomerization therefore must have been in excess of this figure. These calculations assume only one active center. It seems, however, that there are at least three and probably four (Britton, Carreras, and Grisolia, unpublished data). If there are four then for each active center $k_3 > 0.8 \times 10^6$ sec. These calculations make no allowance for activity coefficients and it is assumed that the K_m is unaffected by the ionic strength. It is unlikely that such factors will alter the order of magnitude of the calculation (Britton and Clarke, 1968).

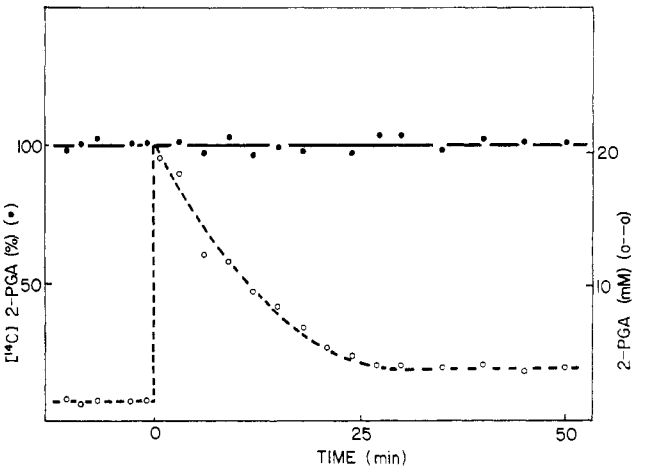

FIGURE 6: Induced transport with ^{14}C-labeled substrate at high substrate concentration. (○) Concentration of 2-PGA, mM, (●) [^{14}C]2-PGA expressed as per cent of initial value before the addition of nonradioactive 2-PGA. 100 μl of a mixture of 3-PGA and 2-PGA (10:1; total concentration 200 mM), 120 μl of DPGA (0.25 mM), 0.53 ml of buffer (imidazole-Cl (pH 5.9, 100 mM) and bovine serum albumin (0.02%)) and 50 μl yeast phosphoglycerate mutase (50 units) were incubated at 30° for 15 min. 200 μl of [^{14}C] 3-PGA (4 μCi) was then added and the mixture was incubated for a further 30 min. Seven samples of 10 μl were taken and at zero times 100 μl of 2-PGA (200 mM) was added. 10-μl samples were taken during the subsequent chemical reaction. 2-PGA and [^{14}C]2-PGA were estimated as described. The reagents with the exception of the DPGA were dissolved in the imidazole buffer. The concentrations immediately after the addition of 2-PGA were 2-PGA (20.5 mM), 3-PGA (16.4 mM), and DPGA (27 μM).

Exchange Experiments at Chemical Equilibrium. At chemical equilibrium ^{14}C and ^{32}P substrates will exchange at different rates if the number of intermediate steps (used in the sense given in the introduction) is different. In the experiments shown in Table I the ratio of the ^{14}C to the ^{32}P fluxes was close to 2. This is the expected result for Schemes C and C′ provided that isomerization of the enzyme is rapid: if there were a rate-limiting isomerization the ratio would be greater. Scheme B would give a ratio of 3:2 if there were no rate-limiting isomerization; this would increase to 2 if the isomerization were the major rate-limiting step (Britton and Clarke,

1968; Britton *et al.*, 1971). Thus, as with the induced transport experiment at intermediate concentrations the results are compatible either with Schemes C and C′ without a rate-limiting isomerization or with Scheme B with the isomerization as the major rate-limiting step.

Measurement of K_m for 3-PGA at pH 7.4. Figure 7 illustrates some initial velocity measurements made with the enolase-coupled assay (Rodwell *et al.*, 1956). To maintain the free Mg^{2+} constant at 3.33 mM additional $MgCl_2$ was added to allow for Mg^{2+} binding by 3-PGA and DPGA, assuming affinity constants for 3-PGA at pH 7.4 of 255 M^{-1} (H. G. Britton and J. B. Clarke, unpublished data) and for DPGA of 1111 M^{-1} (Rose, 1968). The results indicate a K_m for 3-PGA of about 200 μM. However, at the lower concentration of DPGA there is pronounced substrate inhibition which would suggest that the 3-PGA at relatively low concentrations competes with DPGA at the DPGA binding site. The maximum forward velocity, V_F, is about 262 nmoles/min per unit of enzyme.

Measurement of Maximum Reverse Velocity. To calculate the highest value of the K_m for which it would be possible to explain the induced transport data by Scheme B, the ratio of the maximum reverse to maximum forward velocity is required (see Appendix). To measure the maximum reverse velocity (V_R) various quantities of DPGA were added to a solution of 3 mM 2-PGA in buffer (Tris-Cl^-, 16.7 mM, pH 7.4; $MgCl_2$, 3.33 mM; bovine serum albumin, 0.02%). The reaction at 30° was started by the addition of enzyme and 100-μl samples were taken to assay 2-PGA. The first 30% of the reaction was linear with respect to time. There was essentially no difference in rate (±6%) between 100 and 200 μM DPGA and a mean value for V_R of 597 μmoles/min per unit of enzyme was obtained. Similar results were obtained when the reaction was started with substrate.

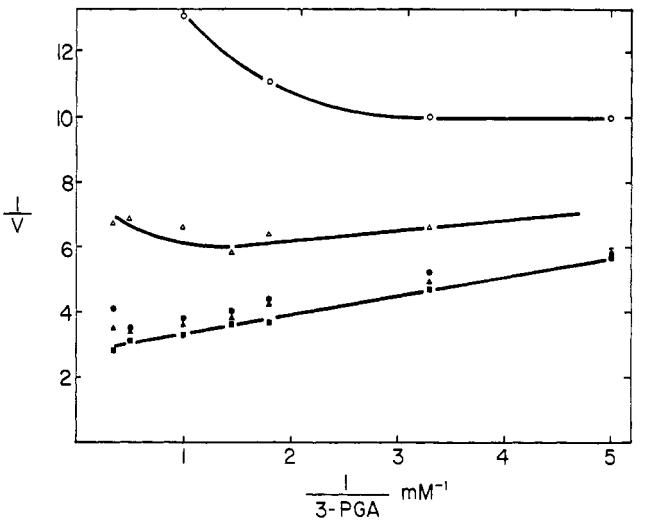

FIGURE 7: The influence of substrate and cofactor concentrations on initial velocities. Each cell (1-cm light path) contained in 3.0 ml Tris-Cl^- (pH 7.4 16.7 μM), the indicated concentrations of 3-PGA (DPGA free) and DPGA, $MgCl_2$ to give a free Mg^{2+} concentration of 3.33 mM (see text), and 25 units of enolase. 0.35 unit (Grisolia, 1962) of yeast mutase (10 μl) was added to start the reaction. The initial velocities are given in mutase units (1 unit = 250 nmoles of 2-PGA/min). Concentration of DPGA: (○) 0.1 μM, (●) 25 μM, (▲) 50 μM, and (■) 100 μM. Temperature 30°.

Discussion

The difference in the behavior of the ^{14}C and ^{32}P isotopes in the various tests excludes unequivocally any intramolecular mechanism (Scheme A, Figure 1). Concerted transfers of phosphate between two or more molecules of substrate, considered for the DPGA-independent mutases (Grisolia and Joyce, 1959; Pizer, 1962), are also excluded. To explain the induced transport data at low substrate concentrations by Scheme B the K_m for 3-PGA cannot be higher than 1.7 μM. Further, at this maximum value, the isomerization of the enzyme must be the slowest step and the transphosphorylation must then occupy only a small part of the enzymic cycle. The turnover number for the enzyme at pH 7.4 is at least 0.5×10^3 sec^{-1} (assuming four active centers) and it seems unlikely that the transphosphorylation would occur much more rapidly. Consequently, the K_m required to explain plausibly the results by Scheme B must be even smaller than 1.7 μM. In this regard Chiba *et al.* (1970) reported a value for the K_m of 0.6 mM at pH 5.9; more recently Sasaki *et al.* (1971a) gave a range of 0.1–0.3 mM at pH 7.5 and in the present study at pH 7.4 a value of 0.2 mM was obtained. The very large differences between the experimental and theoretical figures appear to exclude Scheme B. In comparison, all of the data are entirely in accord with the phosphoenzyme mechanism provided any isomerization of the phosphoenzyme is rapid, with a rate constant in excess of $\sim 10^6$ sec^{-1}. Since the tests are relatively insensitive to isotope effects and nonideal behavior of the solutions none of the conclusions should be affected by such factors (Britton and Clarke, 1968). The findings however give no information about the nature of the phosphoenzyme and it may be that an enzyme–DPGA complex is involved as in Scheme C′ (Figure 1). Recent spectrophotometric evidence for phosphoenzyme formation and the isolation of a phosphoenzyme with little or no bound [^{14}C]-DPGA (H. G. Britton, J. Carreras, and S. Grisolia, unpublished data) nevertheless favor a true phosphoenzyme. This latter work indicated at least three active centers to each enzyme molecule and the molecular weight suggests that there may be four. In this context the lack of induced transport with ^{14}C substrates over a very wide concentration range enables certain types of interaction between the active centers to be excluded (Britton, 1966, 1967). For example cotransport would have been expected, particularly at low substrate concentrations, if the conversion of 2-PGA to 3-PGA occurred more readily at one center if the same reactions were taking place at the other centers.

In contrast to the above, Chiba *et al.* (1970), who obtained a converging line initial velocity pattern, proposed that the reaction proceeds by the sequential mechanism, Scheme B. The converging pattern however may be given by the Ping-Pong or phosphoenzyme mechanism provided that the phosphoenzyme is unstable. If, for example, the rate constant for the hydrolysis of the phosphoenzyme were equal to the rate constant for dissociation of DPGA from the enzyme–substrate complex, the lines would meet on the 1/[3-PGA] axis. The spectrophotometric evidence which has been obtained for the formation of a phosphoenzyme indicates that it is unstable with a half-life of 1–2 min (H. G. Britton, J. Carreras, and S. Grisolia, unpublished data), and the enzyme has DPGA diphosphatase activity (Joyce and Grisolia, 1958; Sasaki *et al.*, 1971b). Although the rate of breakdown of the phosphoenzyme is small when compared to the mutase activity, dissociation of DPGA from the enzyme–substrate complex is also known to be infrequent except in the presence of high concentrations of salt (Grisolia and Cascales, 1966). Consequently it is possible that spontaneous hydrolysis of the phos-

phoenzyme in the experiments of Chiba *et al.* (1970) may have been sufficient to influence appreciably the initial velocity patterns. There is the further possibility in these experiments that there may have been a tendency to underestimate the initial velocities when both the 3-PGA and DPGA concentrations were low. Under these conditions the effective K_m for 2-PGA will be very small and significant product inhibition may have occurred. Relatively small errors in determining the initial velocities would give the degree of convergence observed. It may finally be noted that the rapid equilibrium sequential mechanism favored by Chiba *et al.* (1970) is incompatible with the slow rate of exchange of ^{32}P between DPGA and the substrate (Grisolia and Cascales, 1966) and it must be assumed that DPGA dissociates only infrequently from the enzyme.

Since with the present technique phosphoglycerate mutase from rabbit muscle and yeast both appear to possess a phosphoenzyme mechanism and when examined by the same techniques a similar mechanism was found for the phosphoglucomutases from rabbit muscle and *Micrococcus lysodeikticus* (Britton and Clark, 1968, and unpublished), it seems possible that all of the mutases which require a diphosphate as a cofactor may have a common type of mechanism. In all cases the isomerization of the phosphoenzyme appears to be extremely rapid suggesting at most only a very minor rearrangement of the enzyme molecule. This may support a mechanism suggested for rabbit muscle phosphoglycerate mutase which does not involve an isomerization step (H. G. Britton and J. B. Clarke, unpublished data). In this scheme the enzyme has only one point of attachment for phosphate and the enzyme–substrate complex rearranges so either the 2 or the 3 position of the substrate can be attacked. If this should be the case these mutases may be closely related mechanistically and perhaps evolutionarily to DPGA and 1,6-diphosphoglucose diphosphatases. This does not necessarily mean, however, that the phosphoglycerate and phosphoglucomutases have evolved from a common ancestor. Indeed, the differences in metal requirement and in the phosphoenzymes suggest that the two groups may have arisen by parallel evolution.

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Appendix

Permissible Values for K_m (H. G. Britton and J. B. Clarke, unpublished data). From Britton and Clarke (1968)

$$\frac{\alpha K_{2\text{-PGA}}}{V_R} = \frac{1}{k_3} + \frac{1}{k_{-3}} \tag{3}$$

$\alpha = k_{+1}k_{+2}/k_{-3}(k_{-1} + k_{+2})$, where k_{+1}, k_{+2}, etc., are rate constants as shown in eq 1 and 2, $K_{2\text{-PGA}}$ = Michaelis constant for 2-PGA, and V_R = maximum reverse velocity (2-PGA as substrate) per mole of enzyme. Since the overall velocity cannot exceed the velocity of the slowest step

$$V_R < k_{+3} \tag{4}$$

$$V_F < k_{-3} \tag{5}$$

where V_F = maximum forward velocity.
From eq 3, 4, and 5 and the Haldane relationship

$$K_{3\text{-PGA}} < \frac{1}{\alpha K}\left(1 + \frac{V_F}{V_R}\right) \tag{6}$$

when K = equilibrium constant and $K_{3\text{-PGA}}$ = Michaelis constant for 3-PGA. From the definition of $K_{2\text{-PGA}}$ given in Britton and Clarke (1968), it follows that eq 6 refers to the value of $K_{3\text{-PGA}}$ measured at saturating DPGA concentration. For curve B* (Figure 4), $\alpha = 10^4$ mM^{-1}. Since $V_F/V_R = 1/2.3$ and $K = 1/11.33$ (H. G. Britton and J. B. Clarke, unpublished data) from eq 6, $K_{3\text{-PGA}} < 1.63$ μM.

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