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Mechanism of the 2,3-Diphosphoglycerate-Dependent Phosphoglycerate Mutase from Rabbit Muscle

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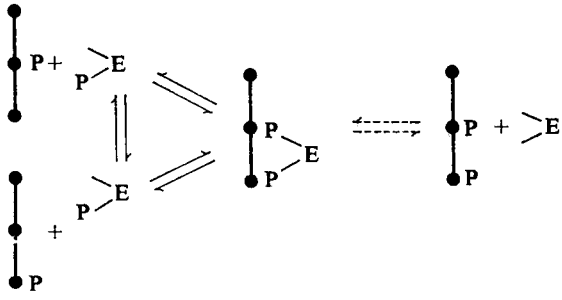
1. The properties and kinetics of the 2,3-diphosphoglycerate-dependent phosphoglycerate mutases are discussed. There are at least three possible mechanisms for the reaction: (i) a phosphoenzyme (Ping Pong) mechanism; (ii) an intermolecular transfer of phosphate from 2,3-diphosphoglycerate to the substrates (sequential mechanism); (iii) an intramolecular transfer of phosphate. It is concluded that these mechanisms cannot be distinguished by conventional kinetic measurements. 2. The fluxes for the different mechanisms are calculated and it is shown that it should be possible to distinguish between the mechanisms by appropriate induced-transport tests and by comparing the fluxes of ^{32}P- and ^{14}C-labelled substrates at chemical equilibrium. 3. With ^{14}C-labelled substrates no induced transport was found over a wide concentration range, and with ^{32}P-labelled substrates co-transport occurred that was independent of concentration over a twofold range. ^{14}C-labelled substrates exchange at twice the rate of ^{32}P-labelled substrates at chemical equilibrium. The results were completely in accord with a phosphoenzyme mechanism and indicated a rate constant for the isomerization of the phosphoenzyme of not less than $4\times10^6 s^{-1}$. The intramolecular transfer of phosphate (and intermolecular transfer between two or more molecules of substrate) were completely excluded. The intermolecular transfer of phosphate from 2,3-diphosphoglycerate would have been compatible with the results only if the K_m for 2-phosphoglycerate had been over 7.5-fold smaller than the observed value and if an isomerization of the enzyme-2,3-diphosphoglycerate complex had been the major rate-limiting step in the reaction. 4. The very rapid isomerization of the phosphoenzyme that the experiments demonstrate suggests a mechanism that does not involve a formal isomerization. According to this new scheme the enzyme is closely related mechanistically and perhaps evolutionarily to a 2,3-diphosphoglycerate diphosphatase.

Three possible mechanisms for rabbit muscle phosphoglycerate mutase are illustrated in Fig. 1. Scheme 3, the intramolecular transfer of phosphate, was suggested by Meyerhof & Kiessling (1935). This might involve the formation of a cyclic ester, but Harrison *et al.* (1955) found no transfer of ^{16}O from water or from glycerate to the substrates; Pizer & Ballou (1959) showed that the cyclic monophosphoglycerate ester was not a substrate, activator or inhibitor, nor did it accept ^{32}P from 2,3-diphosphoglycerate. These findings make the involvement of a cyclic ester unlikely but they do not necessarily exclude an intramolecular transfer. Wheat-germ phosphoglycerate mutase has recently been shown to catalyse an intramolecular transfer of phosphate yet the mechanism does not seem to involve a cyclic ester (Britton *et al.*, 1971).

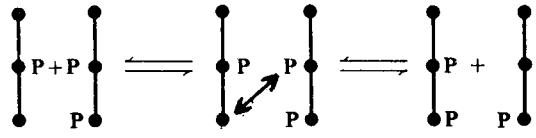
Scheme 2 (Fig. 1), the intermolecular transfer of phosphate by the sequential mechanism, was proposed by Sutherland *et al.* (1949), since they found that 2,3-diphosphoglycerate is a cofactor for the enzyme and that ^{32}P exchanged between 2,3-diphosphoglycerate and the substrates. Subsequently Grisolia & Cascales (1966) and Cascales & Grisolia (1966) found that the exchange of ^{32}P between 2,3-diphosphoglycerate and the substrates is much slower than the mutase reaction except perhaps in the presence of salts. If Scheme 2 (Fig. 1) should represent the mechanism, therefore, 2,3-diphosphoglycerate must be tightly bound to the enzyme, from which it dissociates only infrequently.

Scheme 1 (Fig. 1) is based on the mechanism of rabbit muscle phosphoglucomutase (Najjar & Pullman, 1954; Ray & Roscelli, 1964*a*,*b*; Britton & Clarke, 1968). It was proposed as an alternative to Scheme 2 (Fig. 1) by Grisolia & Cleland (1968), since they obtained parallel rather than converging lines when they plotted the reciprocal of the initial velocity against the reciprocal of the 3-phosphoglycerate concentration at constant 2,3-diphosphoglycerate concentrations and vice versa. However, their measurements were complicated by binding of the substrates by Mg^{2+}, and further such measurements do not unequivocally distinguish between the

Scheme 1



Scheme 2



Scheme 3

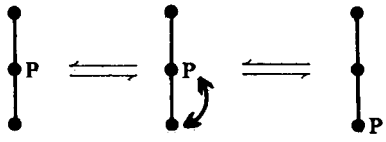


Fig. 1. *Possible schemes for the phosphoglycerate mutase reaction*

Scheme 1 illustrates the phosphoenzyme mechanism. The enzyme E is shown with two bonds representing two alternative positions for phosphate attachment. Scheme 2 represents the sequential addition mechanism, with the enzyme catalysing an intermolecular transfer of phosphate from 2,3-diphosphoglycerate to 2-phosphoglycerate at its surface. Scheme 3 shows an intramolecular transfer of phosphate from the 2- to the 3-position of the glycerate residue; 2,3-diphosphoglycerate in this case plays an indirect role and acts as an activator.

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mechanisms. Scheme 2 may give parallel lines if 2,3-diphosphoglycerate dissociates from the enzyme–substrate complex, and Scheme 3 may given converging lines if the phosphoenzyme is unstable (Britton & Clarke, 1968). Scheme 1 explains the requirement for 2,3-diphosphoglycerate and the exchange of ^{32}P between 2,3-diphosphoglycerate and the substrates, and if the phosphoenzyme slowly hydrolyses also explains the known 2,3-diphosphoglycerate phosphatase activity of the enzyme (Rodwell *et al.*, 1957). A variant of Scheme 1 that may also be considered as a possible mechanism is one in which an enzyme–2,3-diphosphoglycerate complex is formed but behaves as the phosphoenzyme in Scheme 1. This would require that the enzyme should labilize one of the phosphate groups of the 2,3-diphosphoglycerate and that this plays the role of the phosphate in the phosphoenzyme.

Other mechanisms that might possibly be considered for the enzyme are the concerted intermolecular transfer of phosphate between two and three molecules of substrate, suggested by Pizer (1962) and Grisolia & Joyce (1959) respectively for the 2,3-diphosphoglycerate-independent mutases.

With Scheme 1 the active form of the enzyme will be a phosphoenzyme, whereas with Scheme 2 the intermediate will be an enzyme–2,3-diphosphoglycerate complex. However, attempts to isolate the active intermediate have given conflicting results. The findings by Grisolia *et al.* (1961) and Rose (1970), and a spectrophotometric and gel-filtration study (H. G. Britton, J. Carreras & S. Grisolia, unpublished work), appear to indicate a phosphoenzyme, whereas results of Jacobs & Grisolia (1966) and Zwaig & Milstein (1966) suggest an enzyme–2,3-diphosphoglycerate complex.

Scheme 1 (and its variant) and Scheme 2 appear to be the most probable mechanisms because of the requirement for 2,3-diphosphoglycerate and the exchange of ^{32}P between 2,3-diphosphoglycerate and the substrates, although Scheme 3 would explain why some mutase activity may persist in the absence of 2,3-diphosphoglycerate (Grisolia, 1968).

In the different schemes a radioactive label will be transferred differently. Suppose that in Scheme 1 the substrate is labelled with ^{32}P. When one molecule of 2-[^{32}P]phosphoglycerate reacts with the enzyme the label will be transferred to the phosphoenzyme, and to transfer the label to 3-phosphoglycerate it is necessary for a second molecule of 2-phosphoglycerate to react with the phosphoenzyme. The phosphate is therefore initially transferred to an intermediate site, the phosphoenzyme. In contrast, if the substrate is labelled with ^{14}C the label will appear in the product without passing through such an intermediate site. With the same argument, there are two intermediate sites in the transfer of ^{32}P and one intermediate site in the transfer of ^{14}C from substrate to product by Scheme 2. These intermediate sites are located on the enzyme-bound 2,3-diphosphoglycerate. For Scheme 3 there are, of course, no intermediates. In the work described below the flux approach (Britton, 1965, 1966, 1967; Britton & Clarke, 1968) has been used, in which the net chemical reaction is considered as the resultant of two flows (or fluxes) in opposite directions. This analysis is the counterpart of the usual treatment of permeability data. As is discussed in the Theory section this approach leads to tests that distinguish between the mechanisms according to the number of intermediate sites. It should be noted in this context that the term 'intermediate site' is being used in a

specialized sense and does not refer to the number of intermediate enzyme–substrate complexes involved in the transition state.

A brief account of some of the results has already been published (Britton & Clarke, 1969).

Theory

It is assumed in this section that isotope effects and non-ideal behaviour of the solutions can be ignored. They are discussed in Britton & Clarke (1968).

For Schemes 1, 2 and 3 the steps may be formally written:

$$2\text{-}P\text{-Gri} + E_1 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E_2 + 3\text{-}P\text{-Gri} \quad (1)$$

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

where 2-*P*-Gri and 3-*P*-Gri represent 2-phosphoglycerate and 3-phosphoglycerate respectively and $E_2 \rightleftharpoons E_1$ represents an isomerization of the enzyme. For Scheme 1, E_1 and E_2 will be the two forms of the phosphoenzyme (Fig. 1). For Scheme 2, E_1 and E_2 will be different forms of an enzyme–2,3-diphosphoglycerate complex that interconvert without dissociation of 2,3-diphosphoglycerate. This follows because at low ionic strength isotopic studies indicate that 2,3-diphosphoglycerate dissociates from the enzyme only once in every 100 catalytic cycles (Cascales & Grisolia, 1966; Grisolia & Cleland, 1968). The 2,3-diphosphoglycerate concentrations in the experiments described below were sufficient to saturate the enzyme, but the above will also hold at lower concentrations. Consider the enzyme at equilibrium but with subsaturating concentrations of 2,3-diphosphoglycerate. The enzyme–substrate complex most frequently dissociates (>100:1) to give enzyme–2,3-diphosphoglycerate complexes, and by the principle of microscopic reversibility 2-phosphoglycerate and 3-phosphoglycerate will most frequently interconvert by reacting with the enzyme–2,3-diphosphoglycerate complexes. If the reaction is now considered displaced from equilibrium it will be found that the same must apply. The effect of a low concentration of 2,3-diphosphoglycerate is therefore only to increase the amount of enzyme in a non-active form.

Since the enzyme catalyses a reversible transfer of phosphate from 2-phosphoglycerate to 3-phosphoglycerate, at any one time there will be a flow of phosphate from the former to the latter and a flow in the opposite direction. The net rate of formation of 3-phosphoglycerate will be equal to the difference between the flows. By analogy with permeability these flows have been termed 'fluxes' (Britton, 1966). In a similar way there will be a flux of the glycerate moiety of the 2-phosphoglycerate to 3-phosphoglycerate and vice versa. With these definitions of fluxes it is possible to write, by analogy with Britton & Clarke (1968), for Scheme 1:

$$\frac{\text{Flux of phosphate from 2-}P\text{-Gri to 3-}P\text{-Gri}}{\text{Flux of phosphate from 3-}P\text{-Gri to 2-}P\text{-Gri}} = \frac{[2\text{-}P\text{-Gri}]^2(1+\alpha K[3\text{-}P\text{-Gri}])}{K^2[3\text{-}P\text{-Gri}]^2(1+\alpha[2\text{-}P\text{-Gri}])} \quad (3)$$

$$\frac{\text{Flux of glycerate from 2-}P\text{-Gri to 3-}P\text{-Gri}}{\text{Flux of glycerate from 3-}P\text{-Gri to 2-}P\text{-Gri}} = \frac{[2\text{-}P\text{-Gri}](1+\alpha K[3\text{-}P\text{-Gri}])}{K[3\text{-}P\text{-Gri}](1+\alpha[2\text{-}P\text{-Gri}])} \quad (4)$$

and at chemical equilibrium:

$$\frac{\text{Flux of glycerate from 2-}P\text{-Gri to 3-}P\text{-Gri}}{\text{Flux of phosphate from 2-}P\text{-Gri to 3-}P\text{-Gri}} = 2+\alpha \quad (5)$$

where [3-*P*-Gri] etc. represent concentrations, K is the equilibrium constant and α is given by the expression:

$$\alpha = \frac{k_{+1}k_{+2}}{k_{-3}(k_{-1}+k_{+2})} = \frac{Kk_{-1}k_{-2}}{k_{+3}(k_{-1}+k_{+2})} \quad (6)$$

If the isomerization of the enzyme is very rapid $k_{+3} \to \infty$ and $k_{-3} \to \infty$ and thus α will be zero. Under these conditions the flux ratios given by eqns. (3)–(5) are independent of rate constants and depend only on the concentrations of the substrates and the equilibrium constant.

For Scheme 2, by analogy with Britton & Clarke (1968):

$$\frac{\text{Flux of phosphate from 2-}P\text{-Gri to 3-}P\text{-Gri}}{\text{Flux of phosphate from 3-}P\text{-Gri to 2-}P\text{-Gri}} = \frac{[2\text{-}P\text{-Gri}]^3(1+\alpha K[3\text{-}P\text{-Gri}])}{K^3[3\text{-}P\text{-Gri}]^3(1+\alpha[2\text{-}P\text{-Gri}])} \quad (7)$$

$$\frac{\text{Flux of glycerate from 2-}P\text{-Gri to 3-}P\text{-Gri}}{\text{Flux of glycerate from 3-}P\text{-Gri to 2-}P\text{-Gri}} = \frac{[2\text{-}P\text{-Gri}]^2(1+\alpha K[3\text{-}P\text{-Gri}])}{K^2[3\text{-}P\text{-Gri}]^2(1+\alpha[2\text{-}P\text{-Gri}])} \quad (8)$$

and at chemical equilibrium:

$$\frac{\text{Flux of glycerate from 2-}P\text{-Gri to 3-}P\text{-Gri}}{\text{Flux of phosphate from 2-}P\text{-Gri to 3-}P\text{-Gri}} = \frac{3+2\alpha}{2+\alpha} \quad (9)$$

where α is given by eqn. (6). Thus again, when the isomerization of the enzyme is very rapid, the flux ratios are independent of rate constants.

For Scheme 3 the ratio (flux of phosphate from 2-phosphoglycerate to 3-phosphoglycerate)/(flux of phosphate from 3-phosphoglycerate to 2-phosphoglycerate) and the ratio (flux of glycerate from 2-phosphoglycerate to 3-phosphoglycerate)/(flux of glycerate from 3-phosphoglycerate to 2-phosphoglycerate) will both be given by eqn. (4). At chemical equilibrium the ratio (flux of glycerate from 2-phosphoglycerate to 3-phosphoglycerate)/(flux of phosphate from 2-phosphoglycerate to 3-phosphoglycerate) will be unity.

In deriving eqns. (3)–(9) it has been assumed either that 2,3-diphosphoglycerate exchanges very rapidly and that a steady state is achieved or that the rate of exchange is negligible. The experimental result lies between the two extremes, but as the quantity of 2,3-diphosphoglycerate at present is small the effect is relatively unimportant. It is discussed in the Results section.

To measure the flux ratios of the type shown in eqns. (3), (4), (7) and (8) the induced-transport test may be used. Appropriately labelled substrate is incubated with the enzyme until equilibrium has been reached. Unlabelled 2-phosphoglycerate is then added and the radioactivity in the 2-phosphoglycerate is observed during the reaction that follows. The unlabelled material can be regarded as separate species, and consequently (provided that the equilibrium constant does not change) the total radioactivity in the 2-phosphoglycerate should remain constant. However, there may be an interaction between the flows. The radioactivity may be transiently carried in the direction of the chemical reaction (co-transport) or a flow may be induced in the opposite direction (counter-transport). In either case there must be a transfer of energy between the labelled and the unlabelled species. Qualitatively, if the equilibrium constant is unaffected by the addition of the unlabelled 2-phosphoglycerate, the conditions for no induced transport, co-transport and counter-transport are:

$$\frac{\text{Flux from 2-}P\text{-Gri to 3-}P\text{-Gri}}{\text{Flux from 3-}P\text{-Gri to 2-}P\text{-Gri}} = \frac{[2\text{-}P\text{-Gri}]}{[3\text{-}P\text{-Gri}]K} \quad (10)$$

$$\frac{\text{Flux from 2-}P\text{-Gri to 3-}P\text{-Gri}}{\text{Flux from 3-}P\text{-Gri to 2-}P\text{-Gri}} > \frac{[2\text{-}P\text{-Gri}]}{[3\text{-}P\text{-Gri}]K} \quad (11)$$

$$\frac{\text{Flux from 2-}P\text{-Gri to 3-}P\text{-Gri}}{\text{Flux from 3-}P\text{-Gri to 2-}P\text{-Gri}} < \frac{[2\text{-}P\text{-Gri}]}{[3\text{-}P\text{-Gri}]K} \quad (12)$$

where K is the apparent equilibrium constant (Britton & Clarke, 1968). Quantitatively the amount of induced transport can be derived from the flux ratios by calculating the incremental change in radioactivity as the reaction progresses (Britton & Clarke, 1968). These calculations require that the apparent equilibrium constant should be known at each stage in the reaction. It was clear that there was no large change in this constant when 2-phosphoglycerate was added since the constant was the same at the end of the reaction as at the beginning. However, Mg^{2+}-binding studies (H. G. Britton & J. B. Clarke, unpublished work) showed a small difference in the Mg^{2+}-binding constants for 2-phosphoglycerate and 3-phosphoglycerate at pH7.4. The effect of this difference in the binding constants was taken into account as follows. The free and bound forms of the substrates are related by the expression:

$$K^{Mg}_{3\text{-}P\text{-Gri}} = \frac{[3\text{-}P\text{-Gri Mg}]}{[3\text{-}P\text{-Gri}][Mg^{2+}_{free}]} \quad (13)$$

$$K^{Mg}_{2\text{-}P\text{-Gri}} = \frac{[2\text{-}P\text{-Gri Mg}]}{[2\text{-}P\text{-Gri}][Mg^{2+}_{free}]} \quad (14)$$

where $K^{Mg}_{3\text{-}P\text{-Gri}}$ and $K^{Mg}_{2\text{-}P\text{-Gri}}$ are the respective affinity constants (at pH7.4).

From eqns. (13) and (14) the cubic eqn. (15) for the concentration of free Mg^{2+} may be derived:

$$K^{Mg}_{2\text{-}P\text{-Gri}} K^{Mg}_{3\text{-}P\text{-Gri}} [Mg^{2+}_{free}]^3 + \{K^{Mg}_{2\text{-}P\text{-Gri}} + K^{Mg}_{3\text{-}P\text{-Gri}} + K^{Mg}_{2\text{-}P\text{-Gri}} K^{Mg}_{3\text{-}P\text{-Gri}} ([2\text{-}P\text{-Gri}_T] + [3\text{-}P\text{-Gri}_T] - [Mg^{2+}_T])\} [Mg^{2+}_{free}]^2 + \{1 + K^{Mg}_{2\text{-}P\text{-Gri}} ([2\text{-}P\text{-Gri}_T] - [Mg^{2+}_T]) + K^{Mg}_{3\text{-}P\text{-Gri}} ([3\text{-}P\text{-Gri}_T] - [Mg^{2+}_T])\} [Mg^{2+}_{free}] - [Mg^{2+}_T] = 0 \quad (15)$$

The suffix T in eqn. (15) indicates total concentration of that particular substance. Eqn. (15) was solved for each integration step by successive approximations. The free Mg^{2+} concentration was then used to calculate the effective equilibrium constant from eqn. (16), which follows from eqns. (13) and (14):

$$K = K^0 \frac{(1 + K^{Mg}_{2\text{-}P\text{-Gri}} [Mg^{2+}_{free}])}{(1 + K^{Mg}_{3\text{-}P\text{-Gri}} [Mg^{2+}_{free}])} \quad (16)$$

In eqn. (16) K^0 is the equilibrium constant in the absence of Mg^{2+}. From H. G. Britton & J. B. Clarke (unpublished work), at pH7.4: $K^0 = 1/11.92$, $K^{Mg}_{2\text{-}P\text{-}GrI} = 0.286\,mM^{-1}$ and $K^{Mg}_{3\text{-}P\text{-}GrI} = 0.255\,mM^{-1}$.

The induced-transport patterns to be expected for the different schemes are summarized in Fig. 2. If the isomerization of the enzyme is rapid ($\alpha = 0$) then each scheme gives characteristically different patterns, which are independent of all rate constants. If the isomerization should be rate-limiting ($\alpha \neq 0$) the curves become dependent on the term α. When α is large the patterns to be expected of Scheme 2 approximate to those for Scheme 1 with $\alpha = 0$, and there is therefore an ambiguity in the interpretation of the induced-transport data if α is not known. It is possible, however, to set a limit on the maximum value of α by the following argument. From Britton & Clarke (1968):

$$\frac{\alpha K_{2\text{-}P\text{-}GrI}}{V_F} = \frac{1}{k_{+3}} + \frac{1}{k_{-3}} \qquad (17)$$

where $K_{2\text{-}P\text{-}GrI}$ is the Michaelis constant for 2-phosphoglycerate and V_F is the maximum velocity/mol of enzyme with 2-phosphoglycerate as a substrate. Since the overall velocity must be slower than the fastest step:

$$V_F < k_{+3}$$

and

$$V_R < k_{-3}$$

where V_R is the maximum reverse velocity/mol of enzyme.

From eqn. (17):

$$\alpha < \frac{1}{K_{2\text{-}P\text{-}GrI}}\left(1 + \frac{V_F}{V_R}\right) \qquad (18)$$

The minimum value for α required to make eqn. (7) approximate to eqn. (3) (with $\alpha = 0$) increases as the concentrations of substrates are lowered. Thus at sufficiently low substrate concentrations α cannot be large enough for the co-transport given by eqn. (7) to approximate to that given by eqn. (3), and the interpretation of the data will no longer be ambiguous. Eqn. (18) also gives the maximum value of α compatible with a positive solution for the rate constants (H. G. Britton, unpublished work).

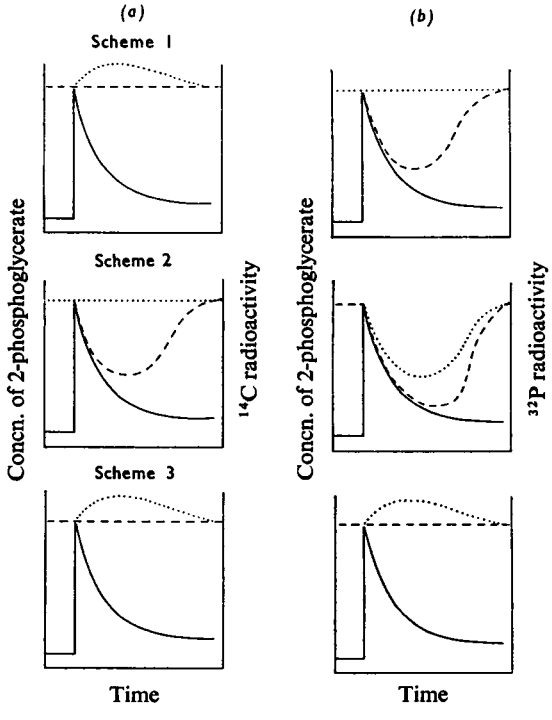

Fig. 2. *Diagram to show the induced-transport pattern to be expected of the Schemes for the phosphoglycerate mutase reaction shown in Fig.* 1

(*a*) Induced transport of ^{14}C; (*b*) induced transport of ^{32}P. ——, Concn. of 2-phosphoglycerate; – – – –, theoretical curves if isomerization of the enzyme (eqn. 2) is not rate-limiting; ....., theoretical curves if the isomerization of the enzyme (eqn. 2) is very rate-limiting. For reasons of space the scale of the counter-transport curves has been considerably reduced. The initial rise in 2-phosphoglycerate concentration corresponds to the addition of unlabelled 2-phosphoglycerate.

Thermodynamic significance of the induced-transport test

When induced-transport occurs (provided that there is no change in the equilibrium constant) the radioactive label is displaced against its free-energy gradient and there must be a transfer of free energy between the unlabelled and the labelled species. This is a severe restriction on possible mechanisms. Co-transport produced by the indirect transfer of label may be discussed in connexion with the transfer of phosphate by Scheme 1. To label the phosphoenzyme a molecule of ^{32}P-labelled substrate must react with enzyme. To transfer this label to 3-phosphoglycerate, a molecule of 2-phosphoglycerate (which need not be labelled) must then react with the labelled phosphoenzyme; or to transfer the label to 2-phosphoglycerate a molecule of 3-phosphoglycerate must react. When an excess of 2-phosphoglycerate is added, in the induced-transport test, the phosphoenzyme most frequently reacts with 2-phosphoglycerate and the radioactivity is therefore preferentially transferred to 3-phosphoglycerate. The only alternative mechanism giving rise to co-transport would appear to be a polyvalent enzyme

with an interaction between active centres such that conversion of substrate into product at one site increases the rate of the reaction in the same direction at another (Britton, 1966, 1967). Counter-transport occurs with a slow isomerization of the free enzyme, since a net flow of 2-phosphoglycerate to 3-phosphoglycerate 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. Radioactive molecules of 3-phosphoglycerate therefore have a greater chance of finding E_2 molecules than radioactive 2-phosphoglycerate molecules have of finding E_1. Consequently a net flow of radioactivity from 3-phosphoglycerate to 2-phosphoglycerate results. The only alternative mechanism giving counter-transport would again appear to be interaction between active centres of a polyvalent enzyme (Britton, 1966, 1967).

Materials and Methods

Reagents and materials

2-Phosphoglycerate and 3-phosphoglycerate (as the trisodium salts), 2,3-diphosphoglycerate (as the cyclohexylamine salt), ADP (as the free acid), ATP and NADH (as the sodium salts) and the enzymes (all from rabbit muscle) phosphoglycerate mutase [specific activity 30 units/mg; crystalline suspension, 2mg/ml in 2.6M-$(NH_4)_2SO_4$ soln.], enolase [specific activity 27 units/mg; 2mg/ml in 2.8M-$(NH_4)_2SO_4$ soln.], pyruvate kinase [specific activity 150 units/mg; 2mg/ml in 2.1M-$(NH_4)_2SO_4$ soln.] and lactate dehydrogenase [specific activity 360 units/mg; 5mg/ml in 2.2M-$(NH_4)_2SO_4$ soln.] were obtained from C. F. Boehringer and Soehne G.m.b.H. (Mannheim, Germany). Glassware was cleaned with Decon 75 (A. Gallenkamp and Co. Ltd., London E.C.2, U.K.) before use.

Preparation of labelled substrates

Equilibrium mixture of 2-[^{32}*P*]*phosphoglycerate and* 3-[^{32}*P*]*phosphoglycerate.* A mixture of 2-[^{32}P]phosphoglycerate and 3-[^{32}P]phosphoglycerate of high specific radioactivity was prepared by a procedure based on that of Grisolia *et al.* (1961). A solution containing 1mCi of sodium [^{32}P]phosphate (carrier-free; supplied by The Radiochemical Centre, Amersham, Bucks., U.K., as a sterilized solution, pH3) was evaporated to dryness overnight in a vacuum desiccator containing NaOH. Fresh human blood (0.1ml) containing 0.5mg of dry heparin as anticoagulant was added and the solution was agitated for 6h at 37°C in an atmosphere of 'alveolar air' and 0.2ml of 10% (w/v) trichloroacetic acid was then added. After standing overnight at room temperature the mixture was centrifuged, the supernatant was removed, the precipitate was washed with 0.2ml of 10% trichloroacetic acid and the two supernatants were combined. The trichloroacetic acid in the supernatant was largely removed by three extractions with twice its volume of ether saturated with water. The solution was neutralized with 2–3 drops of NaOH (approx. 0.3M). Then 0.1ml of a solution of 3-phosphoglycerate (3mg/ml) in 50mM-tris–HCl buffer, pH7.4, and 2μl of phosphoglycerate mutase solution (0.3 unit) were added. The solution was incubated at 37°C for 2h and allowed to stand overnight. 2-[^{32}P]Phosphoglycerate and 3-[^{32}P]phosphoglycerate were separated from 2,3-di[^{32}P]phosphoglycerate and [^{32}P]P_i by paper chromatography on Whatman no. 4 paper, once with di-isopropyl ether–90% formic acid (3:2, v/v) (Eggleston & Hems, 1952) and once with butan-1-ol–acetic acid–water (12:3:5, by vol.). The marker spots of 3-phosphoglycerate, 2,3-diphosphoglycerate and P_i were developed with Molybdenum Blue (Burrows *et al.*, 1952). The mixture (yield approx. 100μCi) was finally eluted with water and stored at −20°C. A sample was chromatographed on Whatman no. 4 paper with di-isopropyl ether–90% formic acid (3:2, v/v) and the chromatogram was scanned for radioactivity (Packard scanner). At least 95% of the radioactivity was found in the 3-phosphoglycerate and less than 5% was associated with P_i or with 2,3-diphosphoglycerate.

Preparation of 3-[^{32}*P*]*phosphoglycerate.* 3-[^{32}P]-Phosphoglycerate free of 2-phosphoglycerate was prepared by removing the latter enzymically from the mixture obtained in the above method. After incubation with 3-phosphoglycerate and phosphoglycerate mutase, 0.1ml of 0.25M-$HClO_4$ was added. The solution was evaporated to dryness in a desiccator over NaOH and the residue was dissolved in 0.2ml of triethanolamine buffer [triethanolamine–HCl buffer, pH7.6 (0.2M), KCl (0.29M), $MgSO_4$ (10mM) and ADP (1mM)], 0.05ml of NADH soln. (3mg/ml) and 0.01ml of a mixture of equal parts (by vol.) of enolase, pyruvate kinase and lactate dehydrogenase solutions was added, and the solution was then incubated at 30°C for 30min. After standing overnight at 4°C the mixture was chromatographed on Whatman no. 4 paper, first with di-isopropyl ether–90% formic acid (3:2, v/v) and secondly with butan-1-ol–acetic acid–water (12:3:5, by vol.). After elution with water the 3-[^{32}P]phosphoglycerate was stored at −20°C. A sample was chromatographed on Whatman no. 4 paper with di-isopropyl ether–90% formic acid (3:2, v/v) and the chromatogram was scanned. At least 95% of the radioactivity was found in the 3-phosphoglycerate and less than 5% was associated with P_i or with 2,3-diphosphoglycerate. This procedure was used since no satisfactory chromatographic system for the separation of 2-phosphoglycerate and 3-phosphoglycerate was found.

Preparation of 3-phospho[^{14}C]glycerate. 3-Phospho-[G-^{14}C]glycerate (specific radioactivity 28mCi/mmol) was obtained from Calbiochem, Los Angeles, Calif., U.S.A. For the induced-transport experiments it was usually used without further purification. For the equilibrium-flux experiments 2-phospho[^{14}C]-glycerate, which was also present, was removed by conversion into lactate as described for 3-[^{32}P]phosphoglycerate and the mixture was chromatographed with butan-1-ol–acetic acid–water (12:3:5, by vol.) on Whatman no. 4 paper. The 3-phospho[^{14}C]-glycerate was eluted with water and stored at −20°C.

Analytical methods

Determination of enzyme protein. The total enzyme protein was determined from the extinction at 280nm by using the extinction coefficient $E^{1\%}_{1cm} = 12.5$ (Zwaig & Milstein, 1963).

Determination of 2-phosphoglycerate. 2-Phosphoglycerate was determined by a modification of the method of Czok & Eckert (1963). Samples (0.10ml) were taken from the reaction mixture and each was added to 0.10ml of 0.25M-$HClO_4$ in stoppered test tubes. Then 0.02ml of 1M-NaOH was added to each tube to neutralize the acid, and next 0.5ml of triethanolamine buffer [triethanolamine–HCl buffer, pH7.6 (0.2M), KCl (0.29M), $MgSO_4$ (10mM) and ADP (1mM)], 0.10ml of freshly prepared NADH solution (3mg/ml), 1.45ml of water, 10μl of lactate dehydrogenase solution and 10μl of pyruvate kinase solution (3 units) were added. Each solution was transferred to a 1cm cuvette and its extinction measured at 340nm, and then 10μl of enolase solution (0.5 unit) was added and the extinction redetermined 13min later.

In the original method (Czok & Eckert, 1963) the buffer contained 40mM-EDTA, but this was found to inhibit the enzymic reaction, presumably because it formed a complex with Mg^{2+}.

Determination of 2-[^{32}P]phosphoglycerate and 3-[^{32}P]phosphoglycerate. The radioactivity of 2-[^{32}P]-phosphoglycerate was counted after enzymic production of [^{32}P]ATP, hydrolysis and precipitation of P_i (Sugino & Miyoshi, 1964). 2-[^{32}P]Phosphoglycerate was converted into lactate with the production of [^{32}P]ATP as described above. A portion (0.6ml) of the solution was transferred to a 15ml conical centrifuge tube containing 0.6ml of 1.8M-$HClO_4$ and 50μl of 10mM-sodium phosphate. The stoppered tubes were placed in a boiling-water bath for 10min. Then 1.2ml of a freshly prepared mixture of 57mM-ammonium molybdate and 74mM-triethylamine (1:1, v/v) was added and the tubes were heated again at 100°C for 90s. After standing overnight at 4°C the tubes were centrifuged (2000*g* for 10min), the supernatants were removed and the precipitates were washed three times with 1.0ml of a mixture of 57mM-ammonium molybdate, 74mM-triethylamine, 0.9M-$HClO_4$ and water (1:1:2:6, by vol.). The precipitates were dissolved with gentle warming in 0.2ml of 1M-NaOH and each solution was transferred quantitatively, together with three washings of 0.2ml of water, to a scintillation vial containing 15ml of scintillation medium [60g of naphthalene, 4g of 2,5-diphenyloxazole, 0.2g of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 100ml of methanol, 20ml of ethylene glycol and dioxan to 1 litre] containing 4% of Cab-O-Sil (Packard Instrument Co., Wembley, Middx., U.K.). The solutions were neutralized with 0.1ml of 1M-HCl and the radioactivities in the vials were counted in a Packard Tri-Carb scintillation counter.

3-[^{32}P]Phosphoglycerate was determined by transferring 0.4ml of the supernatant after precipitation of P_i to a scintillation vial and adding 15ml of scintillation medium containing 4% of Cab-O-Sil. Approx. 0.15ml of 1M-NaOH was then added to discharge the yellow colour before the radioactivity was counted.

The above methods were suitable for concentrations up to 5.0mM-2-phosphoglycerate.

Determination of 2-phospho[^{14}C]glycerate. The determination of 2-phospho[^{14}C]glycerate was based on the method used by Britton & Clarke (1968) to measure [^{14}C]glucose 1-phosphate. Samples (0.1ml) were treated enzymically to convert the 2-phosphoglycerate into lactate with the production of ATP as described above. To 1.0ml samples from the resulting solution were added 0.60ml of $ZnSO_4$ soln. (approx. 5%, w/v) followed by 0.60ml of $Ba(OH)_2$ soln. (approx. 0.15M) and the mixture was thoroughly agitated to precipitate 3-phosphoglycerate. After centrifugation at 2000*g* for 10min 0.6ml of the supernatant (containing [^{14}C]lactate derived from 2-phosphoglycerate) was pipetted into 15ml of scintillation medium in a scintillation vial. 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+} (Nelson, 1944). The method was suitable for concentrations up to 5.0mM-2-phosphoglycerate. For higher concentrations a smaller initial sample was taken, e.g. 20μl for the experiments at 27.5mM.

Total radioactivity in the reaction mixture was determined by the addition of 0.10ml samples to the scintillation medium.

Determination of 2,3-diphospho[^{14}C]glycerate. The sample (0.2ml) was added to ethanol (0.25ml) and the mixture was heated briefly to boiling. It was then applied to a Whatman no. 4 paper (previously washed for 12h with 1M-HCl containing 0.02% of the tetrasodium salt of EDTA), with drying in a stream of cold air. Descending chromatography was carried out for 20h at room temperature with 2-methoxyethanol–butan-2-one–aq. 3M-NH_3 (7:2:3, by vol.) (Mortimer, 1952). The chromatogram was scanned

(Packard scanner) and the relative amounts of radioactivity in the 2,3-diphosphoglycerate, 2-phosphoglycerate and 3-phosphoglycerate were determined by measuring the areas under the peaks.

Induced-transport tests. Rabbit muscle phosphoglycerate mutase solution, diluted in tris buffer (16.7mM-tris–HCl–3.33mM-$MgCl_2$, pH7.4), was added to the reaction mixture, which contained 3-phosphoglycerate and 2,3-diphosphoglycerate made up in the same tris buffer. After incubation for 10min at 30°C to allow equilibrium to be nearly established, a small quantity of 2-[^{32}P]phosphoglycerate+3-[^{32}P]phosphoglycerate or 3-phospho[^{14}C]glycerate was added and the incubation continued for a further 80min to allow complete equilibration. The addition of radioactive substrate was delayed in this way to minimize any induced transport of the labelled material. The total volume of solution was 3.00ml. Four 0.10ml portions were withdrawn at intervals during the last 15min of incubation and assayed for total and radioactive 2-phosphoglycerate. At the end of the equilibration period non-radioactive 2-phosphoglycerate was added in small volume (0.4ml) and further samples (0.10ml) were taken while the system relaxed to its new equilibrium. The concentrations of tris and Mg^{2+} after the addition of 2-phosphoglycerate were 14.4mM and 2.89mM respectively.

Results

In all the experiments described below the reaction was carried out at 30°C in $MgCl_2$–tris buffer, pH7.4. 2,3-Diphosphoglycerate was present in a concentration that exceeded the effective K_m (0.28μM) three- to five-fold (H. G. Britton & J. B. Clarke, unpublished work).

Induced-transport tests at intermediate substrate concentrations

The results of a series of induced-transport tests with ^{14}C- and ^{32}P-labelled substrates are shown in Fig. 3(*a*). Radioactively labelled substrates in these experiments were allowed to come into equilibrium in the presence of enzyme, and then non-radioactive 2-phosphoglycerate was added. The 2-phosphoglycerate contained a small quantity of 2,3-diphosphoglycerate, to maintain the 2,3-diphosphoglycerate concentration approximately constant.

With ^{14}C-labelled substrates the total radioactivity in the 2-phosphoglycerate did not change appreciably (Fig. 3*a*) during the chemical reaction that followed the addition of the non-radioactive 2-phosphoglycerate. Since the addition of the non-radioactive compound caused the specific radioactivity of the 2-phosphoglycerate to fall by a large factor some radioactivity may have been transferred from 2,3-diphosphoglycerate to 2-phosphoglycerate. However, as discussed below, this must have been small. Thus the constancy of the radioactivity in the 2-phosphoglycerate must have reflected a similar constancy in the 3-phosphoglycerate and therefore an absence of induced transport. If any isomerization of the enzyme (eqn. 2) is rapid this result, with ^{14}C-labelled substrates, is to be expected for Schemes 1 and 3, whereas Scheme 2 should give marked co-transport (see theoretical lines in Fig. 3*a*). The theoretical curve for 20% of the flux proceeding by Scheme 2 and 80% by Scheme 1 is also shown and is clearly different from the experimental result. The theoretical curves are independent of rate constants and therefore the conclusions apply very generally. The effects of a rate-limiting isomerization of the enzyme is considered below.

A second series of induced-transport experiments with ^{14}C-labelled substrates were carried out with substrate and cofactor concentrations that were twice those shown in Fig. 3(*a*). An identical result was obtained.

In Fig. 3(*b*) the results of series of induced-transport experiments with ^{32}P-labelled substrates are illustrated. In contrast with the results obtained with ^{14}C-labelled substrates, with ^{32}P-labelled substrates there was a very marked fall in the total radioactivity in the 2-phosphoglycerate followed by a return to the original value at the end of the chemical reaction. As in the experiments with ^{14}C-labelled substrates 2,3-diphosphoglycerate may have contributed some radioactivity to the 2-phosphoglycerate, but, as discussed below, this should have been small. Consequently when the radioactivity in the 2-phosphoglycerate fell there must have been a corresponding rise in the radioactivity in the 3-phosphoglycerate, and since the change was only transient it would appear to have been due to co-transport of the ^{32}P-labelled phosphate. The degree of co-transport approximated closely to that expected for Scheme 1 if any isomerization of the enzyme were not rate-limiting (see theoretical lines in Fig. 3*b*). Scheme 3 would give no induced transport under the same conditions, and the amount of co-transport given by Scheme 2 would be considerably greater. Further, the theoretical curve for 20% of the reaction proceeding by Scheme 3 and 80% by Scheme 1 differs considerably from the experimental results. As with the data for ^{14}C-labelled substrates, these theoretical curves are independent of rate constants and therefore apply very generally. The effects of a rate-limiting isomerization of the enzyme is discussed below.

A second series of induced-transport experiments with ^{32}P-labelled substrates were carried out with twice the substrate and cofactor concentrations shown in Fig. 3(*b*). The degree of co-transport was the same as that seen in Fig. 3(*b*) and the experimental minimum (48.0%) for the radioactivity in the 2-phosphoglycerate agreed well with the theoretical minimum (46.2%) for Scheme 1.

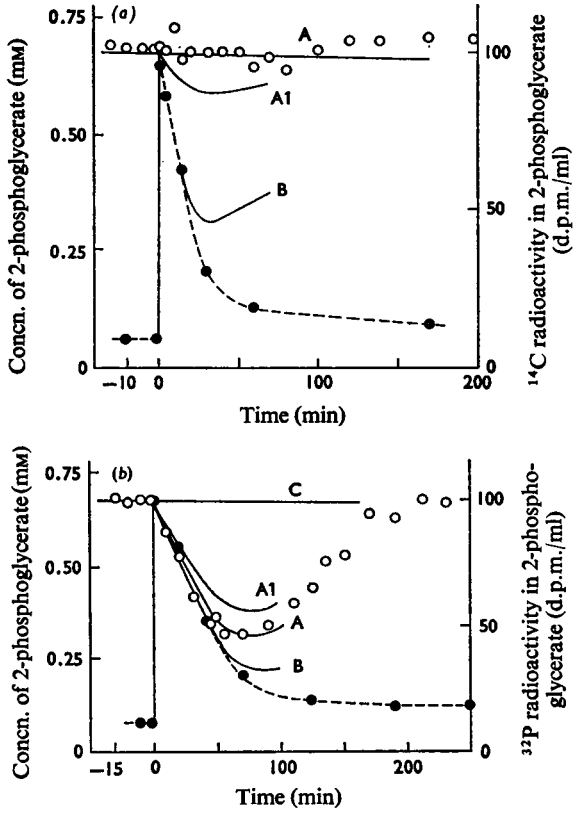

Fig. 3. *Induced-transport tests for the phosphoglycerate mutase reaction at intermediate substrate concentrations and low ionic strength with labelled substrates*

(*a*) Induced-transport tests at intermediate substrate concentrations and low ionic strength with ^{14}C-labelled substrates, showing the absence of induced transport. 3-Phospho[^{14}C]glycerate was incubated at 30°C with rabbit-muscle phosphoglycerate mutase in a $MgCl_2$–tris buffer, pH 7.4, containing 2,3-diphosphoglycerate until equilibrium was reached. Samples were then taken from the reaction mixture and at zero time non-radioactive 2-phosphoglycerate and 2,3-diphosphoglycerate were added. ●, Concn. of 2-phosphoglycerate; ○, ^{14}C radioactivity in 2-phosphoglycerate (corrected for dilution caused by addition of 2-phosphoglycerate). Non-radioactive 2-phosphoglycerate and 2,3-diphosphoglycerate were added in a volume of 0.4ml to 2.6ml of solution. Concentrations before addition: 2-phosphoglycerate 51.5μM; 3-phosphoglycerate, 582μM; 2,3-diphosphoglycerate, 5.0μM; concentrations immediately after addition: 2-phosphoglycerate, 678μM; 3-phosphoglycerate, 504μM; 2,3-diphosphoglycerate, 4.8μM; *I*, 0.028. The points shown represent means of two or three observations. In individual experiments the time-course of the reaction varied slightly, and consequently the time-scale of each was adjusted so that the course of reaction was represented by the curve shown. The continuous curves are theoretical curves: curve A for Scheme 1 or 3 (Fig. 1); curve A1 for 80% of the glycerate flux from 2-phosphoglycerate to 3-phosphoglycerate proceeding by Scheme 1 or 3 and 20% proceeding by Scheme 2; curve B for Scheme 2. (*b*) Induced-transport tests at intermediate substrate concentrations and at low *I* values with ^{32}P-labelled substrates. The details of the procedure and the concentrations were those described in (*a*) except that 3-[^{32}P]phosphoglycerate was added instead of 3-phospho[^{14}C]glycerate. ●, Concn. of 2-phosphoglycerate; ○, ^{32}P radioactivity in 2-phosphoglycerate (corrected for dilution after addition of the non-radioactive substrates). The continuous lines are theoretical curves for ^{32}P-labelled substrates: curve A for Scheme 1 (Fig. 1); curve B for Scheme 2; curve C for Scheme 3; curve A1 for 80% of the flux from 2-phosphoglycerate to 3-phosphoglycerate proceeding via Scheme 1 and 20% proceeding via Scheme 3 (Fig. 1). It is assumed in the calculations for both (*a*) and (*b*) that any isomerization of the free enzyme is not rate-limiting ($\alpha = 0$). The curves do not depend on rate constants. For details of calculations see the Theory section.

Transfer of radioactivity from 2,3-diphosphoglycerate to 2-phosphoglycerate

To investigate the contribution of 2,3-diphosphoglycerate to the radioactivity in the 2-phosphoglycerate an induced-transport experiment was carried out with ^{14}C-labelled substrates similar to that of Fig. 3(*a*) except that the samples (0.1ml) were added to 0.25ml of ethanol and chromatographed and the relative sizes of the 2,3-diphosphoglycerate and monophosphoglycerate peaks then measured. The radioactivity in 2,3-diphosphoglycerate expressed as a proportion of that in the monophosphoglycerates fell from about 0.5% to 0.36, 0.20, 0.26 and 0.26% at 5, 15, 25 and 35min respectively after the addition of 2-phosphoglycerate. The 2,3-diphosphoglycerate therefore contributed only about 0.3% of radioactivity to the monophosphoglycerates; taking into account the equilibrium constant (11.3) the maximum contribution to the 2-phosphoglycerate would have been about 3%. However, the contribution to 2-phosphoglycerate is very unlikely to have been as large, since some of the radioactivity must enter the 3-phosphoglycerate. Only about one-fifth would enter the 2-phosphoglycerate according to the data of Mantle & Garfinkel (1969). In the experiments with

^{14}C-labelled substrates therefore the contribution of radioactivity from 2,3-diphosphoglycerate to 2-phosphoglycerate can be ignored. In the experiments with ^{32}P-labelled substrates it might appear that the maximum contribution from 2,3-diphosphoglycerate might be doubled. However, a consideration of the mechanisms leads to the conclusion that only one of the phosphate groups should contribute substantially to the 2-phosphoglycerate (Britton & Clarke, 1968). If the active form of the enzyme is an enzyme–2,3-diphosphoglycerate complex rather than a true phosphoenzyme, it is conceivable that [^{32}P]phosphate might be transferred from the 2,3-diphosphoglycerate to the 2-phosphoglycerate more rapidly than the ^{14}C label. Even in these circumstances, however, the contribution from 2,3-diphosphoglycerate should be small.

Induced-transport experiments at low substrate concentration

In the above it has been assumed that the isomerization of the enzyme (eqn. 2) is rapid, but the possibility that there may be a rate-limiting isomerization must be considered. The effects are discussed in the Theory section and are summarized in Fig. 2. The expected patterns for Schemes 1 and 3 still differ markedly from the experimental results, but with Scheme 2 the patterns will approach the observed results (i.e. the result for Scheme 1 in the absence of a rate-limiting isomerization) as the isomerization becomes very rate-limiting. To resolve the ambiguity, induced-transport experiments were carried out with ^{14}C-labelled substrates at low substrate concentrations. As already discussed above, the value of the parameter α (eqn. 6) for Scheme 2 to give a theoretical pattern approaching Scheme 1 increases as the concentration of substrates is lowered, but there is a maximum value for this constant set by the K_m and the ratio V_F/V_R. In Fig. 4 an induced-transport experiment with ^{14}C-labelled substrate at about 100μM concentration is illustrated. As at intermediate concentrations, the constancy of the radioactivity in the 2-phosphoglycerate must reflect a lack of induced transport. A theoretical curve for Scheme 2 with a value of $\alpha = 6.99 \times 10^2$ mM^{-1} is shown (Fig. 4), and it is apparent that α must be in excess of this value to obtain a curve that is a satisfactory fit to the experimental results. However, from eqn. (18) α cannot exceed 0.92×10^2 mM^{-1}, assuming a K_m for 2-phosphoglycerate of 32.3μM and the ratio V_F/V_R to be 2.2 (H. G. Britton & J. B. Clarke, unpublished work). It seems therefore that Scheme 2 must be excluded as a possible mechanism even if there should be a rate-limiting isomerization.

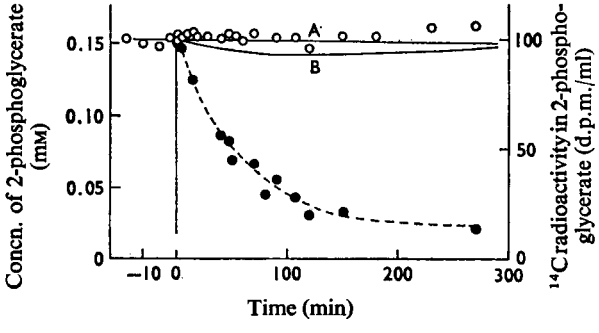

Fig. 4. *Induced-transport tests for the phosphoglycerate mutase reaction at low substrate concentrations and low ionic strength with ^{14}C-labelled substrates*

For symbols and experimental procedure see the legend to Fig. 3(*a*). Concentrations before addition of non-radioactive substrates: 2-phosphoglycerate, 10.8μM; 3-phosphoglycerate, 123μM; 2,3-diphosphoglycerate, 1.33μM. Concentrations immediately after addition: 2-phosphoglycerate, 143μM; 3-phosphoglycerate, 106μM; 2,3-diphosphoglycerate, 1.29μM; *I* 0.023. The continuous lines are theoretical curves: curve A for Schemes 1 or 3 (Fig. 1), assuming that any isomerization of the free enzyme is not rate-limiting ($\alpha = 0$); curve B for Scheme 2, assuming $\alpha = 6.99 \times 10^2$ mM^{-1} (eqn. 6). For details of calculations see the Theory section.

Experiments at very high substrate concentrations

A rate-limiting isomerization of the phosphoenzyme (Scheme 1) would lead to counter-transport with ^{14}C-labelled substrates (Fig. 2), and any such step cannot have been substantially rate-limiting in the experiments just described. The effect of such a step, however, becomes more prominent at high substrate concentrations, and some experiments were therefore carried out at substrate concentration of about 15mM (Fig. 5). The results showed essentially no induced transport. Any contribution of radioactivity from 2,3-diphosphoglycerate to 2-phosphoglycerate would have been small and in any case would have led to a rise in the radioactivity in the 2-phosphoglycerate. A theoretical curve for $\alpha = 0.0133$ mM^{-1} is shown, and it is apparent that any counter-transport substantially in excess of this curve would have been detected. The K_m for 2-phosphoglycerate is 32.3μM under conditions of low ionic strength (H. G. Britton & J. B. Clarke, unpublished work), and, assuming a molecular weight for the enzyme of 60000 (Pizer, 1960; Edelhoch *et al.*, 1957), V_F (2-phosphoglycerate as substrate) is about 1940s^{-1} (H. G. Britton & J. B. Clarke, unpublished work). Insertion of these values into eqn. (17) gives a rate constant for the isomerization of 9×10^6 s^{-1} (assuming $k_{+3} = k_{-3}$). Any isomerization of the phosphoenzyme thus must have a rate constant as high as or higher

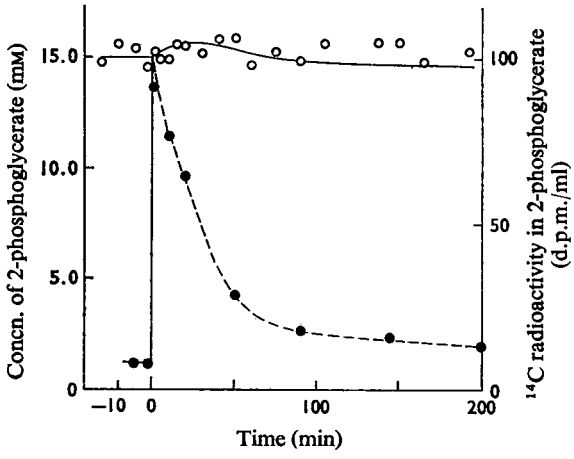

Fig. 5. *Induced-transport test for the phosphoglycerate mutase reaction at high substrate concentration and high ionic strength with ^{14}C-labelled substrates*

For symbols and experimental procedure see the legend to Fig. 3(*a*). Concentrations before addition of non-radioactive 2-phosphoglycerate and 2,3-diphosphoglycerate: 2-phosphoglycerate, 1.14mM; 3-phosphoglycerate, 12.9mM; 2,3-diphosphoglycerate, 0.11mM. Concentrations immediately after addition: 2-phosphoglycerate, 15.0mM; 3-phosphoglycerate, 11.2mM; 2,3-diphosphoglycerate, 0.106mM; *I*, 0.184. The continuous line is the theoretical curve for Scheme 1 or 3 (Fig. 1) assuming that $\alpha = 1.33 \times 10^{-2}$ mM^{-1}. For details of calculations see the Theory section.

than this value. If the enzyme has two active centres, the rate constants for each centre will be 4.5×10^6 s^{-1}. The calculations assume that the K_m is not affected by the high ionic strength, which, because of the high substrate concentrations, was comparable with that in the experiments in the presence of KCl (see below). Grisolia & Cleland (1968) found that the K_m increases with ionic strength with the enzyme from chicken breast: in part at least this would seem to be due to the fall in activity coefficients. The effective K_m for 2-phosphoglycerate may therefore have been higher than the value assumed. However, this does not alter the qualitative conclusion, and it is unlikely that the order of magnitude of the calculations will be changed (Britton & Clarke, 1968). It may be noted that the values of α considered in this section would have a negligible effect on the induced-transport curves at intermediate concentrations.

Induced-transport tests at high ionic strength

At high ionic strength the kinetics of phosphoglycerate mutase are considerably affected (Cascales & Grisolia, 1966; Grisolia & Cascales, 1966; Grisolia & Cleland, 1968), and therefore some induced-transport experiments were carried out under conditions identical with those for Figs. 3(*a*) and 3(*b*) with added KCl (150mM). The ionic strength in these experiments was similar to that in the experiments shown in Fig. 5. The results were similar to those in the absence of KCl; there was no induced transport with ^{14}C-labelled substrates, and with ^{32}P-labelled substrates the radioactivity in the 2-phosphoglycerate fell to 49.3%, in good agreement with the theoretical curve for Scheme 1, assuming a rapid isomerization of the enzyme. The results therefore suggest that there is essentially no change in mechanism when KCl is added.

Exchange at equilibrium

Table 1 shows some data for the fluxes of the phosphate and glycerate moieties at chemical equilibrium. The ratio of the fluxes was close to 2. If it is assumed that any isomerization of the enzyme is rapid, this is the expected result for Scheme 1 (eqn. 5). In contrast Scheme 3 should yield a ratio of 1 and Scheme 2 a ratio of 1.5 (eqn. 9). If any isomerization were rate-limiting the theoretical ratio for Scheme 2 would be greater than 2 and the ratio for Scheme 3 would approach 2. Thus, as with induced-transport experiments at intermediate concentration, the results are consistent either with Scheme 1 and a rapid isomerization of the enzyme or

Table 1. *Radioactive fluxes at chemical equilibrium*

Results are from two experiments, one of which is illustrated in Fig. 6. Details of the experimental procedure and calculations are given in the legend to Fig. 6.

Concn. of phosphates	Concn. of enzyme (μg/ml)	Glycerate flux (μmol/min per μg of enzyme)	Phosphate flux (μmol/min per μg of enzyme)	Glycerate flux/phosphate flux ratio	
2-Phosphoglycerate (0.203mM)	0.0417	0.177	0.0894	1.98	Average = 2.05
3-Phosphoglycerate (2.297mM)					
2,3-Diphosphoglycerate (0.01mM)	0.0417	0.147	0.0700	2.11	

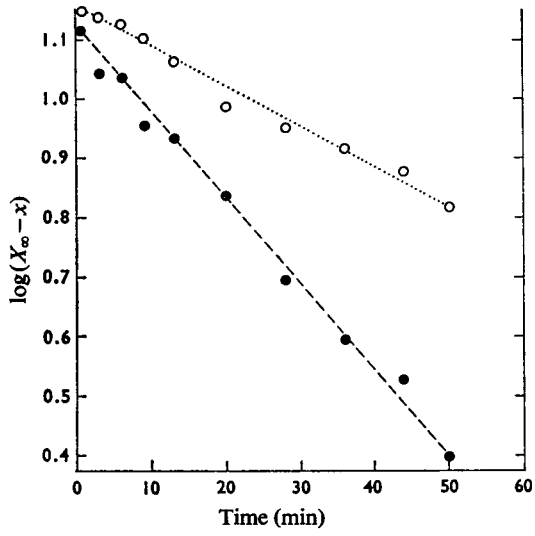

Fig. 6. *Measurement of fluxes at equilibrium*

3-Phosphoglycerate and 2,3-diphosphoglycerate were incubated in tris–HCl buffer, pH 7.4, with the enzyme at 30°C until equilibrium was reached. 3-[^{14}C]Phosphoglycerate and 3-[^{32}P]phosphoglycerate (representing less than 0.5% of the 3-phosphoglycerate already present) of high specific radioactivity were added in small volume to give a final volume of 3.0ml. The final concentrations of tris–HCl and $MgCl_2$ were 16.67 and 3.33mM respectively. 2-[^{14}C]Phosphoglycerate and 2-[^{32}P]phosphoglycerate were assayed at various times as described in the Materials and Methods section. 2-Phosphoglycerate and 3-phosphoglycerate concentrations were assayed at zero time to check that equilibrium had been attained. X_∞ = Final radioactivity in 2-phosphoglycerate; x = radioactivity at time t. o, 2-[^{32}P]Phosphoglycerate determination: •, 2-[^{14}C]phosphoglycerate determination. The straight lines were fitted to the points visually. Fluxes were calculated from the slopes of the lines as described by Britton & Clarke (1968).

with Scheme 2 with an isomerization of the enzyme that is the major rate-limiting step.

Discussion

It was concluded in the introduction that the available evidence did not distinguish between the possible mechanisms for rabbit muscle phosphoglycerate mutase. However, the present experiments have excluded all but the phosphoenzyme pathway. The distinction from Scheme 3, the intramolecular transfer of phosphate, was particularly clear-cut since Scheme 3 would have required that both ^{32}P and ^{14}C labels behave identically in the induced transport tests and in the exchange at equilibrium. For the same reasons the concerted transfer of phosphate between two or more molecules of substrate (Grisolia & Joyce, 1959; Pizer, 1962), which was suggested for the 2,3-diphosphoglycerate independent mutases, can also be excluded. Scheme 2 was excluded by the fact that the K_m would have had to be more than 7.5-fold lower than the measured value to explain the lack of co-transport of ^{14}C-labelled substrates at low substrate concentrations. Further, for Scheme 2, if the K_m were only 7.5-fold lower than the measured value, the rate constants for the transphosphorylation would have to be larger than the rate constants for the isomerization (eqn. 2). Under these conditions the turnover number for the enzyme would be determined by the rate constants for the isomerization and the rate constants for the transphosphorylation would be much greater than the turnover number. The turnover for the enzyme, assuming a molecular weight of 60000 (Pizer, 1960; Edelhoch *et al.*, 1957), is about 1940s^{-1} (H. G. Britton & J. B. Clarke, unpublished work), and it seems unlikely that the rate constants for the transphosphorylation would be much larger. Consequently the K_m would have to be considerably smaller than the maximum permissible value if Scheme 2 were to explain the results plausibly. The present experiments have also shown that any isomerization of the phosphoenzyme must be very rapid with a rate constant of the order of at least $4.5 \times 10^6 s^{-1}$, assuming two active centres. Although not extensively investigated the mechanism seemed to be the same in the presence of KCl, as suggested by Grisolia & Cleland (1968). As has been discussed elsewhere (Britton & Clarke, 1968), none of the conclusions should be substantially affected by non-ideal behaviour of the solutions or possible isotope effects.

Although the present findings indicate a phosphoenzyme mechanism, attempts to isolate a phosphoenzyme have led to conflicting results, and it is possible that the phosphoenzyme may in fact be an enzyme–2,3-diphosphoglycerate complex with one of the phosphate groups of the 2,3-diphosphoglycerate playing the role of the phosphate in the phosphoenzyme. The most recent findings (Rose, 1970; H. G. Britton, J. Carreras & S. Grisolia, unpublished work), however, suggest that the enzyme is a true phosphoenzyme and that it has two active centres. The lack of induced transport with ^{14}C-labelled substrates over a wide concentration range is of particular interest in this context, since, as discussed in the Theory section, it excludes certain types of co-operativity between centres (Britton, 1966, 1967). For example, co-transport would have been expected at low substrate concentrations if the conversion of 2-phosphoglycerate into 3-phosphoglycerate occurred

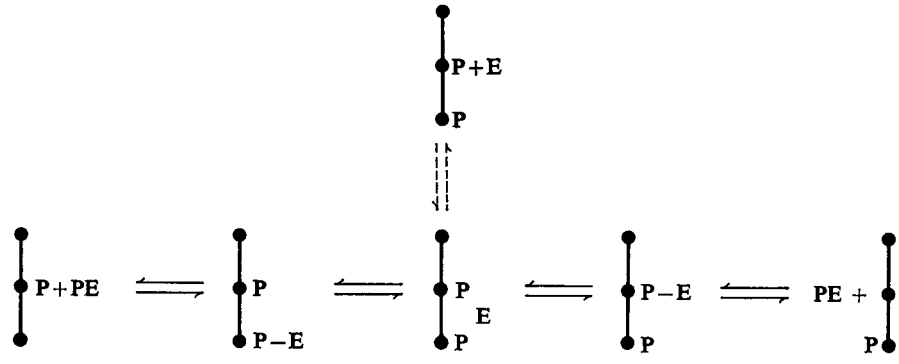

Fig. 7. *Modified phosphoenzyme mechanism for the phosphoglycerate mutase reaction to account for the absence of detectable phosphoenzyme isomerization*

E represents the enzyme and PE the phosphoenzyme. The dashed arrows indicate slow reactions.

more readily at one centre if the same reaction was occurring at the other site.

The very high value for the rate constant for isomerization of the phosphoenzyme ($4.5 \times 10^6 s^{-1}$) indicates that only a very minor rearrangement of the enzyme molecule can be involved, and it suggests that the representation of the mechanism in Fig. 1 is incorrect. An alternative scheme that avoids the need for an isomerization of the phosphoenzyme is shown in Fig. 7. According to this scheme the enzyme may have originated from a 2,3-diphosphoglycerate phosphatase in which the substrate-binding site allowed the substrate to be bound in two different positions so that either the 2- or the 3-phosphate group could be attacked. According to the mechanism shown in Fig. 7 some mutase activity would be expected from such an enzyme, and subsequent evolution might then have rendered the phosphoenzyme relatively stable, decreasing phosphatase activity while increasing mutase activity. If the enzyme should have evolved in this way the active enzyme should be a true phosphoenzyme rather than an enzyme–2,3-diphosphoglycerate complex. It may be of interest that the phosphoglycerate mutases from yeast and kidney (H. G. Britton, J. Carreras & S. Grisolia, unpublished work) and the phosphoglucomutases from rabbit muscle (Najjar & Pullman, 1954; Ray & Roscelli, 1964*a*,*b*; Britton & Clarke, 1968) and *Micrococcus lysodeikticus* (Britton & Clarke, 1972) also have phosphoenzyme mechanisms. The isomerization of the phosphoenzyme with all of these enzymes has been found to be very rapid, and it is possible therefore that Fig. 7 may represent a mechanism that is common to all phosphomutases requiring the appropriate diphosphate as a cofactor. This does not, however, mean that they have necessarily evolved from a common ancestor. Indeed the lack of a requirement for Mg^{2+} (Rodwell *et al.*, 1957) and the evidence for the involvement of a phosphohistidine residue with the 2,3-diphosphoglycerate-dependent phosphoglycerate mutases (Rose, 1970, 1971; H. G. Britton, J. Carreras & S. Grisolia, unpublished work), when compared with the Mg^{2+} requirement of the phosphoglucomutases and the evidence for the involvement of a phosphoserine residue with the enzyme from rabbit muscle (Kennedy & Koshland, 1957; Anderson & Jollès, 1957) and *M. lysodeikticus* (Birch *et al.*, 1972) and its probable involvement with the enzymes from flounder and shark muscle (Hashimoto & Handler, 1966), suggest that the phosphoglycerate mutases and phosphoglucomutases have evolved from different ancestors.

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