The Phosphorylated Intermediate in the Phosphoglyceromutase Reaction

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1. High-voltage paper-electrophoresis methods have been used for the separation of ^{32}P-labelled phosphoesters. 2. Evidence is presented which indicates that ^{32}P-labelled phosphopeptides, obtained after acid hydrolysis of phosphoglyceromutase incubated with impure 2,3-di[^{32}P]phosphoglycerate, are derived from phosphoglucomutase contamination. 3. The hydrolysis of 2,3-di[^{32}P]phosphoglycerate by phosphoglyceromutase has been studied. After an apparent instantaneous hydrolysis of 1 mole of coenzyme/mole of enzyme the reaction proceeds at a very low rate. 4. This hydrolysis seems to be due to the destruction of an enzyme–coenzyme complex. The proportions of the decomposition products of the complex vary according to further handling (pH of ionophoresis). 5. The inorganic [^{32}P]-phosphate produced by the hydrolysis of the complex and the inorganic [^{32}P]phosphate produced by the slow phosphatase activity can be differentiated by the ability of the former to be incorporated into non-radioactive substrate before enzyme denaturation. 6. The effect of enzyme concentration on the stoicheiometry of the slow phosphatase hydrolysis of the diphosphoglycerate is described and this suggests that it may occur via two independent reactions, one of them being the decomposition of the enzyme–coenzyme complex on standing.

Phosphoglyceromutase (2,3-diphospho-D-glycerate-2-phospho-D-glycerate phosphotransferase, EC 2.7.5.3) catalyses the intramolecular transfer of the phosphate of 2- and 3-phosphoglycerate. Since the discovery by Sutherland, Posternak & Cori (1949) that a diphosphate cofactor (identified as 2,3-diphosphoglycerate) is involved in the reaction, its mechanism has been associated with the mechanism of the phosphoglucomutase (EC 2.7.5.1) reaction. In the latter a phosphoenzyme has been postulated which is able to phosphorylate either glucose 1-phosphate or glucose 6-phosphate to give the cofactor glucose 1,6-diphosphate (Najjar & Pullman, 1954). That phosphate is covalently bound to this enzyme was shown by Anderson & Jolles (1957), who isolated phosphoserine from acid hydrolysates; the amino acid sequence around this particular phosphoserine was shown to be (Milstein & Sanger, 1961) Thr-Ala-phosphoserine-His-[Asp or Asp(NH$_2$)]. A similar mechanism was proposed by Pizer (1960) for the phosphoglyceromutase reaction based on the finding that labelled phosphoserine was obtained from a partially acid-hydrolysed phosphoglyceromutase preparation equilibrated with [^{32}P]diphosphoglycerate.

More recently, Grisolia, Joyce & Fernandez (1961) reported that a fraction of the phospho-

protein produced after incubation with the diphosphoglycerate did not equilibrate with the substrate. The possibility that phosphoserine arose from a contamination with phosphoglucomutase was mentioned (Pizer, 1962). Acid-labile phosphate was shown to remain associated with the muscle enzyme, after incubation with diphosphoglycerate and purification on a basic resin, in the ratio of 2 phosphate residues/mole of protein. This bound phosphate was shown to equilibrate with substrate (Grisolia *et al.* 1961).

Experimental evidence indicating that the acid-stable phosphorylated enzyme is a phosphoglucomutase contaminant and that the acid-labile phosphorylated protein is an intermediate enzyme–coenzyme complex are presented in this paper. The experimental approach used to disclose this complex may be of more general use for obtaining evidence on the existence of rather unstable intermediates. The complex decomposes under mild conditions giving inorganic phosphate and monophosphoglycerate in different proportions depending on the conditions. The 2,3-diphosphoglycerate-phosphatase activity of the enzyme has been reinvestigated. A preliminary report on part of this work has been published (Zwaig & Milstein, 1963).

MATERIAL AND METHODS

[^{32}P]*Phosphoesters.* A crude mixture of ^{32}P-labelled phosphoesters was prepared by two methods.

Method 1. ^{32}P was incorporated into the phosphoesters by using erythrocytes (Prankerd & Altman, 1954). The erythrocytes from 1 ml. of rabbit blood were incubated with 1 mc of ^{32}P (inorganic phosphate) as indicated by Grisolia *et al.* (1961) and were then centrifuged and washed twice with 3 ml. of 0·9% NaCl. The erythrocytes were haemolysed with 5 ml. of water and proteins were precipitated in a boiling-water bath for 30 min. The supernatant was concentrated in an evacuated desiccator containing H_2SO_4.

Method 2. In this method purified or partially purified enzymes were used to incorporate the ^{32}P into the phosphoesters. This gave maximum specific radioactivity and was used at early stages of the work. However, the yield of radioactive esters was rather low and not reproducible, varying with the batch of radioactive phosphate. For this reason method 1 was preferred.

The incubation mixture contained: 15 μmoles of fructose diphosphate, 15 μmoles of $MgSO_4$, 30 mμmoles of K_2HPO_4 containing 1 mc of ^{32}P, 0·2 mg. of NAD, 50 μmoles of tris, pH 8·0, 0·5 μmole of sodium pyruvate, 40 μg. of crystalline aldolase (C. F. Boehringer und Soehne G.m.b.H., Mannheim,

Germany), 25 μg. of crystalline glycerophosphate dehydrogenase (a kind gift of Dr J. Park) and 15 μl. of a water extract of chicken breast (Rodwell, Towne & Grisolia, 1957). The final volume was 1 ml. The mixture was incubated for 1 hr. at 38° and the reaction was then stopped with a few drops of acetic acid. Up to 7% of ^{32}P was converted into 2,3-diphosphoglycerate.

Purification of 2,3-di[^{32}P]*phosphoglycerate.* The crude mixture of ^{32}P-labelled phosphoesters prepared by either method 1 or method 2 was subjected to paper electrophoresis at pH 3·5 with Whatman 3MM paper in a 20 cm.-wide band. The position of the esters was located by using suitable markers and the diphosphoglycerate, free from radioactive contaminants, was eluted with distilled water. At most pH values the diphosphoglycerate was well separated from other phosphoesters and also from inorganic phosphate (Fig. 1). This purification procedure was considered better than paper chromatography in propanol–NH$_3$–water as used by Pizer (1960) since, in the latter, glucose 1,6-diphosphate and 2,3-diphosphoglycerate are not separated.

Labelling of phosphoglyceromutase. Labelling of the enzyme was attempted by several procedures with labelled diphosphoglycerate or a mixture of labelled phosphoesters. Two methods are described in detail.

Method (*a*). A crude mixture of ^{32}P-labelled phosphoesters was used in this method. A portion (2·5 ml.) of the haemolysed erythrocytes containing ^{32}P (prepared as indicated in method 1 above) was placed in a 10 ml. conical tube and allowed to equilibrate for 48 hr. at 4° with 2·5 mg. of phosphoglyceromutase, which was dissolved in a minimum volume of water and placed in a small dialysis bag. The enzyme was then dialysed with stirring for 24 hr. against distilled water, which was changed at 30 min. intervals by a siphoning procedure. The dialysis bag was fixed at one end and a trapped air bubble ensured good mixing of its content during the process of siphoning and refilling of the outside container.

Method (*b*). [^{32}P]Diphosphoglycerate purified by ionophoresis at pH 6·5 was used in this method. Phosphoglyceromutase (2 mg. dissolved in 0·033 M-tris, pH 7·0) was incubated with about 0·2 μmole of 2,3-di[^{32}P]phosphoglycerate, containing about 7·5 × 10^5 counts/min. The total volume was 0·6 ml. The incubation was carried out for 3 hr. at 31°. The preparation was then dialysed until negligible radioactivity was detected in the diffusate.

Partial acid hydrolysis. This was carried out at 100° with 5·6 N-HCl for 30 min. (Milstein & Sanger, 1961).

Trypsin digestion. ^{32}P-labelled phosphoglyceromutase (1 mg.; method *b*) was denatured by incubation for 10 min. in a boiling-water bath. Ammonium hydrogen carbonate was added to give a final concentration of 0·5% and 10 μg. of trypsin (crystalline; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was added. Digestion was carried out at 37° for 20 hr. The material was dried *in vacuo* over NaOH. Residual salt was removed by two successive additions of water, the material being dried after each addition. The products were subjected to ionophoresis and radioautography (Milstein, 1964). A mixture of dyes was used as visual indication of the run (Milstein & Sanger, 1961).

Enzyme activity. Phosphoglyceromutase activity was estimated by the method of Sutherland *et al.* (1949). Phosphoglucomutase activity was measured as described by Milstein (1961).
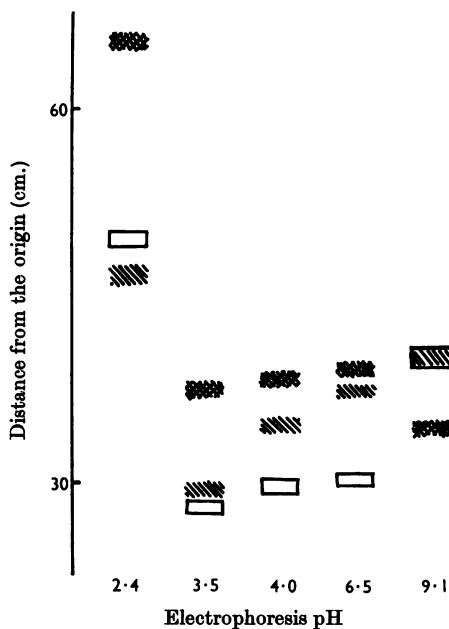

Fig. 1. Diagram showing the separation of monophosphoglycerate from 2,3-diphosphoglycerate and inorganic phosphate by paper electrophoresis. ▭, Inorganic phosphate; ▨, 2- and 3-phosphoglycerate; ▩, 2,3-diphosphoglycerate.

pH 2·4: formic acid–pyridine–water (3:0·5:96·5, by vol.);
pH 3·5: pyridine–acetic acid–water (1:10:189, by vol.);
pH 4·0: from pH 3·5, by adding pyridine;
pH 6·5: pyridine–acetic acid–water (10:0·4:90, by vol.);
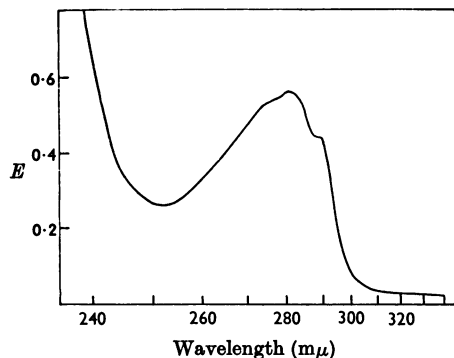pH 9·1: ammonium carbonate–water (1:9, w/w).

Fig. 2. Ultraviolet spectrum of phosphoglyceromutase (1·344mg. dry wt. dissolved in 3·00ml. of water in a 1cm.-path cell). The spectrum was determined in a Beckmann DK-2A automatic recorder spectrophotometer.

Inorganic phosphate. This was estimated by the method of Fiske & Subbarow (1925) or by the method of Bartlett (1959) when only very small quantities were available.

Protein concentration. This was estimated from the extinction at 280mμ. The u.v. spectrum of the protein is shown in Fig. 2. The extinction coefficient of crystalline phosphoglyceromutase was determined on a dry-weight basis: $E_{280\,m\mu}^{1\%}$ 12·5.

Other methods. Paper electrophoresis, radioautography and radioactive measurements were carried out as described by Milstein (1964).

Phosphoglyceromutase. This enzyme, from rabbit muscle, was obtained as a crystalline suspension in 2·6м-$(NH_4)_2SO_4$ from C. F. Boehringer und Soehne G.m.b.H. and dialysed against glass-distilled water before use. ^{32}P-labelled phosphoglucomutase was prepared according to Milstein & Sanger (1961). Glucose 1,6-diphosphate was a kind gift of Dr C. Cardini and 2,3-diphosphoglycerate was kindly provided by Dr S. Grisolia.

RESULTS

Binding of ^{32}P to phosphoglyceromutase

Preliminary experiments to study the binding of ^{32}P to phosphoglyceromutase were made with crude muscle extracts, equilibrated with a mixture of ^{32}P-labelled phosphoesters by the dialysis technique employed to label phosphoglucomutase (Milstein & Sanger, 1961). Separation of phosphoglyceromutase and phosphoglucomutase activities can be achieved on a DEAE-cellulose column at pH 8·3 and a gradient of tris from 0·02 to 0·2м. Phosphoglucomutase is retained in the column and phosphoglyceromutase comes straight through. These experiments did not show any radioactivity associated with the phosphoglyceromutase activity. The ionograms of radioactive peptides obtained after partial hydrolysis of the labelled protein material (which eluted together with the phospho-

glucomutase activity) were the same as the one obtained from pure ^{32}P-labelled phosphoglucomutase. More thorough experiments were made with crystalline preparations of phosphoglyceromutase.

Labelling of the crystalline phosphoglyceromutase by method (a) (in which a mixture of ^{32}P-labelled phosphoesters was used) and by method (b) (in which purified 2,3-diphosphoglycerate was used) yielded different products. In method (a) the label was acid-stable but with method (b) no acid-stable phosphoprotein was obtained. An investigation of the acid-stable phosphoprotein obtained by method (a) was undertaken.

When the radioactive peptides obtained after partial acid hydrolysis of a ^{32}P-labelled phosphoglyceromutase preparation (method a) were compared by ionophoresis at pH 3·5 with those obtained from ^{32}P-labelled phosphoglucomutase, the same pattern was obtained (Fig. 3C and D). Although no precise measurements of the acid-stable ^{32}P-labelled protein were made, it became obvious that the yields were considerably lower than those obtained when ^{32}P-labelled phosphoglucomutase was prepared. Therefore the possibility that this acid-stable phosphoprotein was in fact a contaminant (and Fig. 3C and D suggested that the contaminant could be phosphoglucomutase) was considered. Trace amounts of phosphoglucomutase activity were detected in the crystalline phosphoglyceromutase preparation used. The activity present was equivalent to 0·5% (by wt.) of phosphoglucomutase as contaminant. However, the possibility of this activity being due to a lack of specificity of the phosphoglyceromutase rather than to contamination was considered.

The pattern of the ionogram of radioactive peptides obtained after partial acid hydrolysis of ^{32}P-labelled phosphoglucomutase was unchanged if 3-phosphoglycerate was incubated with the radioactive enzyme before partial acid hydrolysis. In contrast, incubation of ^{32}P-labelled phosphoglucomutase with glucose 6-phosphate (equilibration of the radioactive phosphoenzyme with the non-radioactive substrate being allowed) gave rise to a pattern of radioactive bands in which a stronger band 3 (containing glucose phosphate; Milstein & Sanger, 1961) was obtained, and the peptide bands 4, 5 and 7 were no longer present (Fig. 3F). Band 6, on the other hand, was present after this treatment. When this band was run on ionophoresis at pH 6·5 it did not correspond to any of the peptide bands identified by Milstein & Sanger (1961) in the partial acid hydrolysate of ^{32}P-labelled phosphoglucomutase, but rather to the unidentified spot which the above authors called X.

When a similar experiment was carried out with

Fig. 3. Tracing of the radioautograph of the [^{32}P]peptides obtained after partial acid hydrolysis of ^{32}P-labelled phosphoglyceromutase (A, B and C) and ^{32}P-labelled phosphoglucomutase (D, E and F). ^{32}P-labelled enzymes (0·03 μc) were previously equilibrated with 8 μmoles of histidine, pH 7·0, 0·4 μmole of MgCl$_2$ and 2 μmoles of non-radioactive substrate (in 0·2 ml.): A and E, 3-phosphoglycerate; B and F, glucose 6-phosphate; C and D, incubation with non-radioactive substrate omitted. Electrophoresis at pH 3·5. ^{32}P-labelled phosphoglyceromutase was prepared by method (a). Band numbers are as in Milstein & Sanger (1961).

the acid-stable ^{32}P-labelled phosphoglyceromutase (prepared by method a) the same result was obtained, namely that after incubation with glucose 6-phosphate the radioactive peptides characteristic of the sequence around the reactive serine in phosphoglucomutase were lost (Fig. 3B). Incubation with 3-phosphoglycerate on the other hand did not change the radioactive spots on the ionogram after acid hydrolysis (Fig. 3A). These results indicated that

the acid-stable [^{32}P]phosphoprotein obtained by method (a) was indeed a contamination of phosphoglucomutase present in the crystalline phosphoglyceromutase preparation.

State of the labile ^{32}P bound to phosphoglyceromutase

When the ^{32}P-labelled phosphoglyceromutase was prepared by method (b) and dialysed, considerable radioactivity remained associated with the protein. But after partial acid hydrolysis or digestion with trypsin, the radioactivity was recovered as inorganic phosphate after ionophoresis at pH 3·5 and 6·5. The presence of labile bound phosphate to phosphoglyceromutase has been reported by Grisolia et al. (1961).

When 2,3-diphosphoglycerate was incubated with phosphoglyceromutase for long periods, a slow hydrolysis of the ester, leading to the formation of inorganic phosphate and monophosphoglycerate, was observed. The time-course of this extremely slow process is shown in Fig. 4. The formation of inorganic phosphate under these conditions was linear, but extrapolation showed that the line did not pass through the origin. Values obtained at the minimum time of 2 min. indicated that a small but significant amount of diphosphoglycerate was hydrolysed. From this and other similar observations it became obvious that this amount was of the same order of magnitude as the amount (in moles) of enzyme which was being used. This apparently instantaneous hydrolysis of diphosphoglycerate was studied in considerable detail under conditions in which the phosphatase reaction was negligible. This was achieved by reducing the incubation period to a minimum (not more than 2 min.) and the temperature to 0°. The incubation mixture was then immediately applied to a chromatographic paper, dried with a hairdrier and subjected to high-voltage electrophoresis. This procedure involves a loss of enzyme activity, which was shown on a control by eluting with 0·01 M-tris, pH 7·0, a piece of 3MM paper containing dried enzyme and measuring the specific activity before and after application to the paper. The enzyme activity remaining after the procedure was less than 5% of the original. To obtain a reasonable accuracy, very low concentrations of diphosphoglycerate (of the same order of magnitude as the concentration of enzyme) had to be used. Therefore it was essential to work with radioactive diphosphoglycerate of very high specific activity.

When the 'burst' reaction was studied under these conditions the following results were obtained. The radioactivity of the decomposed [^{32}P]diphosphoglycerate recovered as inorganic phosphate and monophosphoglycerate depended on the pH at
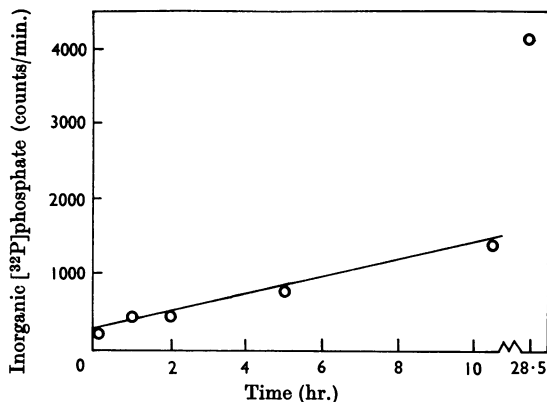
Fig. 4. Time-course of the formation of inorganic phosphate on incubation of 2,3-diphosphoglycerate with phosphoglyceromutase. 2,3-Di[^{32}P]phosphoglycerate (104 mμmoles; 2·7 × 10^6 counts/min./μmole) was incubated with 1·6 mμmoles of phosphoglyceromutase in 0·3 ml. of 0·05 M-tris buffer, pH 7·0. At different times, samples were applied to a sheet of Whatman 3MM paper, immediately dried with hot air and subjected to pH 6·5 electrophoresis. The inorganic [^{32}P]phosphate was located by radioautography and the spots were counted in a gas-flow counter (Nuclear–Chicago model D–47).

which the ionophoretic separation was carried out. Fig. 5(a) shows the proportion of radioactive products recovered by ionophoresis at pH 3·5 and Fig. 5(b) the proportion of radioactive products recovered in the same experiment by ionophoresis at pH 6·5. When the amount of coenzyme was higher than the amount of enzyme present, within the experimental error, stoicheiometric amounts of cofactor decomposed under these conditions. When the slow phosphatase activity was avoided by using very short incubations in the cold the excess of cofactor above a mole to mole ratio with enzyme was always recovered as such. When the amount of coenzyme was lower than the amount of enzyme, no coenzyme could be recovered after electrophoresis at any pH. In this experiment it was observed also that, depending on the pH of ionophoresis, different proportions of inorganic phosphate and monophosphoglycerate were obtained from the diphosphoglycerate 'burst'. This curious effect is discussed below.

The inorganic phosphate which represented 90% of the decomposition products of the 2,3-diphosphoglycerate after ionophoresis at pH 3·5 (see Fig. 5a) did not seem to be present as such in the incubation mixture before ionophoresis was carried out. This was shown as follows. If after incubation of the enzyme with the labelled cofactor (2 min. in the cold) non-radioactive 3-phosphoglycerate was added and the products were separated by ionophoresis,
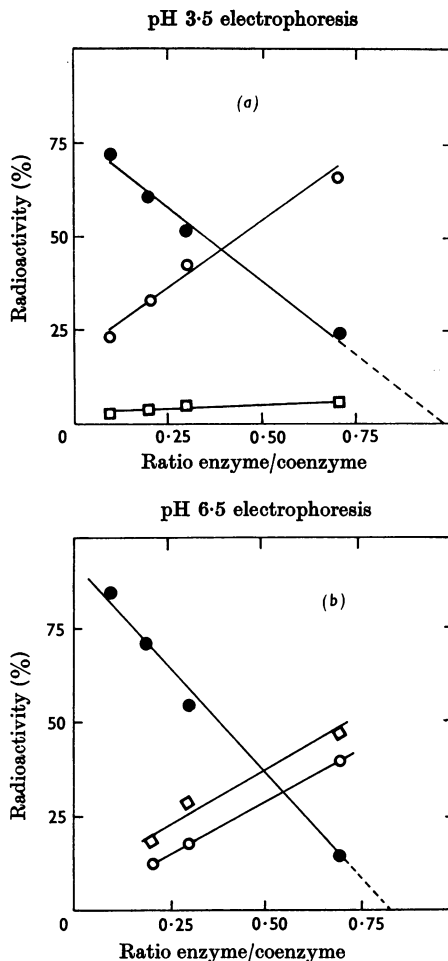

Fig. 5. Effect of enzyme/coenzyme ratio and of the ionophoretic separation procedure on the 'burst' hydrolysis of 2,3-diphosphoglycerate. 2,3-Di[^{32}P]phosphoglycerate (4·4 mμmoles; 4·5 × 10^6 counts/min./μmole) was incubated for 2 min. in an ice bath with different amounts of phosphoglyceromutase in 0·27 ml. of 0·05 M-tris buffer, pH 7·0. Samples were applied on Whatman 3MM paper and dried as fast as possible with a hair-drier. Ionophoresis was carried out at pH 3·5 (Fig. 5a) and pH 6·5 (Fig. 5b). The radioactivity of the spots obtained after radioautography was measured and plotted as a percentage of the total. Radioactivity present as ●, diphosphoglycerate, □, monophosphyglycerate, ○, inorganic phosphate.

no inorganic phosphate was obtained. This was true when the fractionation was carried out at pH 3·5 or at pH 6·5 (Fig. 6). However, when added inorganic [^{32}P]phosphate was incubated with 3-phosphoglycerate and enzyme, all the radioactivity was recovered as inorganic phosphate, and no incorporation into esters was detected. Since the

radioactivity associated with the active enzyme exchanges with the substrate, whereas inorganic [^{32}P]phosphate does not, the above results indicate that hydrolysis takes place during or after the denaturation process occurring at the drying stage before ionophoresis.

The method of analysis shown in Fig. 6 differentiates between two types of phosphate. The one present as protein-bound ^{32}P was incorporated into
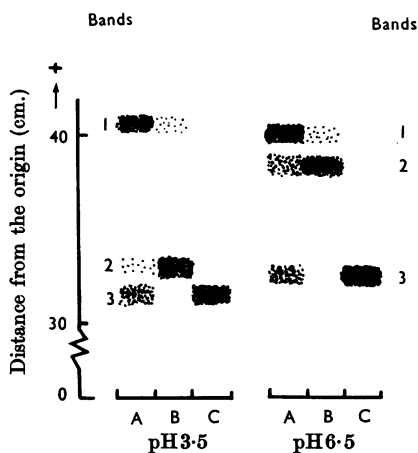

Fig. 6. Diagram of the radioactive products obtained by incubating phosphoglyceromutase and radioactive 2,3-diphosphoglycerate. A, Enzyme and ^{32}P-labelled cofactor (fivefold excess of cofactor) incubated for 2 min. in the cold (0·05 M-tris buffer, pH 7·0). B, Same as A further incubated with excess of non-radioactive 3-phosphoglycerate. C, Enzyme incubated with inorganic [^{32}P]phosphate and 3-phosphoglycerate. In all cases samples of the incubation mixture were applied to a sheet of Whatman 3MM paper and dried down. Electrophoresis (30 v/cm.) was carried out for 2 hr. at pH 6·5 and for 3 hr. at pH 3·5. Band 1, 2,3-diphosphoglycerate; band 2, monophosphoglycerate; band 3, inorganic phosphate.

3-phosphoglycerate before the ionophoresis. The ^{32}P present as inorganic phosphate was not. When the incubation of 2,3-diphosphoglycerate and enzyme was carried out for long periods and at room temperature (as in Fig. 4), inorganic phosphate was produced which could not be incorporated into the substrate by an incubation with 3-phosphoglycerate before fractionation as described in Fig. 6. The latter was a much slower process than the 'burst' hydrolysis, and corresponded to the linear phase of the phosphatase activity of Fig. 4.

Unequal amounts of inorganic phosphate and monophosphoglycerate were observed during the slow hydrolysis of the diphosphate in the presence of the enzyme. This difference could be increased substantially by increasing the enzyme/cofactor ratio and allowing the reaction to go to completion. This is shown in Table 1. It can be seen that, as the enzyme/cofactor ratio increased, more radioactivity was recovered as inorganic phosphate and less as monophosphoglycerate. That this difference is not due to subsequent hydrolysis of the monophosphoglycerate was shown in a control experiment in which the hydrolysis of the 3-phosphoglycerate was measured. The amount of inorganic phosphate formed at the expense of the monophosphoglycerate after incubation with the enzyme was not higher than the amount present in the controls (Table 2). These results suggest that the phosphatase activity involves a stoicheiometric hydrolysis of the diphosphoglycerate into inorganic phosphate plus monophosphoglycerate only when the enzyme concentration is negligible as compared with the diphosphoglycerate concentration.

DISCUSSION

To explain the presence of [^{32}P]phosphoserine in acid hydrolysates of rabbit-muscle crystalline phosphoglyceromutase (Pizer, 1960, 1962) as being

Table 1. *Effect of enzyme concentration on the slow hydrolysis of 2,3-di*[^{32}P]*phosphoglycerate*

2,3-Di[^{32}P]phosphoglycerate (5·4 mμmoles; 3·9 × 10^6 counts/min./μmole) was incubated at room temperature with enzyme in 0·27 ml. of 0·05 M-tris buffer, pH 7·0. Samples were taken at the indicated times and analysed as indicated in Fig. 5(b). The inorganic [^{32}P]phosphate was not incorporated into the substrate when the samples were incubated with non-radioactive 3-phosphoglycerate.

| Enzyme (mμmoles) | Ratio $\dfrac{\text{[enzyme]}}{\text{[coenzyme]}}$ | Incubation time (hr.) | Radioactivity | | Difference |
			Inorganic phosphate (%)	Monophospho-glycerate (%)	
0·25	0·046	4	58·5	41·5	17·0
0·25	0·046	20	58·8	41·2	17·6
0·5	0·093	20	67·9	32·1	35·8
1·0	0·185	20	74·6	25·4	49·2
3·5	0·66	20	77·7	22·3	55·4

Table 2. *Failure of phosphoglyceromutase to hydrolyse 3-phosphoglycerate*

[^{32}P]Diphosphoglycerate (6 mμmoles; $1\cdot2 \times 10^4$ counts/min./mμmole), 76 mμmoles of 3-phosphoglycerate and 10 mμmoles of enzyme were incubated at room temperature for 2 min. in 0·05 M-tris buffer, pH 7·0. Three samples were taken and treated as indicated. The radioactivity associated with the 3-phosphoglycerate fraction and the inorganic phosphate fraction were determined. The calculated ratio [^{32}P]monophosphoglycerate/inorganic [^{32}P]phosphate, as expected from 'burst' hydrolysis, is 6.

		Radioactivity (counts/min.)		Ratio monophosphoglycerate / inorganic phosphate
Sample	Treatment	3-Phosphoglycerate	Inorganic phosphate	
A	Incubated for 20 hr. at room temperature	657	103	6·4
B	10 min. in boiling-water bath and 20 hr. at room temperature	935	187	5·0
C	20 hr. at −20°	675	99	6·8

due to a contamination with phosphoglucomutase, one must assume that: (1) crystalline phosphoglyceromutase is contaminated with phosphoglucomutase, and (2) 2,3-di[^{32}P]phosphoglycerate is contaminated with glucose 1,6-di[^{32}P]phosphate.

In the crystalline enzyme that we used we showed that the first condition was fulfilled and this was probably true also in the above-mentioned experiments (Pizer, 1960), since no special precautions were taken to avoid phosphoglucomutase contamination. The second condition requires that glucose 1,6-diphosphate is not separated from 2,3-diphosphoglycerate by chromatography in the propanol–ammonia–water system used for the separation of the phosphoesters. This was tested by us and shown to be the case. The purification of the diphosphate ester by paper electrophoresis reported in this paper yields a product which is free of such impurity. With this purified coenzyme no acid-stable phosphoenzyme could be obtained.

The presence of acid-labile ^{32}P in rabbit-muscle phosphoglyceromutase after incubation with its coenzyme was reported by Grisolia *et al.* (1961). Our results confirm this report. Furthermore, the methods applied in this work permitted a closer study of the nature of this weakly bound ^{32}P responsible for the diphosphoglycerate 'burst' and its relation to the phosphatase activity.
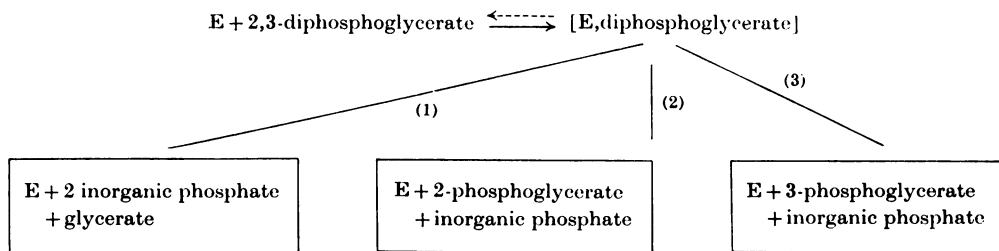
The diphosphoglycerate decomposition 'burst' cannot be attributed to the previously described diphosphoglycerate-phosphatase activity (Rodwell *et al.* 1957; Joyce & Grisolia, 1958) since: (a) In the linear phase hydrolysis of 1 mole of cofactor/mole of enzyme required (see Fig. 4) about 2 hr. at room temperature, whereas the 'burst' hydrolysis of 1 mole of cofactor/mole of enzyme was complete after only 2 min. incubation in an ice bath. (b) Under identical incubation conditions the decomposition products consistently depended on the treatment after application on to the paper. Un-

published results indicated that, after ionophoresis at pH 9, a high proportion of monophosphoglycerate was obtained (as at pH 6·5, see Fig. 5), whereas ionophoresis at pH 2·0 gave negligible amounts of it (as at pH 3·5, see Fig. 5). (c) The stoicheiometry of the 'burst' hydrolysis strongly suggested a specific effect. Any excess of coenzyme, under the conditions at which 'burst' hydrolysis was studied, was recovered as such. (d) After 2 min. in the cold there was no detectable inorganic [^{32}P]phosphate in the incubation mixture, as judged by its ability of being incorporated into the substrate.

We explain the diphosphoglycerate 'burst' by the properties of the intermediary enzyme–coenzyme complex, in which the phosphoester bonds of the diphosphoglycerate have lost their stability. It must be emphasized that the ionophoretic technique which we have used involved denaturation (the material is dried on the paper before the run), which could play an important role in the hydrolysis. Both bonds in diphosphoglycerate are known to resist very strong acid conditions without being hydrolysed (Greenwald, 1925), and the extreme lability of those bonds in the enzyme–coenzyme complex indicates a distortion effect due to binding to the protein. This lability in the denatured complex (as well as in the native complex, see below) explains why previous workers did not find bound co-enzyme in the mutase preparations (Rodwell *et al.* 1957). In fact the coenzyme bound to the enzyme is not a diphosphoglycerate ester in the chemical sense, but an enzyme–coenzyme intermediate in which the chemical properties of the ester bonds are no longer those of the molecular components.

The theoretically possible pathways of the decomposition of the enzyme–coenzyme complex which do not lead to formation of a phosphoenzyme intermediate, are shown in Scheme 1.

The possibility, however, of other pathways is not excluded. When ionophoresis is carried out on the
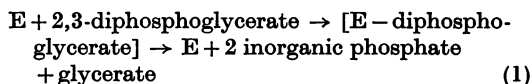
E + 2,3-diphosphoglycerate ⇌ [E,diphosphoglycerate]

(1)   (2)   (3)

| E + 2 inorganic phosphate + glycerate | E + 2-phosphoglycerate + inorganic phosphate | E + 3-phosphoglycerate + inorganic phosphate |

Scheme 1.

denatured complex at pH 6·5, pathways (2) and/or (3) could account for the products. Since under our conditions of analysis no distinction between 2-phosphoglycerate and 3-phosphoglycerate is possible we cannot say whether pathway (2), (3) or both are followed at pH 6·5. However, the results obtained in Fig. 5(b) suggest that, at this pH, pathway (2) might be preferred for the following reason. If the 2,3-diphosphoglycerate were homogeneously labelled, the radioactivity of the products inorganic phosphate and 2-(or 3-)phosphoglycerate ought to be the same (if they were produced in one to one molar ratio), or higher for inorganic phosphate if glyceric acid was also formed (pathway 1). To find a higher value for the monophosphoglycerate as shown in Fig. 5 was very puzzling at first. However, as reported by Prankerd & Altman (1954), the incorporation of ^{32}P into the 2,3-diphosphoglycerate by the erythrocytes, under the conditions used in this work to prepare the coenzyme, gave a diphosphoglycerate in which the specific radioactivity of the phosphate in position 2 is higher than that of position 3. The slightly but consistently higher radioactivity in the monophosphoglycerate fraction than in the inorganic phosphate fraction after ionophoresis at pH 6·5 (Fig. 5), suggests therefore a preference for pathway (2). A closer study of these effects requires the separation of the two monophosphate esters.

On the other hand, under the pH 3·5 conditions (Fig. 5a), most of the radioactivity was recovered as inorganic phosphate, suggesting that pathway (1) was preferred.

The results presented in this paper indicate that the specific hydrolysis of the coenzyme occurs in two clearly different steps. The 'burst' hydrolysis due to the destruction of the enzyme–coenzyme complex is negligible only when the ratio enzyme/coenzyme is negligible and does not represent a true phosphatase activity, since it is observed only after enzyme denaturation. However, after longer incubations a true diphosphoglycerate-phosphatase activity can be detected. This activity produces inorganic phosphate which is no longer in an exchangeable state. This phosphatase activity does not seem to be in itself a single reaction, since the stoicheiometry of the reaction does not follow a simple pattern and is influenced by the enzyme concentration (Table 1). This lack of stoicheiometry should not be confused with the lack of stoicheiometry during the burst reaction since (a) after 20 hr. there is no ^{32}P present which can be incorporated into the substrate and (b) there is an excess of inorganic phosphate produced over the amount of monophosphoglycerate, which is the opposite effect to the one observed during the 'burst' hydrolysis on ionophoresis at pH 6·5. In fact Table 1 suggests that when diphosphoglycerate and native enzyme are incubated for long periods, two simultaneous but independent phosphatase activities are superimposed. One corresponds to pathway (1) and could be due to the instability of the enzyme–coenzyme complex. Thus:

E + 2,3-diphosphoglycerate → [E − diphosphoglycerate] → E + 2 inorganic phosphate + glycerate   (1)

The second phosphatase activity is the hydrolysis of the diphosphoglycerate giving inorganic phosphate and monophosphoglycerate as reported by Rodwell et al. (1957) for the yeast enzyme according to equation (2).

2,3-Diphosphoglycerate + enzyme → inorganic phosphate + 2-(or 3-)phosphoglycerate   (2)
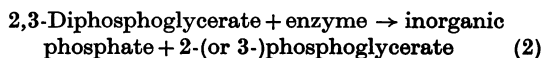
When there is less diphosphoglycerate than enzyme, reaction (2) is mostly suppressed, owing to the very low concentration of free 2,3-diphosphoglycerate, and the main products of the hydrolysis are those of equation (1) (Table 1). When there is a great excess of diphosphoglycerate the reaction proceeds mostly as shown by equation (2). Since the values of Fig. 4 were determined while there was still a great excess of diphosphoglycerate present, the linear phase of that Figure measures within experimental error the velocity of reaction

(2). This second phosphatase activity might be due to contamination with a specific 2,3-diphosphoglycerate phosphatase.

The method used to disclose the presence of the intermediary complex may prove of more general use in other cases in which such complexes have been postulated. The possibility that the hydrolysis of the enzyme–coenzyme complex could be responsible for some of the observed behaviour in some other mutases should be considered. Kinetic data of phosphoglucomutase (Bodansky, 1961) have suggested to Cleland (1963a,b) that the phosphoenzyme is not an intermediate in the reaction. Furthermore, recent results (Ray & Roscelli, 1964) have cast some doubt on the role of a phosphoenzyme intermediate in this reaction. If, as proposed in this paper for phosphoglycerate mutase, the transfer occurs directly via an enzyme–coenzyme complex in which the phosphate ester bonds become distorted, further handling could lead to the formation of inorganic phosphate or to a stable covalent bond with the substrate moiety and/or with an active residue of the protein. A distorted enzyme–glucose diphosphate complex could give a phosphoenzyme with its phosphate attached to the serine in the sequence Thr-Ala-Ser-His-[Asp or Asp(NH₂)].

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