Specificity of Phosphoglyceric Acid Mutase*

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The enzyme phosphoglyceric acid mutase acts in the Embden-Meyerhof glycolytic scheme to catalyze the migration of the phosphate group between the 3- and the 2-position of D-glyceric acid, and so prepare the molecule for dehydration by enolase. Sutherland *et al.* (1), implicated D-glyceric acid 2,3-diphosphate as an activator for the mutase; and they favored, as the mechanism of action, a transfer of phosphate from glyceric acid-2,3-diphosphate to glyceric acid monophosphate rather than an intramolecular migration of the phosphate group. We have substantiated this work with the crystallized enzyme (2), and suggest that the over-all reaction may be treated from two aspects, the donation of phosphate by glyceric acid-2,3-diphosphate and its acceptance by glyceric acid phosphate.

In the present work the substrate specificity of phosphoglyceric acid mutase has been studied in an effort to define the structural requirements for compounds to act as phosphate donors or phosphate acceptors. It has been surprising to find that such compounds as hydroxypyruvic acid 3-phosphate and the bisulfite addition complex of glycolaldehyde phosphate can act as acceptors, but the results as a whole indicate a high degree of specificity for both donation and acceptance of phosphate.[1]

EXPERIMENTAL

The absorbance of solutions was determined with a Beckman model DU Spectrophotometer fitted with a photomultiplier attachment. When the rates of change of absorbance were to be followed, an energy recording adaptor was attached to the above instrument and scales covering both 0 to 100 per cent and 90 to 100 per cent transmittance were used (3). Optical rotations were determined with the Keston polarimetric attachment to the Beckman spectrophotometer and with the Rudolph precision photoelectric polarimeter.

Glyceric acid 3-phosphate was determined by the polarimetric assay of Meyerhof and Schulz (4), while glyceric acid 2-phosphate was determined with the use of the enzyme enolase to convert it to phosphoryl-enolpyruvate which absorbs at 240 mμ (5). Inorganic phosphate was determined by the method of Ernster *et al.* (6). Crystalline mutase (2) and enolase (7) were used throughout this work.

Serine and the nucleotides were obtained from the Nutritional Biochemical Corporation, and creatine phosphate from Mann Research Laboratories. β-Glycerol phosphate was purchased from Eastman Chemicals. Phosphoamidate was a gift from Dr. C. A. Dekker, carbamyl phosphate from Dr. A. B. Pardee, and serine phosphate from Dr. E. E. Snell. The following compounds were synthesized by published procedures: D-glyceraldehyde 3-phosphate (8), D-glyceric acid 2-phosphate (9), D-glyceric acid 3-phosphate (10), D-*erythro*-dihydroxybutyric acid 2-phosphate and 3-phosphate (11), hydroxypyruvic acid 3-phosphate (12), phosphoryl-enolpyruvate (13), D-glyceric acid 2,3-diphosphate (14), and β-chlorolactic acid (12).

L-Glyceric acid 2-phosphate and 2,3-dihydroxyisobutyric acid 2-phosphate and 3-phosphate were synthesized by Dr. F. Wold, while the L-*threo*-dihydroxybutyric acid 2-phosphate and 3-phosphate were synthesized by Mr. G. Mathur. The following compounds were newly synthesized for this work: methyl D-glycerate 2-phosphate and 3-phosphate; D-glyceric acid 2,3-phosphate; D-glyceric acid 3-phosphite; D-*erythro*-dihydroxybutyric acid 2,3-diphosphate; and DL-1,2-dihydroxyethane sulfonic acid 2-phosphate.

D-Erythro-2,3-dihydroxybutyric Acid 2,3-diphosphate—Methyl 2-benzyl-D-*erythro*-2,3-dihydroxybutyrate was prepared as previously described (12). This compound (1.8 gm.) was debenzylated by hydrogenolysis in absolute ethanol (100 ml.) with a palladium catalyst (1.0 gm. of 5 per cent palladium chloride-carbon). The catalyst was removed and the solution was concentrated to a syrup that weighed 1.1 gm. This crude methyl dihydroxybutyrate was phosphorylated in 15 ml. of dry pyridine at 5° with 7.5 gm. of diphenylphosphorochloridate. After 2 days at room temperature the reaction was worked up in the manner used for other reactions of this type to give 3.2 gm. (67 per cent) of a syrup.

This syrupy, methyl 2,3-di-(diphenylphosphoryl)-dihydroxybutyrate, was then hydrogenated in 100 ml. of absolute ethanol at atmospheric pressure with 0.5 gm. of platinum oxide. The hydrogen uptake was 2.4 l. in 1.5 hours. The catalyst was removed and 40 ml. of 1 M sodium hydroxide was added to saponify the methyl ester. The ethanol was evaporated at reduced pressure and the residue was taken up in water. The product was precipitated as the barium salt according to Baer (14). The barium salt was then converted to the cyclohexylamine salt by adding cyclohexylammonium sulfate to an aqueous solution of the salt. The barium sulfate was removed and the water solution was concentrated to dryness. The residue was crystallized from water by adding acetone. The yield was 0.3 gm., m.p. 188–190°. The substance analyzed as a pentacyclohexylamine salt of dihydroxybutyric acid diphosphate.

$C_{34}H_{75}O_{10}P_2N_5$ (775)

Calculated: N 9.0, P 8.0

Found: N 8.5, P 7.9

On chromatography the substance showed one phosphate containing component with R_F similar to glyceric acid diphosphate.

Methyl D-glycerate 2-phosphate—3-Benzyl-D-glyceric acid was

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[1] A preliminary report of this work was presented at the Pacific Slope Biochemical Conference, Berkeley, Calif., August 1957.

prepared, converted to the methyl ester and then to the 2-diphenylphosphoryl ester as has been described previously (9). Of the resulting methyl 2-diphenylphosphoryl-3-benzyl-D-glycerate, 6.7 gm. in 135 ml. of absolute ethanol were debenzylated by reduction with 6.7 gm. of 5 per cent palladium chloride-carbon previously reduced and washed to remove acid (9). After the reduction was complete, the catalyst was removed and replaced with 1.35 gm. of platinum oxide. Reduction was resumed for 60 minutes to remove the phenyl groups, after which time the catalyst was filtered off and 12 gm. of barium perchlorate [$Ba(ClO_4)_2 \cdot 3\ H_2O$] in 45 ml. of 95 per cent ethanol were added. The pH was raised to between 6 and 7, and the precipitate was allowed to form overnight in the cold. The barium salt was collected by centrifugation and washed by centrifugation with 60 ml. of absolute ethanol and ether. After drying in a vacuum the yield was 3.13 gm.

When a solution of the ester was desired the barium was removed by treating a suspension of the compound with Dowex 50(H^+). After filtering off the resin, the pH was brought to 6.5 with KOH. Enzymatic assay of this solution before and after saponification indicated that both methyl ester and free acid were present in the ratio of 4:1 and in amounts sufficient to account for all the phosphate. Solutions standardized in this way were used in the enzymatic tests.

Methyl D-glycerate 3-phosphate—2-Benzyl-D-glyceric acid was prepared by oxidation of 2-benzyl-D-glyceraldehyde (8), and was then converted to methyl D-glycerate 3-phosphate by the procedure used above. Solutions were prepared in a manner similar to that used for the 2-isomer. The enzymatic tests indicated that the preparation was free of 2-isomer, but again contained free acid in one-fourth the amount of the ester.

D-Glyceric Acid 2,3-phosphate—Glyceric acid-3-P obtained from yeast fermentation was treated with dicyclohexylcarbodiimide according to the procedure of Khorana (15) to yield an equilibrium mixture of the acyclic and cyclic esters, which were separated by chromatography.

Ba glyceric acid-3-P, 1.0 gm., was converted to the free acid with Dowex 50(H^+), and, after removal of the resin, pyridine was added to give a neutral pH. The solution was evaporated to dryness with a stream of air and the residue was dissolved in 6 ml. of water. Pyridine, 40 ml., was added followed by 2.5 gm. of dicyclohexylcarbodiimide. After the mixture was shaken for 10 minutes, crystals of dicyclohexylurea started coming out of the solution. After 2 hours these were filtered off and washed with a small portion of cold water. The filtrate was made up to 100 ml. with water and extracted three times with 150-ml. portions of ether. Evaporation of the water gave an oil (1 gm.) which was placed on a cellulose column (diameter 3.5 cm.; length 68 cm.; 200 gm. of cellulose) which had been packed wet with the solvent isopropanol-water-ammonia (70:25:5). The column was developed with the same solvent. After one holdup volume had passed (400 ml.), 15-ml. fractions were collected at a flow rate of 2.5 ml. per minute. Tubes containing phosphate compounds were detected, and the compounds were later identified by paper chromatography. Tubes 28–49 contained cyclic phosphate with the majority of the compound in Tubes 40–48 (166 mg.). The acyclic phosphate esters started appearing in Tube 65 and the majority of the phosphate-containing material was recovered by Tube 80.

Material in Tubes 40–48 was converted to the cyclohexylamine salt by passage through a Dowex 50 (cyclohexylamine) column, and the product obtained on evaporation of the eluate was crystallized from a mixture of absolute ethanol and acetone. This material was very hygroscopic. When exposed to air by filtration, it became an oil. However, collection of crystals by centrifugation and washing with acetone and ether gave a white powder which was dried under high vacuum. As no inorganic phosphate was present, solutions of the cyclic compound were standardized by total phosphate analyses.

The material gave one spot in ammonia-propanol solvents with an R_F approximating those of other cyclic phosphates as reported by Khorana. On treatment with acid or alkali this compound disappeared and was replaced by one that cochromatographed with the starting material (glyceric acid-3-P). Complete absence of glyceric acid-2-P or glyceric acid-3-P in the cyclic compound was established by enzymatic test. The cyclic nature of the compound is indicated by its titration which shows no secondary phosphate dissociation, pK range 5 to 8; but this pK appears after the same solution is saponified.

D-Glyceric Acid 3-phosphite—The synthesis of glyceric acid 3-phosphite was carried out with PCl_3 in a fashion similar to that of Robertson and Boyer (16). Methyl 3-benzyl-D-glycerate, 2.06 gm., in 15 ml. of pyridine, was treated with 2.5 gm. of benzoyl chloride in 10 ml. of pyridine for 17 hours at room temperature. The material was worked up as for a phosphorylation and yielded 2.78 gm. (92 per cent) of syrup. This syrup, in 40 ml. of absolute ethanol, was hydrogenated with 2.0 gm. of 5 per cent palladium chloride-on-carbon as catalyst to remove the benzyl group. The hydrogen uptake in 30 minutes was 205 ml., and the syrup, after removal of the catalyst and solvent, weighed 2.0 gm. This material, in 15 ml. of anhydrous benzene, was added dropwise with stirring to 2.0 gm. of PCl_3 contained in 25 ml. of anhydrous benzene. The 3-necked flask containing the PCl_3 was cooled in ice during the addition, which took 10 minutes, and for 30 minutes thereafter. The ice bath was removed, and the solution was allowed to reach room temperature. A water bath was placed under the flask and the temperature was increased to 50° for 15 minutes, then the ice bath was replaced. To the cooled solution 50 ml. of cold 1 M $KHCO_3$ were added, and after the evolution of gas had ceased, the solvents were removed by distillation under reduced pressure. After benzene was added and removed by distillation to facilitate drying, the syrup was extracted with anhydrous benzene. The benzene soluble fraction was concentrated to a syrup (2.75 gm.) under reduced pressure. This material was dissolved in aqueous ethanol and 20 ml. of 1 M KOH was added for saponification of the compound. After 8 hours, cations were removed from the solution with Dowex 50(H^+), and both the resin and benzoic acid were removed by filtration. Remaining benzoic acid was extracted into ether, the aqueous solution was brought to pH 8 with cyclohexylamine and was then evaporated to dryness. The residue was extracted with ethanol and the alcohol soluble material crystallized on the addition of acetone to the solution. The yield was 1.39 gm. (38 per cent overall). A twice recrystallized sample melted between 147–149°. The rotation of the cyclohexylamine salt in water was $[\alpha]_D^{22} = 9.6°$ and in 10 per cent ammonium molybdate it rose to $[\alpha]_D^{22} = 80°$.

$C_{15}H_{32}O_6N_2P$ (365)

Calculated: P 8.48, N 7.67

Found: P 8.30, N 7.59

DL-1,2-Dihydroxyethane Sulfonate 2-phosphate—The addition of bisulfite to certain aldehydes results in compounds with biological activity (17). Such compounds have the structure of sulfonic acids, *i.e.* a sulfur to carbon bond (18). Glycolaldehyde phosphate produced by hydrolysis of the diethyl acetal (19) forms an addition complex when mixed with 1 equivalent of sodium sulfite for 24 hours. Attempts at crystallization of the sodium salt were unsuccessful, but the complex behaved as a discrete component when chromatographed on Whatman No. 1 filter paper with a methanol, formic acid, water solvent system (80:15:5) (20). In this solvent system the free aldehyde had an R_F of 0.78 while the bisulfite addition complex had an R_F of 0.37. The binding of the bisulfite was followed by determining the quantity of aldehyde free to react with iodine (21). After 1 hour 81.7 per cent of the bisulfite was bound and after 24 hours this figure had risen to 88.7 per cent.

Glyceric acid-3-P labeled with P^{32} was isolated by a modification of the fermentation procedure of Neuberg and Lustig (22).

Transfer Experiments Involving Hydroxypyruvic Acid 3-phosphate and Dihydroxyethane Sulfonic Acid 2-phosphate—Hydroxypyruvic acid 3-phosphate and dihydroxyethane sulfonic acid 2-phosphate cannot be readily assayed polarimetrically; therefore, a transfer experiment with P^{32}-labeled glyceric acid-3-P was carried out to verify that these compounds are substrates. To an incubation mixture containing 1×10^{-3} M glyceric acid-3-P labeled with P^{32}, 5×10^{-6} M glyceric acid-2,3-di-P, 5×10^{-2} M imidazole HCl buffer pH 7.0, and crystalline mutase at 50 μg. per ml. was added either dihydroxyethane sulfonic acid 2-phosphate, hydroxypyruvic acid 3-phosphate, or D-*erythro*-dihydroxybutyric acid 2-phosphate at 3×10^{-3} M. The controls contained no enzyme. After 3.5 hours at room temperature the solutions were frozen. Aliquots were chromatographed on Whatman No. 1 filter paper with picric acid-tertiary butanol as solvent (23). The solvent was run in descending fashion at 36° for 24 hours after the system had equilibrated for 4 hours. The phosphate compounds were detected by the Axelrod and Bandursky (20) modification of the Hanes and Isherwood phosphate spray, and radioactive compounds by radioautography.

Radioactivity was detected in the spots corresponding to glyceric acid-P, R_F 0.57, (glyceric acid-2-P, glyceric acid-3-P, and glyceric acid-2,3-di-P do not separate in the solvent), and inorganic phosphate, R_F 0.43 (present as a contaminant in the radioactive glyceric acid-P), for all the samples and controls. The samples also showed radioactivity in spots corresponding to either dihydroxyethane sulfonic acid 2-phosphate R_F 0.21, hydroxypyruvic acid 3-phosphate R_F 0.21, 0.31 (double spot; low incorporation), or D-*erythro*-2,3-dihydroxybutyric acid 2-phosphate, R_F 0.66. The presence of P^{32} in the phosphate groups of the sulfonic acid phosphate and hydroxypyruvic acid phosphate indicates that these groups were in equilibrium with the phosphate of glyceric acid-P, and therefore verifies that the compounds are substrates for the mutase.

TABLE 1

Acceptors of phosphate from glyceric acid 2,3-di-P

Each cuvette contained 1.0 ml. of 0.1 M imidazole-HCl buffer, pH 7.0; 0.015 M in $MgCl_2$; 0.2 mg. of enolase; and 0.05 mg. of mutase as well as the compound to be tested as acceptor. The final volume was made to 2.9 ml. with water. After equilibrium had been established, 0.1 ml. of 0.015 M glyceric acid-2,3-di-P was added and the absorbance at 240 mμ followed to the new equilibrium. Rate of transfer of phosphate is given relative to D-*erythro*-2,3-dihydroxybutyric acid 2-phosphate.

Compound	Concentration	Glyceric acid-2,3-di-P phosphate transferred at equilibrium	Rate of transfer
	M	%	
β-Glycerol phosphate	5×10^{-3}	0	
D-Glyceraldehyde 3-phosphate	5×10^{-3}	0	
Methyl D-glycerate 3-phosphate	2.5×10^{-3}	0	
Methyl D-glycerate 2-phosphate	2.5×10^{-3}	0	
DL-1,2-dihydroxyethane sulfonic acid 2-phosphate	5×10^{-3}	45	8
L-Glyceric acid 2-phosphate	5×10^{-3}	90	15
Hydroxypyruvic acid 3-phosphate	5×10^{-3}	80	23
D-*Erythro*-dihydroxybutyric acid 3-phosphate	5×10^{-3}	90	55
D-*Erythro*-dihydroxybutyric acid 2-phosphate	5×10^{-3}	90	100
L-*Threo*-dihydroxybutyric acid 2-phosphate	5×10^{-3}	0	
L-*Threo*-dihydroxybutyric acid 3-phosphate	5×10^{-3}	0	
DL-2,3-Dihydroxyisobutyric acid 2-phosphate	1×10^{-2}	0	
DL-2,3-Dihydroxyisobutyric acid 3-phosphate	1×10^{-2}	0	
D-Glyceric acid 3-phosphite	4×10^{-3}	0	
D-Glyceric acid 2,3-phosphate	5×10^{-3}	0	
DL-Serine phosphate	1×10^{-2}	0	
DL-Glyceric acid	1×10^{-2}	0	
DL-β-Chlorolactic acid	1×10^{-2}	0	
DL-Serine	1×10^{-2}	0	

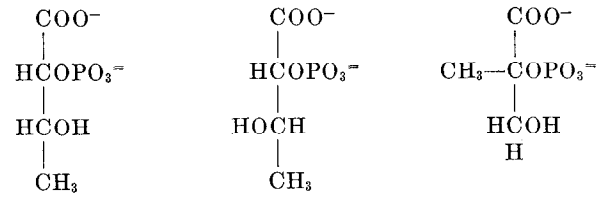
RESULTS AND DISCUSSION

In a previous publication it was shown that the D-*erythro*-2,3-dihydroxybutyric acid monophosphates would accept phosphate from glyceric acid-2,3-di-P to give rise to a mixture of glyceric acid monophosphates and dihydroxybutyric acid diphosphate (2). Moreover, the rate of the reaction was dependent on the mutase concentration. With the use of the same conditions, with the exception that the D-*erythro*-dihydroxybutyric acid monophosphate was replaced by the substrate analogue to be tested, it was possible to determine what structural factors were important in affecting the substrate's ability to accept phosphate. The results of treating a series of analogues in this manner are presented in Table I. It appears that carbon 1 must carry a negative charge, since the sulfonic acid analogue can accept phosphate while the compounds having an aldehyde, alcohol, or methyl ester on carbon 1 cannot. Carbon 2 of glyceric acid is asymmetric, and therefore its hydroxyl group can have either a D- or an L-configuration. The natural substrate is the D-isomer, but the unnatural L-isomer *can* accept phosphate although at a very slow rate. The hydroxyl group on carbon 2 can also be enolic in nature, since hydroxypyruvic acid 3-phosphate appears to accept phosphate. Replacement of the hydrogen on carbon 2 with a methyl group causes loss of activity, perhaps because of steric hindrance by the methyl group.

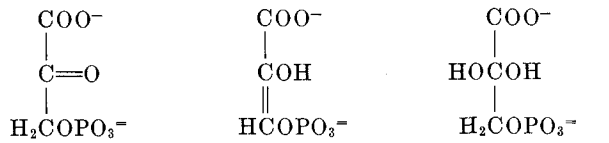
In contrast to carbon 2, carbon 3 can carry a methyl group in place of one of its hydrogens, so that the hydroxyls have a D-

erythro-configuration; but replacement of the other hydrogen to form the L-*threo* isomer results in loss of activity.

Finally, modifications of the phosphate group were tried. Removal of the phosphate group, replacement with a phosphite group, or cyclization to form D-glyceric acid 2,3-phosphate produced an inactive compound. Cyclic phosphates are intermediates in the acid-catalyzed migration of glycerol phosphate (24), but the inactivity of D-glyceric acid 2,3-phosphate as an activator, acceptor, or substrate probably excludes it from any role in the mutase-catalyzed interconversion of the two isomers. Replacement of a hydroxyl with an amino group (serine phosphate) also leads to loss of activity. The acceptor specificity indicates a requirement for three groups: a carboxyl (or charged equivalent), phosphate, and hydroxyl.

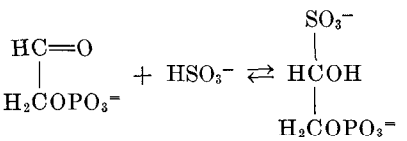


D-*erythro*-2,3-Dihydroxybutyric Acid 2-phosphate | L-*threo*-2,3-Dihydroxybutyric Acid 2-phosphate | D-2,3-Dihydroxyisobutyric Acid 2-phosphate



Keto | Enol | Hydrated

Hydroxypyruvic Acid 3-phosphate



1,2-Dihydroxyethane Sulfonic Acid 2-phosphate

In order to determine the relative affinities of the D- and L-glyceric acid monophosphates and D-*erythro*-dihydroxybutyric acid phosphate, the conversions of the 2-phosphates to the 3-isomers were followed by the polarimetric assay. Here glyceric acid-2,3-di-P was present in only catalytic amounts. The results of this experiment are shown in Fig. 1. Under these conditions the L-isomer reacts only very slowly, even with a 1000-fold increase in enzyme concentration over that used for the D-isomer. The butyric acid phosphate is intermediate in rate. The very large difference in rate explains why Meyerhof (25) concluded that only the D-isomer in a DL-mixture of glyceric acid phosphates was acted upon by the enzyme.

The transfer experiments with P^{32}-labeled glyceric acid-P showed that, in the presence of hydroxypyruvic acid 3-phosphate, both inorganic phosphate and an acid labile organic phosphate compound were produced. Likewise, with dihydroxyethane sulfonic acid 2-phosphate as the acceptor, radioactive phosphate was transferred from glyceric acid-P. By analogy with hydroxypyruvic acid, hydroxypyruvic acid 3-phosphate can exist in either the enol, keto, or hydrated form (26). Which form is the substrate for the mutase is of interest for steric reasons: the enol form would be unique among the known substrates for the mutase in having all its functional groups coplanar. On the other hand, the hydrated form, being tetrahedral about carbon 2, is sterically similar to the natural substrates. Acceptance of phosphate by the different forms should give rise to compounds of different stability. Acceptance by the enol hydroxyl would produce an enol phosphate, which would be expected to be acid labile (12, 13),

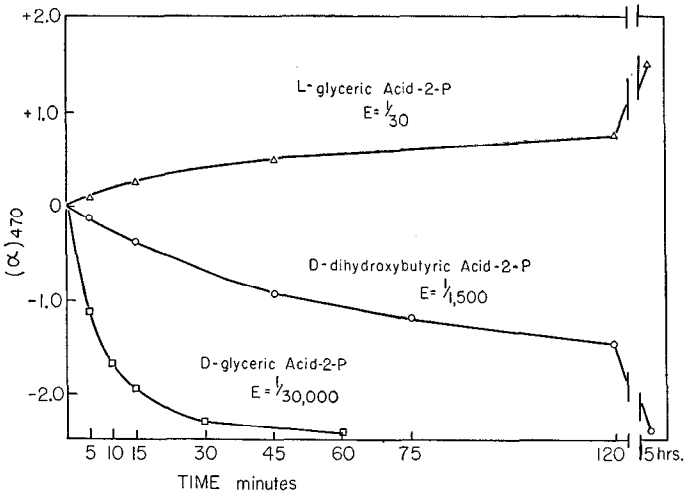


FIG. 1. Rotation changes as the 2-phosphates of D- and L-glyceric acid and dihydroxybutyric acid are acted upon by the mutase. Mixtures containing 0.033 M imidazole-HCl buffer pH 6.8, glyceric acid-2,3-di-P at 5×10^{-5} M and D- and L-glyceric acid-2-P, or D-*erythro*-2,3-dihydroxybutyric acid 2-phosphate at 0.05 M were incubated at 22°. Enzyme was added to give the dilutions indicated in the figure, and aliquots of 0.5 ml. were removed at the desired times and added to 0.5 ml. of 20 per cent ammonium molybdate solution, which serves both to stop the reaction and to provide ions for the "exaltation" of rotation. Rotations were determined on the resulting solution. E refers to the dilution used for that compound of an enzyme solution containing 30 mg. of protein per ml.

TABLE II

Formation of inorganic phosphate from glyceric acid-2,3-di-P

To 2 ml. of 0.5 M imidazole-HCl buffer pH 7.0 were added 0.85 ml. of 0.035 M hydroxypyruvic acid 3-phosphate, 0.40 ml. of 0.015 M glyceric acid-2,3-di-P, and water to give a final volume of 6.0 ml. Aliquots were taken as zero time samples; at 15 minutes the reaction was started with 10 μl. of crystalline mutase solution (40 μgm. of protein). All aliquots were 0.5 ml. in volume and were added directly to 2.5 ml. of cold acid-molybdate reagent.

Time	P per 0.5 ml.	
	Immediately	After 60 minutes
min.	*μgm.*	*μgm.*
0	4.3	6.3
5	4.4	6.5
7	4.0	6.7
12	4.6	6.5
16	5.5	7.8
21	11.1	18.0
25	11.5	20.3
30	12.6	23.0
45	14.0	21.0
60	14.2	22.9

TABLE III

Phosphate donors

Each 1-cm. cuvette contained 1 ml. of 0.1 M imidazole-HCl buffer pH 7.0, 0.015 M in $MgCl_2$, 0.5 ml. of 3×10^{-3} M phosphoryl-enolpyruvate, 0.1 mg. of enolase, and the compound to be tested as activator at 1×10^{-5} M concentration. The volume was made up to 2.90 ml. with water, and sufficient time was allowed for the enolase equilibrium to be established before 50 μl. of crystalline mutase diluted 1:5000 with distilled water were added. The change in absorbance at 240 mμ was then followed. If no activity was present after 10 minutes glyceric acid-2,3-di-P was added to the cuvette to check that the system was capable of the mutase reaction. Activities are given relative to glyceric acid-2,3-di-P which is taken as 100.

Compound	Activity	Activity on addition of glyceric acid-2,3-di-P
D-Glyceric acid diphosphate	100	
D-*Erythro*-dihydroxybutyric acid diphosphate	83	
D-Glyceric acid 2,3-phosphate	0	94
ATP	0	90
ADP	0	93
CTP	0	85
UTP	0	96
Phosphoamidate	0	98
Creatine phosphate	0	91
Carbamyl phosphate	0	96

while if the water of hydration accepts the phosphate, the effect is acceptance by water, that is, formation of inorganic phosphate.

The method of Ernster (6) for phosphate determination was chosen, as it allows inorganic phosphate to be determined in the presence of acid-labile phosphate. Glyceric acid-2,3-di-P and hydroxypyruvic acid 3-phosphate were incubated with the mutase, and aliquots were removed and added to acid molybdate previously cooled in ice. The mixture was immediately extracted with a cold benzene-butanol mixture, and the phosphate in the organic layer was determined. After an hour's standing at room temperature, the acid molybdate was again extracted with benzene-butanol and the phosphate determined. The results of this experiment are shown in Table II. They show that both inorganic and acid-labile phosphate are formed. Their sum (16.3 μg. P per 0.5 ml.) is approximately equal to the value expected (15.0 μg. P per 0.5 ml.) if one phosphate from glyceric acid-2,3-di-P is transferred during the reaction. Since both inorganic and acid-labile phosphate were formed, both enol and hydrated forms of hydroxypyruvic acid 3-phosphate must have accepted phosphate. This explains the low incorporation of P^{32} from glyceric acid-P into hydroxypyruvic acid 3-phosphate in the radioactive transfer experiment. Hydroxypyruvic acid 3-phosphate, in effect, produces in the mutase a phosphatase activity towards glyceric acid-2,3-di-P.

The specificity of the donor compound was tested by assaying its ability to activate the enzyme. In order to eliminate the possibility of glyceric acid-2,3-di-P contamination of the monophosphate substrate, synthetic enol-pyruvic acid 2-phosphate was used, and glyceric acid-2-P was generated from it with enolase. Mutase activity was measured by its effect on the absorbance at 240 mμ of the equilibrated mixture.

$$\text{(Enol-pyruvic acid-2-P} \xrightleftharpoons{\text{enolase}} \text{glyceric acid-2-P} \xrightleftharpoons{\text{mutase}} \text{glyceric acid-3-P)}$$

Of the compounds tested as phosphate donors (Table III) only the diphosphates of D-glyceric acid and D-*erythro*-dihydroxybutyric acid were active. They activated over the range 10^{-4} to 10^{-7} M, and the K_m for the glycerate was 2×10^{-6} M. No synergistic effects were detected between the compounds in Table III and glyceric acid-2,3-di-P when the latter was present at the concentration required for half-maximal activity.

SUMMARY

The substrate specificity of phosphoglyceric acid mutase has been studied by use of structural analogues of the natural substrates. It was found that a negatively charged group was required on carbon 1, that carbons 2 and 3 had to carry hydroxyls which could take up a D-*erythro*-configuration, and that one of these hydroxyls had to be esterified with phosphate if the compound were to be active as an acceptor. Both hydroxyls had to be phosphorylated if the compound were to act as a donor.

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