The Enzymatic Reaction between Ribose 1-Phosphate and Glucose 1,6-Diphosphate[1]

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Received May 17, 1955

Introduction

It has earlier been shown (1) that the phosphoribomutase reaction can be catalyzed by crystalline phosphoglucomutase preparations from muscle extract. It was found that the reaction could be activated by addition of glucose 1,6-diphosphate (G 1,6-P_2). From incubation mixtures of G 1,6-P_2, ribose 1-phosphate (R 1-P), and phosphoglucomutase, a compound was isolated, the composition and properties of which suggested it to be ribose 1,5-diphosphate (R 1,5-P_2). It was, therefore, presumed that the following reaction took place:

ribose 1-phosphate + glucose 1,6-diphosphate $\rightleftharpoons$

ribose 1,5-diphosphate + glucose 6-phosphate (1)

This reaction has now been studied spectrophotometrically in the presence of triphosphopyridine nucleotide (TPN) and an excess of Zwischenferment.

Materials and Methods

Phosphoglucomutase

This was assayed either as described by Najjar (2) or spectrophotometrically at 340 mμ in glycylglycine (10^{-2} *M*)-cysteine (6×10^{-3} *M*) buffer (gg-c buffer) pH 7.4, containing glucose 1-phosphate (G 1-P) (5×10^{-4} *M*), TPN (10^{-5} *M*), $MgCl_2$ (3×10^{-3} *M*), G 1,6-P_2 (10^{-7} *M*), and an excess of Zwischenferment.

The enzyme was prepared in the crystalline form from rabbit muscle extract according to the method of Najjar (2). The enzyme was stored under 60% satu-

[1] This project has been supported by grants from Carlsbergfondet; Rockefeller Foundation; Lederle Laboratories Division, American Cyanamid Company; and The Lilly Research Laboratories.

rated ammonium sulfate. Before use the protein was collected by centrifugation and dissolved in a volume of water equal to the original aliquot of protein suspension. This solution usually contained 3.5 mg. protein/ml. and 0.2 mmoles amonium sulfate/ml.

Zwischenferment

This was assayed spectrophotometrically at 340 mμ in gg-c buffer, pH 7.4, in the presence of TPN (10^{-5} *M*) and glucose 6-phosphate (G 6-P) (6×10^{-4} *M*).

This enzyme was prepared from "Kongens Bryghus" dry yeast according to the procedure of LePage and Mueller (3). This preparation was used directly or after fractionation of an aqueous solution of the enzyme by collecting the precipitate obtained between 55 and 80% saturation with ammonium sulfate. When dissolved in and dialyzed against gg-c buffer, pH 7.4, this preparation was stable for several weeks at 0°C., whereas it deteriorated rapidly when dissolved in water. The preparation used was free of 6-phosphogluconate dehydrogenase, phosphoglucomutase, phosphoribomutase, and phosphatase activity.

Xanthine Oxidase

This enzyme was assayed spectrophotometrically at 293 mμ as described by Kalckar (4). One unit of activity was defined as the amount of enzyme required to give an increase in optical density at 293 mμ of 1.0/min./cm. light path, when assayed in 1 ml. of a 0.05 *M* phosphate buffer, pH 7.4, containing 0.1 μ*M* of Versene[2] and 0.25 μ*M* of hypoxanthine. The xanthine oxidase was prepared from cream. The solubilization of the enzyme was facilitated by treatment with butanol as suggested by Morton (5). The procedure was as follows: The cream was separated from fresh milk while this was still above 25°C. The cream was cooled and kept at 0°C. overnight, and churned the next day at 15°C. To the buttermilk was added 0.6 vol. of 0.2 *M* Na_2HPO_4, and this mixture was incubated at 35°C. for 3 hr. with 600 mg. of commercial trypsin per liter solution. The following steps were performed at 0°C. While stirring 1 vol. of aqueous butanol was slowly added; after centrifugation at 10,000 × *g* for 15 min. the transparent yellowish aqueous phase was siphoned off and fractionated with ammonium sulfate. To each 100 ml. of solution was added 47 ml. of saturated ammonium sulfate (0°C.). After 15 min. most of the protein which had been salted out had collected at the surface; it was separated by centrifugation and discarded. To each 100 ml. of the clear aqueous solution was added 47 ml. of saturated ammonium sulfate. After an hour the brown protein had collected at the surface. It was collected by centrifugation and dissolved in water to give a final concentration of about 8 mg. protein/ml. Any insoluble protein present at this stage was removed by centrifugation. This preparation is completely free of turbidity and has a deep red–brown color. The ratio of optical densities at 280 mμ and 450 mμ was usually 6.5. The preparation was found, however, to contain considerable amounts of phosphatase activity toward R 1-P. The solution was therefore treated with 1.2 ml. of calcium phosphate gel (6) (16 mg./ml.) for each milliliter of enzyme solution. The gel collected by cen-

[2] Versene or cysteine were found to be required for full activity of the purified enzyme.

TABLE I

Purification of Xanthine Oxidase

Starting material: 20 l. of fresh (warm) cow's milk.

Stage	Volume *ml.*	Total units	Specific activity *units/mg. protein*	Yield %
Buttermilk	440	9100	0.36	(100)
Treatment with trypsin and butanol	585	5850	4.0	64
Ammonium sulfate fractionation	80	4800	6.9	53
Gel treatment	240	3800	11.0	42
Ammonium sulfate precipitation	80	3400	11.0	38

trifugation was eluted three times with 1 ml. of 0.2 *M* phosphate buffer, pH 7.4, and a fourth time with 1 ml. of 0.3 *M* K_2HPO_4 for each milliliter of initial enzyme solution. These eluates were completely free of phosphatase activity. In order to free the enzyme from most of the orthophosphate, the combined eluates were precipitated by addition of 1.1 vol. of saturated ammonium sulfate.

The enzyme was stable for more than 6 months when stored at −15°C. The yield and the purification during the fractionation procedure are given in Table I. When the enzyme preparation obtained in this way was dissolved in enough water to give a concentration of *ca.* 1 mg. protein/ml., and fractionated with saturated ammonium sulfate, the fraction obtained between 37.5% and 41% saturation showed a pronounced schlieren effect. This suggests that the enzyme may be in the crystalline state. The ratio E_{280}/E_{450} of the dissolved precipitate was 5.2–5.5, which is close to the ratio recently reported by Avis *et al.* (7) for a xanthine oxidase preparation obtained in the crystalline form by precipitation with alcohol.

Nucleoside Phosphorylase

This was assayed as described for xanthine oxidase except that the substrate was inosine, and the reaction mixture contained an excess of xanthine oxidase. The enzyme was prepared according to Price, Otey, and Plesner (8). One unit of enzyme was defined as described for xanthine oxidase.

Ribose 1-Phosphate

This compound was prepared by phosphorolytic cleavage of inosine (9). The incubation mixture consisted of 32.2 ml. of 0.1 *M* tris–formic acid buffer, pH 7.6, 90 units of nucleoside phosphorylase, 130 units of xanthine oxidase, 1430 μM of inosine, and 1800 μM of phosphate buffer, pH 7.4. The total volume was 53 ml. The reaction was followed by measuring the formation of acid-labile phosphate. After 6 hr. of incubation at room temperature with shaking in an atmosphere of oxygen, 1010 μM of R 1-P had formed, and the mixture was passed through a Dowex-1 formate column which was eluted according to the method of Hurlbert

et al. (10), using a gradient of ammonium formate, pH 5.0. The fractions containing acid-labile phosphate were pooled, the pH was adjusted to 8.0 with ammonia, and barium acetate in excess of the phosphate was added. The precipitate obtained by addition of 3 vol. of alcohol contained the barium salt of R 1-P. The purity was 80%, and the yield 975 μM calculated as the monobarium salt. The crystalline cyclohexylamine salt was obtained as earlier described (1); the final yield based on the content of acid-labile phosphate/mg. of the dry material was 47% of the initial amount of inosine. The purity was 98–99%, calculated for dicyclohexylammonium salt and based on the content of acid-labile phosphate. This preparation was used for all the experiments.

Glucose 1,6-Diphosphate

During the preparation, this compound was assayed for its coenzyme activity toward the phosphoglucomutase present in dialyzed yeast autolyzates. A calibration curve was obtained by using a sample of synthetic G 1,6-P_2 (11) (kindly furnished by Professor T. Posternak, Geneva). The procedure was essentially as described by Cardini *et al.* (12). When the compound was obtained in about 10% pure state, it could be assayed by measuring the content of acid-labile phosphate.

G 1,6-P_2 was prepared from yeast with some modifications of the method of Cardini *et al.* (12). Different kinds of yeast were tested for their ability to produce G 1,6-P_2. Fresh baker's yeast, when incubated as described by Cardini *et al.* (12), yielded about 10 μM/g. yeast (dry weight). Anheuser-Busch's dry yeast and "Kongens Bryghus" dry yeast yielded, when incubated as described below, about 30 and 50 μM/g. dry yeast, respectively.

The incubation mixture consisted of 200 g. of "Kongens Bryghus" dry yeast, 400 g. sucrose, 73 g. $NaH_2PO_4.H_2O$, 220 g. $NaHCO_3$, and 1.6 l. water. After 21 hr. at 37°C. about 1000 μM of G 1,6-P_2 had formed. For obtaining the 10–15% pure barium salt of G 1,6-P_2, the incubation mixture was fractionated according to the procedure of Cardini *et al.* (12), except that the total removal of orthophosphate as magnesium ammonium salt was omitted, since this step in our hands caused considerable losses. After the barium salt had been dissolved in weak acid and treated with Norit, the barium was removed as the sulfate, the pH was adjusted to 8 with ammonia, and the solution was adsorbed on a column of Dowex-1 formate. The column was eluted with a gradient of formate, pH 5.0, according to the method of Hurlbert *et al.* (10) using 1 M sodium formate in the reservoir. The salt was isolated as the dibarium salt. This was brought into solution as described by Posternak (11). After rechromatography the compound is 60–70% pure, and the ratio of total to acid-labile phosphate was about 2.07. The compound was free of absorption in the ultraviolet light, did not contain any ribose, and moved as a single spot on the paper chromatogram using the propanol–ammonia–water mixture described by Hanes and Isherwood (13). Attempts to prepare a crystalline cyclohexylamine salt of G 1,6-P_2 were unsuccessful.

Glucose 1-Phosphate was the dipotassium salt of a commercial product.

Deoxyribose 1-Phosphate. The barium salt of this compound was obtained essentially as described by Friedkin (14).

Galactose 1-Phosphate was a gift from Professor Leloir, Buenos Aires, to Dr. Kalckar.

Phosphate. Orthophosphate was determined as described by Lowry and Lopez (15). Orthophosphate plus acid-labile phosphate was determined as described by Fiske and SubbaRow (16).

Ribose was determined by the orcinol reaction as described by Mejbaum (17).

Triphosphopyridine Nucleotide was an 80% pure commercial product.

Protein was determined by the turbidimetric method of Bücher (18).

Experimental and Results

Assay

The reaction between R 1-P and G 1,6-P_2 was followed spectrophotometrically by measuring the G 6-P formed. This process was followed at 340 mμ by the oxidation of G 6-P in the presence of Zwischenferment and TPN, the latter being simultaneously reduced. The incubation mixture was usually composed as described in the legend to Fig. 1. Control experiments with omission of G 1,6-P_2 or R 1-P showed no change in the absorption at 340 mμ (Fig. 1). The rate of the reaction was found to be proportional to the amount of phosphoglucomutase used, provided that the amount of Zwischenferment present was capable of causing the oxidation of G 6-P (under standard conditions) at a rate which was at least five times the rate of the reaction to be measured. The reaction proceeded as a zero-order reaction until about 80% of the initial amount

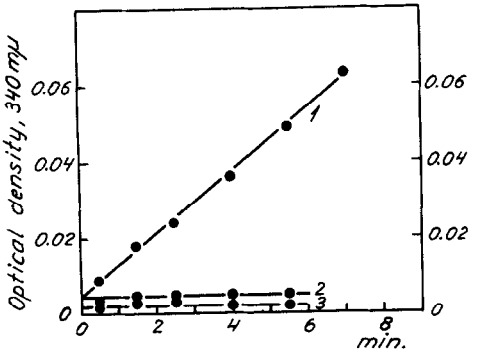

Fig. 1. Spectrophotometric assay of the reaction between R 1-P and G 1,6-P_2. The reaction mixture contained glycylglycine (10^{-2} *M*) and cysteine-HCl (6×10^{-3} *M*) adjusted to pH 7.4 with NaOH; TPN, 10^{-4} *M*; G 1,6-P_2, 6×10^{-5} *M*; R 1-P, 2.6×10^{-4} *M*; $MgCl_2$, 2.5×10^{-3} *M*; Zwischenferment, about 0.5 mg. protein; phosphoglucomutase, about 0.05 mg. protein. The total volume was 1 ml. Curve 1: complete; curve 2: G 1,6-P_2 omitted; curve 3: R 1-P omitted.

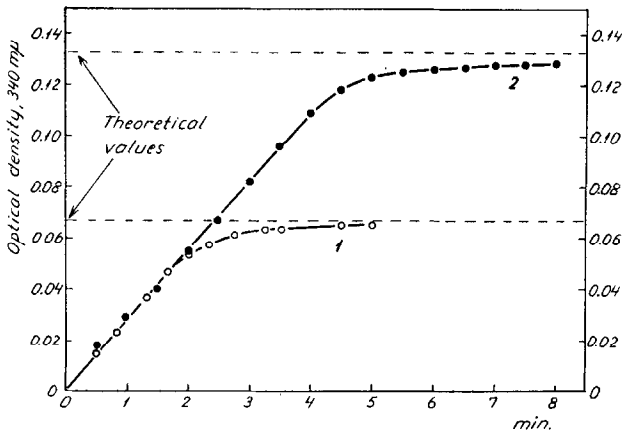

FIG. 2. Quantitative spectrophotometric determination of G 1,6-P_2. Conditions as in Fig. 1, curve 1, except for G 1,6-P_2. Curve 1: R 1-P: 5×10^{-4} *M* and about 0.0105 μM of G 1,6-P_2/ml. Curve 2: R 1-P: 8×10^{-4} *M* and about 0.021 μM of G 1,6-P_2/ml.

of G 1,6-P_2 and about 10% of the initial amount of R 1-P had been utilized.

The high affinity of the enzyme for G 1,6-P_2 (19) made possible a convenient quantitative spectrophotometric assay for this compound. As seen in Fig. 2, the reduction of TPN may reach a stable end point within a few minutes. The total increase in density is proportional to the amounts of G 1,6-P_2 added.

Effect of the Sequence of Addition of the Compounds

The reaction velocity was found to be dependent on the sequence of addition of some of the components. As can be seen from Table II, the rates obtained fall into two categories. The low rates were always obtained when the enzyme was added last, and the fast rates were obtained when one of the two substrates was added last. Similar results were obtained for the phosphoglucomutase reaction when assayed as described by Najjar (2). In the experiments reported here, the components are added in the sequence used in Expt. 6 in Table II.

The Effect of Concentration of Cysteine

The effect of glycylglycine buffers containing increasing amounts of the hydrochloride of cysteine adjusted to pH 7.4 with sodium hydroxide

TABLE II

Effect of the Sequence of Addition of the Components

The reaction mixture consisted of glycylglycine, 10^{-2} *M*, and cysteine-HCl, 6×10^{-3} *M*, adjusted to pH 7.4 with NaOH; $MgCl_2$, 2.5×10^{-3} *M*; TPN 10^{-4} *M*; Zwischenferment, 0.5 mg. of protein; G 1,6-P_2, 8×10^{-5} *M*; R 1-P, 1.6×10^{-4} *M*; phosphoglucomutase about 0.05 mg. protein. Total volume, 1 ml. To a mixture of the first five components, the last three components were added at varying sequences.

Sequence of addition	Rate ($\Delta E_{340} \times 1000$/minute)
1. Enzyme, G 1,6-P_2, R 1-P	16.7
2. R 1-P, G 1,6-P_2, enzyme	11.3
3. R 1-P, enzyme, G 1,6-P_2	17.1
4. G 1,6-P_2, enzyme, R 1-P	17.0
5. Enzyme, R 1-P, G 1,6-P_2	15.6
6. G 1,6-P_2, R 1-P, enzyme	11.0

is seen in Fig. 3. It was found that the reaction in the presence of 1.2×10^{-1} *M* cysteine and sodium chloride had a very pronounced lag period which lasted up to 10 min. under the test conditions used. After the lag period the reaction obtained a constant velocity which was somewhat larger than the reaction rate at 3×10^{-2} *M* cysteine. If, however, potassium chloride is added to the buffer containing 3×10^{-2} *M* cysteine to

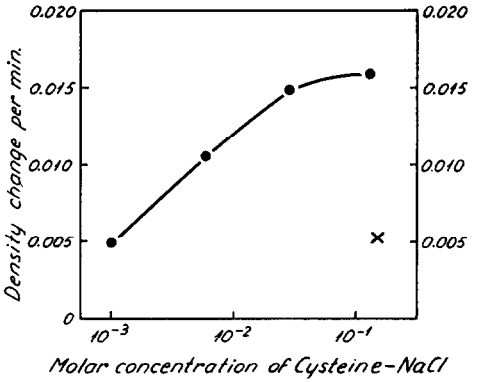

FIG. 3. Effect of the concentration of neutralized cysteine-HCl. Conditions as in Fig. 1, curve 1, except for cysteine-'NaCl'. At the point marked x the mixture contained 1.2×10^{-1} *M* KCl and 3×10^{-2} *M* cysteine-'NaCl', i. e., the chloride concentration is the same as that of the last point of the curve, and the cysteine concentration is that of the second last point of the curve.

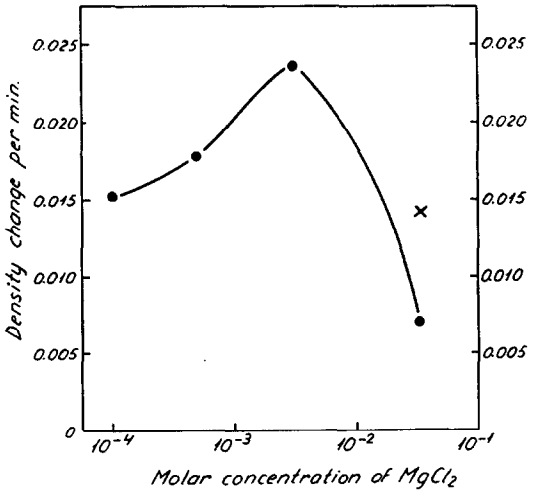

FIG. 4. Effect of the concentration of $MgCl_2$. Conditions otherwise as in Fig. 1, curve 1, except for $MgCl_2$. At the point marked x the mixture contained 6.6×10^{-2} *M* KCl and 3.3×10^{-3} *M* $MgCl_2$, i.e., the chloride concentration is that of the last point of the curve and the magnesium-ion concentration is that of the next to the last point of the curve.

give a final chloride concentration of 1.2×10^{-1} *M*, the reaction velocity is even lower than the initial velocity at 1.2×10^{-1} *M* cysteine. Since it has been shown (19) that anions inhibit the reaction studied, these findings indicate that cysteine itself has no inhibitory effect; it seems on the contrary to activate the reaction even at 0.1 *M* concentration. Cysteine could be replaced by 8-hydroxyquinoline. The highest rate was obtained in the presence of about 10^{-3} *M* 8-hydroxyquinoline.

Effect of the Concentration of Mg^{++}

The effect of increasing amounts of magnesium chloride is seen in Fig. 4. The optimum concentration is about 4×10^{-3} *M*. When the chlorides of magnesium or potassium are added to the reaction mixtures containing 4×10^{-3} *M* magnesium chloride, it is seen that at equal chloride concentrations the magnesium salt has a somewhat more pronounced inhibitory effect than the potassium salt, suggesting a specific inhibitory action of the former cation.

Stoichiometry of the Reaction

Incubation mixtures consisting of R 1-P, G 1,6-P_2, and phosphoglucomutase would catalyze both reaction (1) and the reaction:

ribose 1-phosphate + ribose 1,5-diphosphate $\rightleftharpoons$

ribose 1,5-diphosphate + ribose 5-phosphate (2)

In order to obtain information on the relative rates of the two reactions under these conditions, the concentrations of some of the components of the reactions during the incubation were measured. In heat-denatur-

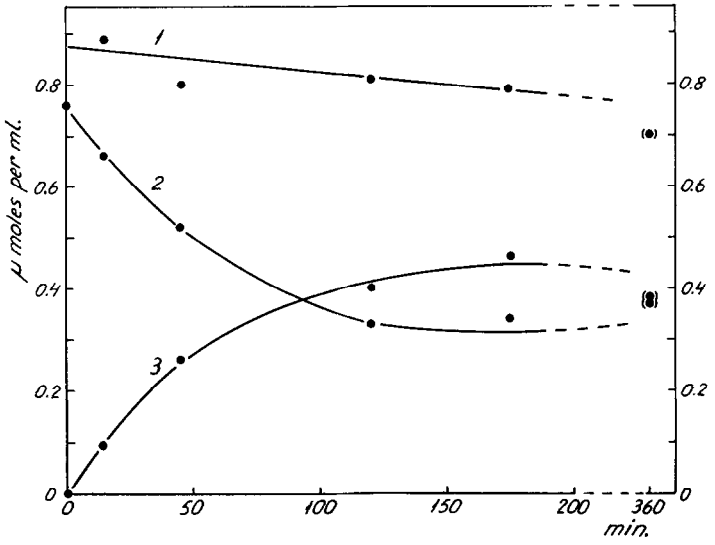

FIG. 5. Stoichiometry of the reaction between R 1-P and G 1,6-P_2. The incubation mixture consisted of glycylglycine 2.5×10^{-2} *M*, adjusted to pH 7.4 with NaOH; 8-hydroxyquinoline 10^{-3} *M*, G 1,6-P_2 10^{-3} *M*. R 1-P 9×10^{-4} *M*; $MgCl_2$ 2.5×10^{-3} *M*; phosphoglucomutase about 0.25 mg. protein. After heat denaturation of aliquots of 0.05 ml., the amount of G 6-P present was determined by measuring the increase in density at 340 mμ after addition of TPN (0.1 μmole), Zwischenferment (0.5 mg. protein), and gg-c buffer to 1 ml. When this reaction had gone to completion, R 1-P (0.8 μmole) and phosphoglucomutase (0.05 mg. protein) were added, and the further increase in density at 340 mμ was recorded as a measure of the amount of G 1,6-P_2 present. The sum of the concentration of R 1-P and R 1,5-P_2 was determined in aliquots of 0.1 ml. as orthophosphate according to Fiske and SubbaRow (16). Curve 1: R 1-P + R 1,5-P_2; curve 2: G 1,6-P_2; curve 3: G 6-P.

ated aliquots of the reaction mixture, G 6-P was measured spectrophotometrically at 340 mμ in the presence of TPN and Zwischenferment. When this reaction was finished, the amount of G 1,6-P_2 present in the aliquot could be determined by the further increase in absorption at 340 mμ after addition of phosphoglucomutase and excess R 1-P. The sum of R 1-P and R 1,5-P_2 can be determined in aliquots of the reaction mixture as orthophosphate by the method of Fiske and SubbaRow (16), since the glycosyl phosphate bond of both compounds is very acid-labile. A decrease in this sum is ascribed to the conversion of R 1-P to R 5-P according to Eq. (2). Under these conditions of phosphate determination, G 1-P, G 1,6-P_2, G 6-P, and R 5-P will not be hydrolyzed. It appears from Fig. 5 that the disappearance of G 1,6-P_2 is approximately equivalent to the formation of G 6-P as would be expected from Eq. (1). It appears further that the disappearence of G 1,6-P_2 is proceeding much faster than the disappearence of R 1-P.

Reaction of Other Monophosphate Esters with G 1,6-P_2

Under the assay conditions used here, also other aldose phosphate esters than R 1-P react with G 1,6-P_2. It was found that deoxyribose 1-phosphate reacted at a rate which was more than twice than that of R 1-P, whereas galactose 1-phosphate reacted somewhat more slowly, and ribose 5-phosphate reacted very slowly compared with R 1-P.

Discussion

Since anions in general have been shown to inhibit the reaction studied here (19), the effect of the concentrations of both magnesium ions and cysteine (when a salt of this compound is employed) will be influenced by the effect of the accompanying anion. Thus, the inhibition caused by high concentration of $MgCl_2$ was found to be due partly to the magnesium ion and partly to the chloride ion. For the phosphoglucomutase reaction, Najjar (2) found a much more pronounced apparent inhibitory effect of high magnesium ion concentrations than is found for the reaction studied here. This difference might partly be ascribed to the use of $MgSO_4$ in Najjar's experiments, since sulfate is a stronger inhibitor than is chloride (19). Since anion inhibition is competitive with regard to R 1-P, the optimum concentration of a magnesium salt will probably depend not only on the nature of the anion, but also on the concentration of R 1-P. Cysteine appears to have a pronounced activating effect even at high concentrations. The inhibition of, e.g., 0.1 *M* chlo-

ride can apparently be overcome by the activating effect of the same concentration of cysteine under the assay conditions used here and provided that the maximal rate obtained during the reaction is taken into account.

From the measurements of concentration of the components of Eqs. (1) and (2) (see Fig. 5), it appears that the concentration of G 1,6-P_2 decreases to a minimum value, whereafter it slowly increases again. Likewise, G 6-P slowly decreases after having reached a maximum value. This apparent reversal of reaction (1) can be ascribed to the steady slow removal of one of the components in this reaction, i.e., R 1-P according to Eq. (2). The relatively slow decrease in the sum of the concentration of R 1-P and R 1,5-P_2 suggests that reaction (1) proceeds with a faster rate than does reaction (2), i.e., that R 1-P reacts faster with G 1,6-P_2 than with R 1,5-P_2. The minimum value obtained for G 1,6-P_2 and the maximum value obtained for G 6-P suggest that the equilibrium of Eq. (1) is close to 50%.

For the preparation of R 1,5-P_2 from R 1-P and G 1,6-P_2, these experiments suggest that in order to get a high yield of this compound from G 1,6-P_2 it is necessary to start with an excess of R 1-P over G 1,6-P_2 and to stop the reaction when the formation of G 6-P has reached maximum. Under such conditions it has been found that a yield of the barium salt of R 1,5-P_2 of about 70% of the original amount of G 1,6-P_2 present can be obtained, when it is isolated as earlier described (1).

The experiments in which R 1-P has been replaced by galactose 1-phosphate, deoxyribose 1-phosphate, or R 5-P suggest that the enzyme is highly unspecific with regard to aldose monophosphate. It is assumed that the reactions in these cases proceed by analogy with Eq. (1), i.e., that the monophosphate ester is esterified to give the appropriate diphosphate ester. This is in accordance with findings in other laboratories (20, 21) that phosphoglucomutase preparations from rabbit muscle catalyze the mutation of a number of different aldose monophosphate esters and that these reactions can be enhanced by addition of G 1,6-P_2.

Summary

The properties of the reaction between ribose 1-phosphate and glucose 1,6-diphosphate as catalyzed by crystalline phosphoglucomutase have been studied with a spectrophotometrical assay, which can be used for a specific quantitative determination of glucose 1,6-diphosphate.

It was found that the rate of the reaction depends on the sequence of addition of some of the components.

The reaction required both cysteine and magnesium ions. High concentrations of magnesium ions were inhibitory, whereas no such effect was found for high cysteine concentrations.

The stoichiometry of the reactions catalyzed by phosphoglucomutase in the presence of ribose 1-phosphate and glucose 1,6-diphosphate has been worked out, and the optimum conditions for formation of ribose 1,5-diphosphate have been determined.

Under the conditions used here also deoxyribose 1-phosphate, galactose 1-phosphate, and ribose 5-phosphate react with glucose 1,6-diphosphate.

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