ARCHIVES OF BIOCHEMISTRY
Vol. 22, p. 87, 1949

THE ISOLATION OF THE COENZYME OF PHOSPHOGLUCOMUTASE

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Received November 23, 1948; revised January 14, 1949

INTRODUCTION

Previous papers (1,2) reported the existence of a coenzyme for phosphoglucomutase and its probable identity with glucose-1,6-diphosphate. The isolation and properties of the substance and its role in animal tissues are now described.

The rate of the reaction: glucose-1-phosphate → glucose-6-phosphate is, under certain conditions, proportional to the concentration of coenzyme which can thus be easily estimated (Fig. 1).

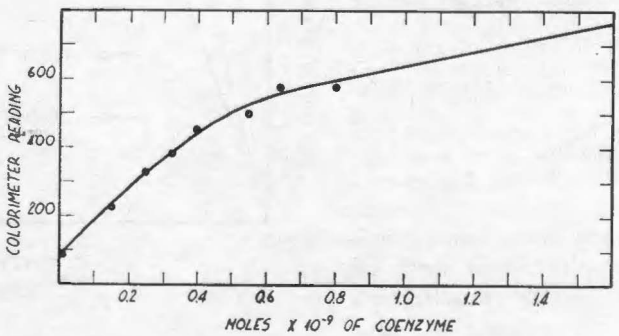

FIG. 1. Relationship between the amount of coenzyme and the activity of yeast phosphoglucomutase.

TABLE I

Results of the Puritzation of the Coenzyme

Step	Amount of extract	Micromoles of coenzyme	Relation P total/P acid-labile
1. Filtered yeast extract	1700 ml.	650 *a*	226 *b*
2. After Pb and H_2S	1300 ml.	680 *a*	130 *b*
3. Alkali, Mg++, Pb, H_2S	580 ml.	—	15 *b*
4a. First Ba salt	5.06 g.	515 *a* 680	10
4b. Fractionated Ba salt	1.85 g.	450	6.5
5a. Acetone precipitation	—	F_1 50	2.2
		F_2 128	2.1
		F_3 107	2.5
		F_4 28	3.8
5b. Final Ba salt ($F_1 + F_2$)	150 mg.	140	2.07

a Estimations by enzymatic method
b P total/μM of coenzyme.

During a search for the best starting material, and following the observation of Kendal and Stickland (3) that the phosphoglucomutase of rabbit muscle is activated by fructose diphosphate, this substance was tested. The sample used was found to contain considerable amounts of coenzyme. It was next observed that, during the incubation of yeast with sugar, phosphate and ether, as described by Neuberg and Lustig (4) for the preparation of fructose diphosphate, the coenzyme content increased enormously.

The separation of these two substances proved to be very difficult. For instance, a preparation which had been purified by barium, lead and alcohol fractionation, gave the same relation of coenzyme to fructose as the starting material. The only procedure by which the fructose ester could be removed was the destruction by heating at alkaline reaction. Large amounts of inorganic phosphate are then liberated, since the amount of coenzyme is only about 0.5 % of the other phosphoric esters. The removal of the inorganic phosphate with magnesia mixture often resulted in great losses by coprecipitation. This difficulty was finally overcome, and, after purification of the product by lead, barium, and acetone fractionation, a substance of about 70 % purity was obtained.

About half the phosphate present in these preparations is hydrolyzed by mild acid with loss of the coenzymatic activity. The hydrolysis constant for the first phosphate at 37ºC. in 0.25 *N* acid found to be $3.1 \times 10\text{-}4$ (Table II). The hydrolysis of glucose-1-phosphate is about four times faster under the same conditions ($K = 1.29 \times 10\text{-}3$) (5).

The fact that the value of *K* remained constant up to 65 % hydrolysis shows that the preparation was not appreciably contaminated with other labile esters.

The connection between coenzymatic activity and acid-labile phosphate was revealed during the last stages of the purification where both values were always parallel. This parallelism was also evident during the acid hydrolysis (Table II).

Heating in 0.1 *N* acid at 100ºC. leads to complete liberation of the labile phosphate in 9-10 min. Fig. 2 shows the course of the hydrolysis compared with that of glucose-1-phosphate.

After it fas proved that the labile phosphate is related to the activity it became necessary to investigate the nature of the rest of the molecule.

The intact coenzyme gives a barium salt which is insoluble in water, but after hydrolysis most of the part bearing the second phosphate becomes water soluble. This indicates that the labile and stable phosphate belong to the same molecule.

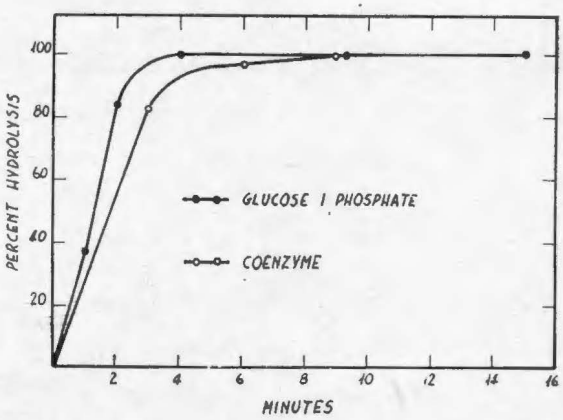

FIG. 2. — Hydrolysis of the labile phosphate of the coenzyme and of glucose1-phosphate in 0.1 *N* H_2SO_4 at 100ºC.

TABLE II

Hydrolysis of the Coenzyme in 0.25 N HCl at 37ºC.

$$K = \frac{1}{t_2 - t_1} \log_{10} \frac{100 - x_1}{100 - x_2}$$

Time	Liberation of P inorg. Per cent of acid-labile	$K \times 10^4$	Decrease in coenzymatic activity
min.			*per cent*
60	3.50	2.67	—
180	11.4	3.08	0
360	22.0	3.11	23
540	32.5	3.50	37
720	39.7	2.78	34
1440	64.5	3.25	77
		Mean: 3.06	

The estimation of aldose by titration with hypoiodite revealed that after mild acid hydrolysis one equivalent appeared per molecule of phosphate split off. None was reactive in the intact molecule.

These facts indicated that the second phosphate belonged to an aldose phosphate, and the enzymatic detection of glucose-6-phosphate was attempted. The procedure was based on the fact that muscle extracts contain enzymes which catalyze the equilibrum glucose-1- phosphate $\rightleftharpoons$ glucose-6-phosphate $\rightleftharpoons$ fructose-6-phosphate. Using aged and fairly dilute extracts, it is unlikely that other reactions will interfere. Moreover, since fructuose can be easily estimated, this affords a method for detecting any one of the 3 esters. The coenzyme before and after hydrolysis was incubated with the extract, and fructose was then estimated. As shown in Table III, the expected amount of fructose was formed from the hydrolysed coenzyme, and none from the nonhydrolyzed.

The correct identification of glucose-6-phosphate with relatively small amounts of material is a problem which has not been worked out satisfactorily. Two experiments were carried out in which polarimeter readings were taken before and after hydrolysis. The values obtained before hydrolysis gave $[\alpha]_D = +63^\circ$ to $+70^\circ$ in acid solution and calculated for an hexose diphosphoric acid. After hydrolysis, $[\alpha]_D = +30^\circ$ to $+32^\circ$ for an hexose monophosphoric acid. For glucose-6-phosphoric acid, the corresponding value is $+34.2^\circ$ to $+35.1^\circ$ (6,7).

The solution was then separated into water-soluble and insoluble barium salts. The soluble fraction gave $[\alpha]_D = +12^\circ$ to $+15^\circ$, calculated for the barium salt of an hexose monophosphate. The value given by Robison and King (6) for glucose-6-phosphate is $+16.6^\circ$.

TABLE III

Formation of Fructose Phosphate from the Hydrolyzed Coenzyme
(details in text)

Incubation for 20 min. at 37°C. of 0.1 ml. of enzyme solution plus additions. Total volume 0.5 ml.

Additions	Colorimeter readings
6.2 μM of coenzyme	12
6.2 μM of coenzyme hidrolyzed	128
2 μM glucose-1-phosphate	45
5 μM glucose-1-phosphate	98
Enzyme alone	8

Since these results were not considered conclusive, additional information was sought in the acid and alkaline hydrolysis and by preparing the osazone. The results were as follows:

	Soluble Ba salt from hydrolysis of coenzyme *per cent*	Glucose-6-phosphate *per cent*
Hydrolysis, 1 N acid at 100°C., 1 hr.	0—1	1.8 (6)
Hydrolysis, 1 N acid at 100°C., 2 hr.	4—5	3.5 (6)
Hydrolysis, 0.2 N alkali at 100°C., 3 min.	54	60 (10)

The osazone was undistinguishable microscopically from that of glucose-6-phosphate, and its melting point was 150-153°C. The value given by Robison and King [6] being 154°C.

If it is admitted that glucose-6-phosphate is formed by hydrolysis of the coenzyme, then in the intact substance the position of the labile phosphate can be fixed in 1 because of its facile hydrolysis with acid and because it masks the reducing group. The coenzyme would be, therefore, glucose-1,6-diphophate, and its high dextrorotation, which decreases on hydrolysis, indicates the α-anomer.

The presence of an aldose component in fructose diphophate preparations has been discussed by Neuberg, Lustig and Rothenberg [26]. Due to the small proportion of the glucose ester, it may have escaped detection by chemical methods or it may have been removed in the highly purified preparations used by Neuberg *et al.*

The synthesis of glucose diphosphate was attempted by treating 1,6-dibromotriacetylglucose with Ag_3PO_4, using the procedure of Cori, Colowick and Cori [5], which proved successful for the synthesis of glucose-1-phosphate. While an active preparation was obtained, thus adding more evidence to the proposed structure, the yield was so low, and purification so difficult, that the method was abandoned. The synthesis starting with glucose-1-phosphate and phosphorus oxychloride was unsuccessful.

All the facts which have been mentioned agree with the proposed structure of the coenzyme, but, since analytically pure preparations have not been obtained, other methods of synthesis are being investigated.

The structure of the coenzyme is important from the point of view of the mechanism of action of the enzyme. Meyerhof *et al.* [11] had foud that during the reaction there was no interchange between the hexose phosphates and radioactive inorganic phosphate. Schlamawitz and Greenberg [12] foud that labeled glucose did not interchange with the phosphorylated glucose, and postulated that a 1,6-glucose monophosphte was formed as an intermediary in the reaction.

A simple explanation for the mechanism, is that the enzyme would catalyse the transfer of the phosphate-1 of the coenzyme position 6 of glucose-1-phosphate. The reaction products would thus be glucose-6-phosphate and glucose-1,6-diphosphate. In the reaction the coenzyme would be regenerated at the expense of the substrate.

Another intriguing point has been settled. Gori, Colowick and Cori [13] had found that rabbit muscle phosphoglucomutase coul be electrodialyzed without losing activity. They could not reproduce the activation which Kendal and Stickland [9] had observed on adding fructose diphosphate preparations. Moreover, phosphoglucomutase has been purified by Schlamowitz and Greenberg [14], and crystallized by Najjar [15], and the necessity of a coenzyme was not noticed.

Since the coenzyme was found to activate yeast phosphoglucomutase but hardly at all that rabbit muscle, it seemed posible that the mechanism of action of the two enzymes might be different. However, it has been found that, if the muscle enzyme is tested in the presence of cysteine, as described by Najjar [15], then the addition of coenzyme produces a great increase in activity (Fig. 4, left).

If the muscle phosphoglucomutase is treated with acid in $(NH_4)_2SO_4$ solution as described by Warburg and Christian [16], for the reversible splitting of flavoproteins, then the activity without coenzyme becomes negligible (Fig. 4, right).

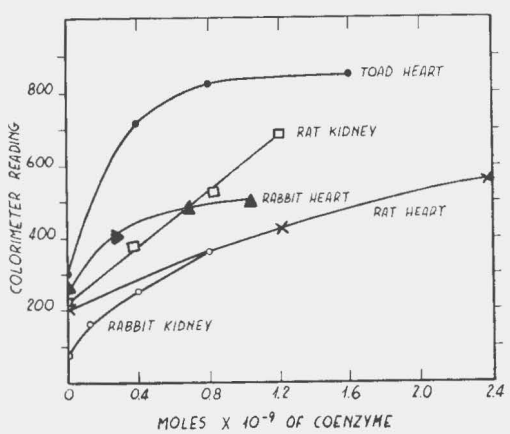

FIG. 3. — The activation of phosphoglucomutase from different organs by different amounts of coenzyme. Measurement by the copper reduction method. No cysteine used.

In extracts of heart and kidney the effect of the coenzyme can be observed even without the addition of cysteine (Fig. 3).

The coenzyme has been found in every animal tissue thus far investigated (Table IV), and it can be concluded that it is present wherever phosphoglucomutase is present.

EXPERIMENTAL

Enzymatic Estimation of the Coenzyme

Dried brewer's yeast was suspended in 3 vol. of water, incubated 2 hr. at 37°C., and then centrifuged. The supernatant was then diluted with 4 vol. of water. The activity was found to be unchanged on storage in the frozen state for several months. This crude extract was found to be activated by the coenzyme as much as dialyzed or $(NH_4)_2SO_4$-purified preparations.

The measurements were carried out in 12 × 100 mm. test tubes graduated to 7.5 ml. The reaction mixture contained 2 μM of sodium or potassium glucose-1-phosphate 1 μM $MgSO_4$, 0.02 ml. of enzyme solution and coenzyme solution. Total volume 0.3 ml. The reaction was started by adding the enzyme and, after 10 min. at 37°C., it was interrupted by addition of 1.5 ml. of Somogyi's sugar reagent (17).

The tubes were heated 10 min. in a boiling water bath, and then cooled. After addition of 1.5 ml. of Nelson's arsenomolybdic reagent (18) and water to complete the volume the color was measured with a Klett photocolorimeter fitted with filter 52. The values obtained with increasing amounts of coenzyme are shown in Fig. 1.

Since no buffer was used, all the samples were adjusted to pH 7.4-7.5. Unknown solutions were compared with a standard coenzyme, blanks without glucose phosphate were run at the same time. These blanks are necessary because crude solutions may reduce initially or may develop reducing power during incubation. For instance, samples containing saccharose give a high blank, due to the invertase present in the yeast extract. If blanks are high, it is convenient to purify partially by precipitation with lead acetate.

The most frequent cause of error is the presence of salts which inhibit the enzyme, as was observed by Cori, Colowick and Cori (13). Sometimes a precipitation with lead acetate is useful in this case also.

Substances with SH groups hardly affect the activity with the yeast preparations and, moreover, they can be destroyed by heating in alkaline solution.

Usually, 2 or 3 different amounts of the unknown, were compared with a curve obtained in the same series using 2 or 3 samples of the standard solution. The error amounted to about 10 %.

The Isolation of the Coenzyme

1. *Preparation of the Yeast Extract.* The technique of Neuberg and Lustig for preparing fructose diphosphate (4) was used with fresh baker's yeast, saccharose, and ether. The coenzyme content increased during incubation at 30°C. up to 24 hr., and slowly decreased thereafter:

Hours	0	2	5	24
Coenzyme content (μM/1.)	11	185	415	445

The substitution of saccharose for glucose or starch did not improve the yield.

After incubation, the proteins were coagulated by heating, and the mixture filtered through fluted paper.

2. *Precipitation with Lead Acetate.* To the filtrate of step 1,2 ml.-% of glacial acetic acid were added, and then excess of 25 % lead acetate. The suspension was filtered on Buchner funnels using "Celite super cell." The cake was suspended in water and decomposed with H_2S. After filtration and aeration, the liquid was made alkaline to phenolphthalein with NaOH.

Comments. The treatment with H_2S should be carried out rapidly, since the coenzyme is acid-labile. The omission of step 2 did not give good results.

3. *Destruction of Fructose Diphosphate.* The liquid from step 2 was treated with 0.05 vol. of 5 N NaOH, and then heated in a large boiling water bath. Half an hour after the thermometer had reached 90°C., the liquid was cooled. Acetic acid was added to pH 7, and then an excess of magnesium acetate. The mixture was left overnight in the icebox. The $Mg_3(PO_4)_2$ was filtered off and NH_4OH added until phenolphthalein gave a rose color. After filtration, the absence of inorganic phosphate in the filtrate was checked analytically. Excess lead acetate was then added. The suspension, which was slightly alkaline to litmus, was filtered, and the precipitate decomposed with H_2S, filtered and aerated.

Comments. During the alkaline treatment the liquid becomes dark brown, and nearly all the organic phosphate is hydrolyzed. The Seliwanoff reaction for fructose (19) becomes negative. The amount of alkali can be increased up to 1 N and the heating up to 3 hr. without appreciable destruction of coenzyme.

The elimination of the inorganic phosphate with magnesia mixture in the usual manner resulted sometimes in a loss of about 40 % of the coenzyme by coprecipitation. Excess NH_4OH should be avoided, since it interferes in the subsequent lead precipitation.

4. *Barium Fractionation.* Excess barium acetate and 0.2 vol. of alcohol were added, and the liquid was filtered using Celite super cel. The dry precipitate (4a, Table I) was suspended in 10 vol. of water, cooled in ice, and adjusted to pH 3.5 (bromo-phenol blue) with HCI. The precipitate was discarded, and the liquid was treated with 3 vol. of 96 % ehtyl alcohol. The acid barium salt was separated wtih hardened filter paper, dissolved in water, adjusted with

$Ba(OH)_2$ to pH 8, ond 0.2 vol. of alcohol was added. The precipitate was then centrifuged and dried with alcohol and ether (4b, Table I).

Comments. This fractionation has been described by McFarlane (20) for the purification of fructose diphosphate. In some cases, the procedure was repeated, and in others the acid barium salt was dried and treated directly as described in 5. The latter procedure is to be preferred.

After this step it can be considered that all the acid-labile phosphate corresponds to the coenzyme since all he fructose diphosphate has been destroyed and glucose-1 phosphate is separated as the barium soluble salt.

5. *Precipitation with Acetone.* The barium salt wa's suspended in water as a thick paste, cooled, and 5 *N* H_2SO_4 was added until thymol blue gave a rose color. After checking for the absence of barium ions, the suspension was filtered on a Buchner funnel through Celite and a small amount of Norit. The filtrate was treated with 10 vol. of acetone and centrifuged. The oily precipitate was dissolved in a small volume of water and stored in ice (Fraction 1). To the supernatant a drop of concentraded NH_4OH was added followed by centrifugation. Three or four fractions were collected in this manner and analysed separately for inorganic, acid, acid-labile and total phosphate (5a, Table I). The fractions which showed a relation total phosphate/acid-labile near 2 were mixed, acidified with acetic acid and treated with $BaCl_2$. The $BaSO_4$ was centrifuged off, and washed. The combined supernatant and washings were adjusted to pH 8 with filtered saturated $Ba(OH)_2$, 0.2 vol. alcohol added and centrifuged. The precipitate was washed with 20 % alcohol until the supernatant gave no reaction for chloride and then dried with alcohol-ether (5b). The yield and purification in different steps are shown in Table I. One of the barium salts obtained in this manner and dried *in vacuo* over $CaCl_2$ had an acid-labile phosphate content of 3,48 % and 8.5 % of total phosphate. For an anhydrous dibarium hexose disphosphate the theoretical total phosphate is: 10.15 %. The Seliwanoff reaction (19) for fructose gave values which were equal to those given by equivalent amounts of glucose. In some preparations, the values were slightly higher but could be lowered by a second treatment with alkali.

Acid Hydrolysis

The hydrolysis was carried out in 0.25 *N* HCl at 37° ± 0.1°C., and both the phosphate split off and the coenzymatic activity were determined. The preparation which had a P total/P acid-labile ratio of 2.4 and contained only about 3 % fructose [Roe (19) procedure] was freed from barium by adding the exact amount of H_2SO_4, after centrifuging it was diluted with one volume of 0.5 *N* HCl. It was then immersed in a thermostat, and the samples taken at intervals were pipetted into tubes containing sufficient amount of 0.3 *N* NaOH to neutralize the acid. Both inorganic and acidlabile phosphate were estimated by the method of Fiske and SubbaRow (21). The hydrolysis, which occurs during the development of color is negligible, and no correction was applied for it.

As a check on the whole procedure an experiment was run with glucose-1-phosphate, the value for *K* was found identical to the one given in the literature (5).

For analytical purposes the hydrolysis in 0.1 *N* H_2SO_4 at 100°C. was also studied. The results in Fig. 2 show that after 9-10 min. the phosphate liberated reaches a constant value.

Aldose Estimation

The method of Macleod and Robinson (22) was used, and a solution of the sodium salt of the coenzyme. Hydrolysis was carried out in I *N* HCl at 100°C. during 7 min. In one experiment, 0.71 ml. 0.02 *N* I_2 was used, and the phosphate split off in the same amount solution was 7.0 μM. Another experiment gave 0.75 ml iodine for 7.5 μM phosphate. That is the ratio aldose/labile phosphate was 0.9 and 1.0 respectively. No iodine was used by the nonhydrolyzed coenzyme. A check of the method with the same amounts of glucose gave 96 % of the theoretical value.

Enzymatic Identification of Glucose-6-Phosphate

An extract of rabbit muscle was prepared by mincing and extracting with 3 vol. of water. It was used after keeping several days in the ice box and the amount of enzyme necesary was determined in a preliminary experiment. One sample of the coenzyme was hydrolysed in 0.1 *N* acid 10 min. at 100°C., and then neutralized. Another sample was used intact. The tubes were incubated at 37°C., and then fructose was estimated as described by Roe (19). Results appear in Table III and show that the amount of fructose formed from 6.2 μM of the hydrolyzed coenzyme corresponds to about 6 5 μM of hexose phosphate.

Rotatory Power before and after Hydrolysis

A 2 dm. tube and sodium light were used in all the experiments.

Experiments I. A solution at pH 8 of the sodium salt of a preparation with a ratio total phosphate/labile phosphate = 2.02 was prepared. Concentration estimated by P content was 1.85 %, $\alpha = +1.72°$. Therefore, $[\alpha]_D = +46.4°$, calculated for $C_6H_{10}O_6(Na_2PO_2)_2$.

The solution was made 1 *N* with HCl. Concentration = 1.18 g-% $\alpha = +1.50°$ Therefore, $[\alpha]_D = +63.5°$ calculated for the free acid: $C_6H_{10}O_6(H_2PO_2)_2$.

The same solution was heated 10 min. at 100°C. and cooled: $\alpha = +0.58°$, which gives: $[\alpha]_D = +32.2°$, calculated for: $C_6H_{11}O_6PO_3H_2$. The values in the literature for glucose-6-phosphoric acid are: $[\alpha]_D = +35.1°$ (6) and $+34.2°$ (7).

The liquid was then neutralized, and excess barium acetate was added. The precipitate was centrifuged off and the supernatant precipitated with two volumes of ethyl alcohol. The precipitate was dried, redissolved in water and clarified by centrifugation $\alpha = +0.17°$, concentration = 0.586 g-% calculated from phosphate content. This gives $[\alpha]_D = +15°$ for $C_6H_{11}O_6PO_3Ba$. The value is somewhat lower than that of barium glucose-6-phosphate. Robinson and King (6) give $[\alpha]_{5461} = +19,6°$; from this $[\alpha]_D = +16°6$, ($[\alpha]_D = [\alpha]_{5461}/1.18$) at 0.5 % concentration and $+18°$ at 8,4%. Other values given in the literature are: 17.9° (9), + 17.4 (8), + 16.6 (7).

Experiment II. A preparation of the barium salt with a ratio total phosphate/labile phosphate = 2.29 was dissolved in 0 07 *N* HCl Concentration calculated from the labile phosphate was 1.86 % $a = + 2.66^{\circ}$. Therefore, $[\alpha]D = + 71.5^{\circ}$ for the free acid.

The liquid was made 0.2 *N* with HCl ond heated 10 min. at 100°C. Concentration = 1.32 %, and $\alpha = + 0.79^{\circ}$. This gives $[\alpha]D = + 30.0^{\circ}$ for an hexose monophosphoric acid.

The solution was then separated into water soluble and insoluble barium salts, as in Exp. I. Both were dried and studied. From 242 μM of starting material 183 of inorganic phosphate were recovered, 150 of barium soluble ester, and 51 of barium insoluble ester.

The barium soluble fraction gave $[\alpha]D = + 13^{\circ}$, calculated for a barium hexose monophosphate.

The barium insoluble fraction dissolved in 0.2 *N* HCl gave $\alpha = + 0.26^{\circ}$, and concentration was 0.0134 *M*.

Properties of the Product of Hydrolysis

The water-soluble barium fraction from the previous experiments was heated at 100°C. in 1 *N* H_2SO_4. The phosphate liberated was 0 in 1 hr., and 4 % in 2 hr. In another exp., 1 and 5 % respectively. For glucose-6-phosphate, Robison and King (6). give 1.8 and 3.5 %.

Alkaline hydrolysis was carried out in 0.2 *N* NaOH. Phosphate was estimated with higher acid concentration so as to decrease the interference silicate (23). Blanks run under identical conditions were substracted. Robison and MacFarlane (10) give a value of 60 % under the same conditions.

The osazone was prepared as described by McCready and Hassid (24); a copious precipitate was formed, which was compared with the osozone of a mixture of glucose-6-phosphate and fructose-6phosphate. By microscopic examination both samples were undistinguishable.

After recrystallization from alcohol-chloroform the melting point wa 150°-153°C. Robison and King (6) give 154°C.

Synthesis from 1,6-Dibromotriacetylglucose

4.5 g. of 1,6-dibromotriacetylglucose (25) were dissolved in 30 ml. of anhydrous benzene, and refluxed 2 hr. with 7 g. of Ag_3PO_4. In other experiments, longer times of heating were tried with no better results. After filtration the benzene was removed under reduced pressure. The solid was then dissolved in 40 ml. methanol plus 1.6 ml. of 5 *N* HCl. After 15 hr. at room temperature, the solution was neutralized, and excess barium acetate was added. After 2 hr., the precipitale was separated, washed with 20 % alcohol and dried. Obtained: 1 g. of a substance containing 0.66 of inorganic P, 0.85 of acid-labile and 0.29 of acid-stable phosphate (μM/mg.) The substance was active as coenzyme, 1 mg. having the same activity as 0.05 μM of the pure coenzyme. Yield calculated from the bromoacetyl compound 0.5 %. Four different preparations were carried out varying some details, but the yield was not improved.

Distribution of the Coenzyme in Animal Tissues

The organs from freshly killed animals were weighed, minced and suspended in 2 vol. of water. The proteins were removed by heating and centrifuging, the liquid was heated 20-30 min. at pH 9, and then adjusted to pH 7.4. The coenzyme was then estimated on suitable dilutions by the yeast phosphoglucomutase test. Results in Table IV.

TABLE IV

Coenzyme Content of Animal Tissues
μM/g.

Tissue	Rat	Rabbit	Toad
Liver	0.012-0.014	0.017	0.06
Kidney	0.009-0.0028	0.0025	—
Muscle	0.006-0.024	0.01-0.04	0.017
Brain	0.005-0.011	—	—
Heart	0.0028	0.05-0.09	0.0014

Action of the Coenzyme on the Phosphoglucomutase of Animal Tissues

Many experiments were carried out with the enzyme of animal tissues using essentially the same technique described for the estimation of the coenzyme. The extracts were obtained with 2 vol. of water and then adjusted to pH 7.5. The amount used was 0.005-0.02 ml.

As shown in Fig. 3, a considerable activation was obtained with the phosphoglucomutase of heart and kidney.

With muscle, the results were variable until use was made of the test described by Najjar (15), in which cysteine is used as an activator. The test was carried out as described by him with minor variations.

As shown in Fig. 4 (left), under these conditions,

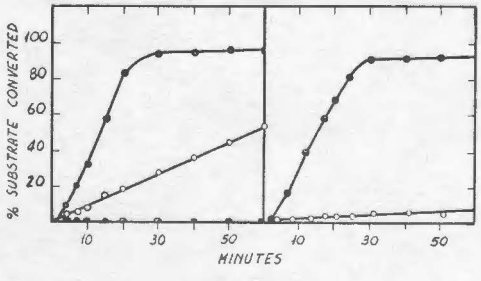

FIG. 4. — The action of the coenzyme on rabbit muscle phosphoglucomutase. Left: crude extract. Right: acid-treated extract. Upper curves: 0.1 ml. enzyme solution (see text), 2.3 μM glucose-1-phosphate, 5 μM cysteine, 1 u*M* $MgSO_4$ and 0.001 u*M* coenzyme, pH 7.5. Total volume, 0.5 ml. Temperature, 30°C. Lower curves (empty circles): the same without coenzyme. Lowest curve on left figure (black squares) same as upper but without cysteine.

the addition of coenzyme increases the rate of reaction about 300 % as compared with cysteine alone.

Still more demonstrative results were obtained when the method of Warburg and Christian (16) was applied. The preparations obtained in this manner showed practically no activity in the absence of coenzyme (Fig. 4, right). The procedure was as follows. A rabbit muscle extract was obtained by mincing and extracting with 2 vol. of ice cold water. After half an hour it was strained through muslin. For the experiment shown in Fig. 4 (left), this extract was diluted 100 times with cold water immediately before use. For the acid splitting: to 2 ml. of this extract plus 0.7 ml. of saturated $(NH_4)_2SO_4$, 3 ml. of 0.1 *N* HCl were added with stirring with the pipette submerged in the liquid. These operations, as well as the centrifugation, were carried out at 0ºC. The precipitate was then dissolved in 2 ml. of water and neutralized in the cold. After centrifuging off denatured protein, the preparation was ready for use and could be stored several days in the frozen state without loss of activity. In the experiment shown in Fig. 4 (right), this solution was diluted with 1 vol. of cold water.

SUMMARY

Methods for the estimation and isolation of the coenzyme of phosphoglucomutase are described. The substance contains two phosphate groups. The first phosphate can be hydrolyzed by mild acid with the simultáneous liberation of an aldose group (hypoiodite titration).

The hydrolysis constant for the first phosphate in 0.25 *N* acid at 37ºC. was found to be 3.1×10^{-4}.

The coenzyme preparations gave a specific rotation $[\alpha]_D = +63º$ to $+70º$ calculated for an hexose diphosphoric ester. On hydrolysis the dextrorotation decreased to values comparable to those of glucose-6-phosphate. Other properties of the substance remaining after removing the first phosphate, such as behavior toward enzymes, acid and alkaline treatment, and the formation of an osazone, agreed with the properties of glucose-6-phosphate.

An active preparation was obtained by treating 1,6-dibromotriacetylglucose with Ag_3PO_4.

The structure of the coenzyme is, therefore, postulated as α-1,6-glucose diphosphate.

The reversible splitting of the phosphoglucomutase from rabbit muscle is described, as well as the distribuiton of the coenzyme in some animal tissues.

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