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Glucose 1,6-Diphosphate Formation by Phosphoglucomutase in Mammalian Tissues*

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SUMMARY

Glucose 1,6-diphosphate concentrations in 10 mouse tissues were found to range from 70 to 80 μmoles per kg in brain and red blood cells to 7 to 10 μmoles per kg in testes and pancreas. There was no discernible correlation between levels of glucose 1,6-diphosphate and levels of glucose 6-phosphate, uridine diphosphoglucose or phosphoglucomutase, or Michaelis constants of phosphoglucomutase for its coenzyme. Ischemia caused a rapid decrease in glucose 1,6-diphosphate level in brain, whereas there was little or no effect of starvation on the level in liver, or of maximal electrical stimulation on the level in muscle.

Evidence is presented to show that glucose 1,6-di-P in muscle can be synthesized by P-glucomutase itself from fructose-1,6-di-P and glucose-6-P (or glucose-1-P). The rate of synthesis is only about 0.015% of the mutase rate for glucose-1-P, but this would be sufficient to synthesize the glucose-1,6-di-P in mouse muscle in 3 min. The equilibrium constant is about 12 for (fructose-1,6-di-P) (glucose-6-P):(fructose-6-P) (glucose-1,6-di-P).

In the 20 years since the discovery of glucose 1,6-diphosphate (1) and its role as coenzyme for phosphoglucomutase (2), there have been very few investigations of the level of this substance in tissues. By accident, it was discovered that in mouse brain there is as much glucose 1,6-diphosphate as there is glucose 6-phosphate or uridine diphosphoglucose. This has prompted further study of levels of this cofactor in brain.

Ischemia causes a rapid fall in brain coenzyme levels. It therefore seemed possible that there might be more of the coenzyme than previously supposed in other tissues as well. Consequently, normal levels have been measured in eight other organs with precautions to prevent change during sample preparation. In brain, the degradation and synthesis of glucose-1,6-di-P were examined to see why this substance is so labile *in vivo*, and what process may normally maintain the tissue level.

It appears that, in both brain and muscle, glucose-1,6-di-P can by synthesized by P-glucomutase from fructose-1,6-di-P and glucose-6-P. The results indicate that this was also what actually took place when Paladini *et al.* (3) observed glucose-1,6-di-P formation from glucose-1-P and ATP in muscle extracts. Very recently, Alpers (4) has found that glucose-1,6-di-P can also be synthesized by P-glucomutase from 1,3-diphosphoglycerate and glucose monophosphate. The relative significance of these two mechanisms for formation of the coenzyme will be discussed.

A companion paper provides additional kinetic information concerning the reaction of fructose phosphates with P-glucomutase (5).

EXPERIMENTAL PROCEDURE

Materials and Experimental Animals—Glucose-1,6-di-P and UDP-glucose dehydrogenase were obtained from Sigma. P-glucomutase, P-glucoisomerase, and glucose-6-P dehydrogenase were from Boehringer und Soehne (Mannheim, Germany). Imidazole was the low fluorescence grade from Sigma. Glucose-1-P preparations, as Sutherland *et al.* (6) and others have found, are contaminated to a variable degree with glucose-1,6-di-P. A lot from Sigma with minimal contamination was selected (see below). White male mice (Ha/ICR) were from A. R. Schmidt (Madison, Wisconsin).

Preparation of Tissues for Analysis—Mice (or in some cases the heads only) were rapidly frozen in Freon 12 (CCl_2F_2) brought to its freezing point, −150°, with liquid N_2. If organs other than brain were to be analyzed, the mice were anesthetized, and to accelerate freezing, the abdominal and thoracic cavities were quickly opened just before immersion. Organ samples were removed without thawing at −15°. Before and after removal, the tissues were held at −80°. Samples (usually about 100 mg) were prepared for analysis according to the method of Nelson, Lowry, and Passonneau (7). In this procedure, the tissue is first dispersed in a small amount of methanol-HCl at −15°, which removes the water without thawing, after which proteins are removed with $HClO_4$ and the extract is neutralized.

Analytical Methods—All analyses were conducted in the Farrand Model A fluorometer. Glucose-6-P was measured

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with the aid of glucose-6-P dehydrogenase and TPN^+ (8). UDP-glucose was measured by a modification (9) of the UDP-glucose dehydrogenase method of Strominger, Maxwell, and Kalckar (10).

Glucose-1,6-di-P was measured with P-glucomutase in a manner similar to that of Paladini *et al.* (3), except that with the use of a fluorometer, instead of a spectrophotometer, blank values could be reduced, and sensitivity and useful range could be increased. The basic reagent (stored at −50°) consists of 0.05 M imidazole-HCl buffer at pH 7.0, containing 1 mM magnesium acetate, 0.1 mM EDTA, and 0.01% bovine plasma albumin. Within a few hours of use, these are added: 1 μg per ml of yeast glucose-6-P dehydrogenase (with an activity of 125 μmoles per min per mg of protein), 0.01 μg per ml of crystalline muscle P-glucomutase, and TPN^+ to give 0.05 mM concentration. This P-glucomutase concentration represents a 1:1,000,000 dilution of the usual 1% suspension commercially available. For achievement of maximal activation (11), the original suspension is first diluted 5,000-fold in a solution of the same composition as the basic reagent.

Glucose-1,6-di-P is added to give an estimated concentration of 2 to 8 × 10^{-9} M. Instrumental sensitivity is adjusted so that full scale on the galvanometer is equivalent to about 5 × 10^{-6} M TPNH. The reaction is started by adding 15 μl of 1 mM glucose-1-P and is followed for 10 or 15 min with two or three intermediate readings. Six or eight samples can be followed simultaneously. Calculation is made from a standard curve constructed from four or five levels of the cofactor between 1.5 and 10 × 10^{-9} M. Because the reaction has a high temperature coefficient (see below), it is very important that samples and standards be run at exactly the same temperature.

The glucose-1,6-di-P was standardized by conversion to glucose-6-P by heating 10 min at 100° in 0.1 N HCl, after which the glucose-6-P was measured with glucose-6-P dehydrogenase and TPN^+.

Comment on Method for Glucose-1,6-di-P—The over-all blank has consistently been equivalent to about 2 × 10^{-10} M glucose-1,6-di-P. Part of this is contributed by the phosphorylated enzyme itself which is present at approximately 10^{-10} M. It is for this reason that the P-glucomutase concentration is kept as low as it is. The glucose-1-P level is also kept low because preparations usually contain some of the coenzyme. (Some preparations are so seriously contaminated as to be unsuitable.) Keeping the blank low not only permits great sensitivity but also permits use of the foot of the activity curve where rates are almost linear with cofactor concentration.

P-glucomutase is stable for several hours, at least, in the reagent, but only in the absence of glucose-1-P. Thus, incubation in the reagent for 30 min with 15 μM substrate present reduced activity by one-third. This might be because addition of substrate would convert most of the enzyme at this high dilution into the relatively unstable diphosphorylated form (12). A small amount of EDTA is incorporated in the reagent, because this increased the rate of reaction about 30%.

The assay reaction has an unusually high temperature coefficient. The rates at 25°, 30°, and 38° were 193, 298, and 438%, respectively of the rate at 20°, *i.e.* the Q_{10} is nearly 3 in the 20–30° range.

TABLE I

Formation of glucose-6-P from glucose-1,6-di-P in brain acid extracts with heat

$HClO_4$ extracts, prepared from three mouse brains, were acidified with 0.11 volume of 1 N HCl, heated 10 min at 100°, and analyzed ("Heated"). "Cold" samples were analyzed without adding HCl or heating.

	Extract 1		Extract 2		Extract 3	
	Cold	Heated	Cold	Heated	Cold	Heated
	μmoles/kg					
Glucose-6-P	39	126	48	124	60	135
Glucose-1,6-di-P	77	1	79	0	79	1
Sum	116	127	127	124	139	136

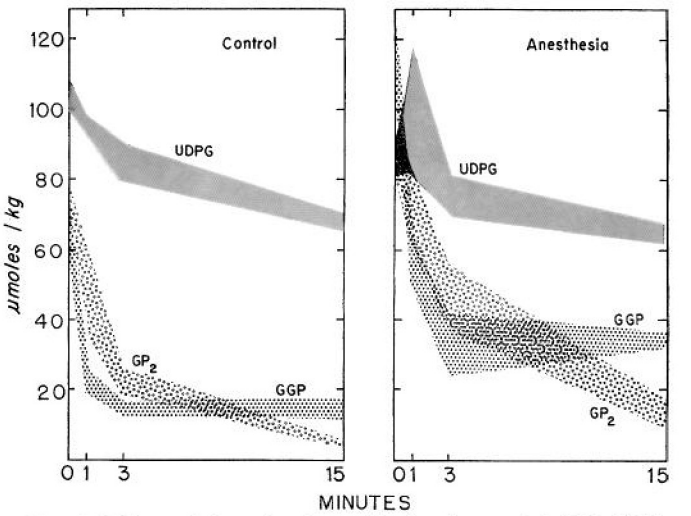

FIG. 1. Effect of decapitation on brain glucose-1,6-di-P (GP_2), glucose-6-P (*GGP*), and UDP-glucose (*UDPG*). Half the mice were deeply anesthetized with phenobarbital (200 mg per kg or, if necessary, 250 mg per kg). Zero time animals were frozen without decapitation. There were four mice at each time interval. Band widths represent 2 standard errors. In the anesthetized group, 1 glucose-6-P value (104 μmole per kg) was omitted from the 3-min average.

RESULTS

Validation of Analytical Procedure for Glucose-1,6-di-P—The relatively high levels of coenzyme in brain were accidentally discovered when glucose-6-P was measured in extracts prepared in two ways: by extraction with cold $HClO_4$ and by heating for a short time at 100° in 0.03 N HCl. The heating procedure gave much higher values. The rate of increase on heating was compatible with the acid lability of authentic glucose-1,6-di-P. A balance experiment with an acid extract of brain indicated that the glucose-1,6-di-P which disappeared on heating was accounted for as glucose-6-P appearing (Table I). This rules out the possibility that part of the P-glucomutase stimulation might result from ribose-1,5-di-P (13), or that the α-glucose-1,6-di-P standard might contain substantial amounts of the β anomer (unless, fortuitously, one should just balance the other). In all three cases of Table I, glucose-1,6-di-P levels were higher than those of glucose-6-P.

Breakdown of Glucose-1,6-di-P in Brain, in Vivo and in Vitro—As a test of the stability of glucose-1,6-di-P in intact brain, the levels were measured after 1, 3, and 15 min of complete ischemia (Fig. 1). For comparison, the related metabolites, glucose-6-P

and UDP-glucose, were also measured. In nonanesthetized mice, the coenzyme fell progressively to 6% of the initial level with intermediate points that approximately fit a first order decay curve with half-time of 1.7 min. Under deep anesthesia, the levels fell about half as fast. The slower rate under anesthesia indicates some kind of direct or indirect control of the breakdown rate. In both control and anesthetized mice, glucose-6-P fell more rapidly than glucose-1,6-di-P, but did not fall as far. UDP-glucose fell much more slowly than the other two metabolites.

As Paladini *et al.* (3) showed, glucose-1,6-di-P is also unstable in brain homogenates. A mouse was decapitated and the brain was removed 15 min later, giving time for most of the hexose metabolites to disappear. A 1:10 homogenate was prepared in 0.02 M Tris-HCl buffer at pH 8. Glucose-1,6-di-P was added to give a 100 μM concentration, *i.e.* a level approximating that originally present *in vivo*. Upon incubation at 25°, the coenzyme disappeared to the extent of 9, 24, 40, and 62% after 5, 15, 30, and 60 min, respectively. These data give a good fit to a first order decay curve with half-time of 40 min. A similar experiment with a 1:50 dilution of brain (instead of 1:10) gave a half-time of about 100 min instead of 200 min, the figure expected if activity had been proportional to tissue concentration. This suggests that there was better access of substrate to enzyme(s) at the higher dilution. The rate for the 1:50 dilution would correspond to a half-time of 2 min if extrapolated back to undiluted brain. This rate *in vivo*, at 25° and pH 8, may be compared to the half-time of 1.7 min *in vivo* observed above for unanesthetized ischemic brain (at 38°).

The products of breakdown *in vitro* were examined (Table II). Almost 80% of the glucose-1,6-di-P that disappeared could be accounted for as hexose monophosphate and 16% as glucose.

TABLE II

Breakdown products of glucose-1,6-di-P produced by incubation in brain homogenate

The brain from each of three mice was removed 30 min after decapitation and homogenized in 10 volumes of 0.02 M Tris-HCl buffer, pH 8. Portions of each homogenate were incubated for 60 min at 38° with and without addition of glucose-1,6-di-P to give an initial concentration of 103 μM. After incubation, the samples were heated for 5 min at 100° and analyzed for glucose-1,-6-di-P, glucose-6-P, and glucose. The sum of hexose monophosphates was calculated on the basis of equilibrium ratios of 3.06:1 for glucose-6-P to fructose-6-P, and 17:1 for glucose-6-P to glucose-1-P.

Substance measured	Glucose-1,6-di-P added	Glucose-1,6-di-P and products found			
		Brain A	Brain B	Brain C	Average
	μM				
Glucose-1,6-di-P	0	0	0	0	0
	103	8	9	8	8
Hexose monophosphate	0	114	127	144	128
	103	205	194	206	202
Glucose	0	36	39	43	39
	103	55	51	55	54
Glucose-1,6-di-P disappeared		95	94	95	95
Products recovered		110	79	74	89

TABLE III

Glucose phosphates and P-glucomutase in mouse tissues

Mice for the metabolite analyses were deeply anesthetized with phenobarbital (250 mg per kg) and the tissues were frozen *in situ* 30 min later (see "Experimental Procedure"). For the enzyme assays, mice were exsanguinated, and the tissues were homogenized in 10 volumes of 0.02 M imidazole-HCl, pH 7.0, containing 1 mM $MgCl_2$ and 0.1 mM EDTA. Further dilution if necessary was made in the same reagent. The assay medium was 1 ml of 0.05 M imidazole-HCl, pH 7.0, containing 1 mM $MgCl_2$, 0.1 mM EDTA, 0.06 mM TPN^+, 0.5 μM glucose-1,6-di-P, 0.2 mM glucose-1-P, 0.01% bovine plasma albumin, and 1 μg per ml of glucose-6-P dehydrogenase. Samples were assayed at 26° with two different amounts of homogenate in each case. For liver, homogenate equivalent to 1.2 and 2.4 μg of tissue was used; for brain, the equivalent of 15 and 30 μg of tissue was used, etc. For the metabolite assays, there were 5 groups of mice, 1 for red cells (from heparinized blood), 1 for fasted liver (24 hr), 1 for pancreas, 1 for stimulated muscle, and 1 for the rest of the samples. The muscle was stimulated maximally for 60 sec with electrodes from an inductorium applied to the exposed inguinal region. Breathing was not interrupted. Flexor muscles of the thigh were used for both control and stimulated mice. Each entry is the mean from 4 mice with its standard error.

	Glucose-1,6-di-P	Glucose-6-P	UDP-glucose	Mutase
	μmoles/kg			*moles kg^{-1} hr^{-1}*
Brain	72±5	65±6	104±5	0.84±0.02
Red blood cells	80±7	17.8±1.4	25.2±1.8	0.025±0.004
Spleen	57±3	174±10	126±11	0.51±0.03
Muscle	45±6	118±55	33.5±1.9	11.3±0.5
Muscle, stimulated	40±4	6290±360	15.9±2.6	
Lung	33.9±3.4	78±22	54.9±2.6	0.74±0.07
Liver	14.1±0.8	98±6	277±24	6.22±0.02
Liver, starved	15.4±1.5	118±25	177±13	
Kidney	14.4±0.8	49±12	144±9	1.93±0.03
Heart	12.2±1.3	76±12	45±9	3.91±0.20
Pancreas	9.9±1.5	95±16	209±28	
Testes	7.2±0.4	49±3	26.3±2.8	0.63±0.02

Glucose-1,6-di-P in Other Tissues—Of 10 tissues examined (Table III), brain and red blood cells are richest in glucose-1,6-di-P. Spleen, muscle, and lung come next. Spleen and lung contain relatively large amounts of red cells that might contribute a substantial portion. Testes, at the low end, has only 10% as much of the coenzyme as brain. Cardini *et al.* (14) reported levels of this coenzyme in rat tissues as follows (micromoles per kg): liver, 12 to 14; kidney, 9 to 2.8 (28?); muscle, 6 to 24; brain, 5 to 11; heart, 2.8. Thus, except in the liver, the levels were substantially lower than those of Table III. Bartlett (15) found 190 to 240 μM glucose-1,6-di-P in human red cells.

No correlation is to be seen among the tissues between glucose-1,6-di-P and either glucose-6-P, UDP-glucose, or P-glucomutase (Table III). In liver and pancreas the UDP-glucose to glucose-1,6-di-P ratio is 20:1, in muscle it is 0.7:1. In pancreas, the glucose-6-P to glucose-1,6-di-P ratio is 9:1; in red cells, it is 0.2:1. Finally, the ratio of glucose-1,6-di-P to P-glucomutase activity varies 1,500-fold with liver at one extreme and red cells at the other. If the turnover number of P-glucomutase

under conditions of measurement is taken as 20,000 per min,[1] the molar ratio of glucose-1,6-di-P to mutase is 2.7:1 in liver, 105:1 in brain, and 4,000:1 in red blood cells.

The differences in glucose-1,6-di-P levels are probably not related to differences in dissociation constants. This possibility was examined for P-glucomutase activities in mouse skeletal muscle, brain, and liver. The respective apparent Michaelis constants, measured in the presence of 200 μM glucose-1-P, were 0.060, 0.090, and 0.090 μM. Michaelis constants for glucose-1-P were also measured (in the presence of 0.5 μM glucose-1,6-di-P), and had the respective values of 8.3, 7.2, and 9.5 μM.

Enzymatic Synthesis of Glucose-1,6-di-P—Two different mechanisms were reported some time ago for the synthesis of glucose-1,6-di-P in muscle, (*a*) phosphorylation of glucose-1-P by ATP (3) and (*b*) transphosphorylation between 2 molecules of glucose-1-P (17). Initial attempts with brain and muscle failed to achieve significant production of the coenzyme by either reaction. These experiments had been made with rather dilute homogenates or extracts. Therefore, an extract was prepared from rabbit muscle as described by Paladini *et al.* (3) and incubated with ATP and glucose-1-P, and with relatively little dilution, according to their directions. Glucose-1,6-di-P was formed as expected. However, when the incubation was performed with more dilute extract, activity was much less than proportional, which suggests that more than one tissue component might be involved (Table IV, Experiment A). Because Paladini *et al.* had mentioned that P-glucomutase contaminated all their preparations, this enzyme was tried as an additive. The result was marked enhancement of activity, especially at high dilutions of the muscle preparation (Table IV, Experiment B).

With glucose-1-P as substrate, a very marked lag in glucose-1,6-di-P synthesis was observed (Experiments B and C). The lag was partially overcome by substituting glucose-6-P for glucose-1-P (Experiment C). However, the fact that glucose-6-P was the better substrate did not prove to be the explanation for the P-glucomutase requirement. Added mutase was an absolute necessity at high dilutions of the muscle extract, even with both glucose-6-P and glucose-1-P present (Table IV, Experiment D).

It seemed conceivable that the mutase (*E*) might be directly phosphorylated by ATP, forming *E*P, and that the role of the glucose-6-P was merely to remove the phosphate by the reaction discovered by Najjar and Pullman (12):

$$E\text{P} + \text{glucose-6-P} \rightarrow E + \text{glucose-1,6-di-P} \qquad (1)$$

This was shown not to be the case in an experiment performed with substrate amounts of P-glucomutase, previously dephosphorylated as described by Najjar and Pullman. Thus, P-glucomutase at 1.2 μM was incubated under the conditions of Table IV with ATP and muscle extract but no glucose-6-P. After incubation, the samples were brought to 0°, glucose-6-P was added to a concentration of 5 mM, and 30 sec later the reaction was terminated with heat. (This brief incubation at 0° was shown to be adequate to carry Reaction 1 to completion.) No phosphorylation was detected (less than 0.05 μM), whereas a control sample incubated at 38° with glucose-6-P present produced 3.4 μM glucose-1,6-di-P.

A series of eight tissues were assayed for activity in forming glucose-1,6-di-P from glucose-6-P, ATP, and P-glucomutase (Table V). Muscle was the most active, liver the least, with a range in activity of 150-fold. The distribution of activity seemed to be better correlated with capacity for glycolysis than with capacity for glycogenolysis. Also, the activity was rapidly lost from tissue homogenates made in water or imidazole buffer at pH 7 but was completely protected in 0.05 M phosphate buffer at the same pH. This suggested that if a known kinase were concerned it might be P-fructokinase. A similar conclusion was reached by Eyer and Pette (18).

Subsequent experiments showed that in the absence of tissue extract glucose-1,6-di-P could be synthesized from glucose-6-P in a system containing P-glucomutase, P-fructokinase, ATP, and

[1] This value for the commercial P-glucomutase may be too low for pure enzyme. Ray and Roscelli (16) reported a turnover number of 45,000 per min at a higher temperature (30°) and in a somewhat different assay medium.

TABLE IV

Formation of glucose-1,6-di-P by muscle extract with various additives

Rabbit muscle was passed through a meat grinder; 100 g were extracted for 1 hour at 0° with 200 ml of H_2O and then reextracted with 100 ml of H_2O. The combined extracts (285 ml) were brought successively to 1.1 and 1.6 M $(NH_4)_2SO_4$ with a 3.3 M solution, and the precipitates were discarded. The supernatant fluid was brought to 2.5 M with solid $(NH_4)_2SO_4$ and the pH adjusted to 7.0 with 1 M NH_4OH. The precipitate was taken up in 0.05 M potassium phosphate buffer, pH 7.4 (total volume 15 ml). This extract was incubated at 38° with 100 or 200 μl of 0.05 M imidazole-HCl buffer, pH 7.0, containing 10 mM $MgSO_4$ plus the other ingredients shown. The reaction was stopped by heating in a water bath at 100° for 2 min. Glucose-6-P was destroyed by adding 0.1 volume of 2 N NaOH and heating for 15 min at 100°. A suitable aliquot (0.5 to 5 μl) was assayed for glucose-1,6-di-P. "Muscle dilution" indicates the equivalent weight of muscle per ml of incubation medium represented by the amount of extract used. The results are calculated both as the concentration of glucose-1,6-di-P found in the incubated sample and as the amount formed per kilogram of muscle per hour. Corrections (usually small) have been made for any glucose-1,6-di-P found in unincubated samples.

Muscle dilution	Time	Substance added				Glucose-1,6-di-P formed	
		ATP	Glucose-1-P	Glucose-6-P	P-Glucomutase		
mg/ml	*min*	*mM*			*μg/ml*	*μM*	*mmoles/kg/hr*
Experiment A							
11	30		5			0.24	0.04
11	30	3	5			8.80	1.58
2.3	30	3	5			0.26	0.23
Experiment B							
1.2	30	3	5			0.00	0.00
1.2	10	3	5		20	0.25	1.32
1.2	30	3	5		20	1.31	2.31
Experiment C							
4.2	10	3	2		20	0.00	0.00
4.2	30	3	2		20	0.63	0.30
4.2	10	3		2	20	1.09	1.57
4.2	30	3		2	20	4.06	1.95
Experiment D							
0.9	30	3	0.25	5		0.06	0.1
0.9	30	3	0.25	5	200	2.65	5.8
0.9	30	1	0.25	5	200	5.05	11.0
0.22	30	1		5	200	1.45	13.3
0.046	30	1		5	200	0.39	16.6

fructose-6-P. However, it was also found that the last three components could be replaced by fructose-1,6-di-P.

This makes it apparent that P-glucomutase can catalyze the reaction

$$\text{Fructose-1,6-di-P} + \text{glucose-6-P} \rightarrow \text{fructose-6-P} + \text{glucose-1,6-di-P} \quad (2)$$

It is concluded that the reaction occurring in muscle extracts, and represented by Paladini *et al.* as

$$\text{Glucose-1-P} + \text{ATP} \rightarrow \text{glucose-1,6-di-P} + \text{ATP} \quad (3)$$

is in fact the result of the sequence

$$\text{Glucose-1-P} \rightarrow \text{glucose-6-P} \rightarrow \text{fructose-6-P} \rightarrow \text{fructose-1,6-di-P} \quad (4)$$

followed by Reaction 2.

Judging from the findings of Najjar and Pullman (11) with glucose phosphates, net Reaction 2 would be the result of two reactions:

$$E + \text{fructose-1,6-di-P} \rightarrow EP + \text{fructose-6-P} \quad (5)$$

$$EP + \text{glucose-6-P} \rightarrow E + \text{glucose-1,6-di-P} \quad (1)$$

Reaction 5 was readily shown by measuring the fructose-6-P formed by substrate amounts of *E* (Fig. 2). The yield of fructose-6-P was consistently less than the yield of glucose-6-P from glucose-1,6-di-P (reverse of Reaction 1), which suggests that a second product was formed from fructose-6-P, presumably fructose-1-P. The lower yield from fructose-1,6-di-P was not caused by an incomplete reaction, inasmuch as the addition of glucose-1,6-di-P after the reaction had stopped did not result in glucose-6-P formation (Fig. 2). This experiment also made it seem likely that both diphosphates react with the same enzyme site, and this was confirmed by the converse experiment in which pretreatment with glucose-1,6-di-P prevented subsequent fructose-6-P formation from fructose-1,6-di-P (Fig. 2).

Effect of Beryllium—The conclusion that P-glucomutase itself, rather than a contaminant, is responsible for the glucose-1,6-di-P synthesis is supported by experiments with Be^{++}.

TABLE V

Glucose-1,6-di-P formation from glucose-6-P and ATP in homogenates of eight mouse tissues

Tissues from each of four mice were dispersed at 0° in glass homogenizers with 20 volumes of 0.05 M potassium phosphate buffer, pH 7.4. Further dilutions, when necessary, were made in the same buffer. Aliquots of 2 to 7 µl, representing 10 to 500 µg of tissue, were diluted with 100 µl of 0.05 M imidazole-HCl buffer, pH 7.0, containing 1 mM ATP, 10 mM $MgSO_4$, 5 mM glucose-6-P and 200 µg per ml of P-glucomutase. These were incubated for 10 and 30 min at 38°, stopped with heat, and prepared for glucose-1,6-di-P analysis as described in Table IV. The rates were linear with time for all tissues except kidney and testes.

Tissue	Velocity	Tissue	Time of incubation	Velocity
	mmoles kg⁻¹ hr⁻¹		*min*	*mmoles kg⁻¹ hr⁻¹*
Muscle	213 ± 12	Testes	10	20.5 ± 1.9
Heart	145 ± 12	Testes	30	16.0
Brain	110 ± 8	Kidney	10	3.6 ± 0.3
Spleen	31.5 ± 2.4	Kidney	30	1.8 ± 0.3
Lung	16.5 ± 1.0	Liver		1.46 ± 0.17

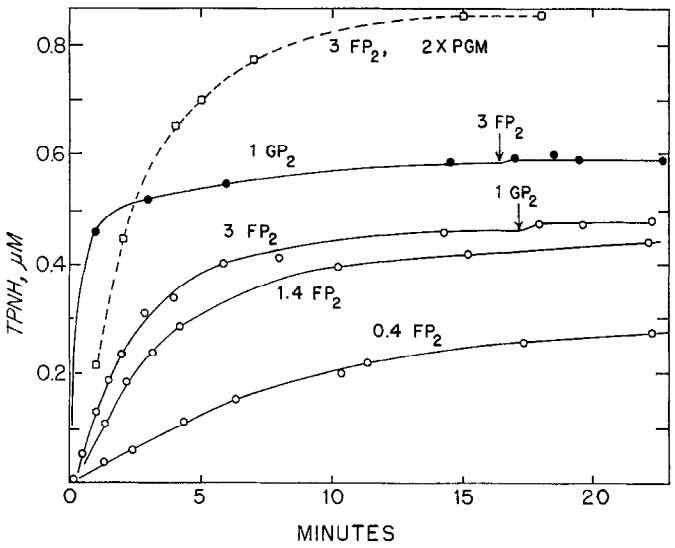

FIG. 2. Phosphorylation of P-glucomutase by glucose-1,6-di-P (GP_2) and fructose-1,6-di-P (FP_2). The basic reagent consisted of 0.05 M imidazole-HCl (pH 7), 1 mM $MgCl_2$, 0.1 mM EDTA, 0.05 mM TPN^+, 0.01% bovine plasma albumin, and 1 µg per ml of glucose-6-P dehydrogenase. To this were added 3.7 µg per ml of P-glucoisomerase. Each tube contained 0.6 µM dephospho-P-glucomutase (*PGM*), except that the top curve contained twice this amount. The numbers on the curves indicate micromolar concentrations. The curves are corrected for minor contamination of the diphosphates with glucose-6-P or fructose-6-P. The *arrows* indicate time of addition of the alternate substrate to two of the samples.

TABLE VI

Inhibition of P-glucomutase phosphorylation and P-fructomutase activity by beryllium

The basic reagent was 0.05 M imidazole-HCl, pH 7.0, with 0.05 mM TPN^+, 100 µg per ml of P-glucomutase and 3.6 µg per ml of P-glucoisomerase. After pretreatment with Be^{++} at about 25° under the conditions shown, fructose-1,6-di-P (40 µM) or glucose-1,6-di-P (1.1 µM) was added, together with $MgCl_2$ (5 mM), and the total increase of fluorescence measured.

Pretreatment conditions			Substrate	Inhibition
Be^{++}	Mg^{++}	Time		
µM		*min*		%
10	10	3	Glucose-1,6-di-P	41
20	10	4	Glucose-1,6-di-P	74
20	10	4	Fructose-1,6-di-P	72
20	50	4	Fructose-1,6-di-P	59
20	5000	25	Fructose-1,6-di-P	25

Selective and practically irreversible inactivation of P-glucomutase can be produced by low levels of Be^{++} salts (19–21). The rate of inactivation depends on the time of exposure and Be^{++} concentration, and further inhibition can be blocked by Mg^{++} addition. In our work, comparison was made between the extent of the stoichiometric reaction of the *E* form of mutase with either glucose-1,6-di-P or fructose-1,6-di-P (Table VI). It proved possible in both cases to block the reaction by pretreatment of the enzyme with Be^{++}. Moreover, the rate of inactivation was about the same for each.[2]

[2] Hashimoto *et al.* (21) reported that, in contrast to Be^{++}, Zn^{++} did not block phosphorylation of P-glucomutase by glucose-1,6-

TABLE VII

Equilibration between fructose-1,6-di-P, glucose-6-P, fructose-6-P, and glucose-1,6-di-P

The sugar phosphates in various proportions were incubated at 38° in 0.05 M imidazole-HCl, pH 7.0, containing 1 mM $MgCl_2$, 0.1 mM EDTA, and 10 μg per ml of P-glucomutase. The reaction was stopped by heating 100-μg samples for 1.5 min in a boiling H_2O bath. Glucose-6-P and fructose-6-P were analyzed fluorometrically with glucose-6-P dehydrogenase and P-glucoisomerase. Fructose-1,6-di-P was measured fluorometrically with the aid of aldolase, triose-P isomerase, glycero-P dehydrogenase, and DPNH. Glucose-1,6-di-P was measured by the procedure described under "Methods." The zero time sample actually was incubated for 1.5 min at 0° to permit equilibration between glucose-6-P and glucose-1-P.

Incubation	Fructose-1,6-di-P (A)	Glucose-6-P (B)	Fructose-6-P (C)	Glucose-1,6-di-P (D)	(A)(B)/(C)(D)
min	*μM*				
Experiment A					
0	90	77	0	1.6	
5	80	71	4	8	178
20	74	66	10	14	35
60	71	60	14	20	15
Experiment B					
0	70	14	1	1	
45	67	10	5.8	7.4	16
90	64	10	6.8	7.9	12
Experiment C					
0	37	202	0	1.6	
90	21	200	10	18	23[a]
Experiment D					
0	2	6	95	102	
45	38	46	67	48	0.6
90	55	65	30	30	4
180	72	73	26	18	11

[a] This value is not very reliable because of analytical uncertainties arising out of the large ratio between glucose-6-P and fructose-6-P.

Equilibrium between Glucose and Fructose Phosphates—The equilibrium between the mono- and diphosphates of glucose and fructose was approached from both directions (Table VII). Control reactions were performed with each component alone for the same time periods. Fructose-1,6-di-P and glucose-6-P were not detectably changed in the control samples; glucose-1,6-di-P fell not more than 5 or 10% in 90 to 180 min. Fructose-6-P controls, however, consistently fell 25 to 30%. The difference was not accounted for by an increase in glucose-6-P; presumably, the balance of the missing fructose-6-P had been converted to fructose-1-P. (Because all four components were measured, partial conversion of fructose-6-P to fructose-1-P would not distort the calculated equilibrium ratio.)

The results for the complete system indicate an equilibrium constant of about 12 (Table VII). Because the equilibrium ratio for glucose-6-P to fructose-6-P at 38° is close to 3:1 (22), the equilibrium ratio for fructose-1,6-di-P to glucose-1,6-di-P must be about 4:1.

TABLE VIII

Formation of glucose-1,6-di-P from several precursors by muscle extract

Rabbit muscle was extracted with H_2O as described in Table IV. To this was immediately added 0.1 volume of 1 M phosphate buffer, pH 7. This extract was used without fractionation in a concentration equivalent to a 50-fold muscle dilution. The reagent consisted of 0.05 M imidazole-HCl buffer, pH 7.0, with 10 mM $MgSO_4$ plus the additions shown. Samples were incubated for 10 min at 38° and analyzed for glucose-1,6-di-P as described in Table IV. The last two columns represent, respectively, the glucose-1,6-di-P found in the incubation mixture and the rate of formation calculated back to the muscle.

Substance added				Glucose-1,6-di-P formed	
Glucose-1-P	Glucose-6-P	ATP	Fructose-1,6-di-P		
mM				*μM*	*mmoles kg⁻¹ hr⁻¹*
5				0.2	0.1
5		3		6.4	1.9
	5			0.9	0.3
	5	3		6.2	1.8
	5		1	12.7	3.8
	5	3	1	6.2	1.9

Formation of Glucose-1,6-di-P by P-glucomutase in Unfractionated Tissue—The foregoing indicates that glucose-1,6-di-P can be synthesized by P-glucomutase from fructose-1,6-di-P and glucose-6-P. This does not, however, establish the importance of this reaction for synthesizing the coenzyme in the tissues. Therefore, an unfractionated H_2O extract of muscle was incubated with glucose-1-P, glucose-6-P, fructose-1,6-di-P, and ATP in several combinations (Table VIII). Glucose-1-P alone gave a negligible yield of glucose-1,6-di-P. ATP in combination with either glucose-1-P or glucose-6-P gave a substantial yield of coenzyme, but the amount was only about half that obtained with glucose-6-P and fructose-1,6-di-P. ATP addition actually reduced the yield from these substrates. ATP also decreased the yield obtained with crystalline P-glucomutase when it was incubated under similar conditions with glucose-6-P and fructose-1,6-di-P (not shown).

A second experiment was performed with the H_2O extract of rabbit muscle and with whole homogenates of mouse brain and muscle. In this case, the P-glucomutase activities were compared with the capacities to synthesize the coenzyme from glucose-6-P and fructose-1,6-di-P (Table IX). It is seen that the ratios between the two activities are very similar for rabbit muscle extract, whole mouse brain, and crystalline mutase. The yield of glucose-1,6-di-P from mouse muscle was disproportionately low, but this can probably be explained by rapid decrease in fructose-1,6-di-P in the whole homogenate.

In a third experiment, muscle extract was treated with Be^{++} until P-glucomutase was 99.5% inactivated (a water extract, equivalent to a 1:40 dilution of muscle, was made 0.2 mM in EDTA and 1 mM in $BeCl_2$ in 0.05 M imidazole-HCl at pH 7.5 and was incubated for 18 min at 25°). This extract was now diluted 20-fold in reagent similar to that of Table IV, containing ATP and glucose-1-P. The yield of glucose-1,6-di-P was reduced 92% by the Be^{++} treatment.

di-P. They left open the question of whether or not this was a fundamental difference or whether Zn^{++} was rapidly dissociated under their experimental conditions. In a direct test, conducted in a manner similar to that of Table VI, we have found that Zn^{++} does in fact prevent liberation of glucose-6-P from glucose-1,6-di-P by P-glucomutase, *i.e.* phosphorylation is prevented.

TABLE IX

Comparison of P-glucomutase and glucose-1,6-di-P synthetic activities for muscle, brain, and crystalline mutase

The mutase activity was measured at 26° under essentially the same conditions as for Table III. The glucose-1,6-di-P synthesis was measured by a 30-min incubation at 38° in 0.05 M imidazole-HCl, pH 7.0, with 10 mM $MgSO_4$, 1 mM glucose-6-P, and 0.5 mM fructose-1,6-di-P. The reaction was stopped with heat, and samples were analyzed in the same manner as for Table IV. The rabbit muscle extract described in Table IV was incubated at a dilution of 1:2,000. The mouse tissues were whole homogenates incubated at dilutions of 1:220 and 1:50 for muscle and brain, respectively. The crystalline mutase was incubated at a concentration of 7 μg per ml.

	P-glucomutase activity	Glucose-1,6-di-P synthesis	Ratio of activity
	moles kg^{-1} hr^{-1}	*mmoles kg^{-1} hr^{-1}*	
Rabbit muscle	34.8	4.2	8,300
Mouse muscle	16.7	1.1	15,200
Mouse brain	1.17	0.16	7,300
P-glucomutase	1.2×10^4	1.9×10^3	6,300

These experiments show that the P-glucomutase system offers a sufficient explanation for the capacity of muscle and brain to synthesize the coenzyme, and that P-glucomutase is probably required whether the precursors are fructose-1,6-di-P and glucose-6-P, or ATP and glucose-1-P. However, one experiment too many was performed. Levey and Alpers (23) had found that suitable preparations from liver or muscle were inactive in forming glucose-1,6-di-P from glucose-6-P and ATP unless 3-P-glycerate, 2-P-glycerate, or 2,3-diphosphoglycerate was added. When, in this study, 3-P-glycerate (1 mM) was tested on the capacity of fructose-1,6-di-P to phosphorylate crystalline P-glucomutase, the rate was actually inhibited 60%. However, 1 mM 3-P-glycerate, added to crude muscle extract during incubation with ATP and glucose-1-P, enhanced activity 6-fold. Moreover, pretreatment with Be^{++} as above (99.5% inhibition of P-glucomutase) reduced this activity in the presence of 3-P-glycerate by only 60% (see also "Discussion").

DISCUSSION

Tissue Levels of Glucose-1,6-di-P—The results presented raise a question as to whether the tissue levels of glucose-1,6-di-P bear much relationship to its function as a coenzyme. There is almost a total lack of correlation, among the tissues examined, between P-glucomutase activity and coenzyme concentration. Liver, with P-glucomutase activity 8 times higher than that in brain, has only one-fifth the concentration of glucose-1,6-di-P. In all tissues examined, the coenzyme level is at least 100 times greater than the Michaelis constant and, in most cases at least, is of an order of magnitude greater than P-glucomutase on a molar basis. In most tissues, the amount is small compared to other carbohydrate supplies, but it may be significant that in brain, where the level is unusually high and total carbohydrate is low, the glucose-1,6-di-P is made available during ischemia to the metabolic pool.

Synthesis of Glucose-1,6-di-P in Vivo—The results presented show that glucose-1,6-di-P can be synthesized by P-glucomutase itself from glucose-6-P and fructose-1,6-di-P, at a sufficiently rapid rate reasonably to account for observed tissue levels of the coenzyme. Nevertheless, certain of the results, as well as the work of others, indicate a more complicated situation. The observations of Paladini *et al.* (3) present little difficulty on this score. Their finding of glucose-1,6-di-P formation in muscle extract from glucose-1-P and ATP is satisfactorily attributed to partial conversion of glucose-1-P to fructose-1,6-di-P, with subsequent reaction between fructose-1,6-di-P and glucose-1-P or glucose-6-P to yield the coenzyme. The same is also probably true of the recent report of Eyer and Pette (18), who attributed "glucose 1-phosphate kinase" activity to P-fructokinase. The observation of Sidbury *et al.* (17), of glucose-1,6-di-P formation from glucose-1-P without ATP, is not so easily explained. We have not tried to repeat their experiments exactly, but have never found significant coenzyme formation in crude muscle extracts with glucose-1-P alone (*e.g.* Table IV). The level of activity reported by Sidbury was quite low, however, and might have been missed in these experiments. Their preparation, like that of Paladini *et al.*, was rich in P-glucomutase. It is not impossible that triose-P might have been formed, in which case fructose-1,6-di-P would arise via the aldolase reaction.

As mentioned above, Levey and Alpers (23) more recently reported preparations from liver and muscle (containing P-glucomutase) which made glucose-1,6-di-P from ATP and glucose-1-P or glucose-6-P, and which had a requirement for 2-P-glycerate or 3-P-glycerate. Recently, Alpers (4) has apparently explained this by finding that P-glucomutase can generate glucose-1,6-di-P from 1,3-diphosphoglycerate and glucose-1-P. Presumably, the first step consists of phosphorylation of the enzyme by 1,3-diphosphoglycerate just as seems to be true for fructose-1,6-di-P.

The question is, "What is the relative significance of these two alternative mechanisms for glucose-1,6-di-P generation?" In the fructose-1,6-di-P mechanism, the equilibrium constant sets an upper limit of the coenzyme level attainable with given levels of fructose-1,6-di-P, glucose-6-P, and fructose-6-P. The equilibrium constant reported here for this reaction is about 12. If glucose-6-P and fructose-6-P are kept in equilibrium by P-glucoisomerase at a ratio of 3:1, the upper limit for glucose-1,6-di-P would be one-fourth the fructose-1,6-di-P level. In fact, however, glucose-1,6-di-P levels may nearly equal or exceed those of fructose-1,6-di-P. Thus, in normal mouse brain, with 70 μM glucose-1,6-di-P, fructose-1,6-di-P concentrations are only about 100 μM (8). Data are not available for other mouse tissues, but in rat liver, the concentration of fructose-1,6-di-P is only about 20 μM (24), and in perfused rat heart, it is 25 μM (25). In red blood cells, Bartlett (15) found a glucose-1,6-di-P to fructose-1,6-di-P ratio of 2:1 or 3:1.

Energetically, the situation would be much more favorable for synthesis from 1,3-diphosphoglycerate. However, the tissue levels of this substance, although never satisfactorily measured, are certainly several orders of magnitude lower than those of fructose-1,6-di-P. From the incomplete kinetic evidence thus far available (4), the rate of glucose-1,6-di-P synthesis with probable tissue levels of 1,3-diphosphoglycerate would be very slow, less than 5 μmoles per kg of muscle per hour, *i.e.* a great deal slower than with fructose-1,6-di-P.

Finally, a single but disturbing observation, mentioned earlier, is the substantial glucose-1,6-di-P synthesis found in Be^{++}-treated muscle extract incubated with ATP, glucose-1-P, and

3-P-glycerate. Thus, P-glucomutase may not be the only enzyme capable of synthesis of glucose-1,6-di-P from compounds derived from glucose-1-P and 3-P-glycerate.

It is of historical interest that Kendal and Stickland (26) originally obtained results which made them believe that fructose-1,6-di-P was the coenzyme for P-glucomutase. Leloir (27) concluded that these results were attributable to contamination of the fructose-1,6-di-P with glucose-1,6-di-P. The present experiments, however, indicate that Kendal and Stickland were correct in attributing activity to fructose-1,6-di-P.

Glucose-1,6-di-P Breakdown in Vivo—The degradation of glucose-1,6-di-P, like its synthesis, could be catalyzed in tissues by P-glucomutase, through reversal of Reaction 2. However, this reaction is too slow to explain the rapid decrease observed in brain when the blood supply is cut off. Moreover, it occurs at a time when fructose-1,6-di-P is rising rather than falling as the mutase reaction would demand. The study in brain *in vitro* suggested breakdown by a phosphatase; this concurs with the findings of Hashimoto and Yoshikawa (28) for liver. Something must control the degradative process, however, because under anesthesia, with lowered metabolic rate, the rate of breakdown (during ischemia) is decreased.

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