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THE FORMATION OF GLUCOSE DIPHOSPHATE BY *ESCHERICHIA COLI*

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INTRODUCTION

Glucose-1,6-diphosphate[1] has been found to act as a coenzyme in the reaction:

Glucose-1-phosphate $\rightleftharpoons$ glucose-6-phosphate

when it is catalyzed by yeast or animal tissue phosphoglucomutase (2). During this latter investigation it was observed that glucose-1-phosphate solutions became contaminated with appreciable amounts of glucose diphosphate when stored for some time in the cold. From such solutions it was possible to isolate several microorganisms. Of these, *Escherichia coli* was found to be responsible for the formation of glucose diphosphate. Several strains of this organism were examined, as well as *Aerobacter aerogenes* and *Klebsiella pneumoniae*. The synthesis of glucose diphosphate occurred in the presence of any of these, whereas of glucose synthesis was obtained in preliminary tests with *Bacillus cereus, Bacillus alkaligenes, Sarcina conjunctivae, Serratia marcescens* and *Staphylococcus aureus*.

This paper represents the results of a study of the formation of glucose diphosphate and the fermentation of phosphoric esters by living *E. coli*. Extracts of *E. coli*, wich catalyzed the synthesis of glucose diphosphate, were prepared and purification of the active system was attempted. The mechanism of the reaction and the action of factors which influenced its rate were also studied.

[1] The structure of this substance has been confirmed by synthesis from silver phosphate and 1-bromo-2,3,4-triacetyl-6-diphenylphosphonoglucose by Reese *et al.* (1).

METHODS

Cultures

For small scale experiments, *E. coli* was cultivated on agar slants. After 24 hr. incubation, the bacterial growth was washed off. centrifuged and resuspended in water to a density corresponding to an extinction coefficient of E. = 0.8 for 1 cm. at 470 mμ. For larger scale experiments, the bacteria were harvested from peptone broth cultures after 20 hr. of incubation at 30ºC with aeration. The bacterial mass was separated by means of a continuous centrifuge and then stored in the frozen state until used. Extracts obtained after some days of storage were often more active than those obtained from fresh bacteria.

SUBSTRATES

Glucose-1-phosphate was prepared as described by Sumner and Somers (3), glucose-6-phosphate according to Colowick and Sutherland (4), and fructose diphosphate after Neuberg and Lustig (5). The preparation of glucose diphosphate has been previously described (2).

Analytical Methods

Glucose diphosphate was estimated by determination of its coenzymatic activity with a yeast phosphoglucomutase (2). Inorganic phosphate was determined by the procedure of Fiske and SubbaRow (6). The inorganic phosphate liberated during 7 min. of hydrolysis at 100ºC by 1 *N* acid is referred to as acid-labile phosphate, and that portion of the organic phosphate not hydrolyzed under similar conditions is referred to as acid-stable phosphate. Glucose was determined by the Somogyi (7) procedure using the Nelson reagent (8). Fructose was determined according to the directions of Roe (9).

RESULTS

The Formation of Glucose Diphosphate by Living E. coli

When the bacteria were suspended in a glucose-1-phosphate solution, an accumulation of glucose diphosphate occurred which rea-

ched a maximum and then decreased. The time at which the maximum was obtained was variable and was found to depend on the amount of bacteria added and on the initial concentration of the substrate. A graphic representation of a typical experiment appears in Fig. 1.

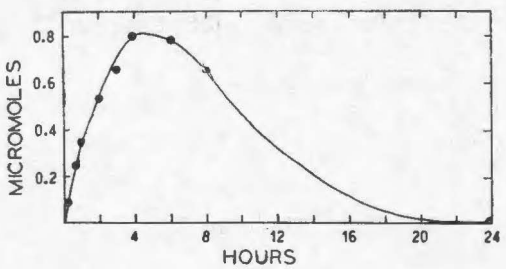

FIG. 1. — Glucose diphosphate formation by living E. Coli. Incubation at 37°C of 20 μM of glucose-1-phosphate, 1 ml. of M/15 phosphate buffer of pH 6.5 and 0.25 ml. of a suspension of E. coli. Total volume, 3.25 ml. Results in μM.

The formation of the diphosphate under aerobic and anaerobic conditions was aproximately the same. The maximum amount of diphosphate formed corresponded to the conversion of about 4 % of the initial amount of monophosphate added.

The formation of glucose diphosphate was measured with substrates other than glucose-1-phospate. No formation was detected from glucose (with or without added inorganic phosphate), glucose-6-phosphate, fructose diphosphate, saccharose, lactose, or maltose. The culture liquid remaining after centrifuging off the bacteria did not catalyze the formation of glucose diphosphate from the monophosphate.

The Fermentation of Phosphoric Esters

Manometric experiments showed that glucose-1-phosphate, glucose + inorganic phosphate, and glucose were rapidly fermented by *E. coli* (Table I) and also by *Aerobacter aerogenes* and *Klebsiella pneumoniae.*

Usually, glucose-1-phosphate was utilized at a rate slightly faster than the others. The rate of fermentation of glucose-6-phosphate was lower, while that of fructose diphosphate and glucose diphosphate was nearly undetectable.

TABLE I

Acid Formation by Living E. coli

CO_2 evolution measured in Warburg manometers containing: 0.025 *M* $NaHCO_3$, 4μ*M* of substrate and 0.4 ml. of a suspension of *E. coli.* Total volume: 2 ml. Temperature: 37°C. Results in μl. Gas: Nitrogen with 5 % CO_2.

Substrate	25 min.	50 min.
None	0	0
Glucose	30	60
Glucose + phosphate	33	67
Glucose-1-phosphate	37	75
Glucose-6-phosphate	17	35
Fructose diphosphate	7	8
Glucose diphosphate	8	10

These organisms differ from yeast, where glucose-1-phosphate is not fermented by intact cells. Evidently, there is a difference in permeability, since in *E. coli* glucose-1-phosphate is not hydrolyzed before entering the cells, for, if such were the case, glucose plus inorganic phosphate should be equivalent to glucose-1-phosphate in all respects. However, only the latter gives rise to glucose diphosphate.

Glucose Diphosphate Formation in Cell-Free Extracts

To study the mechanism of formation of glucose diphosphate from glucose-1-phosphate, it was deemed necessary to separate the enzyme system involved in this reaction. Crude extracts prepared from *E. coli,* acting on glucose-1-phosphate, were found to produce glucose diphosphate, together with considerable amounts of inorganic phosphate and reducing substances.

If the reaction is formulated as:

2 glucose-1-phosphate → glucose-1,6-diphosphate + glucose,

the molar ratio: glucose diphosphate/glucose should be 1. Actually, with the crude extracts, this ratio was found to be 0.01. The amount of inorganic phosphate liberated was roughly the same as that of reducing substances calculated as glucose, evidence which suggested that the main contaminating enzyme was a phosphatase. The increase in value of this

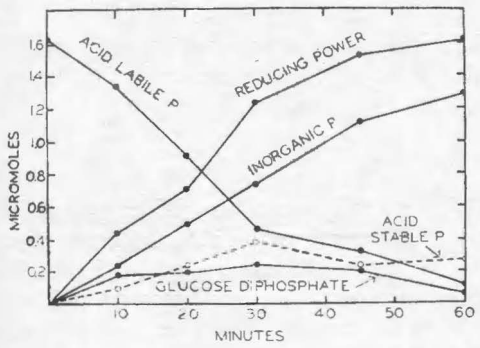

FIG. 2. Chemical changes produced by the enzyme 0.25 ml. samples containing 1.7 μM glucose-1-phosphate, 2 μM cysteine and 0.03 ml. of purified enzyme. Temperature, 37°C. Reducing power referred to a glucose standard.

ratio was, therefore, utilized as an index of the degree of purification of the diphosphate-forming enzyme. As can be observed in Fig. 2, the value of this ratio, with the same enzyme preparation, was found to depend on the length of incubation, and was approximately constant only during the first few minutes of the reaction.

Preparation of Cell-Free Extracts

Several procedures were attempted: Cytolysis with toluene, grinding with glass powder (10), and extracion of acetone-dried cells. The latter procedure was adopted, since it was found to yield more reproducible results. The dried bacteria. obtained as described by Harden (11) for the preparation of zymin, were extracted with 12 volumes of distilled water at 5°C. After 30 min., the suspension was centrifuged at 6000 r.p.m. The supernatant fluid was adjusted to pH 7, and solid $(NH_4)_2SO_4$ added to 0.5 saturation. The precipitate was removed by centrifuging and more solid $(NH_4)_2SO_4$ was added to the supernatant to bring the latter to 0.8 saturation wih respect to this salt. The resulting precipitate was separated and dissolved in water so that the volume was reduced to about 1/5 that of the original extract. This solution was then dialyzed 2-3 hr. in the cold. The dialyzed fluid contained about 50 % of the total activity of the original extract. Such preparations could be stored frozen for several weeks without deterioration.

The ratio: glucose diphosphate/glucose formed was 0.01 with the crude extract and 0.15 to 0.20 with the final dialyzed solution after activation by cysteine (see below). Repeated fractionation by $(NH_4)_2SO_4$ precipitation resulted in exttracts with a glucose diphosphate/glucose ratio of 0.35 to 0.50. Great losses of activity were encountered during this process of purification.

ACTIVATORS AND INHIBITORS

The Action of Cysteine and Magnesium

Dialysis of the extracts against cold water resulted in a gradual reduction of their activity so that, at the end of 6 or more hours of dialysis, the activity might have disappeared completely. The addition of magnesium ion was tested because it activates many of the enzymes which act on phosphoric esters. As shown in Table II, no increase in activity

TABLE II

The Action of Cysteine and Magnesium Ions on the Formation of Glucose Diphosphate

Incubation of: 2 μM glucose-1-phosphate with 0.03 ml. purified enzyme 10 min. at 37°C. Total volume, 0.25 ml.

	μM glucose diphosphate formed	Ratio: glucose diphosphate/glucose
Undialyzed enzyme	0.023	0.06
Dialyzed 4 hr.	0.015	0.02
Dialyzed + 1 μM Mg^{++}	0.015	0.02
Dialyzed + 1 μM cysteine	0.14	0.3
Dialyzed + 1 μM Mg^{++} + 1 μM cysteine	0.11	0.3

could be detected. However, when cysteine was added to the dialyzed extracts, the activity was increased 5- to 10-fold. On the other hand, the addition of cysteine had no effect on the liberation of reducing substances or inorganic phosphate. The optimum activating effect of cysteine was observed at a concentration of about 8×10^{-3} M.

The addition of boiled extracts of *E. coli* had no effect on the activity of the dialyzed enzyme.

The Action of Adenosine Phosphate

Glucose diphosphate has been found to be formed in muscle by transphosphorylation between glucose-1-phosphate and adenosinetriphosphate (12). The effect of the latter was investigated in the reaction as catalyzed by the extracts of *Escherichia coli.*

The effect of adenosinetriphosphate was tested at concentrations ranging from 10^{-5} to 10^{-2} M, and in no case could activation be detected. Concentrations higher than 10^{-3} produced an inhibition of the formation of

glucose diphosphate. Moreover, the maximum amount of adenine compounds present in the enzyme was calculated from the extinction at 260 mμ and the results showed that the amount of glucose diphosphate formed was at least 10 times larger than the maximum amount of adenine compounds present. No acid-labile phosphate could be detected.

It does not seem likely that any reaction which leads to phosphorylation of the adenosine compound takes place under these experimental conditions. In some experiments, the reaction was allowed to take place in Warburg manometers at pH 7 in bicarbonate and a nitrogen-CO_2 gas phase. No acid formation was detectable and, therefore, reactions which would give rise to phosphorylation, such as the oxidation of glyceraldehyde or the formation of phosphopyruvic acid, could be excluded. These results appear to indicate that glucose diphosphate is not formed by transphosphorylation between ATP and glucose-1-phosphate.

Fluoride

Fluoride, at a concentration of 5.8×10^{-4} *M*, inhibited the phosphatase action to the extent of 75 % and retarded the rate of glucose diphosphate formation to about the same degree.

Phloridzin

Phloridzin at a concentration of 3×10^{-4} *M* produced no appreciable change on the course of the reactions.

pH Optimum

The rate of formation of glucose diphosphate was found to be affected only slightly by fairly large changes in pH (Table III). The greatest activity was between pH 5 and pH 6 with acetate, maleate, or phosphate buffers.

Chemical Changes Produced by the Enzyme Preparation

The course of the reaction involving the conversion of glucose-1-phosphate to glucose diphosphate by the purified enzyme was similar to that by intact cells. There was first an increase in glucose diphosphate followed by its gradual disappearance. It has not been possible to separate the formation from the destruction of the diphosphate by purification of the enzyme. Fig. 2 illustrates the changes which occurred during the reaction. There was a gradual increase in inorganic phosphate and in the reducing power of the reaction mixture, coincident with a decrease in the acid-labile phosphate. At least 80 % of the reducing substances were not precipitated by the $ZnSO_4$-Ba$(OH)_2$ reagent (13), which precipitates the hexose phosphates.

TABLE III

pH Optimum

Incubation of 2 μ*M* of glucose-1-phosphate, 2 μ*M* of cysteine, 0.1 ml. of 0.1 *M* of acetate buffer and 0.03 ml. of purified enzyme. Total volume. 0.35 ml., 15 min. at 37°C. Results in μ*M*.

pH	3.8	4.4	4.7	5.3	5.9	6.2
Glucose diphosphate formed	0.08	0.15	0.17	0.23	0.19	0.15

These changes can be explained by the occurrence of the following reactions:

(a) 2 glucose-1-phosphate → glucose diphosphate + glucose,

(b) glucose-1-phosphate → glucose + inorganic phosphate,

(c) glucose-1-phosphate → glucose-6-phosphate.

Reaction (a) would be a transphosphorylation somewhat similar to the phosphoglucomutase reaction, which has been formulated (2) as follows:

(d) glucose-1-phosphate + glucose-1,6-diphosphate ⇌ glucose-1,6-diphosphate + glucose-6-phosphate.

Reaction (b) might occur directly or with (a) or (c) as intermediates. As to reaction (c), it is relatively slow as compared with the phosphatase (b) and, since large amounts of glucose diphosphate are formed, it was not possible to test whether it takes place by mechanism (d). Another possible mechanism for the formation of glucose-6-phosphate would be the removal of the phosphate at position one of glucose diphosphate by phosphatase.

The Effect of Glucose

Addition of glucose produced striking changes in the course of the reactions (Table IV). Whereas the formation of glucose diphosphate was not appreciably affected, there occurred a great increase in the acid-stable phosphate and a decrease in the liberation of inorganic phosphate. The amount of fructose was also increased.

Sorbitol or ethanol did not produce an effect similar to glucose, whereas fructose was about half as effective at the same concentration.

The stable ester, which was formed in larger amounts in the presence of glucose, was presumably glucose-6-phosphate which was in equilibrium with fructose-6-phosphate. This explains the concomitant increase in the "fructose" content of the reaction mixture.

The action of glucose was similar to that described with liver enzymes (14), where it has been interpreted to be due the inhibition of a specific phosphatase acting on glucose-6-phosphate.

The partially purified enzyme of *E. coli* was found to liberate inorganic phosphate, not only from the glucose phosphates but also from the α- and β-galactose-1-phosphates, α- and β-glycerophosphates, and phenylphosphate. Of these, phenylphosphate was the most rapidly hydrolyzed.

Glucose was found to inhibit also the liberation of inorganic phosphate from α-galactose-1-phosphate.

Discussion

The ready fermentability of glucose monophosphate by live *Escherichia coli* and related organisms reveals a difference with intact yeast cells, which do not measurably utilize phosphoric esters. Presumably, the cell membrane of *E. coli* is permeable to monophosphoric esters and, to a lesser extent, also to diphosphoric esters. Thus, during the fermentation of glucose-1-phosphate, the diphosphate is formed, passes to the medium, and is utilized at the end of the fermentation.

Table IV

The Action of Glucose

10 μM glucose-1-phosphate +0.3 ml. enzyme solution + 10 μM cysteine. Total volume, 2 ml., 30 min. at 37°C. Results in μM. Values for fructose were corrected by subtracting the values found at $t = 0$.

Additions	Glucose diphosphate formed	P inorganic formed	Acid-stable P	Fructose
None	0.79	7.5	1.4	0.15
30 μM glucose	0.67	6.2	3.2	0.80
60 μM glucose	0.71	4.9	4.0	1.40

Glucose diphosphate has been found to be an intermediate in the utilization of glucose-1-phosphate, and this raises several problems, such as the mechanism of its formation and destruction and its role in the normal metabolism of *E. coli*

The formation of glucose diphosphate appeared to take place by a mechanism different from that in animal tissues or yeast, where it has been demonstrated that glucose-1-phosphate is transphosphorylated by adenosinetriphosphate (12). Definite proof of the mechanism of synthesis of glucose diphosphate by *E. coli* must be deferred until the specific enzyme system involved can be separated from interferings systems. However, evidence at hand can best be interpreted by assuming that the conversion of glucose-1-phosphate to glucose diphosphate involves a

transfer of phosphate from position-1 of glucose-1-phosphate to position-6 of another molecule of the same substance.

The utilization of glucose diphosphate will require further investigation. The partially purified preparation which catalyzed its synthesis appeared to break it down mainly by the action of a contaminating phosphatase. Some experiments designed to detect in crude extracts an enzyme similar to aldolase, but which would act on glucose disphophate, were not successful.

Summary

Glucose diphosphate was found to be formed by *Escherichia coli, Aerobacter aerogenes* and *Klebsiella pneumoniae* when incubated with glucose-1-phosphate. No formation was detected from other sugars or their derivatives. Living cells fermented glucose monophosphates and free glucose at about the same rate, and fructose disphosphate or glucose diphosphate hardly at all.

A partially purified enzyme was prepared which transformed glucose-1-phosphate into reducing substances and inorganic phosphate, with the transient formation of glucose diphosphate.

The formation of glucose diphosphate was activated by cysteine and inhibited by fluoride. Glucose decreased the rate of liberation of inorganic phosphate and increased the formation of acid-stable phosphoric esters.

It is postulated that glucose diphosphate is formed by transphosphorylation between two molecules of glucose-1-phosphate.

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