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METABOLISM OF PHOSPHORAMIDATES

II. FURTHER STUDIES ON THE *ESCHERICHIA COLI* PHOSPHORAMIDATE PHOSPHORYL TRANSFER ENZYME

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SUMMARY

An enzyme, phosphoramidic hexose transphosphorylase, which catalyzes a phosphoryl transfer from monophosphoramidate, *N*-phosphorylglycine or monophosphoryl histidine to glucose has been purified about 160-fold from extracts of succinate-grown *Escherichia coli*. Several aldo- and ketohexoses as well as sedoheptulose have been shown to be phosphoryl acceptors with this enzyme. Certain preparations of phosphoramidic hexose transphosphorylase have been shown to produce only glucose 1-phosphate while others produce both glucose 1-phosphate and glucose 6-phosphate, suggesting the presence in the latter fractions of a second phosphoryl transferring system.

INTRODUCTION

Evidence for an enzyme catalyzed phosphoryl transfer from PNH_2 to glucose was presented in a previous paper[1]. While several reports of apparently similar phosphoryl transfer reactions from compounds containing a P–N linkage have appeared[2–4], these have generally involved transfer reactions catalyzed by acid or alkaline phosphatase and have required relatively high concentrations of a hydroxylic acceptor (1–2 *M*). More recently CORI *et al.*[5] have reported a reversible, apparently non-nucleotide mediated, phosphoryl transfer between 1,3-diphosphoglycerate and creatine.

Abbreviations: PNH_2, potassium phosphoramidate; P-Ngly, N-phosphorylglycine; P-hist, monophosphorylhistidine; PPi, inorganic pyrophosphate.

The *Escherichia coli* system reported here appears to be unique since it does not require a high concentration of the hydroxylic acceptor. It is relatively specific for hexoses and although not easily separated from a contaminating phosphoramidase, results reported here suggest that the transferase and the amidase are different enzymes. In preliminary reports from this laboratory[6,7] the reaction catalyzed by the *E. coli* enzyme has been described as shown in Eqn. 1.

$$PNHR + \text{glucose} \rightarrow \text{glucose 1-phosphate} + NH_2R \quad (1)$$

wherein the phosphoryl donor (PNHR) may be PNH_2, P-Ngly or P-hist. Several carbohydrates in addition to glucose are enzymically phosphorylated by PNH_2. This apparently new phosphoryl transfer system has been named phosphoramidic hexose transphosphorylase.

In this paper the purification and some of the properties of phosphoramidic hexose transphosphorylase are described.

MATERIALS AND METHODS

PNH_2, $P(NH_2)_2$ and P-Ngly, and the corresponding ^{32}P-labeled compounds were synthesized as previously indicated. N-phosphoryl histidine was prepared by the method of RATHLEV AND ROSENBERG[8]. Creatine-phosphate, acetyl-phosphate, carbamyl-phosphate, phospho-enolpyruvate and all nucleotides used were obtained commercially. D-3-O-methylglucose was generously supplied by Dr. T. Z. CSAKY; 6-fluoro-6-deoxy-D-glucose and 6-deoxy-D-glucose were gifts from Dr. A. WICKE; all other carbohydrates used were obtained commercially.

Pi was determined by the method of FISKE AND SUBBAROW[9], reducing sugars by the method of NELSON[10], and NH_3 as previously outlined[1]. Hexose phosphates were separated by two dimensional paper chromatography according to BANDURSKI AND AXELROD[11], or as their borate complexes by a modification of the ion exchange procedures of KHYM AND COHN[12].

E. coli, Crookes strain, was grown, harvested, and extracts were prepared as outlined previously[1]. Glucose 6-phosphate dehydrogenase was either obtained commercially (Boehringer) or prepared by the method of KORNBERG AND HORECKER[13].

Enzyme assays

Two standard assays for phosphoramidic hexose transphosphorylase were used. In the first assay PNH_2 disappearance was measured using the FISKE AND SUBBAROW[9] phosphate method in which Pi and PNH_2 are equivalent[1,8]. In a total volume of 1 ml the following additions were made: Tris–acetate or histidine buffer, pH 6.5, 100 μmoles; glucose, 40 μmoles; PNH_2 or other P–N compound, 4 μmoles, and sufficient enzyme to catalyze the disappearance of from 0.5 to 1 μmole of PNH_2 at 37° in 15 min. At the end of the incubation period the reaction was stopped by the addition of 0.5 ml of 5% trichloroacetic acid, any protein precipitate was removed by centrifugation, and a sample of the supernatant fluid was assayed for Pi. Phosphoryl transfer was estimated by comparison of results of Pi measurements on similar reaction mixtures containing no glucose.

The second assay, frequently used alone or in combination with the first, made

use of [^{32}P]PNH_2. In this case when there action was stopped as before, 0.5 ml of the reaction mixture was subjected to the solvent extraction procedure of NIELSON AND LEHNINGER[14] and the radioactivity remaining after extraction, presumably organic phosphate, was measured with an end-window counter. Both assays were proportional to the amount of enzyme added, up to about 1.3 units of phosphoramidic hexose transphosphorylase (Fig. 1). The extent of reaction was linear with time up to 30 min, as shown by separation of the organic and inorganic phosphates of samples taken from a reaction mixture containing [^{32}P]PNH_2 (2300 counts/min/μmole) and glucose at 5, 10, 20 and 30 min. A total of 800, 1710, 3320 and 4860 counts/min respectively were converted to organic phosphate.

One unit of phosphoramidic hexose transphosphorylase is defined as the amount of enzyme required to catalyze the transfer of 1 μmole of PNH_2 under the above conditions in 15 min.

In order to measure the exchange of [^{14}C]glucose into glucose 1-phosphate, the assay was run in 0.3 ml containing 5 μmoles of glucose 1-phosphate (adjusted to pH 6.5) and 3 μmoles of [^{14}C]glucose (12 200 counts/min/μmole) and enzyme. The reaction was started by the addition of enzyme (about 1 unit of phosphoramidic hexose transphosphorylase) and after 15 min incubation at 37° was stopped by the addition of 20 μmoles of barium acetate and 100 μmoles of Tris buffer pH 8.5 followed rapidly by 1 ml of absolute alcohol. The reaction mixture was let stand for 20 min in an ice bath and then the barium glucose 1-phosphate precipitate was packed by centrifugation, resuspended in water (0.5 ml) and reprecipitated with alcohol (1 ml). This procedure was repeated twice more and finally the precipitate was dissolved in 1.0 ml of 0.1 *N* HCl and radioactivity in an aliquot determined with an end window counter. Paper chromatography[11] of a barium precipitate (converted to the free acid with Dowex 50W, H^+ phase) revealed the major amount of radioactivity in glucose 1-phosphate and usually less than 5% accompanied the carrier glucose 6-phosphate. Total observed exchange was calculated and expressed by the method of DUFFIELD AND CALVIN[15].

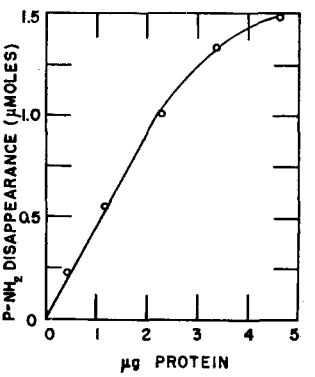

Fig. 1. Phosphoryl transfer as a function of enzyme concentration. The reaction mixture contained in a total volume of 1 ml, Tris–acetate buffer, pH 6.5, 100 μmoles; glucose, 40 μmoles; PNH_2, 4 μmoles; and enzyme (specific activity = 390). Incubation was at 37°.

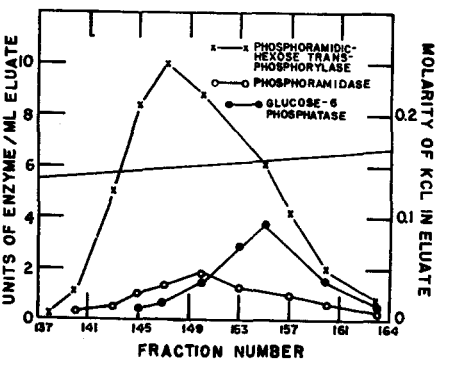

Fig. 2. Elution pattern of phosphoramidic hexose transphosphorylase and the hydrolytic activities from DEAE-cellulose. The conditions used were as described in the text. The solid line indicates the gradient used for elution.

RESULTS

Enzyme purification

Preliminary steps in the purification of phosphoramidic hexose transphosphorylase were the same as the steps reported for phosphoramidase-I (see ref. 1).

The results in Table I summarize the fractionation of phosphoramidic hexose transphosphorylase. Following the usual protamine sulfate treatment, 4 subfractions were obtained with solid ammonium sulfate. Fraction A was precipitated by the addition of 21 g of solid ammonium sulfate/100 ml of the protamine treated supernatant fluid. Fractions B, C and D were obtained by the successive addition of 11, 10 and 21 g of solid ammonium sulfate/100 ml of starting material. All fractions were

TABLE I

PURIFICATION OF PHOSPHORAMIDIC HEXOSE TRANSPHOSPHORYLASE

	Fraction	*Protein*	*Total units**	*Specific activity***
1	Crude	2.1 g	3360	1.6
2	Protamine treated	0.9 g	3600	4.0
3	Ammonium sulfate, fraction C	0.27 g	1240	4.6
4	Ammonium sulfate, fraction D	0.12 g	1300	10.8
5	DEAE-cellulose eluate from fraction 4	10 mg	760	76
6	CM-cellulose	0.26 mg	78	300

* Activity assayed by Pi disappearance as in text.
** Specific activity = units/mg of protein.

collected by centrifugation, dissolved in 0.02 *M* Tris buffer, pH 7.5, and dialyzed against two changes of this buffer. Both fractions C and D were found to contain substantial amounts of phosphoramidic hexose transphosphorylase (Table I) and both fractions were used for further purification on DEAE-cellulose and occasionally also on CM-cellulose.

The specific activity of fraction D in phosphoramidic hexose transphosphorylase was usually 2 to 3 times that of fraction C, and the former fraction was also very rich in phosphoramidase-I and glucose 6-phosphatase. All of these activities tended to overlap on DEAE-cellulose chromatography. Although fraction C was not devoid of either phosphoramidase-I or glucose 6-phosphatase, these activities were markedly reduced on storage of the fraction for 30 days at $-15°$. Most of the experiments reported here have been performed with DEAE-cellulose eluates or CM-cellulose eluates obtained from fraction D, (Table I) but the balance and product isolation experiments were carried out with DEAE-cellulose eluates obtained from fraction C (see Fig. 2).

The chromatographic purification of phosphoramidic hexose transphosphorylase on DEAE-cellulose was performed under the same conditions as used for phosphoramidase-I (see ref. 1). A typical elution pattern for phosphoramidic hexose transphosphorylase obtained from fraction C (Table I) on DEAE-cellulose is shown in Fig. 2. An additional 2 to 3 fold purification of phosphoramidic hexose transphosphorylase was frequently, but not routinely, obtained by passing the eluate from DEAE-cellulose over a CM-cellulose column (H^+ phase) using a linear elution gradient of 0.01 *M* acetate pH 5.1 to 0.08 *M* acetate pH 5.5 (Table I).

The DEAE-cellulose elution pattern for phosphoramidic hexose transphosphorylase depicted in Fig. 2 also shows the presence of smaller amounts of phosphoramidase-I and glucose 6-phosphatase. The marked difference in the ratio of all of these activities over the whole elution pattern and the effect of KF on phosphoramidic hexose transphosphorylase (see below) and the other activities[1] suggests that the transfer enzyme and phosphoramidase-I are not identical proteins.

Storage of phosphoramidic hexose transphosphorylase in the eluting buffer from DEAE-cellulose for as long as 8 months at —15° resulted in no loss of activity. However, storage at 0° or in the pH 5.3 acetate buffer at —15° resulted in a slow loss of activity (about 30% in 6 weeks). Under these conditions phosphoramidase-I activity was reduced by 70 to 80%.

TABLE II

EFFECT OF INHIBITORS ON PHOSPHORAMIDIC HEXOSE TRANSPHOSPHORYLASE

The complete reaction mixture in a total volume of 1 ml contains: EDTA buffer, pH 6.5, 100 μmoles; glucose, 40 μmoles; PNH_2, 4 μmoles; enzyme 15 μg.

Additions	*PNH_2 utilized (μmoles)*
None	0.60
KF 1 μmole	0.14
KF 5 μmoles	0.03
DFP 1.25 μmoles	0.73

TABLE III

SUBSTRATE SPECIFICITY OF PHOSPHORAMIDIC HEXOSE TRANSPHOSPHORYLASE

The reaction tubes contained in a volume of 1 ml: Tris–acetate, pH 6.5, 100 μmoles; P–N compound 5 μmoles [^{32}P]PNH_2 = 49000 counts/min per μmole; [^{32}P]P-Ngly (= 1100 counts/min/μmole carbohydrate), 40 μmoles; enzyme, fraction 5 Table I, 1.3 units. Incubation at 37° for 15 min.

Phosphoryl donor	*Acceptor*	*Total incorporation (counts/min × 10^{-3})*	*PNHR utilized (μmoles)*
[^{32}P]PNH_2	D-glucose	62.2	1.30
[^{32}P]PNH_2	D-fructose	16.0	0.30
[^{32}P]PNH_2	D-fructose	15.0*	—**
[^{32}P]PNH_2	D-mannose	39.0	0.80
[^{32}P]PNH_2	D-2-deoxyglucose	26.3	0.75
[^{32}P]PNH_2	D-3-*O*-methylglucose	49.1	1.0
[^{32}P]PNH_2	D-6-deoxyglucose	19.6	—**
[^{32}P]PNH_2	L-sorbose	22.6	—**
[^{32}P]PNH_2	D-glucosamine	10.4	—**
[^{32}P]PNH_2	D-galactose	9.3	—**
[^{32}P]PNH_2	L-rhamnose	0.2	—**
[^{32}P]PNH_2	Sedoheptulose	12.1	—**
[^{32}P]P-Ngly	D-glucose	1.01	1.10
P-hist	D-glucose	—	0.55
[^{32}P]PNH_2	D-xylose	0.05	0
[^{32}P]PNH_2	Adenosine	0	0

* Enzyme treated with charcoal at pH 5.5.
** Not measured.

pH optimum, cofactors and inhibitors

Phosphoramidic hexose transphosphorylase has a broad pH range of activity with maximal activity at pH 6.5 (Fig. 3) and approx. 50% of maximal activity at pH 5.0 and at pH 8.5. At pH 6.5 equal rates were observed with histidine, maleate, Tris–acetate and EDTA buffers. No stimulation was shown by added metals (Mg^{2+} or Mn^{2+}) and no indication of a nucleotide requirement in the reaction was seen. Treatment of phosphoramidic hexose transphosphorylase with charcoal (pH 5.5) or Dowex-1 (chloride phase) did not reduce the specific activity of the enzyme (Table III) and the addition of nucleoside diphosphates or triphosphates (ADP, GDP, and ATP) to the reaction mixture had no effect on the extent or rate of the reaction.

Phosphoramidic hexose transphosphorylase was markedly inhibited by fluoride ion; at 10^{-3} *M* KF nearly 80% inhibition was observed (Table II). The possibility that fluoride ion prevented phosphoryl transfer to glucose by displacing ammonia from PNH_2 was not conclusively ruled out, but no fluorophosphate could be detected chromatographically in fluoride inhibited reaction mixtures. Furthermore, no ammonia release from PNH_2 dependent on fluoride ion could be detected. Of several other reagents tested, none was found inhibitory. Those tested include: 2,4-dinitrophenol (10^{-5} to 10^{-3} *M*), diisopropylfluorophosphate ($1.25 \cdot 10^{-4}$ *M* to $1.25 \cdot 10^{-3}$ *M*), potassium arsenate (10^{-2} *M*), sodium azide (10^{-2} *M*), PPi (10^{-2} *M*) and *p*-chloromercuribenzoate ($5 \cdot 10^{-4}$ *M*).

Substrate specificity

In most of the experiments reported here glucose has been used as phosphoryl acceptor; however, several carbohydrates are active acceptors (Table III). With the

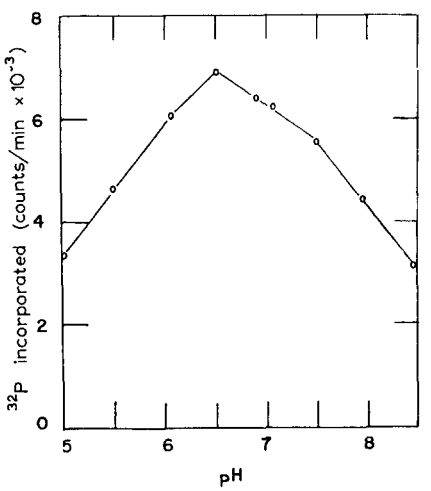

Fig. 3. Effect of pH on phosphoramidic hexose transphosphorylase, The conditions for this experiment were as described in the text. Acetate buffer was used for pH 5.0 and 5.5, Tris–acetate for pH 6.5, histidine for pH 6.8, and Tris buffers for pH 7, 7.8 and 8.5. The [^{32}P]PNH_2 contained 10 500 counts/min/μmole.

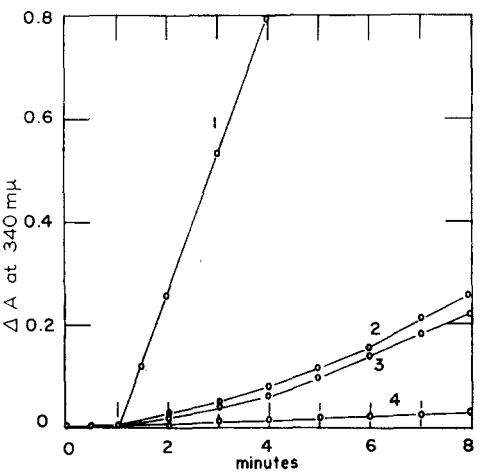

Fig. 4. Phosphoryl donor specificity of phosphoramidic hexose transphosphorylase. The reaction mixture contained in a total volume of 1 ml in 1-cm light path cuvettes, Tris–acetate buffer, pH 6.5, 100 μmoles; glucose, 40 μmoles; TPN^+, 0.5 μmole; phosphoryl donor, 5 μmoles; glucose 6-phosphate dehydrogenase, 2 units[13], phosphoramidic hexose transphosphorylase, 1.5 units. The reaction was started by the addition of phosphoryl donor at 1 min. Curve 1, PNH_2; curve 2, carbamyl-P; curve 3, acetyl-P; curve 4, phospho-enolpyruvate.

exception of sedoheptulose, only hexoses were effective acceptors. Glycerol, dihydroxyacetone, DL-glyceraldehyde, DL-serine, D-ribose, D-xylose, adenosine, guanosine, uridine, cytidine, thymidine, meso-inositol, glycolic acid, glycolaldehyde, L-rhamnose and D-6-fluro-6-deoxyglucose were not phosphorylated by [^{32}P]PNH_2 when incubated with 1 unit of phosphoramidic hexose transphosphorylase for 20 min.

PNH_2 and P-Ngly were equally effective phosphoryl donors and monophosphoryl histidine was somewhat less effective (Table III). No phosphoryl transfer from either creatine phosphate or $P(NH_2)_2$ was observed. Similarly incubation of adenylic 5'-phosphoramidate and glucose with 1 unit of the enzyme led to no new nucleotide materials being formed.

Using glucose as the acceptor and either [^{32}P]PNH_2 or [^{32}P]P-Ngly as phosphoryl donors, experiments were performed with 1 unit of phosphoramidic hexose transphosphorylase to determine the substrate saturations. The results obtained were plotted as reciprocal velocity against reciprocal substrate concentration[16] and extrapolated to Michaelis constants, K_m's, for PNH_2, P-Ngly and glucose respectively. The values obtained were: PNH_2, $3.5 \cdot 10^{-3}$ M; P-Ngly, $3.8 \cdot 10^{-3}$ M; and glucose, $3.8 \cdot 10^{-2}$ M.

Coupling with glucose 6-phosphate dehydrogenase

Most of the phosphoramidic hexose transphosphorylase preparations obtained from fraction D (Table I) by DEAE-cellulose chromatography could be coupled with glucose 6-phosphate dehydrogenase so that phosphoryl transfer to glucose could be measured by following TPN^+ reduction. Thus, not only was a convenient and sensitive method available to test the effectiveness of other phosphate compounds as potential phosphoryl donors in reaction 1 available, but in addition, since (see below) evidence was obtained that the initial product in reaction 1 was glucose 1-phosphate, it was concluded that a second enzyme accounting for the formation of glucose 6-phosphate was present. Using the coupled system described in Fig. 4, it was found that both acetyl-phosphate and carbamyl-phosphate were about 20% as effective phosphoryl donors as were either PNH_2 or P-Ngly with phosphoramidic hexose transphosphorylase. The lag period observed with the acyl phosphates is presumably due to a build-up in glucose 1-phosphate concentration. Creatine phosphate, phosphoenolpyruvate, $P(NH_2)_2$, ATP and sodium hexametaphosphate were completely ineffective as phosphoryl donors in this system.

The presence of a second enzyme in the phosphoramidic hexose transphorphorylase preparation, coupling with glucose 6-phosphate dehydrogenase is shown by the results in Fig. 5. The rate of TPN^+ reduction is shown for 4 separate phosphoramidic hexose transphosphorylase fractions. Transfer activity was measured by the ^{32}P incorporation assay just after the spectrophotometric experiment. The absence of correlation between coupling to glucose 6-phosphate dehydrogenase and phosphoryl transfer from PNH_2 to glucose indicates an additional enzyme is necessary.

Product isolation

Two phosphoramidic hexose transphosphorylase preparations were selected for larger scale experiments from which the phosphorylated product could be isolated. Using [^{14}C]glucose and PNH_2 as substrates, the products isolated were chromatographed[12] as their borate complexes on Dowex-1 (chloride phase) using known glucose

1-phosphate and glucose 6-phosphate as carriers. In the first case, with a phosphoramidic hexose transphosphorylase preparation known to couple with glucose 6-phosphate dehydrogenase, [^{14}C]glucose 1-phosphate and [^{14}C]glucose 6-phosphate were both formed. The ratio of glucose 1-phosphate:glucose 6-phosphate was about 2.5:1.

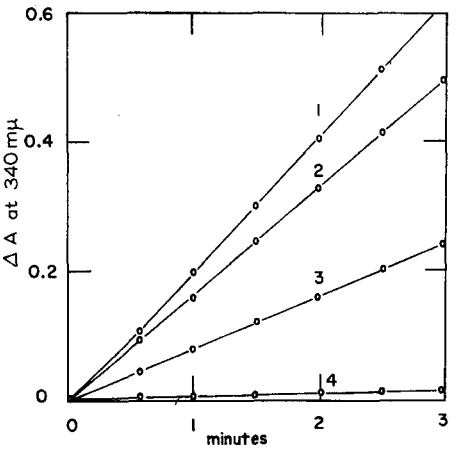

Fig. 5. Lack of correlation between transfer and coupling to glucose 6-phosphate dehydrogenase. The reaction mixture used in this experiment was as described in Fig. 4. The units of phosphoramidic hexose transphosphorylase used for each determination were; curve 1, 0.64; curve 2, 0.58; curve 3, 0.38, curve 4, 0.55.

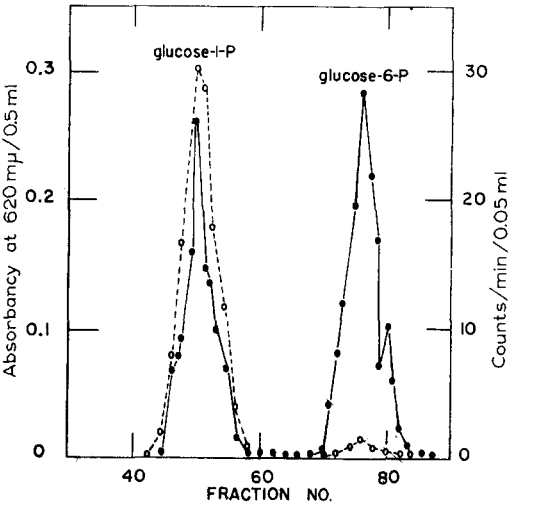

Fig. 6. The separation of glucose 1-phosphate and glucose 6-phosphate. In this experiment a phosphoramidic hexose transphosphorylase preparation unable to couple with glucose 6-phosphate dehydrogenase was used. The reaction mixture contained Tris–acetate buffer, pH 6.5, 400 μmoles; [^{14}C]glucose, 200 μmoles (1220 counts/min/μmole); PNH_2, 40 μmoles; and phosphoramidic hexose transphosphorylase, 7 units. After 20 min at 37° the reaction was stopped by the addition of 0.8 ml of 12% trichloroacetic acid and after 10 min the reaction was neutralized and Pi removed with magnesia. 10 μmoles each of glucose 1-phosphate and glucose 6-phosphate were added as carriers and the mixture applied to a Dowex-I-8X column (0.5 × 12 cm, 100 to 200 mesh). Separation was achieved with a linear gradient consisting of a mixing flask containing 0.01 *M* $Na_2B_4O_7$, 0.02 *M* NH_4Cl and a reservoir containing 0.04 *M* NH_4Cl, 0.005 *M* NH_4OH. ———, Hexosephosphate as measured by the anthrone[17] method. – – – –, Radioactivity.

In the second experiment, (Fig. 6), a phosphoramidic hexose transphosphorylase preparation which was unable to couple with glucose 6-phosphate dehydrogenase was used and essentially only glucose 1-phosphate was formed. Similarly, when [^{32}P]PNH_2 or [^{32}P]P-Ngly were used as donors and glucose as acceptor, the major radioactive material, after removal of ^{32}Pi, was [^{32}P]glucose 1-phosphate, as determined chromatographically on Schleicher and Schüll No. 589 paper by the twodimensional procedure of BANDURSKI AND AXELROD[11].

Several other hexoses and one heptose have been shown to be phosphoryl acceptors in Eqn. 1 (Table III); however, the position of phosphorylation in these other sugars is not yet known.

Reaction stoichiometry

In Table IV the stoichiometry of the Eqn. 1, using several P–N compounds and both glucose and 3-*O*-methylglucose as substrates, is shown. Further evidence that

glucose 1-phosphate is the product is shown by the equivalence of P–N compound and reducing group disappearance. This evidence, together with the paper and column chromatographic analyses and results of acid hydrolysis (Table IV) strongly implicates glucose 1-phosphate as the initial product formed in the case of glucose. In an experiment using D-fructose and PNH_2 as substrates, 0.8 μmole of PNH_2 was converted to non-extractable organic phosphate in 15 min. Complete recovery of this phosphorus as inorganic, extractable phosphate was measured after 10 min at 100° in *N* HCl, suggesting that the more acid labile fructose 1-phosphate rather than fructose 6-phosphate had formed.

TABLE IV

STOICHIOMETRY

Each experimental flask contained in a total volume of 1 ml, EDTA buffer, pH 6.5, 100 μmoles; glucose, 15 μmoles; P–N compound, 5 μmoles [^{32}P]PNH_2 = 7100 counts/min/μmole, [^{32}P]P-Ngly = 4600 counts/min/μmole); enzyme, 1 to 2 units.

Phosphoryl donor	*Utilized*		*Formed*	
	*PNHR**	*Glucose***	*Glucose 1-phosphate*** (μmoles)*	*Amine*
[^{32}P]PNH_2	0.97	1.06	1.01	0.92 §
[^{32}P]P-Ngly	1.15	1.20	1.06	— §§
P-hist	0.46	—	0.39	—
[^{32}P]PNH_2	1.32	1.35 §§§	1.30	—

* Measured as inorganic phosphate.
** Measured by method of NELSON[10].
*** Measured both as radioactive organic phosphate and as acid labile phosphate (10 min 1 *N* HCl).
§ Measured by Nesslerization.
§§ Not measured.
§§§ 40 μmoles 3-*O*-methylglucose used.

TABLE V

EXCHANGE OF [^{14}C]GLUCOSE INTO GLUCOSE 1-PHOSPHATE

The complete system contained in 0.3 ml: glucose 1-phosphate 5 μmoles, adjusted to pH 6.5; [^{14}C]glucose, 3 μmoles (12200 counts/min/μmole); enzyme, 1 unit. Incubation at 37° for 15 min; analysis as in text.

Conditions	*Exchange (μmoles)*
Complete	0.35
Plus NH_3 3 μmoles	0.30
Plus NH_3 10 μmoles	0.33
Plus glycine 3 μmoles	0.26
Plus glycine 10 μmoles	0.30
Plus histidine 3 μmoles	0.27
Plus histidine 10 μmoles	0.25
Minus enzyme	0.001

Reversibility

Since PNH_2 is known as a strong chemical phosphorylating agent, it was suspected that reversibility of Eqn. 1 would be difficult to demonstrate directly. Therefore, a phosphoramidic hexose transphosphorylase preparation virtually free of phosphoramidase-I was used in an attempt to show an ammonia dependent exchange of [^{14}C]glucose into glucose 1-phosphate. The results in Table V clearly show that a rapid exchange between glucose and glucose 1-phosphate can be measured, but the reaction is independent of either ammonia or glycine and, in fact, is inhibited by these reagents.

DISCUSSION

While the present work indicates that an enzyme capable of using a monophosphoramidate as a phosphorylating agent exists, the naturally occurring phosphoryl donor in this reaction is not known. Phosphoguanidates (creatine-phosphate, phosphoarginine, etc.) are well known phosphagens in mammalian systems but are inactive with the *E. coli* enzyme. Monophosphoramidates have been considered from time to time as possible biosynthetic intermediates but their occurrence in biological systems has not been documented. It may be that the marked instability of these compounds[8] precludes their isolation from biological materials by the usual phosphate analysis procedures.

An interesting feature of the phosphoramidic hexose transphosphorylase reaction, and one that tends to differentiate it from the previously studied phosphoryl transfers involving creatine-phosphate[2,3], is its acceptor specificity. In the present work only hexoses, and to a lesser extent sedoheptulose, are phosphorylated, while a wide variety of other hydroxylic compounds were completely inactive.

Phosphoramidic hexose transphosphorylase is associated with phosphoramidase-I (see ref. 1) throughout the extent of the present fractionation of these enzymes. However, the differences in properties of these enzymes suggest that they are not identical proteins. For example, phosphoramidic hexose transphosphorylase is markedly inhibited by fluoride ion, while phosphoramidase-I is slightly stimulated by the same levels of KF. Phosphoramidase-I is relatively unstable and its catalytic activity is lost on storage (50% loss in 14 days at $-15°$) while phosphoramidic hexose transphosphorylase is unaffected by short storage. Finally, the elution pattern shown in Fig. 2 for phosphoramidic hexose transphosphorylase and phosphoramidase-I indicates partial separation of these activities. Phosphoramidic hexose transphosphorylase is not found to any extent in the fractions rich in phosphoramidase-II (see ref. 1).

The observation that certain preparations of phosphoramidic hexose transphosphorylase tend to produce glucose 6-phosphate as well as glucose 1-phosphate suggests that an additional enzyme, accounting for a transformation of glucose 1-phosphate to glucose 6-phosphate, contaminates some of these fractions. When glucose 1-phosphate is used as a substrate in a system containing both fraction D (Table I) and glucose 6-phosphate dehydrogenase, TPN^+ reduction occurs only when free glucose is added to the reaction mixture. No TPNH is formed in the absence of either glucose 1-phosphate or glucose. Further experiments have been performed with this second transfer enzyme and will be reported in a subsequent paper.

Since the exchange experiment (Table V) failed to demonstrate the reversibility

of Eqn. 1, some preliminary experiments on Pi production from glucose 1-phosphate, with preparations known to contain phosphoramidase-I, have been made. Thus far no stimulation of Pi production by ammonia, glycine, or histidine has been observed. If phosphoramidates can be compared to creatine-phosphate, it is possible that the equilibrium position of Eqn. 1 may be sufficiently far to the right that the reverse reaction may not be easily demonstrated by experiments of the type outlined above.

Further work will be required to elucidate the detailed mechanism of the phosphoramidic hexose transphosphorylase reaction. At present the reaction may be pictured as an attack of an acetal hydroxyl group on the P–N linkage, forming in the case of glucose an acetal phosphate, and liberating the amine. Whether this mechanism will also account for the observed transfer from carbamyl-phosphate and acetyl-phosphate is not presently known.

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