METABOLISM OF PHOSPHORAMIDATES

I. ENZYMIC HYDROLYSIS AND TRANSFER REACTIONS

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SUMMARY

Extracts of *Escherichia coli* grown on succinate salts medium contain at least two phosphoramidases which can be largely separated by ammonium sulfate fractionation. Phosphoramidase-I has been purified about 110-fold and shows maximal activity at pH 5.1. This enzyme shows no metal or other cofactor requirements. Phosphoramidase-II shows maximal activity at pH 7.5 and also requires Mg^{2+} ions and a sulfhydryl compound for maximal activity. In addition to the hydrolytic activities, an enzymic phosphoryl transfer from phosphoramidate to glucose was shown.

INTRODUCTION

Since the demonstration by MEYERHOF AND LOHMANN[1] of an enzymic hydrolysis of N-phosphoryl compounds, a number of reports have appeared describing phosphoramidases from various sources[2-6]. While it is clear that a wide variety of P–N compounds are enzymically hydrolyzed, information concerning the possible identity of O-phosphomonoesterases and phosphoramidases is somewhat less conclusive. SINGER AND FRUTON[5] suggested that a phosphoramidase preparation purified from beef spleen, which catalyzed the cleavage of several phosphoramidates as well as phosphoproteins and aryl phosphates, was probably distinct from simple non-specific phosphomonoesterase.

In addition to the hydrolysis of phosphoramidates, enzymic alcoholysis of phosphoramidates has been observed with either glucose or glycerol serving as phosphoryl acceptor molecule. GREEN AND MEYERHOF[7] and more recently MORTON[8] have investigated such transfer activities using either acid or alkaline phosphatase preparations and creatine phosphate as phosphoryl donor. MØLLER[4] has also demonstrated phosphoryl transfer from PNH_2 to glycerol catalyzed by the seminal fluid phosphoramidase.

The enzymic hydrolysis of PNH_2 observed using extracts of *Escherichia coli* was the subject of an earlier report from this laboratory[6]. The presence of two easily separable phosphoramidases has been shown in extracts of succinate grown *E. coli*. One of these enzymes, phosphoramidase-I, has a pH optimum of 5.1 and shows no

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

other requirements. Phosphoramidase-II has a pH optimum of 7.5 and requires the addition of both magnesium ions and a mercaptan for maximal activity. In addition to these hydrolytic activities, phosphoryl transfer from PNH_2 to glucose has also been demonstrated with *E. coli* extracts[9]. The partial purification and properties of the hydrolytic enzymes and the enzymic assay of phosphoryl transfer from PNH_2 are reported in this paper.

MATERIALS AND METHODS

Bacteriological

E. coli, Crookes strain, was grown at 30° in a medium containing in grams per liter: sodium succinate, 5.0; KH_2PO_4, 1.5; Na_2HPO_4, 13.5; $FeSO_4 \cdot 7H_2O$, 0.005; $MgSO_4 \cdot 7H_2O$, 0.2; and yeast extract (Difco) 0.1. Fifteen liters of medium was autoclaved in a 5-gallon Pyrex carboy, cooled, and inoculated with 1 l of a culture grown overnight in the same medium on a shaker. The 15-l culture, when grown in a laboratory fermentor with continuous aeration and agitation, reached maximal growth in about 6 h. The cells were harvested with a Sharples centrifuge and stored frozen until needed; yield: 6–7 g of wet cells per liter.

Extracts of the frozen cell paste were prepared by crushing 20-g batches in a modified Hughes Press followed by suspension of the extract in 100 ml of 0.02 *M* potassium phosphate buffer, pH 7.2, containing 0.5 mg of GSH/ml. Treatment of the suspended extract in a 9 kc Raytheon oscillator for 5 min was found necessary to reduce the viscosity of the preparation. Cellular debris was removed from the extract by centrifugation at 32000 × *g* for 40 min at 5°. All subsequent enzyme fractionations of the supernatant fluid were performed at 0 to 5° and are described in the text.

Enzyme assays

Phosphoramidases were assayed by estimating the difference in rates at which NH_3 was released from PNH_2 in the presence and absence of enzyme. In the case of phosphoramidase-I the assay tube contained 5 μmoles of PNH_2, 100 μmoles of EDTA buffer, pH 5.5, and enzyme in a total volume of 0.5 ml. For phosphoramidase-II the assay tubes contained 100 μmoles of Tris buffer, pH 7.5, 5 μmoles of $MgCl_2$, 10 μmoles of GSH or cysteine, 10 μmoles of PNH_2, and enzyme in a total volume of 1 ml. In both assays the reaction components were preincubated at 37° for 5 min. The reaction was started by the addition of enzyme, and after 10 min incubation was stopped by the addition of an equal volume of absolute alcohol. The reaction mixture was well mixed and a 0.2-ml or 0.5-ml sample was placed in a Conway microdiffusion dish containing 1 ml of 4 *M* K_2CO_3 in the outer chamber and 1 ml of 0.05 *M* H_2SO_4 in the center well. After the lid was sealed in place, the K_2CO_3 and reaction sample were mixed and incubated at 47° for 1 h. In control runs complete recovery of 5 μmoles of NH_3 was obtained after 45 min by this procedure. After the Conway dishes were removed from the incubator, 0.5 ml of the acid solution was removed from the center well and the NH_3 content was estimated by Nesslerization[10]. The proportionality of the phosphoramidase-I assay with respect to time and protein is shown in Fig. 1.

Phosphatase activity of several protein fractions was tested by using the above protocols and substituting the appropriate phosphate compound for phosphoramidate. In this case reactions were stopped by the addition of 0.2 ml of 25% trichloroacetic

acid, and the inorganic phosphate present in a sample of the supernatant fluid was determined[11] after removal of precipitated protein by centrifugation.

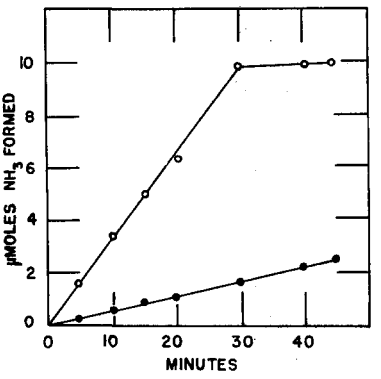

Fig. 1a.

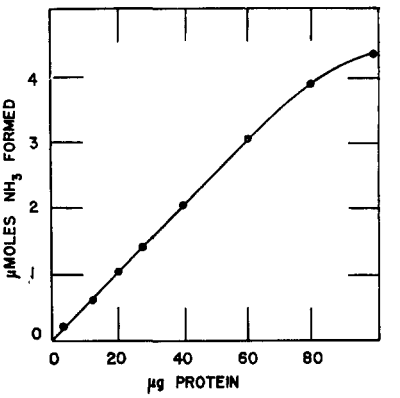

Fig. 1b.

Fig. 1A, Phosphoramidate cleavage as a function of time. The reaction mixture is as described in the text for phosphoramidase-I. O—O, in the presence of enzyme (60 μg protein, specific activity = 50). ●—●, the non-enzymic hydrolysis of PNH_2 at pH 5.5. 1B, Phosphoramidate cleavage as a function of enzyme concentration. Conditions as in Fig. 1A.

Since we were unable to differentiate between Pi and either PNH_2 or P-Ngly by even the mildest methods, such as those of either LOWRY AND LOPEX[12] or BERENBLUM AND CHAIN[13], another procedure was followed. [^{32}P]PNH_2 and [^{32}P] P-Ngly were synthesized (see below) and used as substrates. At the end of the incubation period 10 μmoles of Pi, 50 μmoles of $MgCl_2$, and 250 μmoles of NH_4Cl were added. The reaction mixture was made slightly alkaline with NH_4OH (pH 8.5 to 9.0) and the precipitate formed after 30 min at 0° was packed by centrifugation and washed three times with very dilute ammonium hydroxide. The precipitate was finally dissolved in 2 ml of 0.1 *N* HCl and the radioactivity present determined. Comparisons of this assay method, using [^{32}P]PNH_2, with the ammonia release method outlined above, indicated its validity (see Table II).

One unit of phosphoramidase or phosphatase activity is defined as the amount of enzyme required to form 1 μmole of NH_3 or inorganic phosphate in 10 min in the assay procedures outlined above. Protein measurements were made by either the trichloroacetic acid method of STADTMAN[15] or spectrophotometrically[16].

Chemical preparations

PNH_2 and $P(NH_2)_2$ were prepared by the methods of STOKES[17,18], adenylic 5′-phosphoramidate by the method of CHAMBERS AND MOFFATT[19], and P-Ngly by the method of ZERVAS AND KATSOYANNIS[20]. All other compounds used in these experiments were commercial preparations. [^{32}P]PNH_2 and [^{32}P]P-Ngly were prepared by the above methods after conversion of $H_3{}^{32}PO_4$ to $^{32}POCl_3$ by the method of MURRAY AND SPINKS[21] followed by its conversion to [^{32}P]diphenylphosphorchloridate by the method of BAER[22]. Trimetaphosphimate and diimidotriphosphate were generously supplied by Dr. O. T. QUIMBY.

RESULTS

A rapid liberation of NH_3 from PNH_2 is catalyzed by extracts of *E. coli* grown on succinate as sole carbon source. Paper chromatography (Whatman No. 1 paper; solvent, methanol–formic acid–H_2O (80:15:5, v/v)) of a sample of a reaction mixture after enzymic deamination of [^{32}P]PNH_2 at pH 7.5 with Mg^{2+} ions present revealed ^{32}Pi as the only radioactive reaction product. This result does not exclude the possibility, in analogy with the known non-enzymic cleavage[21], of intermediate PPi formation during the enzymic breakdown of PNH_2. In an additional experiment both [^{32}P]PNH_2 and PPi (5 μmoles each) were incubated with the extract at pH 7.5. After analysis by paper chromatography no radioactivity was found in the remaining PPi.

The phosphoramidase system of the crude *E. coli* extracts is partially inhibited by fluoride (10^{-2} *M*), EDTA (10^{-2} *M*), and by *p*-chloromercuribenzoate ($2 \cdot 10^{-4}$ *M*). Only 60 to 70% inhibition was observed with these reagents even at markedly higher concentrations. Since these results could be interpreted in several ways, further purification of the phosphoramidase activity was undertaken.

Enzyme fractionation

All fractionations of the succinate grown *E. coli* extracts were carried out at 0 to 5° and all fractions were dissolved in potassium phosphate cysteine buffer pH 7.4 (0.02 *M* potassium phosphate and 0.001 *M* cysteine) and dialyzed against 2 changes of this buffer prior to assay.

The first ammonium sulfate fractionation was carried out in the following manner: for each 100 ml of extract 16 g of solid ammonium sulfate was added slowly with stirring. Fifteen minutes after the final addition of ammonium sulfate, the precipitated protein was removed by centrifugation at 25000 $\times$ *g* for 15 min, and discarded. An additional 46 g of solid ammonium sulfate for each 100 ml of original extract was added to the supernatant solution in the same manner and the precipitate was collected by similar centrifugation and dissolved in phosphate buffer.

The solution was carefully adjusted to pH 6.0 by the dropwise addition of 1 *N* acetic acid and about 0.1 volume of a protamine sulfate solution (20 mg/ml) was added slowly with rapid stirring. The whole mixture was dialyzed overnight against the usual phosphate buffer and the protamine nucleate precipitate removed by centrifugation. The supernatant fraction was normally prepared in large quantity and stored at — 15°, with small portions being used for further fractionation.

The second ammonium sulfate fractions were prepared as follows: usually about 300 ml of the protamine treated extract was further fractionated with solid ammonium sulfate into four subfractions. These fractions were obtained as outlined above, after the successive addition of 63 g (fraction A), 33 g (fraction B), 30 g (fraction C), and 63 g (fraction D) of solid ammonium sulfate. Both fraction B and fraction D showed considerable phosphoramidase activity. Fraction D was used for further purification of the phosphoramidase activity. However, fraction B was normally used at this level of purification, since further attempts at fractionation markedly decreased the stability of the activity present.

Fraction D was dialyzed overnight against 0.02 *M* Tris buffer, pH 7.7, at 4° and applied to a column of DEAE-cellulose (2.5 cm by 40 cm) which had been previously

* Obtained from the Brown Company, Berlin, New Hampshire.

converted to the hydroxide phase with 1 l of 1 *M* KOH followed by continued washing with water at 4° until the eluate was neutral. Fraction D (1.25 mg of protein/ml of DEAE-cellulose) was run onto the column followed by 200 ml of distilled water. After the water had run through the column, elution of the enzyme was begun at a flow rate of about 1 ml/min. A linear elution gradient[23] was obtained by the use of a mixing flask containing 1.5 l of 0.02 *M* Tris pH 7.7 and a reservoir containing 1.5 l of the same buffer containing 0.2 *M* KCl. Phosphoramidase activity was generally found in that part of the elution pattern corresponding to a concentration of KCl from 0.1 to 0.12 *M*. At this stage the phosphoramidase preparation was usually about 50 to 75 fold purified. An additional 2 to 3 fold purification was frequently but not routinely obtained by passing the enzyme eluate from the DEAE column over a CM-cellulose column (H^+ phase) using a linear gradient of 0.01 to 0.08 *M* acetate buffer pH 5.1.

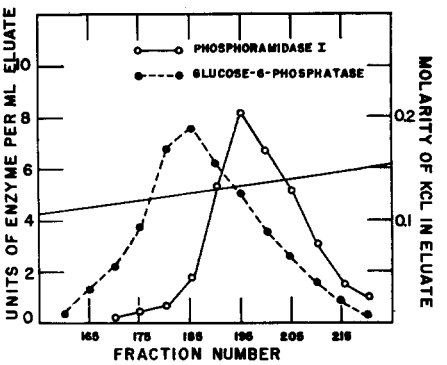

Fig. 2. Elution pattern of phosphoramidase-I and glucose 6-phosphate phosphatase from DEAE-cellulose. Conditions as described in the text. ———, the KCl gradient used in eluting the activities.

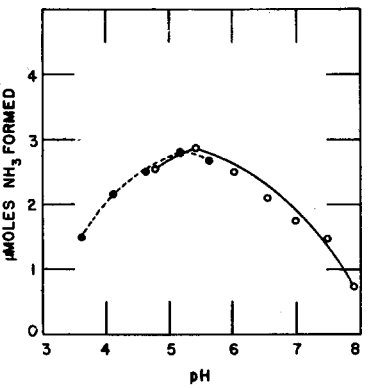

Fig. 3. Effect of pH on the cleavage of phosphoramidate by phosphoramidase-I. Conditions are as described in the text, ●—●, acetate buffer, ○—○, phosphate buffer.

A typical elution pattern for phosphoramidase-I from DEAE-cellulose is shown in Fig. 2. Also shown on this curve is the elution pattern for glucose 6-phosphate phosphatase. These results in addition to inhibitor studies (see below) suggest that phosphoramidase and glucose 6-phosphate phosphatase are different enzymes. Large scale separation of the two enzymes was not routinely achieved. However, phosphoramidase-I generally preceded glucose 6-phosphate phosphatase from the CM-cellulose column and at least several tubes of eluate containing phosphoramidase-I were essentially free of glucose 6-phosphatase. The purification results are summarized in Table I. An overall purification of phosphoramidase-I of about 110 fold is shown although occasionally enzyme fractions as much as 150 to 200 fold purified over the crude material were obtained.

Phosphoramidase-I was stored either at 0° in acetate buffer, pH 5.5 or Tris buffer pH 7.5, or in the same buffers at —15°, and lost 50% of its activity in 14 days under all of these conditions.

Most of the experiments reported in this paper have been carried out either with the phosphoramidase obtained by DEAE-cellulose chromatography (phosphor-

amidase-I) or with fraction B which had been aged (30 days or more at — 15°) and is called phosphoramidase-II.

pH and cofactor dependence

Phosphoramidase-I has a broad pH range of activity with maximal activity at pH 5.1 (Fig. 3) and approx. 50% of maximal activity at pH 3.5 and pH 7.5. Most of our experiments with this enzyme were run in either acetate or EDTA buffer at pH 5.5 since PNH_2 is slightly more stable at this pH than at more acid values. No other requirements could be shown.

TABLE I

FRACTIONATION OF PHOSPHORAMIDASES OF *E. coli* EXTRACTS

	Fraction	Protein (mg)	Phosphoramidase-I		Phosphoramidase-II	
			(units × 10^{-3})	Specific* activity	(units × 10^{-3})	Specific** activity
1	Extract	3500	5.67	1.62	2.1	0.6
2	Ammonium sulfate-I	2620	5.30	2.1	—	—
3	Protamine treated	1820	4.65	2.55	—	—
4	Ammonium sulfate-II, fraction B	512	0.71	1.40	2.14	4.2
5	Ammonium sulfate-II, fraction D	360	1.88	5.20	0	0
6	DEAE-cellulose eluate from fraction 5	20	1.25	63.0	0	0
7	CM-cellulose eluate from fraction 6	3	0.525	175.0	0	0

* Activity of phosphoramidase-I assayed as in text at pH 5.5. Specific activity = units/mg protein.

** Activity of phosphoramidase-II assayed as in text with added $MgCl_2$ and GSH at pH 4. These figures represent an increase in the activity of a given fraction when $MgCl_2$ and GSH were added, but probably do not indicate the total activity of phosphoramidase-II.

TABLE II

SUBSTRATE SPECIFICITY OF PHOSPHORAMIDASE-I

The reaction mixture contained in a total volume of 1 ml, EDTA, pH 5.5, 100 μmoles; substrate as indicated either 5 or 10 μmoles; phosphoramidase-I, 35 μg, specific activity 75.

Substrate	Cleavage in 10 min (μmoles)
Phosphoramidate*	2.6
[^{32}P]phosphoramidate**	2.65
N-phosphorylglycine**	2.7
Creatine phosphate***	0.1
Phosphordiamidate*	0
Trimetaphosphimate*	0.2
Diimidotriphosphate*	0
Adenylic 5'-phosphoramidate*	0.1
Fructose 1-phosphate***	1.3
Glucose 1-phosphate***	1.2
Glucose 6-phosphate***	1.1
Fructose 1,6-diphosphate***	1.0
Ribose 5-phosphate***	0.07
p-Nitrophenylphosphate§	0.1
Casein***	0
Glutamine*	0

* Measured as NH_3 released.

** Measured as ^{32}Pi released.

*** Measured as Pi released[12].

§ Measured as *p*-nitrophenol released.

Substrate specificity

Several phosphoramidates, O-phosphomonoesters, phosphate anhydrides, and amides were tested as substrates for phosphoramidase-I. As shown in Table II, PNH_2 and P-Ngly were rapidly cleaved while hexose phosphates were cleaved at somewhat reduced rate. Some fractions of the eluate from CM-cellulose were virtually inactive toward glucose 6-phosphate and completely inactive toward *p*-nitrophenyl phosphate, but contained a very active phosphoramidase. Other substrates tested with this preparation but not cleaved at a significant rate (less than 0.1 μmole of substrate cleaved in 30 min by 3 units of enzyme) include: phosphopyruvate, acetyl phosphate, UMP, AMP, 3′ AMP, GMP, ATP, CTP, UTP, PPi, tripolyphosphate, trimetaphosphate, tetrametaphosphate, hexametaphosphate, phosphoserine, β-glycerophosphate, monothiolphosphate, 3-phosphoglycerate, and sulfamic acid.

A plot of the reciprocal of velocity versus the reciprocal of PNH_2 concentration was linear and extrapolated to a Michaelis constant, K_m, of $1.1 \cdot 10^{-3}\,M$.

Effect of inhibitors

Phosphoramidase-I was not inhibited by *p*-chloromercuribenzoate (10^{-5} to $5 \cdot 10^{-4}\,M$), diisopropylfluorophosphate ($1.25 \cdot 10^{-3}\,M$) or by fluoride ion (10^{-3} to $5 \cdot 10^{-2}\,M$). Nor were Pi, ammonia or arsenate effective inhibitors of phosphoramidase-I activity. Fluoride ion was partially effective in inhibiting the glucose 6-phosphate phosphatase activity present in the DEAE-cellulose eluates used in this study (Table, III). However, low fluoride ion concentrations stimulated ammonia release from PNH_2 as much as 30%. Although this effect was observed with all phosphoramidase-I preparations tested, it is not readily explainable. Possible formation of monofluorophosphate was considered but neither paper chromatography nor paper electrophoresis of a reaction mixture showed the presence of this compound.

TABLE III

EFFECT OF INHIBITORS ON *E. coli* PHOSPHORAMIDASES

The reaction mixture for phosphoramidase-I contained in 1 ml, acetate buffer, pH 5.5, 100 μmoles; PNH_2, 5 μmoles or glucose 6-phosphate, 8 μmoles; phosphoramidase-I, 35 μg. Incubation 10 min at 37°. The reaction mixture for phosphoramidase-II contained in 1 ml, Tris buffer, pH 7.4, 100 μmoles; $MgCl_2$, 3 μmoles; GSH, 10 μmoles; PNH_2, 10 μmoles; enzyme, 1.35 mg protein, fraction B, Table II. Incubation 10 min at 37°.

Additions		*Cleavage by phosphoramidase-I*		*Cleavage by phosphoramidase-II*
	(μmoles)	*PNH_2 (μmoles)*	*Glucose 6-phosphate (μmoles)*	*PNH_2 (μmoles)*
None		2.7	2.0	5.4
KF	1	3.1	1.9	4.3
KF	3	3.5	1.65	1.1
KF	10	3.1	1.0	0
KF	20	2.9	0.65	0
PCMB*	0.1	2.8	2.05	1.3
Pi	50	2.7	1.9	2.1
EDTA	10	2.8	2.0	2.0

* The complete system lacked GSH when *p*-chloromercuribenzoate was added. In the absence of both PCMB and GSH 3.5 μmoles of PNH_2 was hydrolyzed by phosphoramidase-II.

In an experiment in which 0.5 unit of phosphoramidase-I was incubated with both PNH_2 and glucose 1-phosphate (5 μmoles of each) a 30% inhibition in NH_3 release was observed. Similar experiments in which glucose 6-phosphate or fructose 1, 6-diphosphate were incubated with PNH_2 did not show inhibition of NH_3 release.

Phosphoramidase-II

A second P–N bond cleavage system was found during the preliminary fractionation of the *E. coli* extract with ammonium sulfate. As shown in Table IV, fraction B aged 30 days at 0° showed no phosphoramidase-I activity as measured in the absence of $MgCl_2$ and a mercaptan at either pH 5.5 or 7.5. However, when supplemented with $MgCl_2$ and a mercaptan, considerable phosphoramidase activity was observed.

TABLE IV

HYDROLYSIS OF PHOSPHORAMIDATES BY PHOSPHORAMIDASE-II

The reaction mixture contained in a total volume of 1 ml: Tris buffer pH 7.4, 100 μmoles; $MgCl_2$, 3 μmoles; GSH, 10 μmoles; P-N compound, 10 μmoles; enzyme, 1.35 mg protein, fraction 4, Table I. Incubation 37°.

Conditions	*Substrate*	*Hydrolysis (μmoles/10 min)*
Complete	PNH_2*	5.7
Minus $MgCl_2$	PNH_2*	0.1
Minus $MgCl_2$ (pH 5.5)	PNH_2*	0.15
Minus GSH	PNH_2*	3.3
Minus $MgCl_2$ plus $MnCl_2$	PNH_2*	1.6
Minus enzyme	PNH_2*	0
Complete	P-Ngly**	2.75
Complete	Creatine-P***	0
Complete	AMP-NH_2§*	0.3
Complete	$P(NH_2)_2$*	0.2

* Cleavage was determined as ammonia by micro Conway distillation.
** Determined as ^{32}Pi released.
*** Determined as Pi (see ref. 12).
§ Adenylic 5′-phosphoramidate.

Although other thiols (cysteine, β-mercaptoethanol) could be substituted for GSH in the phosphoramidase-II assay, no other divalent metals tested (Zn^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+}) were effective with this enzyme. Phosphoramidase-II has a fairly broad pH activity curve with a slight maximum at pH 7.5.

Substrate specificity

Both PNH_2 and P-Ngly were rapidly cleaved by phosphoramidase-II (Table IV) while adenylic 5′-phosphoramidate was only slowly cleaved. As with the previous enzyme preparation, neither creatine phosphate nor phosphordiamidate were substrates. Several other enzymes were present in the phosphoramidase-II preparation but further purification of this activity was not attained since the several treatments tested led to complete inactivation of the enzyme.

Effect of inhibitors

Phosphoramidase-II was inhibited by a variety of compounds including fluoride,

Pi, arsenate, and *p*-chloromercuribenzoate (Table III). In spite of their inhibitory effects it was found that either Pi, arsenate, or borate ions were necessary at low concentrations to preserve the phospharamidase-II activity on storage.

Transphosphorylation

Earlier results[9] demonstrated that an enzyme catalyzed phosphoryl transfer from PNH_2 to various hexoses could be observed with extracts of *E. coli*. Further investigation of the system revealed that the transfer reaction could easily be measured by coupling with purified glucose 6-phosphate dehydrogenase when glucose served as an acceptor. Fig. 4 shows the rate of TPN^+ reduction with a coupled system containing PNH_2 or P-Ngly, glucose, glucose 6-phosphate dehydrogenase, and an active protein fraction obtained from *E. coli*, (fraction D, Table I). No TPN^+ reduction was observed if any of the components of the system were omitted, and no increase in the rate of reaction was observed by the addition of either ADP or ATP to the system. When ATP was substituted for PNH_2 in the coupled system, no TPN^+ reduction was observed.

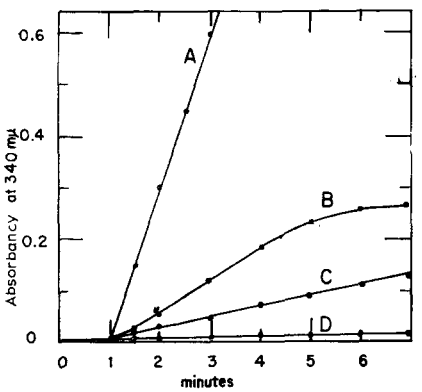

Fig. 4. Rate of TPNH formation with the *E. coli* phosphoryl transfer, glucose 6-phosphate dehydrogenase coupled system. The complete reaction mixture in 1 ml, 1 cm light path cuvettes contained, in μmoles, the following components: Tris buffer, pH 7.4, 100; $MgCl_2$, 5; D-glucose, 30; TPN^+, 0.3; PNH_2 or P-Ngly, 5 (added at 1 min); *E. coli* enzyme, 0.3 mg protein (fraction D, Table II); glucose 6-phosphate dehydrogenase, 2 units[26]. Curve A, complete system; 5 μmoles PNH_2; curve B, 0.05 μmoles PNH_2; curve C, complete system plus 3 μmoles KF; curve D, no PNH_2 or P-Ngly. No TPNH formation was observed in the absence of either glucose or either of the two protein fractions.

Also, when yeast hexokinase was substituted for the *E. coli* fraction, TPN^+ reduction was observed only when ATP replaced PNH_2 or P-Ngly. Also shown in Fig. 4 is the sensitivity of the coupled system to fluoride. Nearly complete inhibition of the transfer reaction occurred with $3 \cdot 10^{-3}$ *M* KF, a level which does not significantly inhibit glucose 6-phosphate dehydrogenase.

Further purification and investigation of the transfer system was undertaken and is the subject of a subsequent paper.

DISCUSSION

The results reported suggest that at least two separate types of phosphoramidases occur in extracts of *E. coli* grown on succinate. Although PPi formation during the chemical hydrolysis of PNH_2 has been observed[24], careful chromatographic and radioisotopic analysis revealed no trace of PPi or phosphate compounds, other than Pi, in the enzymic hydrolyzates of PNH_2 or P-Ngly. Since a wide variety of polyphosphate compounds was found not to be hydrolyzed by phosphoramidase-I, the conclusion that the reaction catalyzed by this enzyme is a direct hydrolysis of the P–N bond, leading to Pi and NH_3, seems justified. PPi is slowly cleaved by fraction B (phosphor-

amidase-II) under the conditions of the standard assay for this activity. However, no incorporation of radioactivity into PPi occurred when 5 μmoles each of [^{32}P]PNH_2 and PPi were incubated with this enzyme under standard conditions. While these results do not entirely rule out possible PPi formation from PNH_2 by phosphoramidase-II, such a reaction would appear to be very minor in comparison to the overall hydrolysis.

The differences in activities toward glucose 6-phosphate and PNH_2, seen over the elution patterns from DEAE and CM-cellulose, and the difference in inhibition of these activities by fluoride, suggest that the amidase and phosphatase activities may be due to different enzymes. Complete separation of these activities will be required to substantiate this statement.

Enzymic phosphoryl transfer from compounds containing P–N bonds to various hydroxylic acceptors has been widely observed and is generally associated with a hydrolytic activity. Two characteristics of the transfer reaction reported here differentiate it from the phosphatase catalyzed phosphoryl transfers. First, only a relatively low carbohydrate concentration is required to demonstrate transfer with the *E. coli* preparation, while generally 1 to 2 *M* acceptor is required in the phosphatase catalyzed transfers[8]. Secondly, as shown in Fig. 4, 85% of the theoretical amount of reduced TPN^+ is observed when limiting PNH_2 is used as phosphoryl donor in the glucose 6-phosphate dehydrogenase coupled reaction.

Although PNH_2 was suggested by SPECK[25] as an intermediate in the glutamine synthetase system, its use in this system led to inconclusive results. In view of the widespread occurrence of phosphoramidase activity[1-6], it seems likely that other metabolic functions of compounds containing P–N bonds may be revealed only after removal of the enzymes which hydrolyze these linkages.

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REFERENCES

1 O. MEYERHOF AND K. LOHMANN, *Biochem. Z.*, 196 (1928) 22.
2 M. ICHIHARA, *J. Biochem, (Tokyo)*, 18 (1933) 87.
3 H. HOLTER AND S. O. LI, *Compt. rend. trav. Lab. Carlsberg*, série chim., 27 (1951) 393.
4 K. M. MØLLER, *Biochim. Biophys. Acta*, 16 (1955) 162.
5 M. F. SINGER AND J. S. FRUTON, *J. Biol. Chem.*, 229 (1957) 111.
6 R. A. SMITH AND D. J. BURROW, *Biochim. Biophys. Acta*, 34 (1959) 274.
7 H. GREEN AND O. MEYERHOF, *J. Biol. Chem.*, 197 (1952) 347.
8 R. K. MORTON, *Nature*, 172 (1953) 65.
9 R. A. SMITH, *J. Am. Chem. Soc.*, 81 (1959) 4758.
10 E. J. CONWAY AND A. BYRNE, *Biochem. J.*, 27 (1933) 419.
11 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
12 O. H. LOWRY AND J. A. LOPEZ, *J. Biol. Chem.*, 162 (1946) 421.
13 I. BERENBLUM AND E. CHAIN, *Biochem. J.*, 32 (1938) 295.
14 T. RATHLEV AND T. ROSENBERG, *Arch. Biochem. Biophys.*, 65 (1956) 319.
15 E. R. STADTMAN, G. D. NOVELLI AND F. LIPMANN, *J. Biol. Chem.*, 191 (1951) 365.
16 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1941) 384.
17 H. N. STOKES, *Am. Chem. J.*, 15 (1893) 198.
18 H. N. STOKES, *Am. Chem. J.*, 16 (1894) 123.
19 R. W. CHAMBERS AND J. G. MOFFATT, *J. Am. Chem. Soc.*, 80 (1958) 3752.

[20] L. ZERVAS AND P. G. KATSOYANNIS, *J. Am. Chem. Soc.*, 77 (1955) 5351.
[21] D. H. MURRAY AND J. W. T. SPINKS, *Can. J. Chem.*, 30 (1952) 497.
[22] E. BAER, *Biochem. Preparations*, 1 (1949) 50.
[23] R. M. BOCK AND N. LING, *Anal. Chem.*, 26 (1954) 1543.
[24] O. T. QUIMBY, A. NARATH AND F. H. LOHMANN, *J. Am. Chem. Soc.*, 82 (1960) 1099.
[25] J. F. SPECK, *J. Biol. Chem.*, 179 (1949) 1405.
[26] A. KORNBERG AND B. L. HORECKER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. I, Academic Press, Inc., New York, 1955, p. 323.

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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.