*PHOSPHATE BOUND TO HISTIDINE IN A PROTEIN AS AN INTERMEDIATE IN A NOVEL PHOSPHO-TRANSFERASE SYSTEM**

By Werner Kundig,† Sudhamoy Ghosh,‡ and Saul Roseman

RACKHAM ARTHRITIS RESEARCH UNIT AND DEPARTMENT OF BIOLOGICAL CHEMISTRY, UNIVERSITY OF MICHIGAN, ANN ARBOR

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Mammalian tissues contain a kinase involved in the intermediary metabolism of the sialic acids.[1, 2] This enzyme has been extensively purified,[3] studied in detail, and catalyzes the following reaction: *N*-Acyl-D-mannosamine + ATP $\xrightarrow{Mg^{++}}$ *N*-Acyl-D-mannosamine-6-P + ADP. To determine whether this kinase occurred in bacteria, such as *Aerobacter cloacae* and *Escherichia coli* K235,[4] that metabolize *N*-acetyl-D-mannosamine, extracts of these organisms were examined and found to contain a novel phospho-transferase system. The system obtained from *E. coli* K235 consisted of two enzymes, I and II, and a histidine-containing, heat-stable protein (HPr). The sequence of reactions is:

$$\text{Phosphoenolypyruvate (PEP)} + \text{HPr} \xrightleftharpoons[Mg^{++}]{I} \text{Phospho-histidine-protein (P-HPr)} + \text{Pyruvate} \quad \text{(A)}$$

$$\text{P-HPr} + \text{Hexose} \xrightarrow[Mg^{++}]{II} \text{Hexose-6-P} + \text{HPr} \quad \text{(B)}$$

$$\text{PEP} + \text{Hexose} \xrightarrow[Mg^{++}]{I + II} \text{Hexose-6-P} + \text{Pyruvate} \quad \text{(A+B)}$$

The intermediate in the system, P-HPr, is protein-bound phosphohistidine.

Materials and Methods.—Unless otherwise specified, all materials were obtained from commercial sources. Previously published methods[1, 5] were used for the preparation, separation, and characterization of C^{12}- and C^{14}-hexosamines, *N*-acylhexosamines, the corresponding 6-phosphate esters, and for the periodate oxidation of the esters and the characterization of glycolaldehyde-phosphate. The following compounds were prepared as described: P-histidine,[6] N-phosphoglycine,[7] phosphoramidate,[8] and PEP.[9] An essential substrate for these experiments, P^{32}-PEP was prepared enzymatically by a published procedure[10] and with the invaluable help of Dr. M. F. Utter and Mr. Douglas Kerr, to whom we are most grateful.[11] The P^{32}-PEP (5–10 μmoles per experiment) contained 200–400 μc of P^{32} per μmole and was purified by ion-exchange chromatography; paper chromatography and electrophoresis indicated that it was homogeneous. It was diluted with unlabeled PEP prior to use.

Purification of enzymes I, and II, and HPr: The organism, *E. coli* K235, was grown to the stationary phase in Todd-Hewitt (Difco) broth supplemented with 1.5% glucose in a New Brunswick fermentor. Maximum yields of the phospho-transferase system were obtained when the culture was stirred during growth but without passage of air through the sparger. After washing with 1% KCl solution, the wet cell paste was stored at −18°. The cells were ruptured by sonic oscillation following suspension in 0.025 *M* phosphate buffer, pH 7.6 (containing 0.1% 2-mercaptoethanol and 10^{-3} *M* EDTA when enzymes I and II were desired).

After centrifugation, the supernatant fluid (crude extract) was treated with charcoal to remove HPr and fractionated for I and II as outlined in Table 1. The critical step was the C_γ alumina gel treatment since I was adsorbed while II was not; after washing the gel with 0.01 and 0.05 *M* phosphate buffers, pH 7.6, I was eluted with 0.10 *M* buffer. These data suggest that both enzymes were purified approximately 300-fold. Since we have not yet determined which enzyme, I or II, was present at rate-limiting concentrations prior to their separation, the purification factor is correct for only one of these enzymes, and is not known for the other. However, the availability of the purified enzymes I and II will now permit accurate analysis for each enzyme.

TABLE 1

PURIFICATION OF ENZYMES I AND II

Fraction	Specific Activity* of I + II	Specific Activity* of I	Specific Activity* of II	Yield (%)
Crude extract	0.30	—	—	100
Charcoal filtrate	0.82	—	—	62
Ammonium sulfate (30–50%)	2.2	—	—	51
Cγ alumina gel:				
Supernatant		0	16	39
Eluate		19	0	41
DEAE-cellulose:				
Cγ supernatant		0	98	24
Cγ eluate		119	0	27

* Specific activity was defined as μmoles of *N*-acetylmannosamine-6-P formed per mg protein per 30 min. Each incubation mixture contained the following (in μmoles) in a final volume of 0.18 ml: C^{14}-acetyl-labeled *N*-acetyl-D-mannosamine, 2.0 (specific activity, 5×10^5 cpm/μmole); PEP, 2.5; $MgCl_2$, 2.5; Tris-HCl buffer, pH 7.4, 10; HPr, 20 μg; enzymes I and II. Following the Cγ alumina step, either I or II was added in rate-limiting amounts, and the other enzyme was added in excess. After incubating for 30 min at 37°, the mixtures were heated at 100° for 3 min, 25-μl samples of the supernatant fluids were spotted on Whatman #3 mm paper and electrophoresed in 0.05 *M* pyridinium acetate "buffer," pH 6.5, at 100 v/cm for 15 min. Under these conditions, the substrate remained at the origin while the product, *N*-acetylmannosamine-6-P, migrated approximately 10 cm. Both areas were cut from the paper strip and counted by liquid scintillation techniques. Controls consisted of mixtures lacking I, II, or HPr or contained heat-denatured I or II. In each case, product formation was shown to be linear with time and proportional to the concentration of I or II (or to I + II in the first 3 fractions).
The 0 values indicate no detectable activity (i.e., less than 0.05).

Based on analysis by a modified biuret protein method,[12] HPr was purified 8,000- to 10,000-fold as follows: the crude extract was heated for 10 min at 100°; the chilled supernatant fluid was adjusted to pH 1; the resulting precipitate was washed with 0.01 *M* HCl; HPr activity was extracted with 0.5 *M* phosphate buffer, pH 7.6, dialyzed 24 hr against 0.025 *M* phosphate buffer, pH 6.5, passed over a column of Ecteola-cellulose that did not adsorb HPr, and finally fractionated by adsorption and elution on two successive columns of DEAE-cellulose. The final elution was conducted with a shallow gradient of 0–0.10 *M* KCl in 0.01 *M* Tris buffer, pH 7.6. Following the elution of inactive protein, HPr activity was eluted in a single symmetrical protein peak where the specific activity of each fraction was essentially constant.[13]

Kinetic Properties and Specificity of the Complete System.—When the assay was conducted as described in Table 1, the addition of 5 μg each of purified enzymes I and II gave 0.5 μmole of *N*-acetylmannosamine-6-P in 30 min. Omission of I, II, HPr, or Mg^{++} gave no detectable (i.e., less than 0.005 μmole) product. Substitution of heated I or II for the active proteins also gave negative results. Each of the three proteins could be made the rate-limiting factor, and in each case (and throughout the purification steps) product formation was linear with time for at least 2 hr. The pH optimum for the complete system was 7.2–7.4, and the approximate K_m values for PEP and *N*-acetylmannosamine were 6×10^{-4} *M*. The following D-sugars could substitute for *N*-acetylmannosamine and exhibited the following approximate K_m values ($M \times 10^4$): glucose, 4; mannose, 20; glucosamine, 30; mannosamine, 10; *N*-acetylglucosamine, 9; *N*-glycolylmannosamine, 20. The ratios of activities obtained with glucose, mannose, *N*-acetylglucosamine, and *N*-acetylmannosamine as phosphate acceptors were approximately constant over the entire range of purification. The following D-sugars did not act as phosphate acceptors in this system: galactose, galactosamine, *N*-acetylgalactosamine, fructose, xylose, arabinose, ribose, glucose-1-P, and glucose-6-P.

The following compounds could not replace PEP as the phosphoryl donor: mono-, di-, and triphosphates (all 5′) of adenosine, deoxyadenosine, guanosine, deoxyguanosine, cytidine, deoxycytidine, thymidine, uridine, and inosine (alone or in mixture with other nucleotides); cyclic 3′,5′-AMP; creatine-P (with and without creatine-P transphosphorylase ± ADP or ATP); PP_i; P_i; phosphor-

amidate; phosphohistidine; *N*-phosphoglycine; thiamine-PP; P-glycerate; P-serine; coenzyme A and glutathione; coenzyme A + succinate ± P_i. Moreover, these compounds did not affect the rate of the reaction in the presence of PEP.

The following divalent cations could either partially or completely replace Mg^{++}: Mn^{++}, Zn^{++}, Co^{++}. In addition, Ca^{++} and Cu^{++} were highly inhibitory in this system at concentrations where the other cations were active.

Nonparticipation of Nucleotides.—As indicated above, the purified proteins exhibited activity only with PEP as the initial phosphoryl donor. By contrast, the crude extracts were fully active with P-glycerate and 10–20 per cent as active with ATP and creatine-P in place of PEP. Only PEP, however, was active in the crude system in the presence of 0.01 *M* KF. Additional evidence suggesting that PEP was the direct phosphoryl donor, and was not involved in a nucleotide triphosphate generating system was obtained as follows: (1) Pyruvate kinase could not be detected in fractions I, II, or HPr. In addition, pyruvate kinase in the presence or absence of varying concentrations of ADP could not substitute for any of the indicated protein fractions. (2) Creatine-P and creatine-P-ATP-transphosphorylase did not substitute for PEP in the presence or absence of ADP. Under the same conditions with ADP, crystalline yeast hexokinase readily phosphorylated glucose. (3) Addition of excessive quantities of fructose and hexokinase to PEP, I, II, and HPr gave no detectable fructose-6-P. The addition of varying quantities of ADP (10^{-6} to 10^{-2} *M*) to this mixture also gave negative results. The presence of fructose, hexokinase, and ADP did not affect the rate of *N*-acetylmannosamine phosphorylation when the latter was added to the mixture. (4) Various concentrations of ATP (10^{-6} to 10^{-2} *M*) did not replace PEP or any of the three protein components necessary for the system; in addition, the ATP did not affect the rate of reaction in the complete system. (5) The addition of purified venom 5′-nucleotidase and/or venom P-diesterase to the complete incubation mixture did not affect the rate of the reaction. (6) Proteins I, II, and HPr were each incubated in the presence and absence of PEP with C^{14}-labeled mono-, di- and triphosphates of adenosine, uridine, guanosine, and cytidine (specific activities: 28, 27, 12, and 13 $\mu c/\mu mole$, respectively; 10 μc total C^{14}-nucleotide were added to each protein fraction). Each of the three proteins retained full catalytic activity but were not radioactive after passing the fractions through Sephadex G-25 followed by pressure dialysis. Under the conditions used for the enzymatic assay (Table 1), C^{14}-nucleotide would have been detected at concentrations above 5×10^{-6} μmole per ml. Based on these experiments, particularly the C^{14} experiment, we conclude either that a nucleotide is not involved in these transfer reactions, or that such a nucleotide is firmly bound to one of the protein fractions and is not in equilibrium with nucleotide in the surrounding solution.

Characterization of Products.—As indicated above, a number of D-sugars of the gluco- and manno-configuration served as phosphate-acceptors. Three of the products have been characterized as the 6-phosphate esters. Analysis of the products isolated after phosphorylation of *N*-acetylmannosamine and *N*-acetylglucosamine showed the following molar ratios: *N*-acetylglucosamine, 1.00, P, 0.95; *N*-acetylmannosamine, 1.00, P, 0.95. These compounds gave the expected *N*-acetylhexosamines on treatment with phosphatase and yielded glycolaldehyde-P on oxidation with periodate. In addition, no P_i was liberated on treatment with

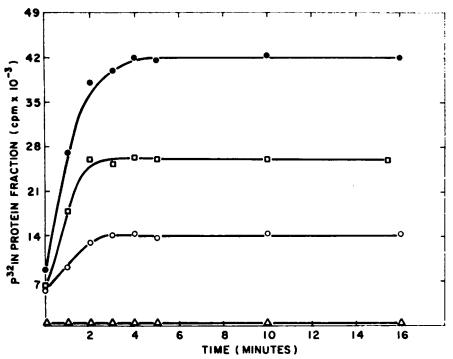

FIG. 1.—Rate of phosphate transfer from P^{32}-PEP to protein as a function of HPr concentration. Each incubation mixture contained the following (in μmoles) in a final volume of 0.45 ml: P^{32}-PEP 0.075 (specific activity, 10^7 cpm/μmole); $MgCl_2$, 1.5; Tris-HCl buffer, pH 7.4, 20; enzyme I, 21 μg; HPr, 50 μg (○), or 100 μg (□), or 150 μg (●). Controls (△) were mixtures lacking either I or HPr, or contained II or heat-denatured I in place of I or HPr. Aliquots of the mixtures (50 μl) were removed at the indicated times, frozen, and stored at −75°. Low-molecular-weight P^{32} components, like P^{32}-PEP, were separated from P^{32}-HPr by subjecting 25 μl of the samples to high-voltage electrophoresis (65 v/cm) in 0.05 *M* citrate buffer, pH 6.5 for 20 min. The product remained close to the origin; all radioactive areas were cut from the paper strips and counted in a gas-flow proportional counter.

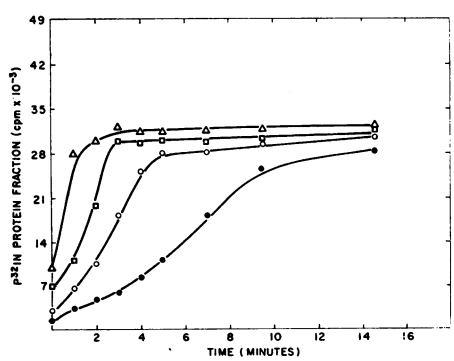

FIG. 2.—Rate of phosphate transfer from P^{32}-PEP to protein as a function of enzyme I concentration. Incubation mixtures were prepared as described in Fig. 1 except that complete mixtures contained 100 μg of HPr and enzyme I at one of the following levels (in μg): 4.5 (●); 9.0 (○); 21 (□); or 45 (△). Controls were the same as those shown in Fig. 1.

1 *M* HCl at 100° for 20 min. Finally, the product obtained from *N*-acetylmannosamine was fully active in the *N*-acetylneuraminic acid-9-P synthetase reaction.[14] The product formed from glucose was shown to be glucose-6-P by including TPN and G-6-P dehydrogenase in the phosphorylating system; TPN was immediately reduced to TPNH as determined spectrophotometrically. Phosphoglucomutase activity was not detected in this system.

Pyruvate was measured with lactic dehydrogenase and DPNH. With each of the seven sugars that served as P-acceptors in the complete system, 1.00 ± 0.03 mole of pyruvate was formed per mole of hexose-P.

Properties of HPr.—The purified material showed a typical protein ultraviolet absorption spectrum, was nondialyzable, and studies with Sephadex gels showed that it was not retarded by G-50, slightly retarded by G-100, and fully retarded by G-200. The following substances were not detected in the preparation and therefore, if present, would have to be at levels (based on protein content) of less than 0.01 per cent for phosphorus, anthrone-reactive hexose, anthrone-reactive pentose, or hexosamine, and less than 0.05 per cent thiobarbituric acid-reactive compounds (such as 2-keto-3-deoxy sugar acids). The factor was stable at 100° at neutral pH for at least 20 min, at pH 1 at room temperature for several hours, and was completely resistant to prolonged digestion and dialysis with the following nucleases: purified venom phosphodiesterase, polynucleotide phosphorylase, pancreatic RNase and DNase, and venom 5′-nucleotidase.

All of the protein[12] and catalytic activity in purified HPr was adsorbed by charcoal but not by mixed-bed ion-exchange resins. It was precipitated with protamine sulfate and lost activity on treatment

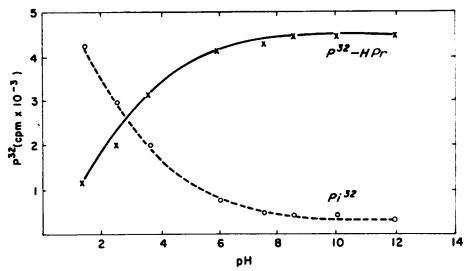

FIG. 3.—Hydrolysis of P^{32}-HPr as a function of pH. Each sample contained 150 μg of P^{32}-HPr (6100 cpm; specific activity 2×10^7 cpm/μmole) in a final volume of 0.25 ml adjusted to the indicated pH. After 30 min at 23°, a 50-μl aliquot was removed to measure the pH, and 0.20 ml was neutralized and spotted on S and S 589 (green) paper. The chromatograms were developed with 0.1 *M* Na_2CO_3, 95% ethanol, 3.5:6.5. P^{32}-HPr remained at the origin while P^{32}-inorganic phosphate migrated close to the solvent front. Radioactivity measurements were performed as described in Fig. 2; the results obtained with the 0.20-ml aliquots are given above.

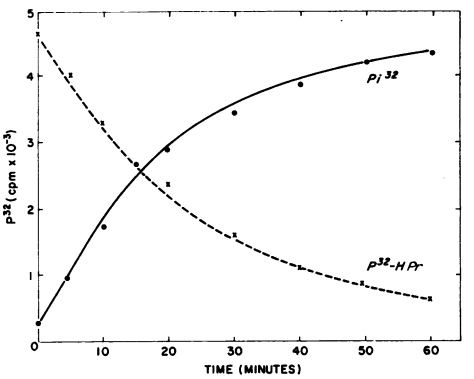

FIG. 4.—Hydrolysis of P^{32}-HPr: pH 3.9 at 40°. The sample, 1.5 mg P^{32}-HPr (47,500 cpm; 1.6×10^7 cpm/μmole), was maintained at 40° after adjusting to pH 3.9 with acetic acid and a final volume of 0.50 ml. Aliquots (50 μl) were removed at the indicated times, neutralized, and assayed by paper chromatography as described in Fig. 3.

with 0.1 *M* alkali for 60 min at room temperature. The activity was completely lost by treating HPr with the following proteinases: chymotrypsin, trypsin, papain, pronase, and pepsin. In each case, suitable controls showed that the loss in activity was a result of proteinase action on HPr, and was not the result of possible residual proteinase action on I and II.

Formation and Properties of P^{32}-HPr.—As shown in Figures 1 and 2, P^{32} was transferred from P^{32}-PEP to protein. While the *rate* of the reaction depended on the concentration of enzyme I, the *extent* of incorporation was directly proportional to the concentration of HPr. Thus, we concluded that P^{32} was transferred to HPr. In these experiments the required components were: I, HPr, P^{32}-PEP, and Mg^{++}. Omission of any of these or substitution for I and/or HPr by II gave no detectable incorporation (i.e., less than 200 cpm compared with 120,000 cpm in the complete system). Further, the addition of II to the complete system did not affect the rate of the reaction. Preliminary experiments indicated that the P-transfer from PEP to HPr was reversible.

To demonstrate reaction B (see above), the following components (in μmoles) were incubated in a final volume of 0.4 ml for 30 min at 37°: *N*-acetylmannosamine, 4.0; $MgCl_2$, 2.5; Tris-HCl, pH 7.4, 25; enzyme II, 0.25 mg; P^{32}-HPr, 22,400 cpm (specific activity, 20×10^6 cpm/μmole). The product, *N*-acetylmannosamine-6-P^{32}, isolated by ion-exchange chromatography, contained 17,500 cpm and gave glycolaldehyde-P^{32} on periodate oxidation. The remaining P^{32} was shown to be P_i^{32}. Omission of enzyme II, or substitution of I for II, gave less than 100 cpm in the product; further, addition of I to the complete system did not affect the transfer of P^{32} to the sugar. In a similar experiment, the incubation was conducted with unlabeled P-HPr and C^{14}-*N*-acetylmannosamine; approximately the same quantity of C^{14}-*N*-acetylmannosamine-6-P was formed as from P^{32}-HPr. In the C^{14}-experiment, the addition of unlabeled PEP to the system did not affect the

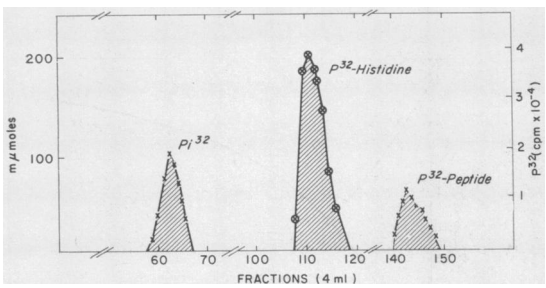

FIG. 5.—Ion-exchange chromatography of alkali-hydrolyzed P^{32}-HPr. The labeled protein was prepared by incubating the following components in 16 ml: 28 mg of purified HPr, 0.2 mg enzyme I, 100 μmoles of P^{32}-PEP (2×10^5 cpm/μmole), 1.0 mmole Tris buffer, pH 7.4, and 0.1 mmole $MgCl_2$. After 30 min at 37°, the mixture was fractionated on a column (2.5 × 80 cm) of Sephadex G-25, previously equilibrated with 0.1 *M* NaCl adjusted to pH 8.5 with Na_2CO_3. The P^{32}-protein fraction (eluted with NaCl solution) contained 1.3×10^5 cpm, and was concentrated by pressure dialysis to 11 ml. It was adjusted with 10 *M* NaOH to 3 *M*, and was hydrolyzed in a sealed tube at 100° for 7 hr. After diluting 10-fold with water, the sample was placed on a column of Dowex-1, hydroxyl form (200–400 mesh; 2.5 × 30 cm), washed extensively with H_2O (no P^{32} was eluted), and eluted with a linear gradient of 0.0–1.5 *M* $NaHCO_3$–Na_2CO_3 buffer,[16] pH 8.5 (total volume, 1 liter). Each fraction (4 ml) was analyzed for P^{32}; the total radioactivity in the 3 peaks (indicated by crosshatching) was over 95% of that placed on the column. All peaks were analyzed for inorganic phosphate[17] ((-X-X) using 2.5-cm cells in the Cary model 14 recording spectrophotometer. Only peak 1 contained inorganic phosphate. However, when 0.5-ml samples of peaks 2 and 3 were pretreated with 0.25 ml of 4 *N* S_2SO_4 at 40° for 30 min, all of the P^{32} was measurable as inorganic phosphate. Histidine (−○−○−) measured fluorometrically,[18] was not detectable in any of the peaks, but was found in peak 2 after the acid treatment described above. Histidine, and 3 or 4 other amino acids were found in peak 3, using paper chromatographic methods, after 6 *N* HCl hydrolysis (12 hr, 100°), of the pooled fractions. Peak 3 was not observed when the 3 *M* NaOH hydrolysis was conducted for 12 rather than 7 hr, and there was a concomitant increase in peaks 1 and 2.

results unless it was added along with enzymes I and II, HPr, and the sugar; in this case the results were similar to those given in Table 1.

The properties of P^{32}-HPr suggested that the P^{32} was attached to a histidine residue; for example, P_i^{32} was immediately formed in acid solution, while none was detected when P^{32}-HPr was treated with 0.1 *M* NaOH for 1 hr at 25°. The results of two studies on the hydrolysis of P^{32}-HPr are given in Figures 3 and 4. The rates of hydrolysis were the same as those observed with P-histidine. These data, along with results indicating that P^{32}-HPr was remarkably susceptible to hydrolysis at pH 6.5 in the presence of pyridine or other organic amines, were typical of the properties reported for P-histidine[6] and for protein bound P-histidine.[15]

To characterize more fully the P^{32}-HPr, it was subjected to alkaline hydrolysis as described by Boyer *et al.*,[16] and the hydrolyzate fractionated as shown in Figure 5. Three radioactive peaks were observed. Peak 1 was identified as inorganic P_i^{32} by colorimetric, paper electrophoretic, and chromatographic methods.

Peak 2 was identified as phosphohistidine by the following criteria: (*a*) It cochromatographed with authentic P-histidine on ion-exchange columns,[15, 16] paper chromatography,[6, 16] and in two electrophoretic systems. (*b*) Inorganic phosphate and histidine were not detected unless the sample was first hydrolyzed with acid. (*c*) Analysis of peak 2 for phosphate by modification of a colorimetric method,[17] and for histidine by a fluorometric method[18] showed a ratio of 1.00 ± 0.06 in each fraction. The specific activity of the P^{32} in peak 2 was the same as the P^{32}-PEP originally used to label the P^{32}-HPr.

Peak 3 appeared to be a peptide containing P^{32}-histidine since (*a*) it liberated P_i^{32} under the same conditions as P-histidine, and (*b*) acid hydrolysis revealed the presence of several amino acids including histidine.

Comments.—Although phospho-proteins have long been known (including such proteins from *E. coli*[19]) and enzymatic transfer of P to protein has been achieved,[20] these have generally involved a serine moiety in the protein. Protein-bound phosphohistidine was first described by Boyer and his co-workers.[16, 21, 22]

The transferase system described here is unique[23] in several respects: (*a*) the initial P-donor is PEP; (*b*) the protein to which the P is transferred serves as a donor for transfer to seven sugars of the gluco- and manno-series when supplemented with the required enzyme (II). Preliminary experiments have shown that more than one enzyme II can be formed by the cells. Thus, when glucose-grown cells were washed and incubated for 6 hr in fresh medium containing galactose, a new enzyme II was isolated that transferred P from P-HPr to galactose and *N*-acetylgalactosamine. No difference in enzyme I or HPr could be detected between glucose and galactose-grown cells. Thus, it seems possible that reaction A (above) serves as a source of phosphate in a variety of phospho-transferase reactions.

While the distribution of the transferase system in various organisms is now under study, it has been detected in strains of *E. coli* (including wild type), *Aerobacter cloacae* and *aerogenes*, and *Lactobacillus arabinosus*.

The biological significance of the new phospho-transferase system is not clear[24] and fruitful speculation must await further experimental results.

Summary.—A novel phospho-transferase system was isolated from *E. coli* K235, and was detected in other bacteria. The system involved a sequential transfer of phosphate from phosphoenolpyruvate to a heat-stable protein to hexoses; the two reactions were catalyzed by two distinct enzyme fractions. Isolation and characterization of the phosphorylated protein showed that the phosphate group was linked to a histidine residue.

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† Postdoctoral fellow, Arthritis and Rheumatism Foundation.

‡ Present address: Department of Biochemistry, University College of Science and Technology, 92 Upper Circular Road, Calcutta 9, India. This work was performed under the tenure of a postdoctoral fellowship from the Helen Hay Whitney Foundation.

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[22] Dr. Boyer has recently informed us that the protein containing phosphohistidine isolated from bovine mitochondria is related to or is, in fact, succinate thiokinase (Mitchell, R. A., L. G. Butler, and P. D. Boyer, *Biochem. Biophys. Res. Commun.*, in press). Similar results were obtained with highly purified succinate thiokinase from *E. coli* (Kreil, G., and P. D. Boyer, *Biochem. Biophys. Res. Commun.*, in press). In the experiments described by these investigators, P_i^{32} was incorporated into protein in the presence of succinyl CoA, or CoA. In the present experiments, enzymes I, II, and HPr exhibited no succinate thiokinase activity, and did not incorporate P_i^{32} into protein in the presence or absence of succinate and CoA. Experiments are in progress to determine whether or not there is any relationship between the succinate thiokinase and the system described in the present paper.

[23] Hexoses can be phosphorylated by an enzyme obtained from *E. coli* where the phosphoryl donor is phosphoramidate, phosphohistidine, or *N*-phosphoglycine [Fujimoto, A., and R. A. Smith, *Biochim. Biophys Acta*, **56**, 501 (1962)]. This enzyme was present in the crude extracts used in the present studies, but was removed during the purification procedure.

[24] As shown above, the heat-stable protein HPr serves as a phosphate "carrier" in the complete system. Another type of heat-stable protein has recently been described [Majerus, P. W., A. W. Alberts, and P. R. Vagelos, these PROCEEDINGS, **51**, 1231 (1964)] and serves to "carry" acyl residues during the biosynthesis of fatty acids. A preparation of this protein was kindly provided to us by Drs. Paul Stumpf and Robert Simoni, and when tested in the phospho-transferase system, could not replace HPr. Similarly, our preparation of HPr was tested by Drs. Stumpf and Simoni, but exhibited no activity in their system.