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STUDIES ON THE PHOSPHOENOLPYRUVATE DEPENDENT PHOSPHOTRANSFERASE SYSTEM OF *ESCHERICHIA COLI*

by
Byron Eugene Anderson

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of the requirements for the degree of
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A B S T R A C T

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Co-Chairmen: Dr. Saul Roseman
Dr. Dale L. Oxender

A number of carbohydrate-specific, inducible, and membrane-bound enzymes, in *Escherichia coli*, have been shown to catalyze a phosphoryl transfer from a common intermediate to various sugars. The intermediate is a phosphohistidine residue in a low molecular weight protein (HPr). The phospho-HPr is formed by an enzyme-mediated phosphoryl transfer from phosphoenolpyruvate. This two-enzyme phosphotransferase system (PTS) synthesizesthe phosphate esters of hexoses, glycosides, and of certain disaccharides, and has been implicated as a component of bacterial carbohydrate transport systems. The reactions of the phosphotransferase system are summarized as follows:

$$\text{Phosphoenolpyruvate} + \text{Histidine-Protein (HPr)} \xrightarrow{\text{Enzyme I}} \text{Phosphohistidine-Protein (P-HPr)} + \text{Pyruvate}$$

$$\text{P-HPr} + \text{Hexose} \xrightarrow{\text{Enzyme II}} \text{Hexose 6-phosphate} + \text{HPr}$$

In the present studies, two of the sugar-phosphate products were characterized, one was synthesized, and the histidine-containing protein, HPr, was purified to homogeneity and studied as discussed below.

D-Galactose 6-phosphate and methyl 1-thio-β-D-galactopyranoside 6-phosphate were isolated from the PTS reaction and crystallized as the dicyclohexylammonium and dipotassium salts, respectively. The position of the phosphate bond in these two

compounds was established by periodate oxidation studies and by their powder X-ray diffraction patterns. Methyl 1-thio-β-D-galactopyranoside 6-phosphate was synthesized by a condensation reaction of methyl 1-thio-β-D-galactopyranoside and cyanoethyl-phosphate in the presence of dicyclohexylcarbodiimide, followed by hydrolysis to remove the cyanoethyl group. The product was isolated by ion-exchange chromatography and the position of the phosphate bond established as above.

Two HPr active proteins (designated HPr-1 and HPr-2) were purified to homogeneity, as judged by electrophoresis in polyacrylamide gels and by ultracentrifuge analyses. The two proteins were similar in molecular weight (9500g/mole) and sedimentation coefficient (1.6). The two HPr's were also identical in amino acid composition, the analyses showing two histidine residues and no half-cystines, tryptophan, or tyrosine. HPr-2 differed from HPr-1 only in containing four less amide nitrogens. This makes HPr-2 more negatively charged than HPr-1 and explains its electrophoretic behavior in polyacrylamide gels and its elution pattern from DEAE-cellulose.

HPr-1 showed twice the activity of HPr-2 in the PTS, and the ratio of the two activities was constant with three Enzymes II. HPr-1 could be partially converted to a protein having the same electrophoretic mobility of HPr-2, either by heating at 100° alone, or by heating and acid precipitation (the first two steps in the purification). These studies indicate that HPr-2 is an artifact of preparation, probably arising as a result of hydrolysis of amide groups.

HPr accepts one mole of phosphate per mole of protein in

the Enzyme I catalyzed transfer reaction. Hydrolysis rates of the phosphorylated HPr, and characterization of the phosphohistidine isolated from the phosphoprotein, indicated that the phosphoryl bond is linked to N-1 of the histidine imidazole ring.

To my wife and son

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ABBREVIATIONS

P_i	orthophosphate
PEP	phosphoenolpyruvate
ATP	adenosine-5'-triphosphate
HPr	histidine-protein
ACP	acyl-carrier protein
TMG	methyl 1-thio-β-D-galactopyranoside
Gal	D-galactose
Glc	D-glucose
ManNAc	N-acetyl-D-mannosamine
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
3-P-histidine	3-phosphohistidine
1-P-histidine	1-phosphohistidine

INTRODUCTION

Recent studies from this laboratory have shown that a number of sugars are phosphorylated by a bacterial phosphotransferase system (PTS) which catalyzes the transfer of phosphate from phosphoenolpyruvate to the sugar; in general, the aldohexoses and their glycosides are phosphorylated at C-6. The system was resolved into two reactions (1), the first being the transfer of phosphate from phosphoenolpyruvate to a histidine residue in a low molecular weight protein (HPr); this step is common to all sugars phosphorylated by the PTS. The second step, the transfer of phosphate from phospho-HPr to the sugars, is catalyzed by a family of membrane-bound sugar-specific enzymes, many of which are inducible. The system has been implicated in the transport of carbohydrates by bacteria, as discussed below.

This thesis will be concerned with the isolation and characterization of two of the products of the PTS, D-galactose 6-phosphate and methyl 1-thio-β-D-galactopyranoside 6-phosphate. The chemical synthesis of methyl 1-thio-β-D-galactopyranoside 6-phosphate is also described.

The purification and characterization of the histidine-containing protein (HPr) will be reported, as well as evidence concerning the nature of the phosphoryl linkage in phospho - HPr.

A. HISTORICAL REVIEW

1. Nucleotide-Independent Phosphotransferases

Interest in non-nucleotide phosphotransferases has recently increased because of their suggested involvement in carbohydrate transport in bacteria, and because bacteria apparently contain only a specific glucokinase (4); the phosphotransferases have therefore been invoked to explain the initial steps in the metabolism of sugars other than glucose (91). Green and Meyerhof first demonstrated that both acid and alkaline phosphatases acted as phosphotransferases. That is, several phosphoryl donors (P-creatine, PEP, acetyl-P) could provide the phosphate for the synthesis of small amounts of sugar phosphates and glycerol phosphate. These activities are probably not of biological significance because the concentrations of alcohol acceptor required for the activity were very high (1-2 M); however, this work points out the need to differentiate, in other transferase studies, between a hydrolyzing and a synthesizing activity.

Fujimoto and Smith (6) reported an enzyme from E. coli that catalyzed a phosphoryl transfer from phosphoramidate to C-1 of glucose; N-phosphoglycine (and to a lesser extent, phosphohistidine) also served as a phosphate donor. Acetyl-P and carbamyl-P exhibited approximately 10% of the activity of phosphoramidate in this system, and a wide variety of hexoses served as acceptors for the transfer. The same laboratory (7) also reported a transferase that utilized glucose 1-phosphate as a donor, and glucose as an acceptor, to form glucose 6-phosphate; these activities were found in E. coli, Aerobacter aerogenes, and Mycobacterium smegmatis. The glucose 1-phos-

phate transferase also utilized methyl α- and β- glucosides as acceptors. Other workers (Kamel and Anderson, (8)) reported an acetyl phosphate or carbamyl phosphate, hexose phosphotransferase from A. aerogenes. However, this activity was not differentiated from the phosphoramidate-hexose transferase mentioned above.

A number of papers have established the common identity of glucose 6-phosphatase, pyrophosphatase, and pyrophosphate-hexose phosphotransferase activities found in liver and kidney microsomal preparations (9-12). Because of the low pH optimum and the high K_M for glucose, this activity may not be physiologically important as a glucose phosphorylating system. However, since the transferase activity was found to be higher in diabetes, and to fall when insulin was given, the transferase was suggested to be important in mammalian carbohydrate transport (13).

Several studies have established the bacterial, two-enzyme, PEP-dependent phosphotransferase system (1) as a key component of bacterial carbohydrate transport systems. These studies are discussed below.

2. Phosphoenolpyruvate-Hexose Phosphotransferase System of E. coli

The PEP-dependent phosphotransferase system (PTS) from E. coli was shown to synthesize the 6-phosphate esters of a number of hexoses and glycosides (1-3). The two enzymes catalyzed the following reactions (1):

$$\text{PEP} + \text{Histidine-Protein (HPr)} \xrightarrow[Mg^{++}]{\text{Enzyme I}} \text{Phosphohistidine-Protein (P-HPr)} + \text{Pyruvate}$$

$$\text{P-HPr} + \text{Hexose} \xrightarrow[Mg^{++}]{\text{Enzyme II}} \text{Hexose 6-Phosphate} + \text{HPr}$$

Enzyme I, which catalyzed the phosphoryl transfer from PEP to HPr, was purified 2-300 fold; this enzyme was located in the soluble fraction of lysed spheroplasts and was also soluble after treating crude extracts with ammonium sulfate to a final concentration of 70%. The extent of labeled ^{32}P incorporation into HPr from ^{32}P-PEP was shown to be dependent on the concentration of HPr, whereas the rate of incorporation was dependent on the concentration of Enzyme I.

Enzyme II, which catalyzes the phosphoryl transfer from P-HPr to the primary hydroxyl at the 6-position of the hexose, was shown to be particulate and membrane-bound. The term "Fraction II" was later used to denote the particulate fraction because it contained a number of carbohydrate-specific Enzymes II (3). Enzyme II activities are mostly inducible and dependent upon the carbon source used for growth; two such activities (D-glucose and D-mannose) are apparently constitutive. When cells are grown on galactose (or D-fucose, an inducer of the galactose operon) or in the presence of the lac operon inducer, isopropylthiogalactoside, the Fraction II phosphorylates galactose or methyl β-thiogalactoside (TMG) (2). In addition, PEP-dependent phosphorylation of pentoses, pentitols and hexitols was demonstrated after growth on these substances.

The HPr protein, purified 10,000-fold, was inactivated by alkali and several proteinases, but was reported to be stable to heat, low pH's and the action of nucleases (1). Phosphate and carbohydrates were not detected in the preparation. ^{32}P-Labeled histidine was isolated from base hydrolyzates of the phosphorylated HPr and characterized by paper chromatography, electrophoresis and colorimetric analyses.

As will be shown later, HPr is remarkably similar to the

acyl carrier protein (ACP) of fatty acid synthesis (14, 15) in composition, size, properties, and function in the sense that both serve as "carriers," one of acyl and the other of phosphoryl groups. Neither HPr nor ACP contain tryptophan or half-cystines, and little or no tyrosine. The molecular weights are about 10,000 and the proteins are relatively heat-stable. Both proteins are enzyme substrates but are also catalytic by virtue of their mediation between two (or more) enzymes in the formation of sugar phosphates or fatty acids.

While most, if not all bacteria contain the PEP-dependent PTS, it is not known whether the proteins synthesized by the different types are or are not identical. Some results along these lines were obtained from experiments where the PTS fractions obtained from E. coli (HPr and Enzymes I and II) were substituted into phosphorylating systems obtained from other organisms. Each of the three protein fractions of the phosphotransferase system could cross-substitute for the corresponding fraction from different strains of E. coli, and from Aerobacter aerogenes and cloacae, Lactobacillus arabinosis, Salmonella typhimurium, and to some extent with Bacillus subtilis. However, the E. coli fractions cross-reacted poorly, if at all, with the PTS from Staphylococcus aureus.

The products of the phosphotransferase system of the following substrates were characterized as the 6-phosphate esters: N-acetyl-D-mannosamine, N-acetyl-D-glucosamine, and D-glucose (1), and methyl α-D-glucopyranoside (17).

3. Involvement of the Phosphotransferase System in Carbohydrate Active Transport

The following evidence has established the PEP-dependent

phosphotransferase system as a necessary component of bacterial carbohydrate transport:

i) The accumulation of phosphate derivatives during carbohydrate transport.

ii) The restoration of glycoside accumulation with HPr, after cells are subjected to an osmotic shock.

iii) The formation of pleiotropic carbohydrate-negative mutants resulting from a loss of HPr or Enzyme I. (These are the components of the PTS common to all sugars phosphorylated.)

i) The first indication of the formation of a phosphate derivative during carbohydrate transport was reported by Rogers and Yu, using the galactose negative E. coli strain A (18). The authors found that as much as 60% of the galactose was accumulated as the 6-phosphate ester. A portion of the α- and β-methylglucosides accumulated by the cells was also isolated as the phosphate esters. In addition, phosphorylated α-methylglucoside has been observed during transport studies with E. coli strains K10 (19), B/r (20) and ML308 (21).

The 6-phosphate ester of TMG has been characterized as the product of TMG accumulation from Staphylococcus aureus (22). Evidence has also recently been presented for the accumulation of phosphorylated derivatives of lactose (23) and orthonitrophenylgalactoside (24), both in S. aureus.

ii) Heppel and his colleagues developed an osmotic shock procedure by which a number of degradative enzymes were released from E. coli (25-27). The cells were first suspended in a high concentration of sucrose in the presence of ethylenediamine tetra-

acetate, and then in a medium of low osmotic strength at 0°. The second step resulted in release of a number of hydrolases. It was suggested that the released enzymes are near or at the cell surface.

The procedure was originally shown by Kundig et al (3) to result in significant loss of transport function by the cells. HPr was found to be released with a concomitant loss in the ability of the F. coli (strain W2244) to concentrate methyl α-glucoside and methyl β-thiogalactoside. Furthermore, the active transport of the two glycosides was restored after preincubation of the shocked cells with purified HPr protein. Other laboratories have since shown that the osmotic shock procedure released the following: a) an amino acid binding protein from E. coli K12 (28); b) a sulphate binding protein from S. typhimurium (29, 30); and c) a galactose binding protein, which could partially restore galactose accumulation to shocked cells (31).

iii) If the phosphotransferase system is essential to carbohydrate transport, mutations affecting its various components would have the following consequences:

a) A mutation affecting any one of the Enzymes II would result in the loss of the organism's ability to utilize the corresponding carbohydrate. Such mutations have been reported for the accumulation and phosphorylation of β-glucosides (93).

b) A mutation effecting the deletion or inactivation of HPr or Enzymes I would result in an inability of the organism to utilize all carbohydrates phosphorylated by the PTS. Such mutants, termed pleiotropic, have been demonstrated in S. aureus (32-34), E. coli (35, 36), A. aerogenes (37) and S. typhimurium (38).

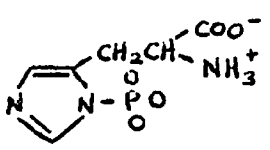
Tanaka and Lin (36) isolated two mutants from A. aerogenes; one lacked HPr and the other, Enzyme I. The mutants were unable to grow on D-mannitol, D-sorbitol, D-glucose, D-mannose, and D-fructose. The authors concluded that the PTS was similar to the ATP-independent phosphotransferases suggested by Fraenkel et al (39), and that the uptake of D-mannitol and the first step in its metabolism are one and the same.

Simoni et al (38) obtained a pleiotropic carbohydrate negative mutant that was unable to grow on, or ferment nine carbohydrates. Also, transport studies indicated that the cells were seriously defective in their ability to transport carbohydrates. The mutation was shown to be the result of a single genetic event that resulted in cells lacking Enzyme I.

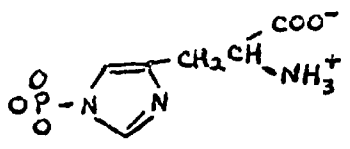
4. Phosphohistidine

Histidine and other imidazole compounds have been phosphorylated using phosphorus oxychloride (40, 41) and phosphoramidate (42-44). The outstanding characteristic of these phosphoimidazoles is their alkali stability and extreme acid lability. The hydrolysis of the N-phosphoryl bond is also catalyzed by cyclic amines via a transphosphorylation mechanism (45).

Hultquist, Moyer and Boyer (43) and Hultquist (44) reported extensive studies concerning the synthesis, isolation, and chemical properties of the 1- and 3-isomers of phosphohistidine. The structures of the two isomers are as follows:



1-P-Histidine



3-P-Histidine

Histidine was phosphorylated using phosphoramidate. At short reaction times, approximately equal amounts of the 1- and 3-isomers are formed. With longer times the 3-isomer predominates and 1-isomer eventually disappears. This phenomenon is the result of preferential transphosphorylation of 1-P-histidine to the 3-isomer, and the greater lability of the 1-isomer. Depending on the conditions of the reaction, 1,3-di and α,1,3-triphosphohistidine can also be formed and isolated.

1-Phosphohistidine has been shown to be very much more labile than 3-phosphohistidine over the pH range of 2 to 5; the hydrolysis rates of the 1-isomer are 10 times greater than those of the 3-isomer. In addition, the 1-isomer cannot be stored for very long periods, even at high pH's or as a solid, without considerable hydrolysis. The influence of the α-amino group was suggested to explain the greater lability at N-1. Because of the ease of transphosphorylations of phosphohistidine, and the lability of the phosphoryl bond, the author advised caution in the interpretation of studies of biological materials where an isomer of phosphohistidine is either detected or reported to be absent (44).

Boyer et al (46) and DeLuca (47) first isolated protein-bound phosphohistidine. The phosphorylated protein was obtained from mitochondria after addition of $^{32}P_i$. After subjecting the protein to pronase digestion followed by alkaline hydrolysis, ^{32}P-labeled phosphohistidine was isolated by chromatography on Dowex-1 resin. The product was identified as 3-P-histidine by its hydrolytic properties, co-chromatography with synthetic 3-P-histidine

and stoichiometric analysis of the P:histidine ratio.

Using soluble extracts of bovine liver mitochondria, Peter et al (48) demonstrated the labeling of protein-bound phosphohistidine by incubation with γ-^{32}P-labeled ATP. The authors suggested that the bound phosphohistidine was an intermediate in one of the steps of oxidative phosphorylation. In later communications, by co-fractionation studies, the phosphoprotein was identified as succinate thiokinase, both in liver mitochondria (49) and in highly purified *E. coli* succinate thiokinase preparations.

Norman et al (51) prepared nucleoside diphosphokinase from artichoke mitochondria, and showed that the enzyme could be labeled with ^{32}P from γ-labeled ATP. The ^{32}P was bound to histidine and the linkage was suggested to be to the 3 position of the histidine imidazole ring.

Zetterquist and Engstrom (52) and Zetterquist (53) studied the phosphohistidine labeling patterns in rat liver fractions using ATP as the phosphate donor. The 105,000 X g supernatant yield of phosphohistidine was 50 times greater than the mitochondrial fraction, and did not contain succinate thiokinase. After alkaline hydrolysis of the ^{32}P-labeled protein, 3-^{32}P- histidine and a much smaller amount of 1-^{32}P-histidine were isolated.

As mentioned above, Kundig et al (1) isolated and characterized phosphohistidine from phosphorylated HPr; the structure of the phosphohistidine (1- or 3-isomer) was not defined.

B. MATERIALS

N-Acetyl-D-mannosamine, D-mannosamine-HCl, D-galactose (substantially glucose-free), and D-galactose 6-phosphate were purchased from Sigma Chemical Co. Pancreatic trypsin, D-fucose and dithiothreitol were obtained from Calbiochem; E. coli alkaline phosphatase and galactostat enzyme kit from Worthington Biochemical Corp.; isopropyl β-thiogalactoside and methyl β-thiogalactoside from Mann Research Laboratories; Whatman ion-exchange celluloses from Reeve Angel Co.; Dowex ion-exchange resins from Bio-Rad Laboratories; all ^{14}C-labeled compounds from New England Nuclear; and $^{32}P_i$ from Tracerlab.

Synthetic 3-phosphohistidine and phosphoramidate were kindly supplied by Dr. Hultquist. All other chemicals were obtained from commercial sources.

E. coli strain W4345 was obtained from Dr. B. Rotman and E. coli W3350 from Dr. M. B. Yarmolinsky.

C. METHODS

1. Protein

Protein concentration was measured by the method of Lowry et al (54), using crystalline bovine serum albumin as a standard. The absorbance of a protein solution at 280 mμ, or the difference in absorbancy at 215 and 225 mμ (55), were also used.

2. Determination of Radioactivity

^{14}C- and ^{32}P-labeled compounds were analyzed in a Packard Tri-Carb Liquid Scintillation Spectrometer. Either a toluene system for counting filter paper, or a dioxane system for counting water solutions was utilized (56, 57).

3. Other Analytical Methods

Carbohydrate-reactive material was assayed by modification of the method of Dubois et al (58). The method of Macpherson (59) was used, at final volumes of 2.5 ml, for the assay of histidine. Ammonia was measured by the ninhydrin method (60), and organic phosphate by the method of Fiske and SubbaRow (61), after digestion with sulfuric acid. Inorganic phosphate was measured by a modification of the Martin and Doty method (66), formaldehyde by the colorimetric method (72), reducing sugar by the method of Park and Johnson (71), hexose by an anthrone method (70), and galactose by the galactose oxidase method (90).

4. Disc Gel Electrophoresis

Electrophoresis in polyacrylamide gels at 7.5% monomer concentration was conducted according to instructions provided by the Canal Industrial Corporation, Bethesda, Maryland. Four systems

were used, with running pH's of 9.5, 6.6, 4.3, and 2.3. The gels were stained for protein with Amido-Schwarz (0.55 g in 100 ml 7.5% acetic acid), and destained by electrophoresis in 7.5% acetic acid. A Canalco model 12 apparatus was used in these studies.

5. Analytical Centrifugation Analyses

All studies were conducted using a Spinco Model E Ultracentrifuge. Sedimentation velocity runs were performed at 59,780 rpm at 4° using the schlieren optical system and a synthetic boundary cell. Sedimentation equilibrium runs were analyzed by Rayleigh interference optics, using the standard double-sector cell with sapphire windows. The runs were performed at speeds of 59,780 rpm and at 4°; y and x displacement measurements were obtained after equilibrium had been reached, and calculations made according to Yphantis (62). All protein samples were equilibrated by dialysis against 0.1 M KCl. The dialysis tubing (Visking No. 8) was pretreated by heating at 90° for one hour in 0.1% $NaHCO_3$.

6. Trypsin Hydrolysis of HPr and Peptide Map

Pancreatic trypsin (1 mg, A grade, Calbiochem) was dissolved in 1 ml of 0.01 M $CaCl_2$ + 0.01 M Tris-HCl (pH 7.8), and used immediately. Ten µl of 0.1 N NaOH was added to 1 mg of HPr dissolved in 0.1 ml of distilled water, the solution incubated at 37° for 1 hour, neutralized with HCl, and 10 µl of 0.1 M Tris-HCl, pH 7.8, were added. After adding 10 µl of the trypsin solution to the HPr, the solution was incubated at 37° for 12 hours; 5 µl of fresh trypsin solution were added at 4 and 8 hours. A control mixture contained all components except HPr.

Each of the reaction mixtures was transferred to a sheet of Whatman 3 MM paper; the complete solutions were placed at one point on the origin by transferring 25 µl aliquots and drying between additions. Each sample was electrophoresed at pH 3.6 in pyridine-acetate buffer (100 ml acetic acid and 10 ml pyridine diluted to 3 liters) for 1 hour at 40 v/cm. After drying the paper, chromatography at 90° to the first dimension was performed, using a solvent system n-butanol:pyridine:glacial acetic acid:H_2O (90:60:18:72). After 10 hours, the paper was dried, dipped in a 0.1% ninhydrin solution in acetone, and heated for 5 minutes in an oven at 90°. The ninhydrin spots were marked and the paper then sprayed with the diazotized sulfanilic acid (Pauly) reagent (63) in order to detect the histidine peptides.

7. ^{32}P-PEP Preparation

^{32}P-labeled PEP was prepared using a PEP carboxykinase enzyme preparation from chicken liver mitochondria, according to the method of Mendicino and Utter (64).

8. Assay for ^{32}P-HPr Formation

An isolated Enzyme I preparation was used to catalyze the phosphoryl transfer from ^{32}P-PEP to HPr. After incubation of a mixture of ^{32}P-PEP, HPr, Enzyme I, Mg^{++} and buffer, the reaction was stopped by freezing in a dry ice-acetone bath. The tubes were thawed at 0°, maintained at this temperature during spotting on S and S 589 (green) paper, and the chromatograms developed by descending chromatography with 0.1 M K_2CO_3:95% ethanol (35:65). ^{32}P-PEP migrated at the solvent front; $^{32}P_i$ exhibited an R_f of about 0.5, and

^{32}P-HPr remained at the origin. After drying, the paper was cut and the ^{32}P in the sections determined by counting in the toluene liquid scintillation solvent.

9. Hydrolysis of ^{32}P-HPr

Buffer at the desired pH was adjusted to the hydrolysis temperature in a 70 x 7 mm test tube, and an aliquot of the isolated, dialyzed ^{32}P-HPr was added; the pH of the mixture was separately determined on an aliquot to ascertain whether or not the pH remained constant. At the end of the hydrolysis time, a predetermined amount of K_2CO_3 solution was added to adjust the pH to 8, and the solution frozen immediately in a dry ice-acetone bath. The tubes were thawed and kept at 0° while samples were transferred to S and S 589 (green) paper. Chromatography and counting were performed as described above.

10. Hydrolysis of 3-Phosphohistidine and Assay for Inorganic Phosphate

The hydrolyses were conducted as described for ^{32}P-HPr. The assay was based on inorganic phosphate appearance, measured by a modification of the Martin and Doty method (66). At the indicated times, an aliquot of the hydrolysis mixture was transferred to a mixture of 1 ml of water and 4 ml of isobutyl alcohol:benzene (1:1), maintained at 0°. When all samples were collected, 4 ml of cold ammonium molybdate solution (1.5 g ammonium molybdate in 100 ml of 0.35 $\underline{N}$ H_2SO_4) were added to each tube, the samples were thoroughly mixed on a Vortex mixer, the phases allowed to separate, and the organic phase removed with a pipette. Absorbancies were determined at 310 mμ.

11. Preparation of N-Acetyl (^{14}C)-D-Mannosamine

^{14}C-Carboxyl-labeled N-acetyl-D-mannosamine was prepared by N-acetylation of D-mannosamine-HCl (Sigma Chemical Co.) using ^{14}C-carboxyl-labeled acetic anhydride (New England Nuclear) according to the method of Distler, Merrick, and Roseman (92).

PART I

SYNTHESIS OF METHYL 1-THIO-β-D-GALACTOPYRANOSIDE 6-PHOSPHATE; CHARACTERIZATION OF BIOSYNTHETIC METHYL 1-THIO-β-D-GALACTOPYRANOSIDE 6-PHOSPHATE AND D-GALACTOSE 6-PHOSPHATE

1. Synthesis of Methyl Tetra-O-Acetyl-1-Thio-β-D-Galactopyranoside

The following procedure was based on the method of Lemieux for the synthesis of the 1-β-thioethyl derivative of D-galactose (67). Dry penta-O-acetyl-β-D-galactopyranoside (40 g; Sigma Chemical Co.) was dissolved in 200 ml of methanethiol containing 20 g of $ZnCl_2$. This mixture was allowed to react at 0° for 12 hours, and then carefully poured into 1 liter of saturated $NaHCO_3$ solution at room temperature. After filtration, the precipitate was extracted three times with 320 ml portions of hot chloroform, the combined solution cooled to room temperature, washed with water, and dried over anhydrous Na_2SO_4. The solution was concentrated in a vacuum, the syrup dissolved in 20 ml of chloroform, treated with an equal volume of ethyl ether, and the methyl tetra-O-acetyl-1-thio-β-D-galactopyranoside crystallized by the dropwise addition of petroleum ether (b.p. 30-60°). The crystals were washed with cold chloroform:ethyl ether (1:1).

The first crop of crystals totaled 17 g (m.p., 106-108°), and the second crop, 12 g (m.p., 104-108°). A melting point of 104-106° has been reported (68).

2. Deacetylation

Methyl tetra-O-acetyl-1-thio-β-D-galactopyranoside (16.5 g) in 50 ml of hot absolute methanol, was treated with 5 ml of 4% (wt./vol.) NaOMe in anhydrous methanol, and the mixture maintained at 45° for 2 hours. After concentrating to approximately 25 ml, the solution was maintained overnight at -20°. The precipitate was collected, and methyl 1-thio-β-D-galactopyranoside (TMG) twice recrystallized from absolute ethanol. The crystals were washed

with ethyl ether and dried in a vacuum over $CaCl_2$. The crystalline material (7.7 g; 84% yield) exhibited a melting point of 176-177.5°, and the following elemental analyses:

	C	H	S
Calculated:	40.11	6.66	15.23
Found*:	39.99	6.71	15.25

A melting point of 170-173° has been reported for TMG (63).

3. Synthesis of Methyl 1-Thio-β-D-Galactopyranoside 6-Phosphate (TMG-6-P)

TMG was phosphorylated at C-6 by the general method of Tener for the synthesis of nucleotide phosphates (69). 2-Cyanoethylphosphate was crystallized as the cyclohexylammonium salt; pyridine was dried over CaH_2.

^{14}C-TMG (10 mmoles; ^{14}C-methyl labeled, specific activity; 1270 cpm/μmole) was dissolved in 10 ml of water and added to 1.5 mmoles of 2-cyanoethylphosphate in 20 ml of pyridine. Water was removed by repeated addition and evaporation of dry pyridine. The reaction was initiated by the addition of 1 g dicyclohexylcarbodiimide in 30 ml of pyridine. After reacting at room temperature for 36 hours with agitation, the mixture was evaporated to a syrup, water (40 ml) added, and the solution filtered. The filtrate was adjusted to 90° in a boiling water bath, 3.5 ml of 1 N NaOH and 0.5 ml of 0.2 N $Ba(OH)_2$ added, and the flask heated for an additional five minutes. After cooling, Dowex-50-H was added to the solution to adjust the pH to 3. The resin was removed, the solution brought

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*The analyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Michigan.

to neutrality by the addition of 1 N KOH and transferred to a Dowex-1-HCO_3 (X-8; 200-400 mesh) column (2.2 x 30 cm). Unreacted TMG was recovered from the water wash of the column.

Using a 4 liter linear gradient of 0 to 0.5 M ammonium bicarbonate, four radioactive peaks were eluted from the column (at 0.05, 0.11, 0.15, and 0.22 M ammonium bicarbonate concentration). On electrophoresis in citrate buffer (pH 4.7) for 30 minutes and 55 v/cm, peaks 1 through 4 migrated 5, 7.5, 10.3, and 11.5 cm, respectively, and the yields from each peak were (in μmoles): 1) 391, 2) 446, 3) 330, and 4) 184. The third peak co-migrated with the biosynthetic TMG-6-P on electrophoresis at pH's of 1.7, 3.5, 4.7, 6.5, and 9.0. The combined fractions of this peak were concentrated in a vacuum, and ammonium bicarbonate removed by the repeated addition and evaporation of water. The resulting syrup was transferred to a conical centrifuge tube in 3 ml of water, and absolute ethanol added (approximately 7 ml) until the solution became slightly turbid. Crystals formed after storage overnight at room temperature, and were collected, recrystallized in the same manner, washed with cold 70% ethanol, followed by washing with acetone and ethyl ether, and finally dried in a vacuum over $CaCl_2$. The crystals analyzed as the monoammonium salt:

	C	H	P	S	N
Calculated:	27.36	5.90	10.08	10.44	4.56
Found*:	27.29	5.99	10.40	10.70	4.39

The melting point of the dipotassium salt of TMG-phosphate,

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*The analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tennessee.

prepared as described below, was difficult to determine because it discolored slightly at 188°. The material decomposed completely at 233-236°. The biosynthetic TMG-6-phosphate (dipotassium salt) behaved in the same way and decomposed at 231-234°. A mixture of the synthetic and biosynthetic crystals also melted at 231-234°. The specific rotation of the synthetic TMG-phosphate was α_D^{22} = + 7.2° (c, 1.1% in water).

The other peaks from the Dowex-1 column may represent diphosphate esters, or cyanoethyl-TMG-phosphodiester, or phosphomonoesters at positions other than C-6. During base hydrolysis some formation of the cyclic-4,6-phosphodiester of TMG is also expected.

4. Biosynthesis and Isolation of Methyl 1-Thio-β-D-Galactopyranoside 6-Phosphate (TMG-6-P) and D-Galactose 6-Phosphate (Gal-6-P)

E. coli (strain W3350) was grown in 12 two-liter flasks, each containing 600 ml of a minimal salts medium*, supplemented with 0.1% Bactotryptone (Difco), 10^{-3} M isopropyl thiogalactoside (Mann Research Laboratories), 2.5 ml glycerol, and 0.6 mg of thiamine. The cells were grown to the middle of the exponential phase, harvested by centrifugation, and washed with 1% KCl (yield, 16 g). After suspension in 100 ml of 0.025 M Tris-HCl buffer (pH 7.6), containing 3 x 10^{-4} M dithiothreitol (DTT), and

*The minimal medium contained: 0.05 M potassium phosphate buffer, pH 7.5; 0.02% $MgSO_4 \cdot 7H_2O$; 0.5 mg% $FeSO_4$; and 0.2% $(NH_4)_2SO_4$.

10^{-3} M EDTA, the cells were ruptured in a French Pressure Cell at 7800 psi. The homogenate was centrifuged at 12,000 X g for 10 minutes, the precipitate discarded, and the supernatant fluid used for the preparation of two incubation mixtures, each containing the following: 40 ml of crude extract, 4 ml of 1.0 M Tris-HCl (pH 8.0), 4 ml of 0.1 M $MgCl_2$, 4 ml of 1% tergitol-4, 4 ml of 0.2 M KF, and 16 ml of 0.2 M PEP. In addition the first incubation mixture contained 16 ml of 0.2 M ^{14}C-TMG (11,600 cpm/μmole) and the second incubation, 10 ml of 0.2 M galactose-1-^{14}C (9,600 cpm/μmole). These were incubated for two hours at 37°, the reactions stopped by heating for 15 minutes in a boiling water bath, the protein precipitates removed by centrifuging, and the supernatant fluids each transferred to 2.25 x 70 cm columns of Dowex-1-HCO_3 (X-8, 200-400 mesh). Each column was eluted with a four liter linear gradient of 0 to 0.3 N triethylammonium bicarbonate. The ^{14}C content of an aliquot of each fraction was determined and the TMG-6-P and Gal-6-P fractions were combined, and evaporated to syrups. Water was repeatedly added and removed by evaporation until the triethylammonium bicarbonate was removed. TMG-6-P was crystallized as the dipotassium salt from a water-ethanol mixture (yield, 580 μmoles). Gal-6-P was crystallized as the dicyclohexylammonium salt from a water-acetone mixture (yield, 450 μmoles).

5. Characterization of D-Galactose 6-Phosphate

a) The dicyclohexylammonium salt of the biosynthetic Gal-6-P gave the following elemental analyses:

	C	H	N	P
Calculated:	47.16	8.57	6.11	6.75
Found*:	46.82	8.67	5.80	6.67

b) The biosynthetic Gal-6-P was treated with E. coli alkaline phosphatase (Worthington Corp.) and the products analyzed with the Martin and Doty inorganic phosphate (66) and galactose oxidase (90) assays. The ratio of galactose to P_i was 1.00:1.01.

c) Both the anthrone (70) and the Park-Johnson (71) reagents gave identical color yields when the biosynthetic and synthetic Gal-6-P's (Sigma Chemical Co.) were compared by the two methods.

d) Synthetic Gal-6-P was crystallized as the dicyclohexylammonium salt and compared with the biosynthetic material by X-ray powder diffraction methods. The patterns were identical as shown in Figure 1.

e) The biosynthetic and the synthetic Gal-6-P's (20 μmoles of each) were separately reduced with 1 ml of 0.1 M $NaBH_4$ for 15 minutes, acetic acid added to a pH of 4 to destroy the borohydride, sodium periodate added (600 μmoles), and the mixture then allowed to react for two hours at room temperature. Ten ml of 0.25 M $NaBH_4$ were added and after 15 minutes the mixture was passed through Dowex-50-H resin and the eluate evaporated in a vacuum. Methanol was repeatedly added and evaporated in order to remove borate, and the residue dried overnight in a vacuum.

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*The analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tennessee.

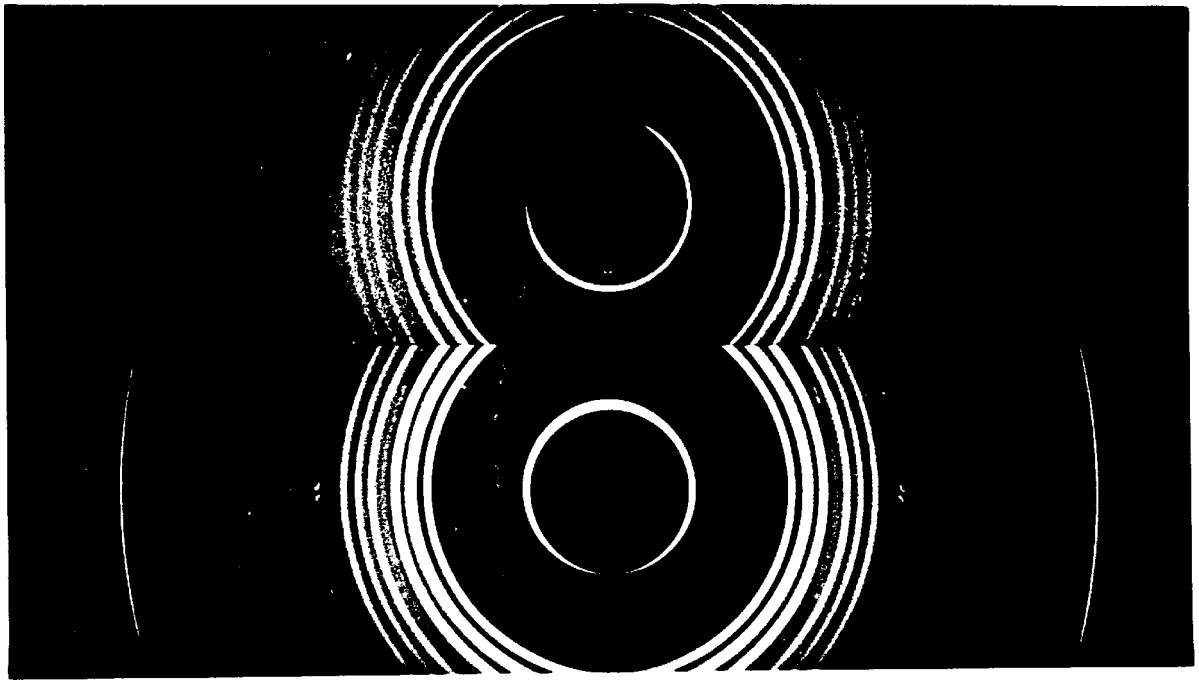
FIGURE 1

Samples of the dicyclohexylammonium salts were pulverized and mounted in capillary tubes in an X-ray powder camera. The samples were exposed to copper radiation with a nickel filter for 12 hours.

Figure 1

X-Ray Powder Diffraction Patterns of Synthetic and Biosynthetic D-Galactose 6-Phosphates

Biosynthetic



Synthetic

Ethylene glycol-phosphate could only have resulted from this procedure if the phosphate group was attached to C-6 of the galactose. The ethylene glycol-phosphate was characterized by its rate of migration in two solvent systems (methanol:formic acid: water, 80:15:5 (94); and nitromethane:pyridine:water, 5:6:4 (95)), and by pH 1.8 oxalate electrophoresis for 25 minutes at 85 v/cm. The phosphate esters were detected on the paper using the Hanes-Isherwood reagent (96).

6. Characterization of Methyl 1-Thio-β-D-Galactopyranoside 6-Phosphate

Elemental analyses and the specific rotation of the chemically synthesized TMG-6-P, and the melting points of the biosynthetic and synthetic TMG-6-P's were given above. The crystals of the dipotassium salt of biosynthetic TMG-6-P gave the following elemental analyses:

	C	H	P	S
Calculated:	22.95	3.58	8.46	8.75
Found*:	23.00	3.67	8.43	8.72

The chemically synthesized TMG-6-P was converted to the dipotassium salt by passage through Dowex-50-K, and crystallized in the same manner as the biosynthetic material. The synthetic and biosynthetic TMG-6-P's, dipotassium salts, were then compared by means of their X-ray powder diffraction patterns, as shown in

*The analyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Michigan

Figure 2. They were identical, and also matched perfectly with the pattern obtained using the crystalline, dipotassium salt of TMG-6-P isolated from Staphylococcus aureus (22).

Periodate oxidation, followed by borohydride reduction and acid hydrolysis would give α-glycerol-phosphate from the 6-phosphate ester of TMG. Further oxidation of the α-glycerol-phosphate would yield one mole of formaldehyde per mole of organic phosphate. Accordingly, the biosynthetic and synthetic TMG-6-P's (5.4 μmoles) were each treated with 0.6 ml of 0.1 M $NaIO_4$ for 12 hours in the dark at room temperature. The oxidation was terminated with sodium borohydride (3 mg), and the mixtures allowed to react for 6 hours. After adding HCl (0.6 N, final concentration), the tubes were heated in a boiling water bath for 30 minutes, the water removed in a vacuum, and methanol added and evaporated twelve times in order to remove borate. The residues were dissolved in 1 ml of water, oxidized with periodate, and formaldehyde determined colorimetrically (72). Organic phosphate was determined by the method of Fiske and SubbaRow (61). The following ratios were obtained:

	Organic-P	:	HCHO
Synthetic TMG-6-P:	1:00	:	1.18
Biosynthetic TMG-6-P:	1:00	:	1.12

The α-glycerol-phosphate was also characterized by its rate of migration on paper chromatography in methanol:formic acid: water, 80:15:5 (94), and by pH 1.8 oxalate electrophoresis for 30 minutes at 85 v/cm. The phosphate esters were detected with the Hanes-Isherwood organic phosphate reagent (96).

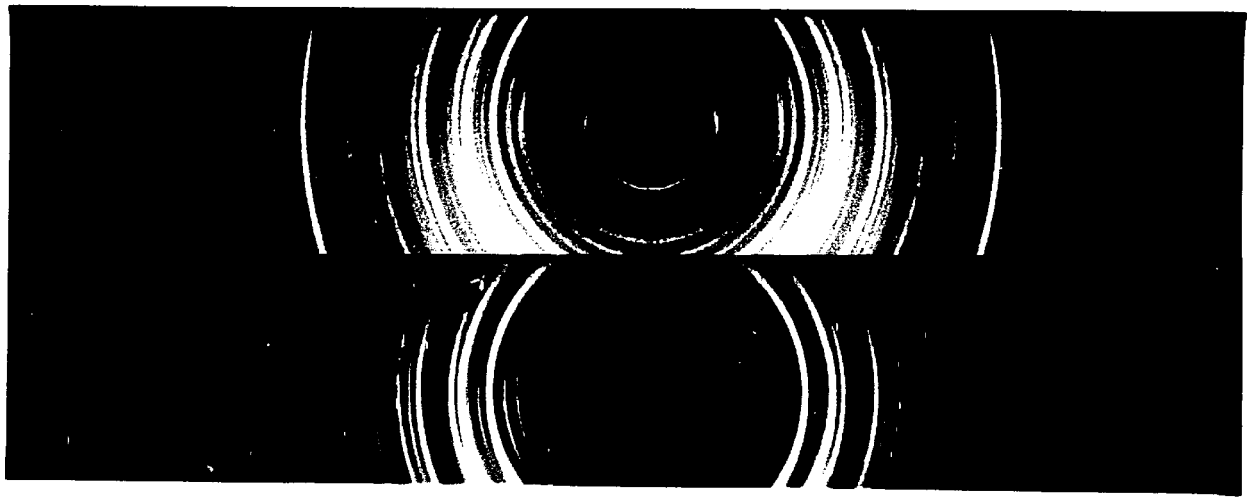
FIGURE 2

(The patterns were obtained by Dr. Peter Laue.) Crystals of the dipotassium salts were pulverized, tapped into 0.5 mm capillaries, and mounted in the X-ray powder camera. Each sample was exposed to copper radiation with a nickel filter at 35 kilovolts and 15 mamps for ten hours.

FIGURE 2

X-Ray Powder Diffraction Patterns of Synthetic and Biosynthetic Methyl 1-Thio-β-D-Galacto-pyranoside 6-Phosphates

Biosynthetic



Synthetic

PART II

PURIFICATION OF THE *E. COLI* PHOSPHOCARRIER PROTEIN (HPR); CHEMICAL AND PHYSICAL CHARACTERIZATION

A. PREPARATION OF ENZYMES FOR ASSAY OF HPR

E. coli K235 (73) was grown to the middle of the exponential phase in a mineral salts medium* supplemented with 0.1% casamino acids and 1% glucose. All remaining steps were conducted at 0-5°. The cells were harvested by centrifugation, washed with 1% KCl, and suspended (1 g wet weight per 2.5 ml buffer) in 0.025 M potassium phosphate buffer, pH 7.5, containing 0.001 M DTT and 0.001 M EDTA (designated KPO_4-DTT-EDTA buffer). The suspension was ruptured in a French Pressure Cell at 7,800 psi, the homogenate centrifuged at 12,000 X g for 10 minutes, and the supernatant treated with 10% charcoal (Darco G-60) for 5 minutes at 0° to remove the HPr (1). The quantity of charcoal required to remove all the HPr (while retaining high levels of enzyme activity) varied, and was determined for each preparation. The charcoal was removed by centrifugation and the supernatant, designated "charcoal-treated crude extract," used for routine assays of column fractions.

In order to separate Enzymes I and II, two volumes of a saturated ammonium sulfate solution were added to the charcoal-treated crude extract. After 10 minutes, the precipitate was collected by centrifugation at 29,000 X g for 15 minutes, suspended in a minimal volume of the KPO_4-DTT-EDTA buffer and dialyzed overnight against the same buffer. The solution was then centrifuged at 100,000 X g for 90 minutes. The supernatant fluid contained Enzyme I (7.6 mg protein/ml).

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*The minimal medium contained: 0.05 M KPO_4 buffer, pH 7.5; 0.2% $(NH_4)_2SO_4$; 0.02% $MgSO_4 \cdot 7H_2O$; 0.5 mg% $FeSO_4$. After sterilization 1 mg of thiamine per liter of media was added, in addition to the glucose which was separately sterilized.

To avoid repeated thawing and freezing, the enzyme was stored in small portions at -18°. The pellet obtained by high speed centrifugation contained Enzyme II and was suspended in the KPO_4-DTT-EDTA buffer, centrifuged at 100,000 X g, and again suspended in buffer (6.2 mg protein/ml). Aliquots were stored as described for Enzyme I.

B. Methods of Assay

All incubations were conducted at 37°. A typical incubation mixture contained the following: 10 µmoles Tris-HCl buffer (pH 7.6), 1 µmole $MgCl_2$, 2 µmoles PEP (monocyclohexylammonium, disodium salt), 1 µmole N-acetyl (^{14}C)-D-mannosamine (^{14}C-ManNAc), HPr fraction (up to 50 µl), and Enzymes I and II (75-150 µl); in a total volume of 150-250 µl. The reaction was terminated either by freezing, or by heating the mixtures in a boiling water bath for 3 minutes. The incubation times varied according to the amount of HPr added but were generally 30 minutes. Control incubations contained heat-inactivated in place of active enzyme, or lack of HPr. One unit of HPr activity was defined as the quanitity required for the conversion of 1 µmole of N-acetyl-D-mannosamine to its 6-phosphate ester in 30 minutes at 37°.

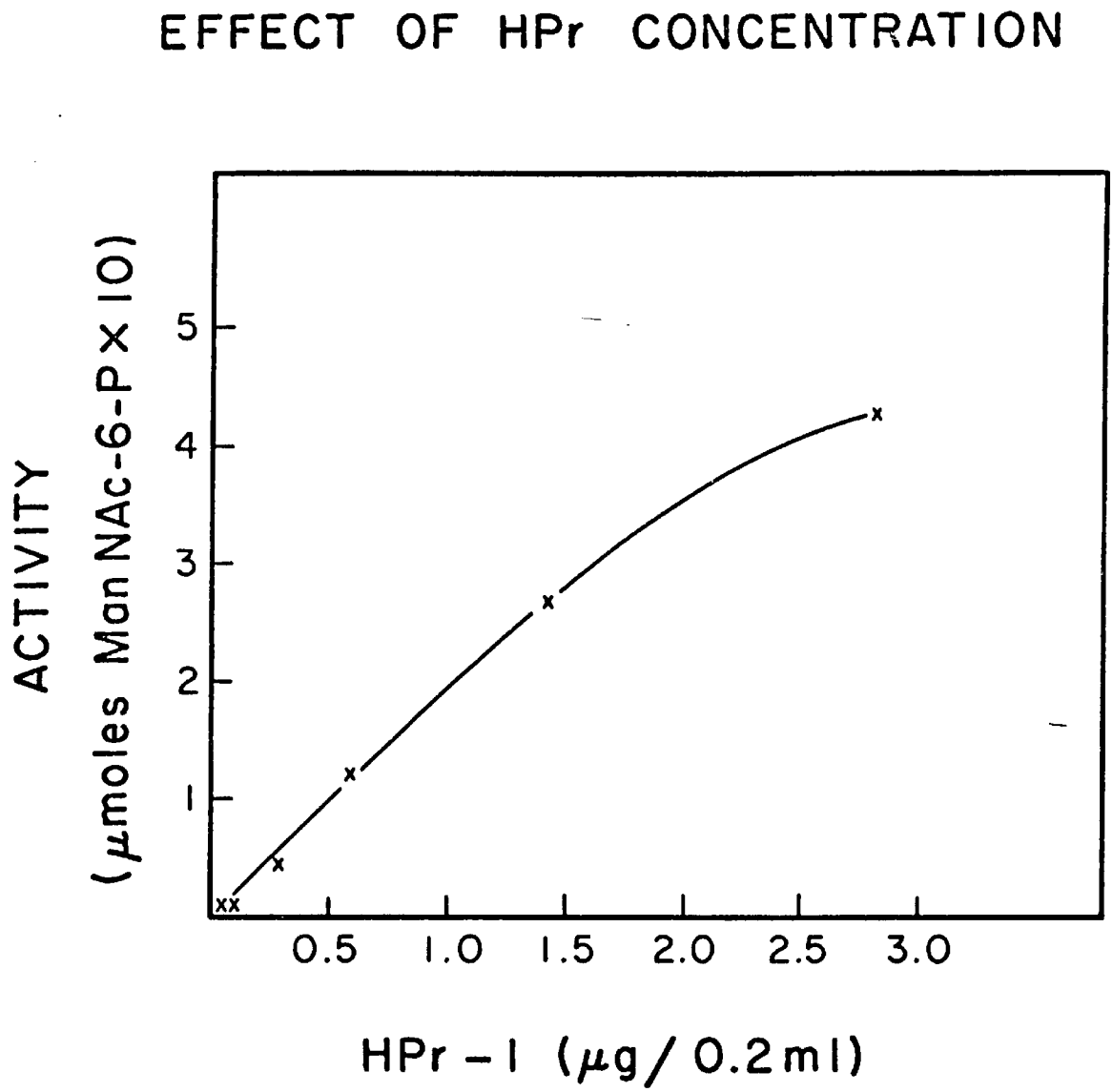
Two methods were used for determining N-acetyl (^{14}C)-D-mannosamine 6-phosphate (^{14}C-ManNAc-6-P). A rapid semi-quantitative method, used for assay of column fractions containing HPr, involved placing 50 or 75 µl aliquots of the assay mixtures on discs (1 cm in diameter) of Whatman DE-81 anion exchange paper (a DEAE-cellulose) and washing four times with 10 ml portions of distilled water on a sintered glass funnel. The papers were dried and counted in a toluene scintillation fluid in the Tri-Carb Liquid Scintillation Spectrometer. The recoveries of sugar-phosphate in this assay depended on the ionic strength used in the incubation mixtures.

Electrophoresis was also used to separate unreacted substrate from the 6-phosphate. Aliquots (75 µl) of the assay mixture were spotted on Whatman 3 MM paper and electrophoresed in 0.05 M pyridine-

acetate buffer, pH 6.5, for 20 minutes at 70 v/cm. The enzymatic product, ^{14}C-ManNAc-6-P, migrated 10-12 cm, while the substrate remained at the origin. After drying, both areas were cut from the paper, and counted as described above.

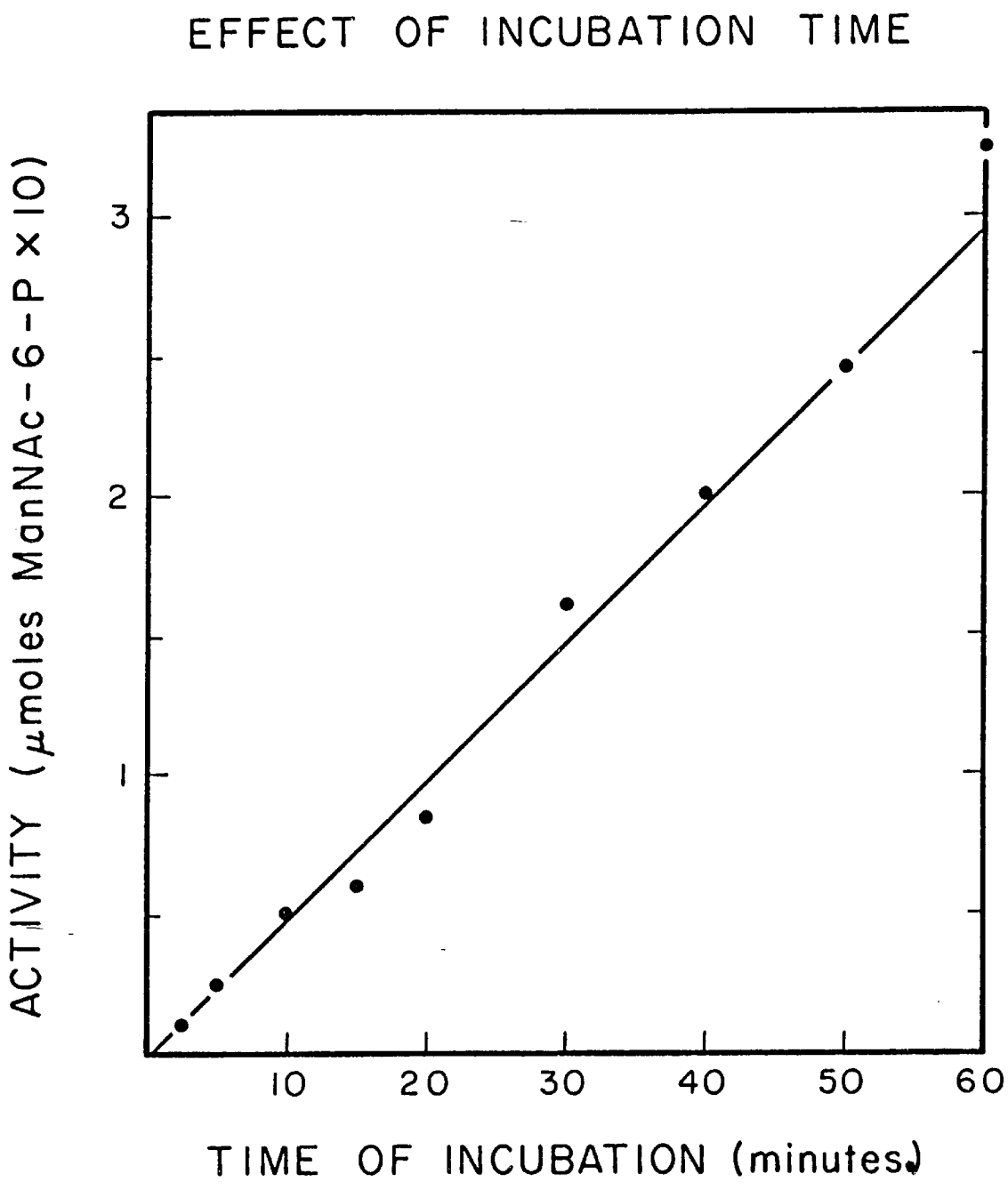
Specific activities of HPr were determined under conditions where the amount of product formed was proportional to the amount of HPr protein added (Figure 3), and to the time of incubation (Figure 4).

FIGURE 3



Each incubation mixture contained the following components (μmoles) in final volumes of 0.2 ml: ^{14}C-ManNAc, 1 (154,000 cpm/μmole); $MgCl_2$, 1; PEP, 2; Tris-HCl buffer, pH 7.6, 10; 0.4 mg of Enzyme I; 0.7 mg of Enzyme II; 0.05 to 2.7 μg HPr-1. Incubations were conducted at 37° for 20 minutes; the reaction was stopped by heating at 100° for 3 minutes and assayed by electrophoresis as described in the text.

FIGURE 4



The incubation mixtures consisted of the components described in Figure 3. Incubations were conducted at 37° for the indicated times and assayed by electrophoresis as described in the text. A charcoal-treated crude extract of E. coli K235 was used as a source of Enzymes I and II.

C. EXPERIMENTAL AND RESULTS

1. Purification of HPr from E. coli K235

i. Growth of E. coli K235 and Preparation of Heat-Treated Supernatant

The following procedures were carried out by the Worthington Biochemical Corporation, Freehold, New Jersey. E. coli K235 was grown at 37° to the middle of the exponential phase in the mineral salts medium described above, supplemented with 1% glucose and 0.12% casamino acids. The pH of the 750 liter culture was maintained at 7.1 to 7.5 by adding NaOH. Growth was stopped by rapid cooling of the culture, and the cells harvested in a Sharples centrifuge. The cells (about 3000 g, wet weight) were suspended in 30 liters of 0.02 M potassium phosphate buffer, pH 7.5, and ruptured in a Mont-Gallon press at 8500 psi. The broken cell preparation was centrifuged in a Sharples to remove debris, the supernatant heated to 95°, and maintained at this temperature for 15 minutes. The mixture was cooled to 25°, centrifuged in the Sharples, the supernatant fluid frozen, and used for purification of HPr in the laboratory.

ii. Acid Precipitation

All subsequent steps were conducted at 0-4°. The frozen heat-treated supernatant fluid was thawed, placed in 10-liter precipitating jars, the pH lowered to 2.4 by the addition of 8.7 N acetic acid, and the precipitate allowed to settle. The supernatant fluid was removed with a siphon, and the remaining slurry centrifuged at 8000 X g for 15 minutes. After suspension of the combined precipitates in 2.5 liters of distilled water, the pH was

adjusted to neutrality with NaOH, the mixture stirred for one hour and centrifuged. The precipitate was re-extracted twice with one liter of distilled water each, and the combined extracts dialyzed against 3 x 10 liter changes of distilled water. A final dialysis was conducted in 0.01 M sodium acetate buffer, pH 4.9; the total period of dialysis was 24 hours, and Visking No. 27 dialysis tubing (Union Carbide Corp., Chicago, Ill.) was used.

Subsequent studies showed that the acid precipitation step, which resulted in poor yields, could be eliminated, and that the crude extract could be placed directly on CM-cellulose.

iii. Batchwise Elution on CM-Cellulose

Whatman CM-23 cation exchange cellulose was equilibrated to pH 4.9 in 0.01 M sodium acetate buffer, according to instructions provided by Reeve Angel Co., Clifton, New Jersey. The equilibrated CM-23 was packed by gravity filtration into a 12 cm diameter column to a height of 50 cm.

The dialyzed protein from the acid precipitation step was centrifuged at 27,000 X g for 15 minutes and the supernatant fluid transferred to the CM-cellulose (CMC) column. Nucleic acids are not adsorbed on CMC at a pH of 4.9, and were removed from the column by washing with 10 liters of the acetate buffer. HPr was then eluted with the acetate buffer containing 0.3 M NaCl. One liter fractions were collected and those containing HPr activity (approximately 9 liters) were concentrated in a rotary evaporator with the bath temperature set at 35°, dialyzed against 2 x 20 liters of distilled water, and finally against 20 liters of 0.01 M Tris-HCl, pH 7.6. Total dialysis time was 24 hours; Visking No.

27 dialysis tubing was used. A ten-fold purification was achieved with 60% recovery of activity.

iv. DEAE-Cellulose Chromatography

Whatman DE-23 was equilibrated to pH 7.6 in 0.01 M Tris-HCl buffer, and packed by gravity filtration in a 5 cm diameter column to a height of 30 cm.

The dialyzed protein of the previous step was applied to the DEAE-cellulose and the column washed with 2.5 liters of 0.01 M Tris-HCl, pH 7.6, followed by a 2 liter wash with the buffer containing 0.02 M KCl. HPr activity was then eluted with 3 liters of 0.10 M KCl in the buffer. The eluate was lyophilized, dissolved in a small volume of distilled water, and desalted on a G-25 Sephadex column (equilibrated in water). A three-fold purification was achieved and 74% of the activity was recovered.

v. Gel Filtration Through Sephadex G-50

An aliquot of the DEAE-fraction (40%) was lyophilized, dissolved in 20 ml of 0.05 M NaCl, adjusted to 5% sucrose concentration, and placed on a Sephadex G-50 (fine mesh) column (5 x 80 cm) previously equilibrated with 0.05 M NaCl. The sucrose solution was layered under a head of 0.05 M NaCl using a peristaltic pump, the sucrose solution allowed to drain into the column, and the column eluted with 0.05 M NaCl. The fractions containing HPr activity were pooled, lyophilized, and solid dissolved in the minimum volume of distilled water and desalted by passage through a Sephadex G-25 column. Eighty percent of the activity was recovered.

Gel filtration separates HPr from most of the remaining contaminating protein (Figure 5). Since large numbers of fractions

FIGURE 5

The sample (20 ml) was placed on a 5 x 80 cm column, and eluted with 0.05 M NaCl as described in the text. Fractions (7.5 ml) were collected at flow rates of 0.5 ml/minute, and aliquots assayed for protein (absorbance at 280 mu: •) and HPr activity (x) as described in the text.

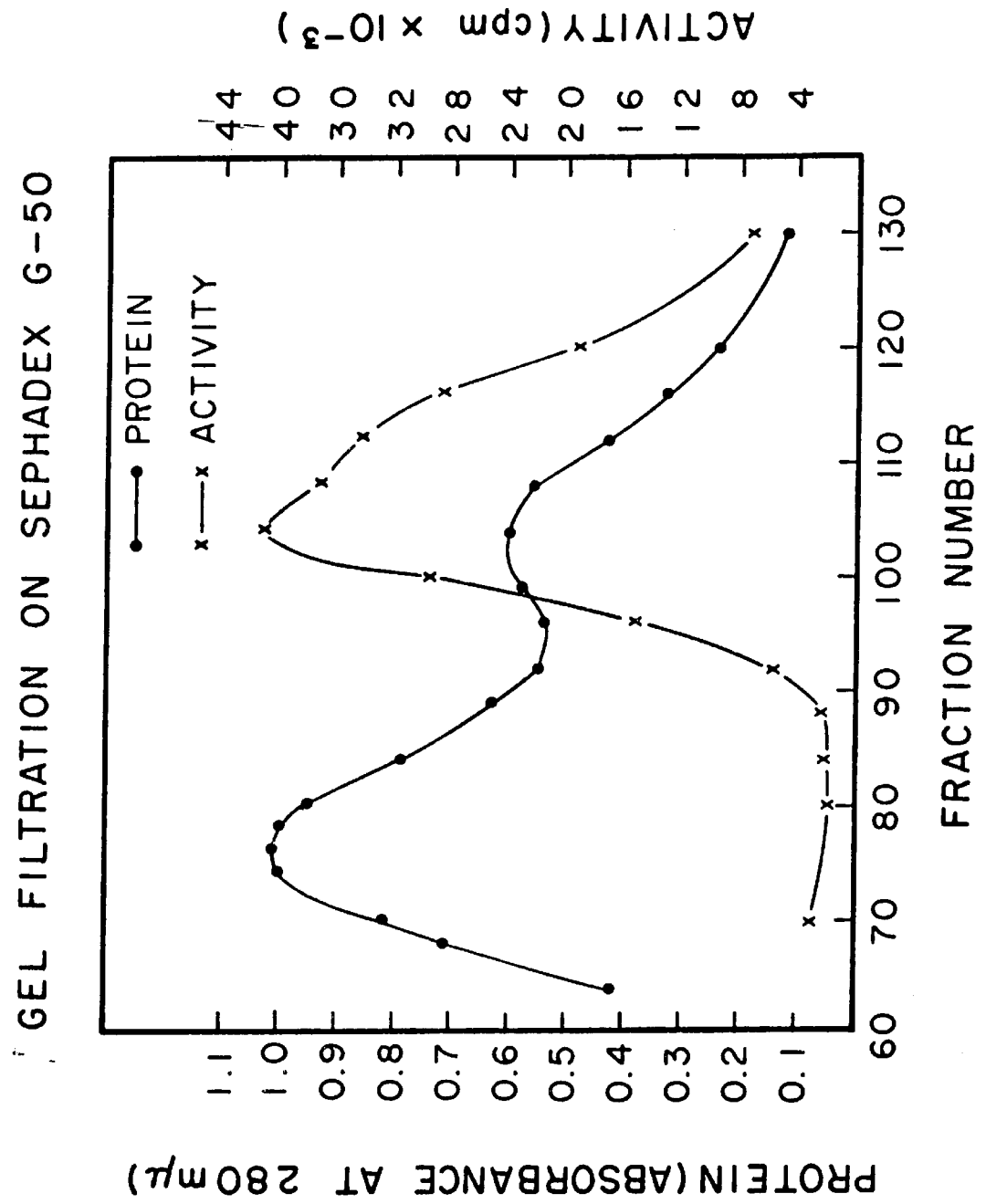

GEL FILTRATION ON SEPHADEX G-50
PROTEIN
ACTIVITY
PROTEIN (ABSORBANCE AT 280mμ)
ACTIVITY (cpm x 10⁻³)
FRACTION NUMBER
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
4
8
12
16
20
24
28
32
36
40
44
60
70
80
90
100
110
120
130

FIGURE 5

were obtained from the Sephadex G-50 and the following DEAE-cellulose column, quantitative assay by paper electrophoresis was impractical, and was conducted with the semi-quantitative DE-81 paper method. As indicated above, the quantity of ^{14}C-ManNAc-6-P that is adsorbed to the ion-exchange paper depends on the salt concentration in the incubation mixture.

vi. Gradient Elution from DEAE-Cellulose, Column 1

Approximately 30 mg of the HPr fraction obtained in the previous step was dissolved in 0.01 M Tris-HCl buffer, pH 7.6, and placed on a column of DE-23 (1 x 10 cm), previously equilibrated with the same buffer. The column was first washed with 5 x 10^{-3} M Tris-maleate buffer, pH 7.7, and the eluate monitored by measuring absorbance at 280 mu. After 100 ml of eluate had been collected, the absorbance increased as maleate anion and protein were eluted. In the next 70 ml, 6-7 mg of protein were eluted containing 10% of the activity placed on the column. Following elution with a total of 250 ml of Tris-maleate buffer, the column was eluted with 0.10 M KCl in 0.01 M Tris-HCl, pH 7.6. Each fraction (3 ml) containing HPr was assayed, and the fractions were electrophoresed in analytical polyacrylamide gel columns. The first two HPr fractions eluted with 0.10 M KCl in the buffer showed a single band, while the other active fractions showed two bands. As detected visually, the relative quantities of the stained protein bands changed; that is, the protein eluted from the DEAE-cellulose in later fractions contained more of the protein component exhibiting the greater electrophoretic mobility on the gel, and decreasing quantities of the material showing lower mobility. The first two fractions containing the single

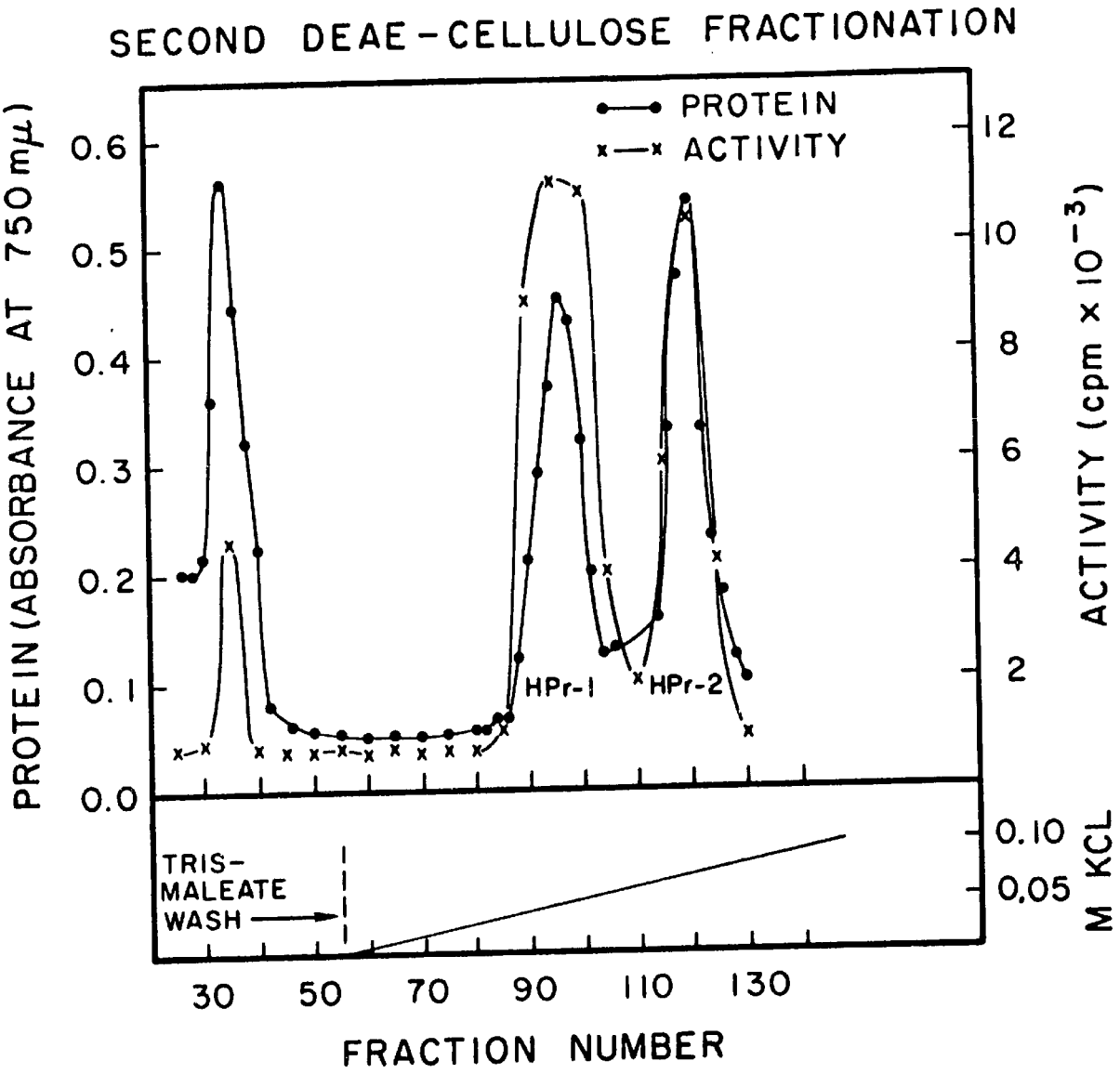
slower component (11 mg of protein), were combined and stored. The remaining fractions were pooled, concentrated by lyophilization, desalted as before, and combined with the remaining HPr fraction from the Sephadex G-50 step.

vii. Gradient Elution from DEAE-Cellulose, Column 2

The combined material was adjusted to 0.01 M Tris-HCl, pH 7.6, and transferred to a DE-23 column (1 x 15 cm), previously equilibrated with the buffer. The column was washed first with 330 ml of 0.005 M Tris-maleate buffer (pH 7.7), and then eluted with a linear gradient of 600 ml (0.0 to 0.10 M) KCl in 0.01 M Tris-HCl (pH 7.6). The elution pattern is shown in Figure 6. Three peaks, containing both protein and HPr activity were detected. The first peak was eluted with the Tris-maleate wash and showed a low specific activity. This fraction may correspond to the slower moving protein band derived from HPr-1 described below (Figure 14); it was not examined further. The major protein fractions showed two distinct and separable species of HPr; each was subsequently shown to be homogeneous. The second peak from the DEAE-cellulose column was designated HPr-1 (yield, 6 mg) and exhibited a specific activity of 307 units/mg. HPr-1 was identical to the single protein (11 mg) isolated from the first DEAE-cellulose column. The third peak was designated HPr-2 and showed a specific activity of 171 units/mg (yield, 7 mg). The active fractions in each peak were pooled, lyophilized, desalted by passage through P-4 polyacrylamide beads (100-200 mesh), and stored at -18°. All further studies were conducted with these two fractions.

A summary of the purification data is given in Table 1. HPr activity, at all stages of the purification, was completely stable to storage at -18° for at least several months.

FIGURE 6



The column was prepared, eluted and samples assayed as described in the text. The fractions (6.5 ml) were collected at flow rates of 0.7 ml/minute; 0.5 ml aliquots were assayed for protein (54), and 0.025 ml for HPr.

TABLE 1

Purification of HPr from *E. coli* K 235

	Step	Specific Activity	Purification Factor	%Yield
1.	Crude Extract	0.058	———	100
2.	Heat Treatment	1.67	29	69
3.	Acid Precipitation	2.03	34.8	40.7
4.	Carboxymethyl-Cellulose	20.2	345	23.2
5.	First DEAE-Cellulose	57.0	966	16.7
6.	Sephadex G-50	198	3380	13.2
7.	Second DEAE-Cellulose			
	HPr-1	307	5410	7.4
	HPr-2	171	2910	1.7

Assays for HPr in each fraction were conducted simultaneously, using a single preparation each of Enzymes I and II, and under conditions where phosphorylation was proportional to the time of incubation and quantity of HPr. A unit of HPr is defined as the quantity required for the phosphorylation of 1 μmole ManNAc at 37° in 30 minutes when incubated with 2.25 units of Enzyme I, and 1.42 units of Enzyme II under the conditions described in the text. Specific activity = units/mg protein.

2. Criteria of Homogeneity; Molecular Weight Determinations

As shown in Figures 7 and 8, HPr-1 and HPr-2 each gave a single protein band in disc gel electrophoresis using polyacrylamide gels at running pH's of 9.5, 6.6, 4.3, and 2.3. The two proteins were separable at pH's 9.5 and 6.6. With the pH 2.3 system, electrophoresis of the gel for 3 hours was required before the protein sample could be applied; multiple bands were obtained if the usual method was employed, an effect found with other proteins, and believed to result from the reaction of protein with persulfate.

Both HPr's were also homogeneous by analysis in the analytical centrifuge. Each gave a single symmetrical sedimentation pattern in velocity runs (Figure 9). From plots of the log of the boundary displacement versus time, sedimentation coefficients (S^{20}_w) for HPr-1 and HPr-2 were calculated to be 1.58 and 1.55, respectively.

The molecular weight of HPr was determined by equilibrium centrifugation with meniscus depletion, according to the method of Yphantis (62). The data is plotted as $\log_{10}$10c (c = concentration) versus x^2 (x = distance from the center of rotation). The slope of the line of best fit was determined by the least squares method, and the weight-average molecular weight ($\overline{M}_w$) was then calculated using a partial specific volume ($\overline{V}$) value of 0.743 (calculated from amino acid analysis data). The calculated values were $\overline{M}_w$ (HPr-1) = 9340 ± 230 (S.D.), and $\overline{M}_w$ (HPr-2) = 9490 ± 300 (S.D.). The linearity of $d\log_{10}10c/dx^2$ (Figure 10) suggested a homogeneous protein solution.

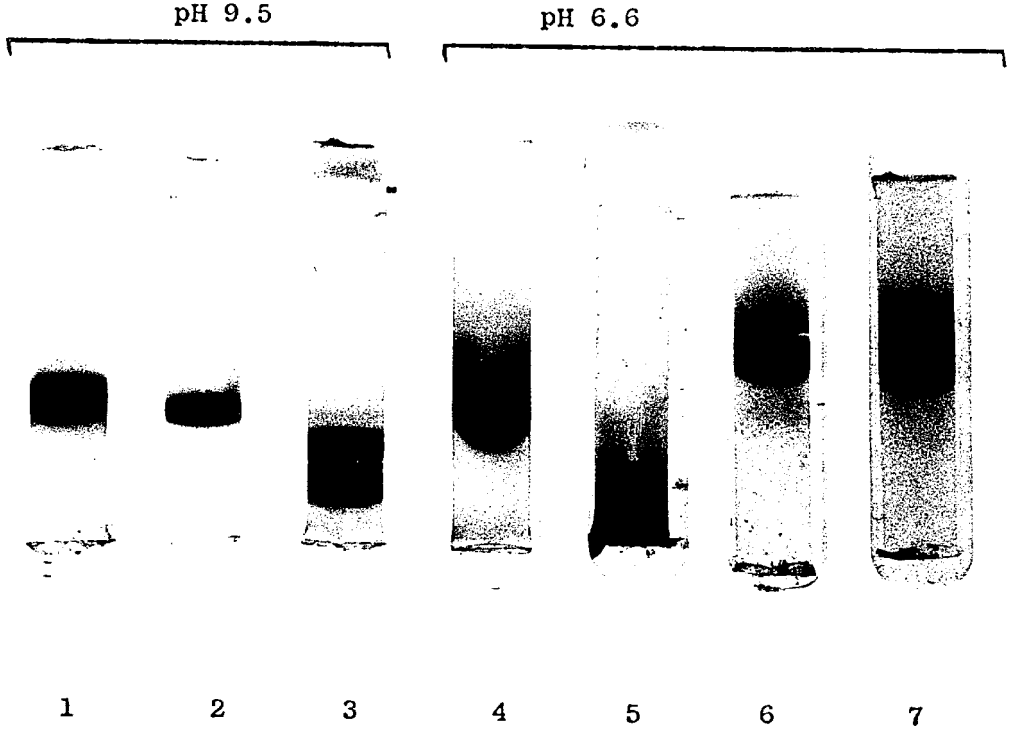
The same analysis was also performed on a protein solution of HPr-1 approximately ten times more concentrated than that used

FIGURE 7

The gels were prepared and protein stained as described in Methods. Numbers 1-3 were electrophoresed for 40 minutes at constant current (4.1 mamps/tube). The voltage increased from 35 to 40 v/tube during the experiment. Numbers 4 and 5 were electrophoresed for 45 minutes at 4 mamps/tube; the voltage increased from 150 to 220 v/tube. Numbers 6 and 7 were electrophoresed for 25 minutes at 3.5 mamps/tube; the voltage increased from 120 to 150 v/tube. Each protein sample (110 µl) contained 25 µmoles DTT, 10 µl of upper gel buffer, and 25 µmoles sucrose. The samples were layered under the reservoir buffer on top of the polymerized upper gel. The gel tubes were 5 mm in diameter. The lower and upper gels were 40 and 5 mm long, respectively. Migration of the protein bands was toward the anode.

FIGURE 7

Disc Electrophoresis of HPr-1 and HPr-2 in Polyacrylamide Gels, Running pH's: 9.5 and 6.6



1. HPr-1
2. HPr-2
3. HPr-1 + HPr-2
4. HPr-1
5. HPr-2
6. HPr-1
7. HPr-2

FIGURE 8

The gels were prepared and protein stained as described in Methods. Migration of the protein bands was toward the cathode. Numbers 1-3 were electrophoresed at a constant amperage of 4 mamps/tube for 60 minutes, and numbers 4-6 at 4 mamps/tube for 25 minutes. The pH 2.3 gels were first subjected to electrophoresis for 3 hours in lower gel buffer; and upper gel was not used. The protein samples (approximately 100 µg), each containing 25 µmoles DTT, 10 µl of upper gel buffer, and 25 µmoles of sucrose (110 µl total volume), were layered under the reservoir buffer.

FIGURE 8

Disc Electrophoresis of HPr-1 and HPr-2 in Polyacrylamide Gels,
Running pH's : 4.3 and 2.3

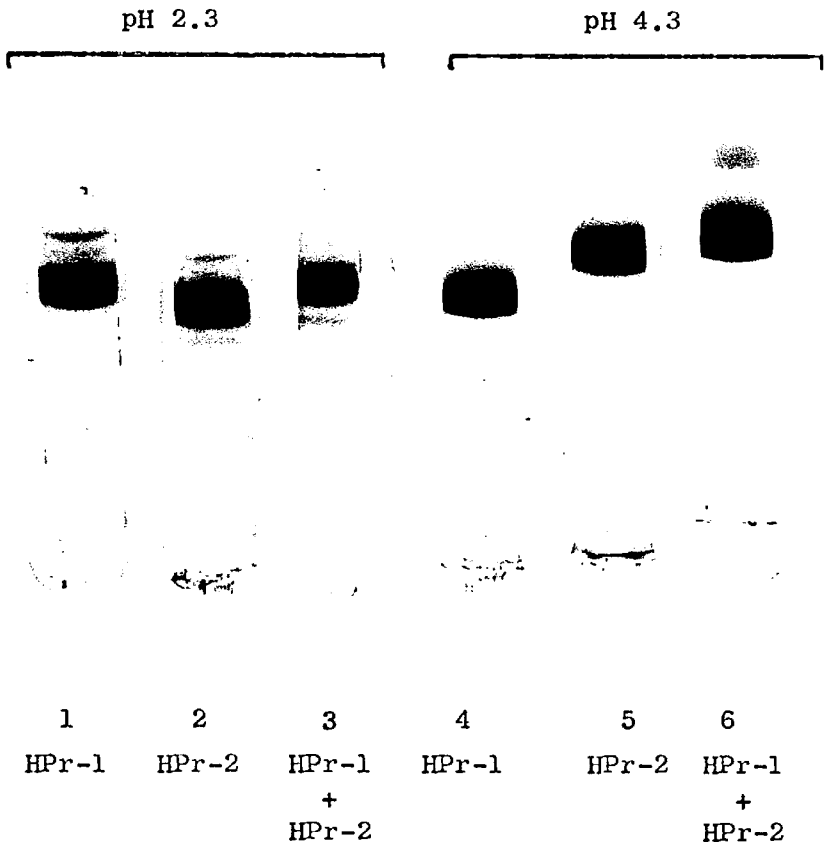
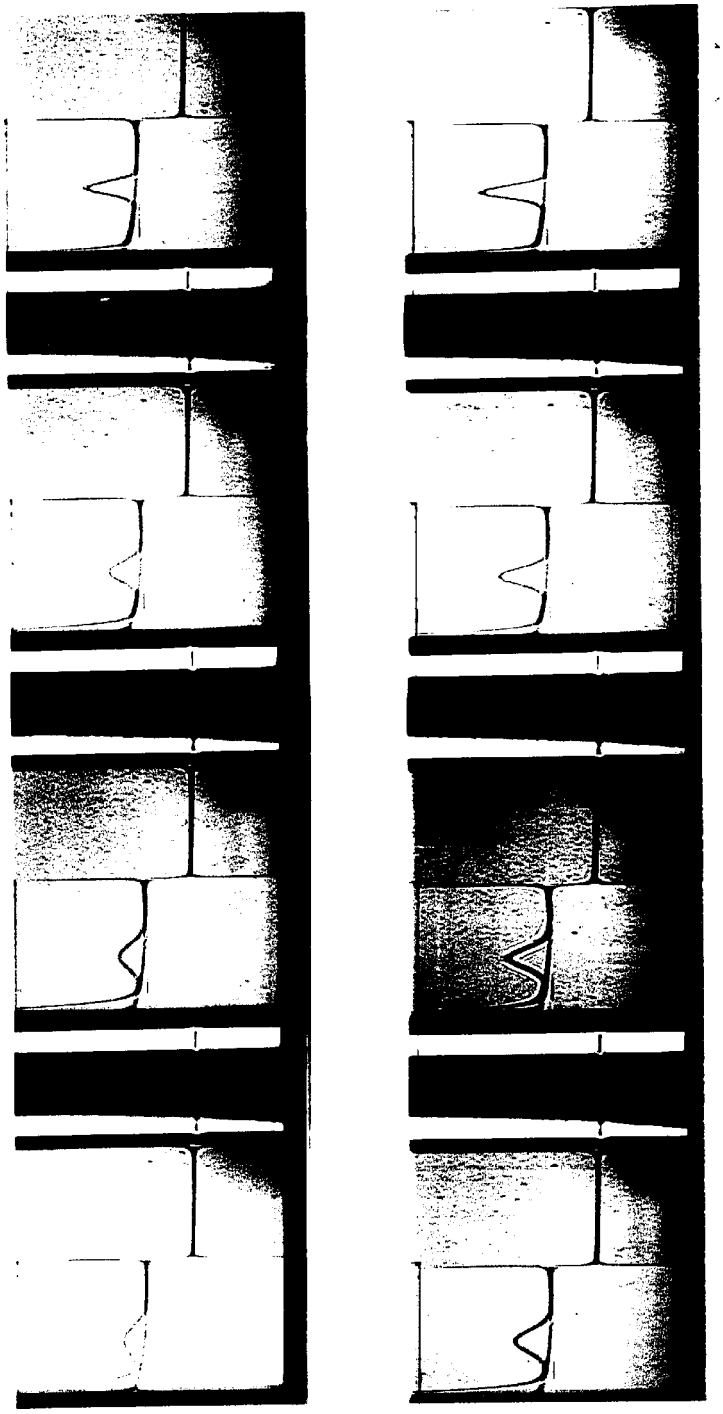


FIGURE 9

Sedimentation velocity runs were made at 59,780 rpm and 7.5° with the use of the schlieren optical system and a snythetic boundary cell. The bar angle was 60°. The protein samples (4 mg/ml) were equilibrated in 0.1 M KCl. The first picture on the right was taken on reaching speed, and succeeding pictures taken at 16 minute (HPr-1), or 8 minute (HPr-2), intervals.

FIGURE 9

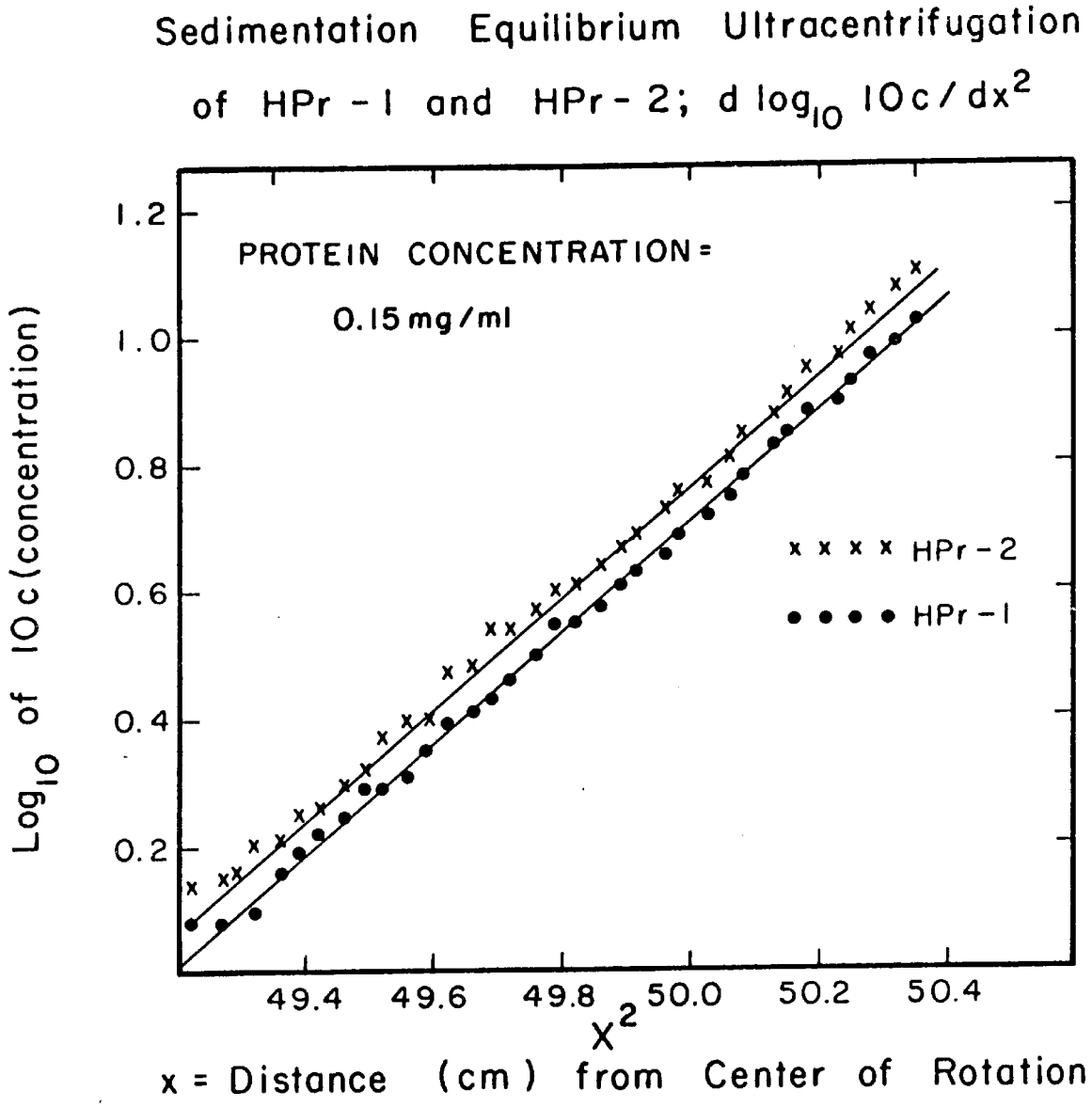
Sedimentation Velocity Studies of
HPr-1 and HPr-2



HPr-1

HPr-2

FIGURE 10



The graphical analyses of the equilibrium runs were made according to the method of Yphantis (62). The speed and temperature of the runs were 59,780 rpm and 5°; HPr-1 and HPr-2 (0.15 mg/ml) were equilibrated by dialysis in 0.1 M KCl before the experiment.

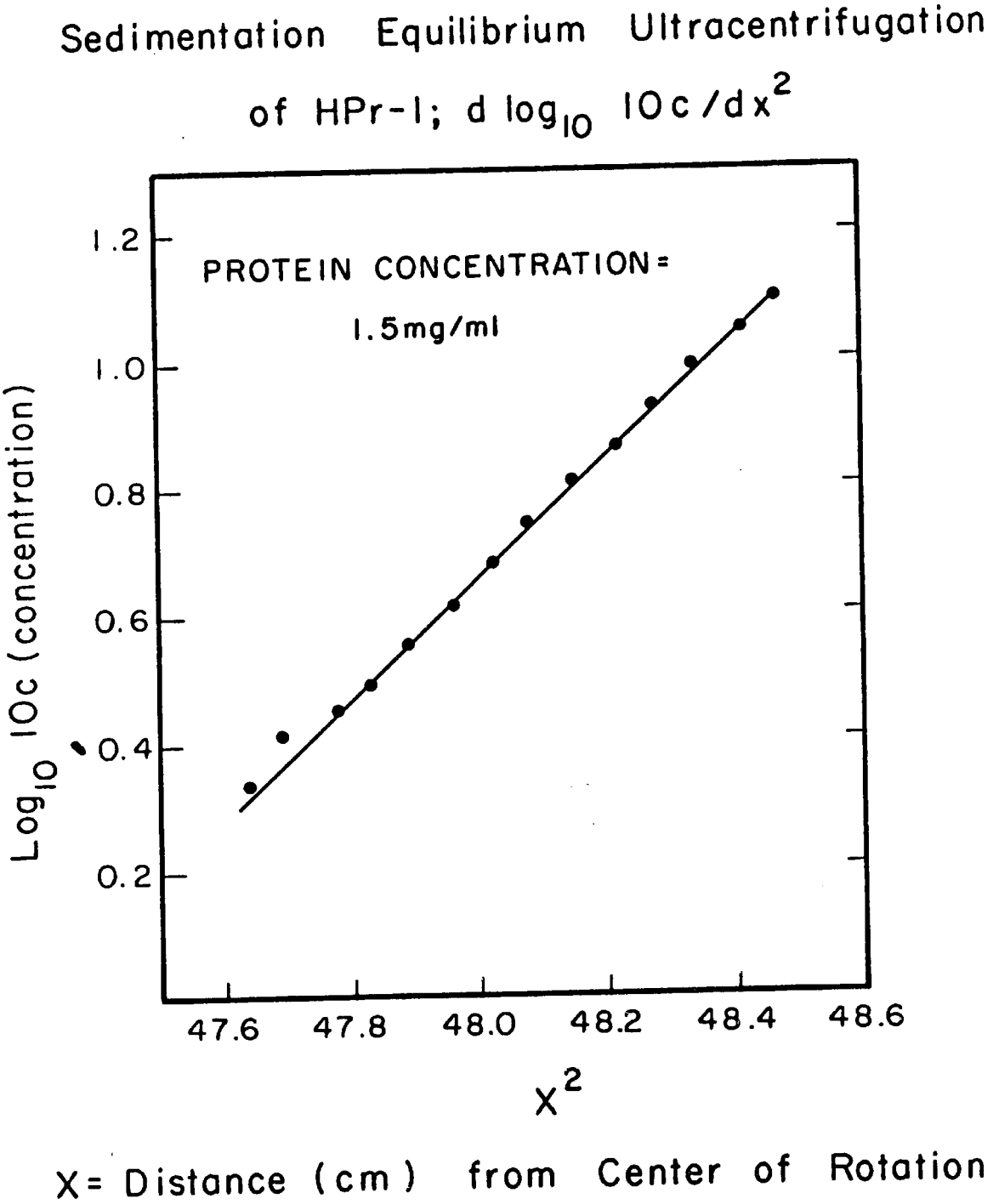
above. The effect of a larger concentration is to shift the measurable portion of the interference fringes toward the center of rotation. Because the protein molecules distribute along the x axis (x = the distance from the center of rotation) as a function of their molecular weight, the larger starting protein concentration will emphasize any smaller molecular weight species in the solution.

The plot of $d\log_{10}10c/dx^2$ for the higher concentration of HPr-1 is shown in Figure 11. The weight-average molecular weight determined using the slope of the line was equal to 9750. This value is very close to the molecular weight calculated from the summation of the amino acid residues. This result shows that there is not any protein species of smaller molecular weight present in the HPr preparation. The difference in molecular weights (9430 versus 9750) determined at two concentrations probably reflects a slight variation in the conditions of the two experiments. (Ideally, the two concentrations should be examined simultaneously, but the appropriate cell was not available). The linearity of the plot obtained at the higher concentration of HPr-1 again emphasizes the homogeneity of the protein sample.

3. Amino Acid, Carbohydrate, and Phosphate Analyses; U. V. Spectra; Peptide Mapping

The amino acid composition was determined with a Spinco Model 120 analyzer. Each hydrolysis was performed in Kontes vacuum hydrolysis tubes, using 400 µg HPr in one ml of constant-boiling HCl. After evacuation, hydrolysis was performed at 110° for 24 hours. Ninhydrin-reactive substances gave absorbancies that were measured between 0 and 0.1.

FIGURE 11



The equilibrium run was conducted and analyzed by the method of Yphantis (62). The speed was 59,780 rpm and the temperature, 3.5°. HPr-1, approximately 1.5 mg/ml, was equilibrated by dialysis in 0.1 M KCl before the experiment.

Within experimental error, the amino acid composition of the two HPr's was identical (Table 2). The values are presented as molar ratios relative to two histidine residues. It is interesting to note that HPr contains no tryptophan, tyrosine, or half-cystines. The absence of the aromatic amino acids, except for phenylalanine, is also confirmed by the U. V. spectra of the two HPr's (Figure 12). One mole of tyrosine or tryptophan per mole of HPr, because of their large extinction coefficients, would have overlapped and obscured the phenylalanine peaks.

Carbohydrate analysis of HPr was conducted with 100 µg of protein. The phenol-sulfuric method of Dubois et al (58) was used, and modified to give a final volume of 1.8 ml. The Fiske and Subba-Row method (61) was used for organic phosphate, after digestion of approximately 3 mg of HPr. In both determinations, absorbancies were determined with a Cary spectrophotometer, using a 50 mm path-length cell.

Amide nitrogen was determined by the method of Wilcox (74), using Conway microdiffusion dishes for distillation of the ammonia, which was assayed by the ninhydrin method (60). HPr-1 contained 15.0 amide residues while HPr-2 contained 10.6. This difference of approximately four amide residues was the only significant difference detected between HPr-1 and HPr-2. This finding explains the difference in the electrophoretic behavior of the HPr's in the polyacrylamide gels and in the elution pattern from DEAE-cellulose.

The number of histidine residues was confirmed by peptide mapping of a tryptic digest as shown in Figure 13. Since HPr contained 8 lysines and 1 arginine, the maximum number of peptides would be ten. Nine ninhydrin reactive spots were found, two of

TABLE 2

Analysis of HPr-1 and HPr-2

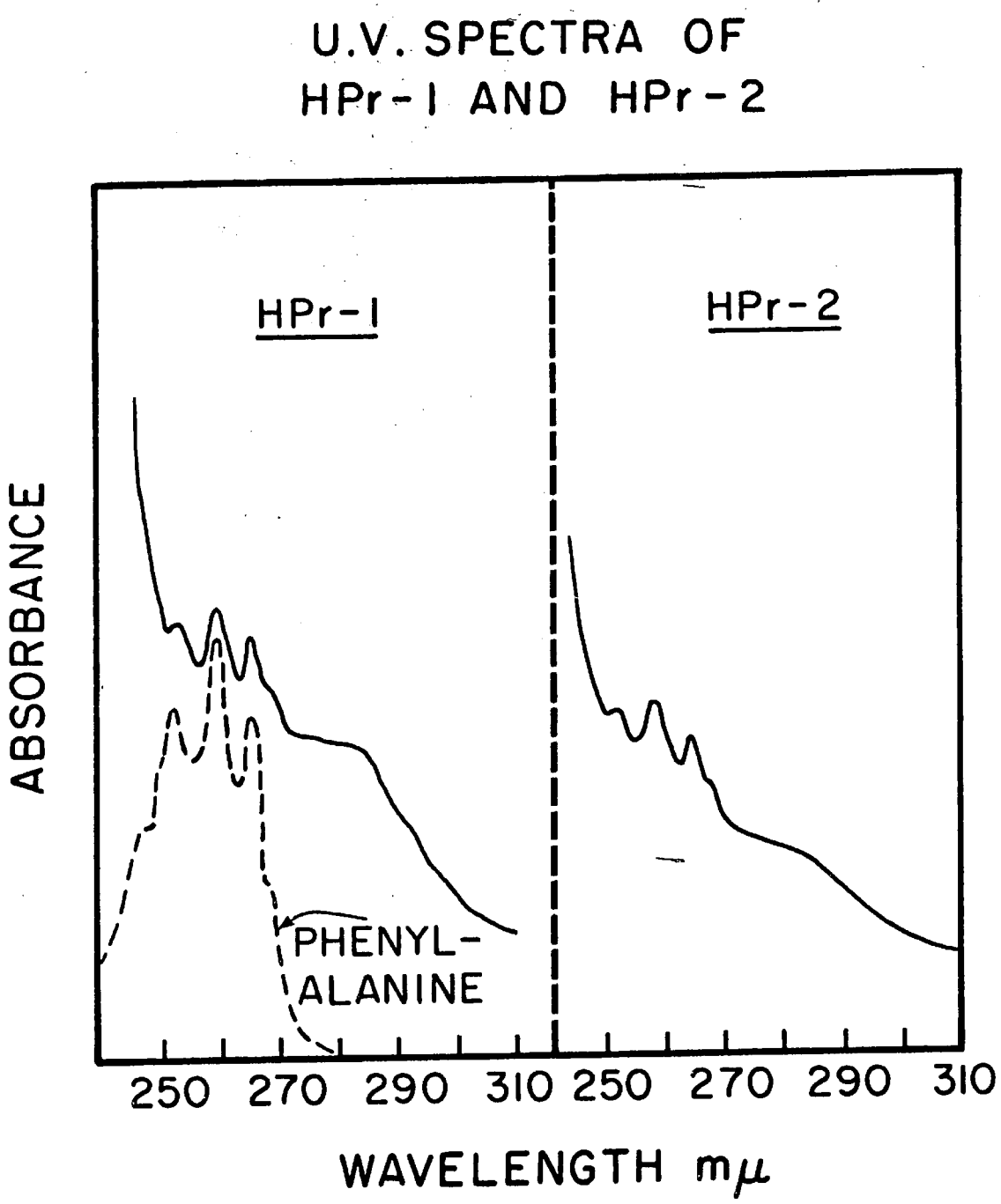
Amino Acid	HPr-1	HPr-2
Lysine	7.8*	7.8*
Histidine	2.0	2.0
Arginine	1.1	1.2
Aspartic Acid	4,2	4,2
Threonine	10.0	10.4
Serine	5.3	5.8
Glutamic Acid	15.2	16.3
Proline	2.3	1.7
Glycine	6.7	6.7
Alanine	9.9	10.2
Half Cystine	<0.05	<0.05
Valine	8.0	8.0
Methionine	2,2	2.1
Isoleucine	3.6	3.2
Leucine	8.3	8.4
Tyrosine	<0.05	<0.05
Phenylalanine	4.1	3.8
Trp (spectrophot.)	<0.1	<0.1
Ammonia (Conway)	15.0 ± 0.2	10.6 ± 0.6
Organic Phosphate	<0.1	<0.1
Carbohydrate	<0.1	<0.1
Molecular Weight	9639 g/mole	9716 g/mole

*Residues/2 moles histidine

TABLE 2 - Cont.

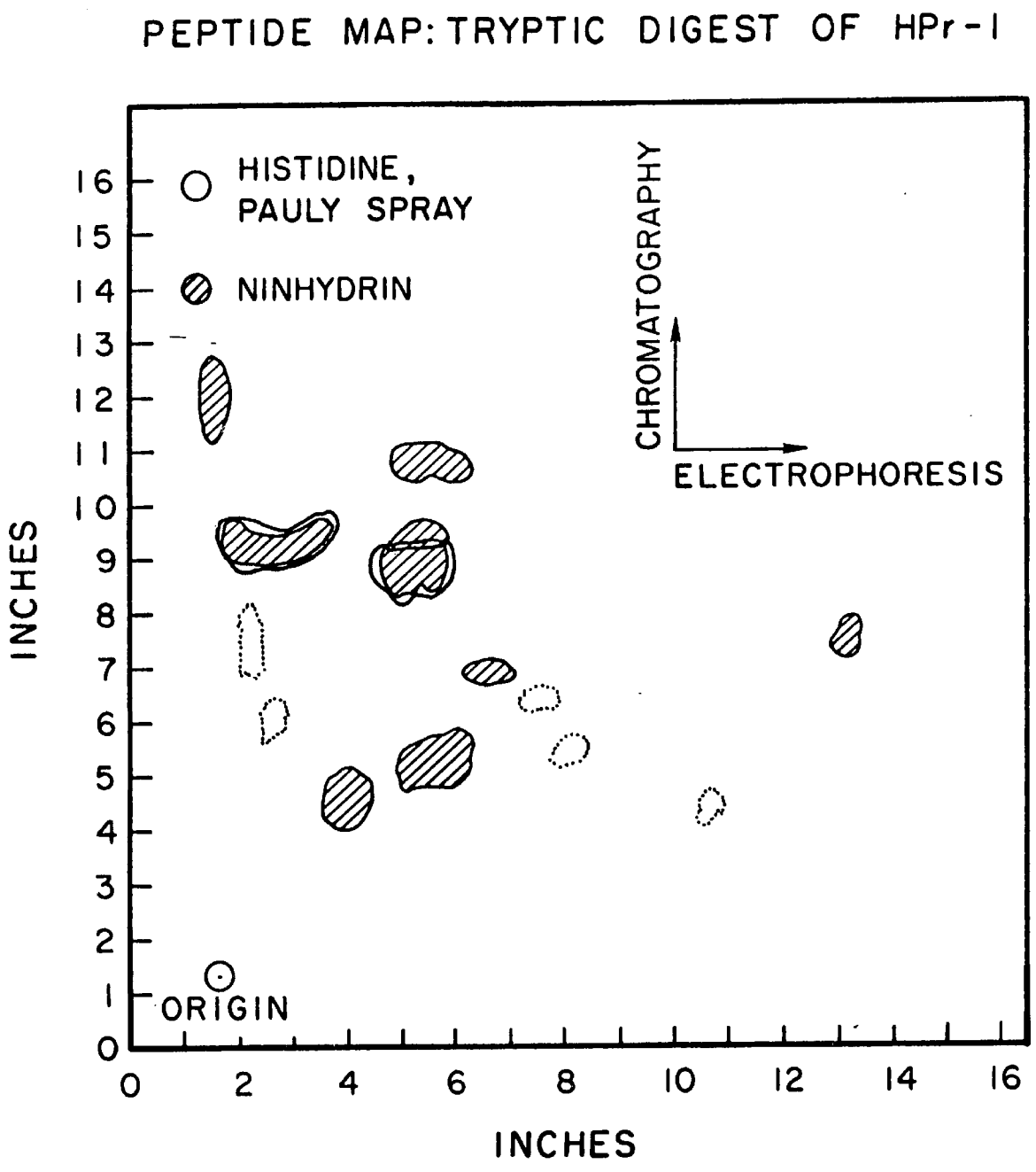
The values given are uncorrected for loss during hydrolysis. Experimental details for amino acid analyses and other determinations are given in the text.

FIGURE 12



The ultraviolet light absorption spectra were recorded on a Cary Spectrophotometer, Model 15, using a 10 mm light path cell. The concentrations (in water) were HPr-1, 1.1 mg/ml, and HPr-2, 0.86 mg/ml. The pH of the solutions were 8.5 and 7.0, respectively.

FIGURE 13



Experimental details are given in Methods. Electrophoresis was in pH 3.6 pyridine-acetate buffer for 1 hour at 40 v/cm. Chromatography in the second dimension was performed, using the solvent system n-butanol:pyridine:glacial acetic acid:water (90:60:18:72). The dotted circles represent faint ninhydrin spots.

which contained histidine, as shown by the Pauly spray (63).

4. Probable Origin of HPr-2

The important question in terms of physiological function is whether HPr-2 is truly a product of the cell, or whether it is an artifact of preparation. Evidence to support the second possibility is the following:

a) Conceivably, a family of HPr's might exist, where each serves as a substrate for different Enzymes II (37). The two HPr fractions, HPr-1 and HPr-2, could result from this *in vivo* requirement. To test this hypothesis, several carbohydrate substrates were used to assay HPr-1 and HPr-2 (Table 3). The particulate Enzyme II preparation used for this experiment contained many Enzyme II activities, each specific for a certain sugar. Therefore, the carbohydrate substrates measured different Enzyme II activities. As shown in the Table, the ratio of activity of HPr-1 and HPr-2 was relatively constant, thereby indicating that neither HPr was specifically required for phosphorylation of particular sugars. Similar results were obtained by assay of the various fractions from the purification steps (Table 4).

b) Strong evidence that HPr-2 was an artifact of isolation is shown in Figures 14 and 15. When purified HPr-1 was treated in a manner similar to the initial steps used in the purification procedure, and the products analyzed by disc gel electrophoresis, HPr-1 was obviously altered. Partial identification of the three bands was demonstrated by co-electrophoresis of the treated material with HPr-1 and HPr-2. The most rapidly migrating band in the treated material showed the same electrophoretic mobility as HPr-2. The

TABLE 3

HPr-1 and HPr-2 Activities with

Different Carbohydrate Substrates

Experiment	Substrate	HPr	6-P-Ester Formed mumoles	Activity Ratio HPr-1/HPr-2
1	ManNAc	1	35.1	2.4
		2	14.6	
		1 + 2	46.0	
2	Glucose	1	7.6	2.5
		2	3.1	
3	Methyl α-Glucoside	1	7.9	2.6
		2	3.0	
4	Galactose	1	7.9	2.2
		2	3.6	

Complete incubation mixtures were prepared and assayed as described in Figure 3. Incubations were conducted for 30 minutes at 37°. HPr-1 (1.14 µg) or HPr-2 (0.86 µg) were used in each incubation. Phosphorylation of galactose was studied with a charcoal-treated crude extract of E. coli W4345. This strain was grown in the minimal salts medium, supplemented with the following: 0.4% glycerol, 0.1% casamino acids, 0.1 mg% thiamine, and 0.001 M D-fucose (to induce galactose permease).

TABLE 4

HPr Activity Throughout Purification with Enzymes: II_{Glc}, II_{ManNAc}, and II_{Gal}

Purification Step	Activity Ratio: Methyl α-Glucoside / ManNAc	Activity Ratio: Gal/ManNAc*
Crude Extract	0.15	0.19
Heat Treatment	0.25	0.26
Acid Precipitation	0.19	0.19
CM-Cellulose	0.26	0.15
Sephadex G-50	0.18	0.14
DEAE-Cellulose		
HPr-1	0.23	0.09
HPr-2	0.24	0.15

*The ratio of the percent conversions for each substrate using the same amount of the HPr fraction.

Complete incubation mixtures were prepared and assayed as in Figure 3. The charcoal-treated crude extract of E. coli W4345 was used as a source of Enzymes I and II.

FIGURE 14

Proteins were analyzed by disc gel eletrophoresis at a running pH of 9.5, as described in Figure 7. HPr-1 was treated in the following manner, simulating some of the purification conditions: 1 mg of salt-free, lyophilized HPr-1 was dissolved in 1 ml of 0.01 M potassium phosphate buffer, pH 7.5, and heated for 10 minutes in a boiling water bath. The pH was lowered to 2.3, at 4°, by the addition of glacial acetic acid and was maintained at this temperature for 30 minutes. The pH was then adjusted to 7 with NaOH, the mixture dialyzed against two changes of 500 ml sodium acetate buffer (0.01 M, pH 4.9) containing 0.3 M NaCl, and the retentate concentrated in a rotary evaporator, with the bath temperature set at 35°, until the salt began to crystallize. Distilled water was added, the solution dialyzed against two changes of 500 ml each of 0.005 M Tris-HCl buffer, pH 7.6, the retentate lyophilyzed and the resulting solid dissolved in 0.5 ml of distilled water; 50 µl of this solution was electrophoresed.

FIGURE 14

Conversion of HPr-1 to HPr-2 by Conditions Used for Isolation

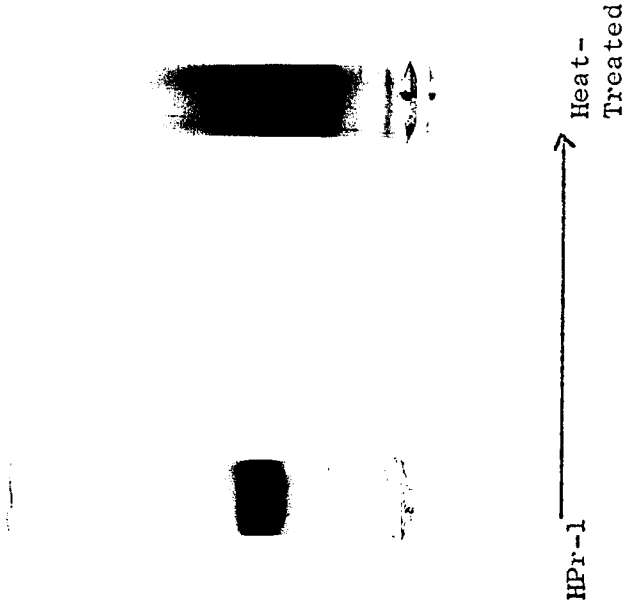


FIGURE 15

Proteins were analyzed by disc gel electrophoresis at a running pH of 9.5 as described in Figure 7. HPr-1 was treated by heating at 100° for 5 minutes in 0.01 M potassium phosphate buffer, pH 7.5.

FIGURE 15

Conversion of HPr-1 to HPr-2 by Heating



middle band migrated with the original HPr-1. The slowest band may correspond to the initial peak eluted from DEAE-cellulose (Figure 6). A later experiment demonstrated that heating alone, at a pH of 7.5, produced the three bands (Figure 15).

HPr was previously reported to be stable to heating at 100° for 20 minutes at neutral pH (1). This was with an HPr preparation that was 10-20% pure. By contrast, purified HPr-1 was not very stable at 100° either at pH 7.5 in potassium phosphate buffer, or at pH 7.6 in Tris-HCl buffer (Figure 16).

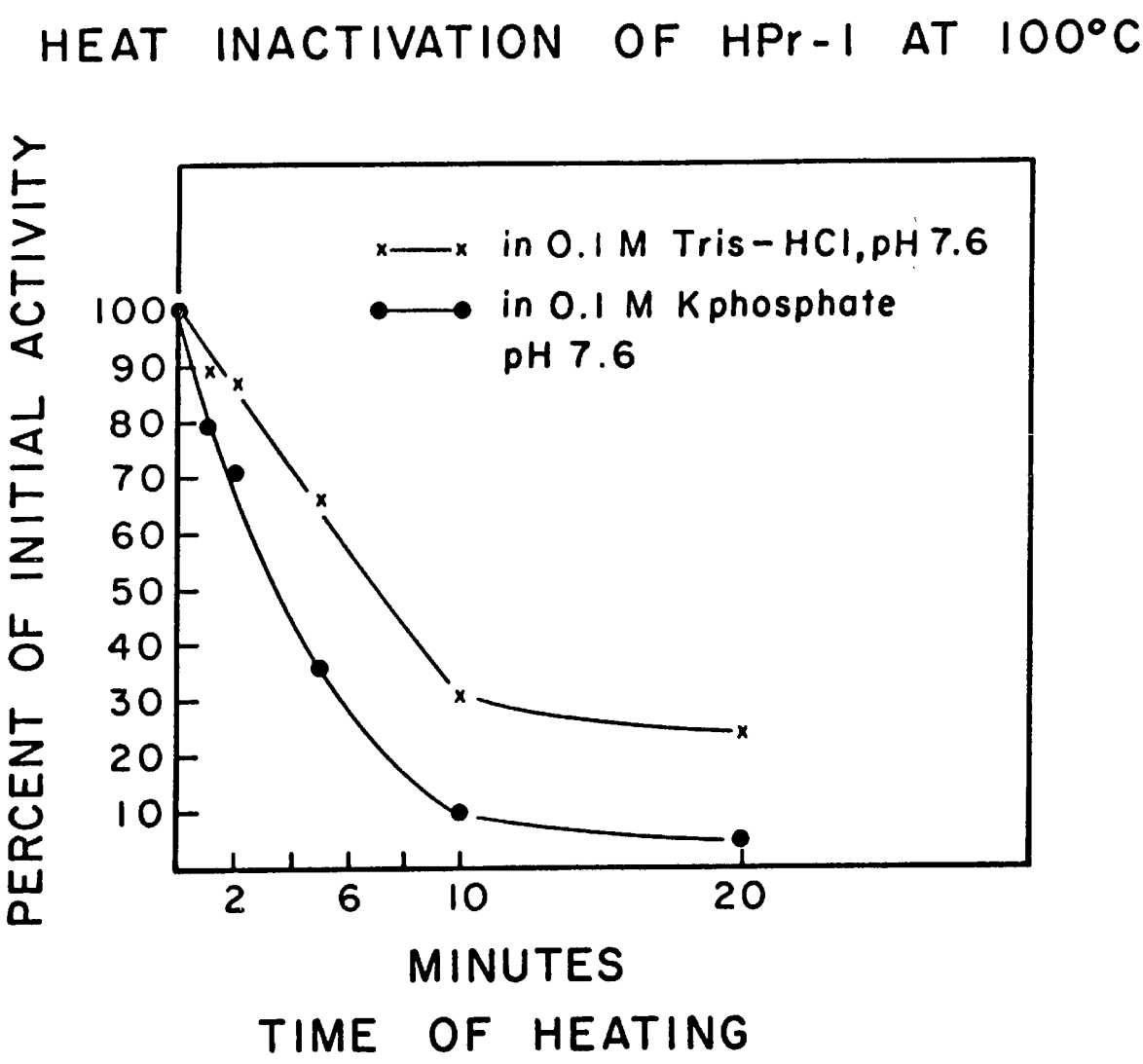
These results therefore suggest that HPr-2 was formed by loss of some of the amide groups from HPr-1, and that this partial hydrolysis probably occured at the initial heat step used in the purification procedure.

5. ^{32}P-HPr Studies; Isolation and Characterization of 1-Phosphohistidine

Since HPr-1 and HPr-2 each contained two histidine residues, and since it was shown that the P in P-HPr is linked to histidine (1), it was important to determine whether one or two phosphate residues were incorporated into the protein. As shown in Table 5, the maximum quantity of P incorporated into HPr by Enzyme I ranged between 0.82-0.95 mole/mole of protein. Therefore, only one of the two histidine residues appears to accept P.

Phosphohistidine had been previously isolated from P-HPr (1). The following studies were performed to determine whether the isolated phosphohistidine was the 1- or 3-isomer, The synthetic 3-P-histidine and phosphoramidate used in these studies were kindly supplied by Dr. Hultquist.

FIGURE 16



After heating for the indicated times, HPr activity was assayed as described in Figure 3, using a charcoal-treated crude extract of E. coli K235 as a source of Enzymes I and II.

TABLE 5

Conversion of HPr to ^{32}P-HPr

$$^{32}\text{P-PEP} + \text{HPr} \xrightarrow[Mg^{++}]{\text{Enzyme I}} {}^{32}\text{P-HPr} + \text{Pyruvate}$$

Each incubation contained the following components (μmoles) in final volumes of 0.095 ml: ^{32}P-PEP (10.1 x 10^6 cpm/μmole), 0.52; $MgCl_2$, 0.5; Tris-HCl buffer (pH 7.6), 5; 0.18 mg Enzyme I. Incubations were conducted at 37° for 15 minutes. The reactions were terminated and assayed as described under "Methods". ^{32}P incorporation into HPr was not increased by longer incubation time or by adding more Enzyme I.

HPr-1	HPr-2	^{32}P-HPr Formed	Molar Ratio ^{32}P/HPr
mμmoles	mμmoles	mμmoles	
2.8		2.7	0.95
2.8		2.3	0.83
5.7		4.8	0.83
5.7		4.7	0.82
	2.5	2.1	0.83
	5.0	4.5	0.90
	5.0	4.4	0.89

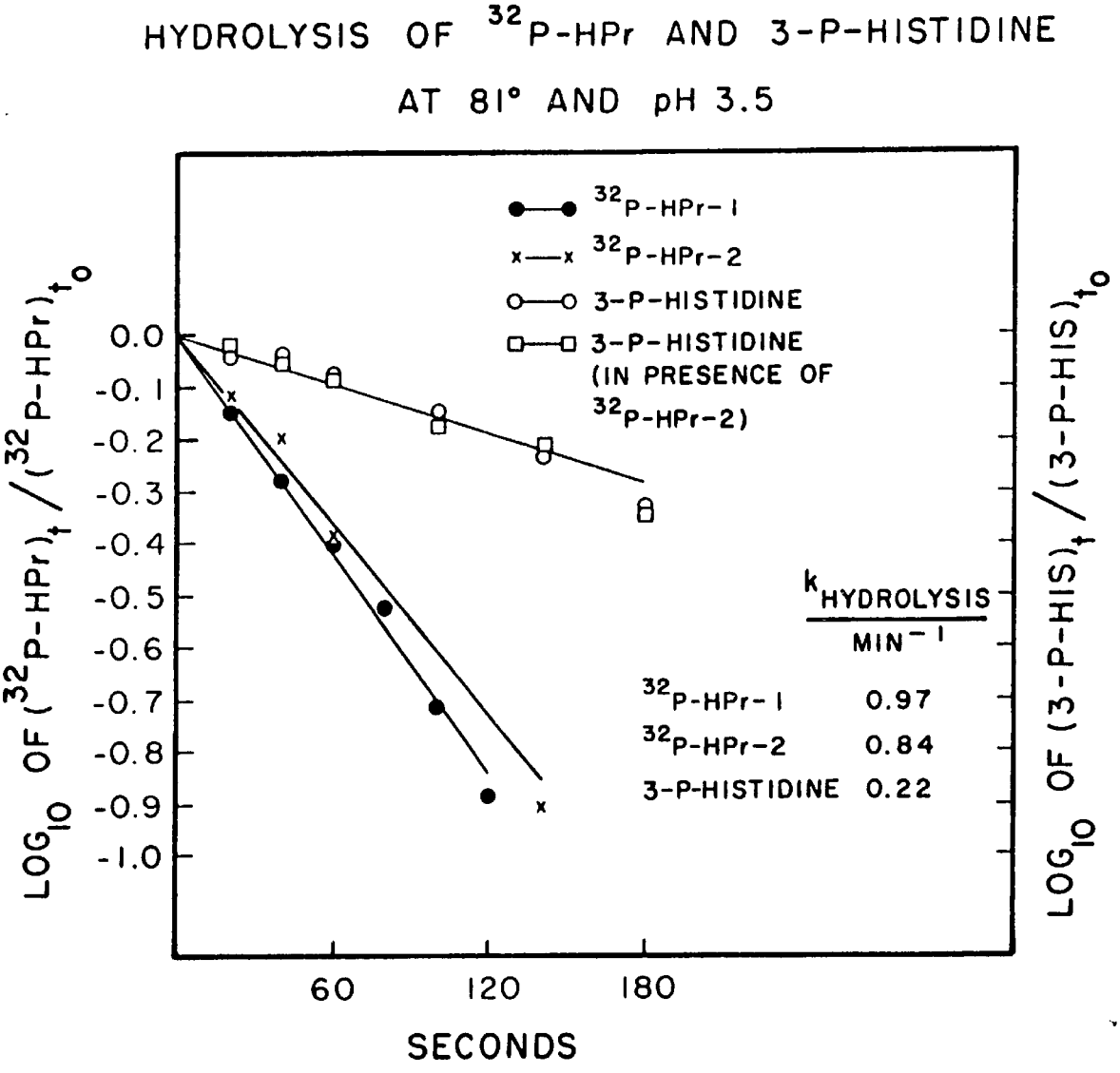
A large incubation mixture (as described in Table 5) containing ^{32}P-HPr was fractionated by passage through a 2 x 50 cm column of Sepadex G-25. The column was first equilibrated and subsequently developed with 0.2 M NaCl-0.1 M Na_2CO_3. The ^{32}P-HPr fractions (separated from $^{32}P_i$ and ^{32}PEP) were pooled, dialyzed to remove salt and stored at -18°.

The rate of hydrolysis of the phosphoryl bond in ^{32}P-HPr was compared to the hydrolysis rate of 3-P-histidine as shown in Figures 17 and 18. A plot of the logarithm of unhydrolyzed ^{32}P-HPr against time, gave a straight line, therefore indicating that the hydrolytic behavior followed pseudo first order kinetics. The rate constants determined for P-HPr were 4-5 times greater than those reported (43) for 3-P-histidine; the rate constants for hydrolysis of 3-P-histidine found in the present studies agree with those previously reported by Hultquist (43, 44). These results are in contrast to those obtained with phosphorylated succinate thiokinase (47, 48) and phosphorylated nucleoside diphosphokinase (51); the hydrolysis rates of these phosphoproteins were the same as those of synthetic 3-P-histidine. Furthermore, 3-P-histidine was isolated from these phosphorylated enzymes.

To reduce the possibility of artifacts (for example that the rate of ^{32}P-HPr hydrolysis was increased by contaminants in the preparation), the ^{32}P-HPr and 3-P-histidine were mixed and the hydrolysis rate of 3-P-histidine determined. As shown in Figure 17, the rate was not affected, and it is concluded that the observed rate constants for the hydrolysis of ^{32}P-HPr are correct.

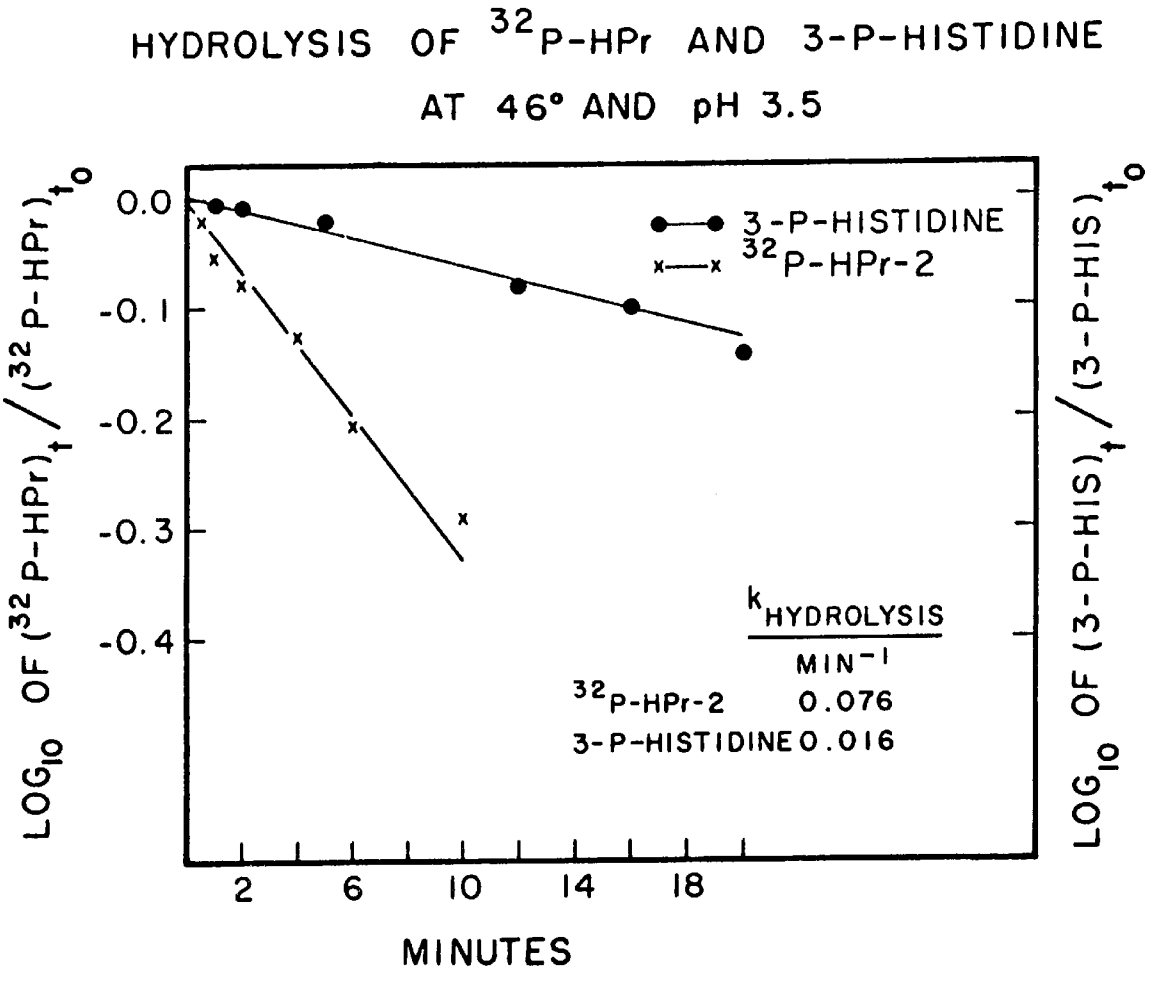
At 46°, the rate constant for P-HPr (0.076 min^{-1}) is less than that reported for synthetic 1-P-histidine (0.12 min^{-1}) (44).

FIGURE 17



^{32}P-HPr-1 and ^{32}P-HPr-2 (approximately 10^{-4} µmole), and 3-phosphohistidine (5×10^{-3} M) were hydrolyzed at 81° in 0.05 M citrate-borate buffer (pH 3.5), and the mixtures assayed for $^{32}P_i$ or P_i as described under "Methods," Experimentally determined values were extrapolated to 0 time to obtain blank values.

FIGURE 18



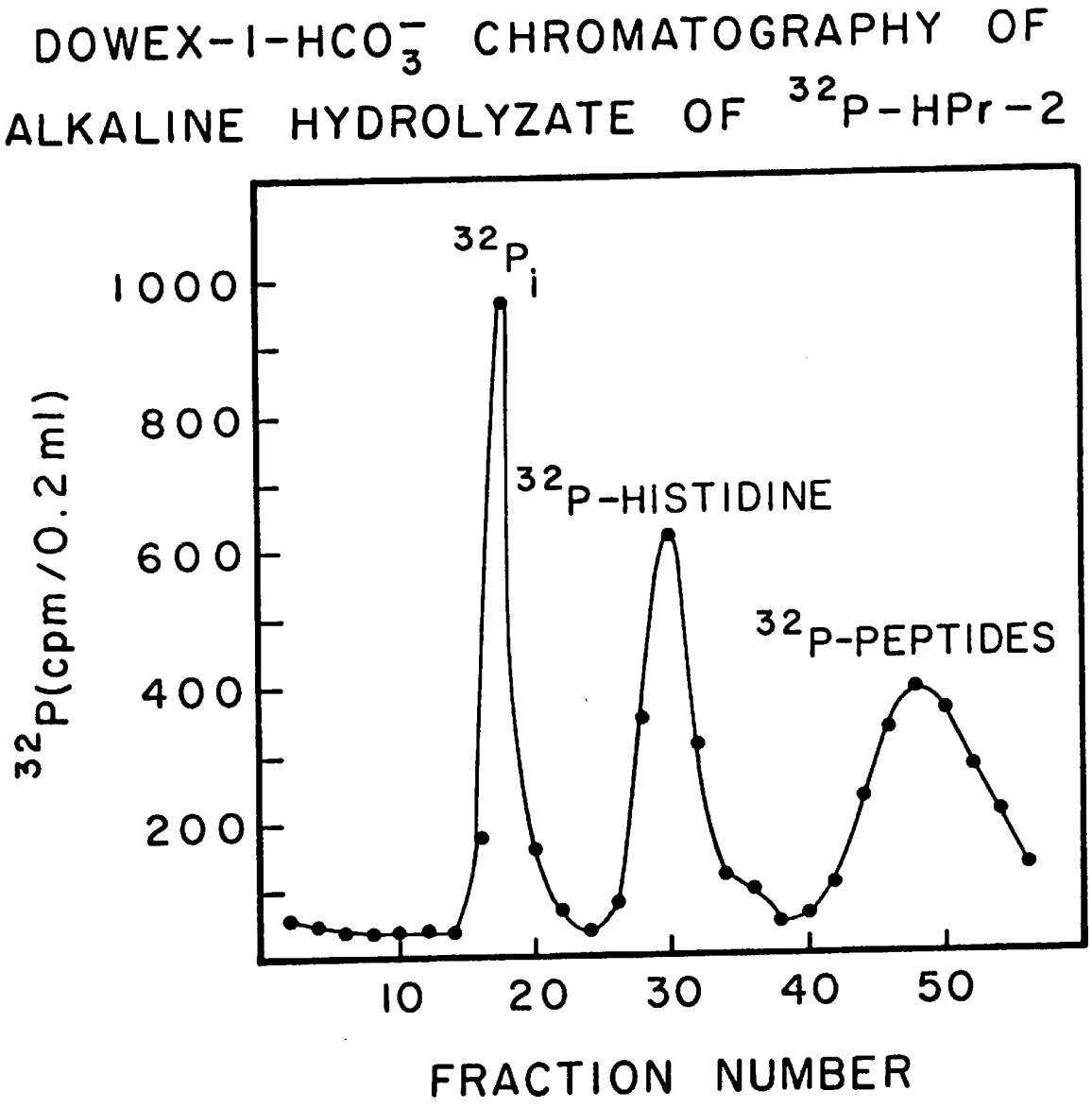
Hydrolyses were conducted at 46° in 0.05 M citrate-borate buffer as described in Figure 17.

The synthetic 1-P-histidine contains its α-amino group in a position close to the phosphoimidazole linkage, and this amino group presumably increases the rate of hydrolysis (44). Since the α-amino group in the protein is acylated, the two substances cannot be strictly compared. A proper interpretation of the lability of ^{32}P-HPr may require knowledge of the configuration of the protein. For example, it would be important to know whether the basic groups of lysine or arginine residues are close to the phosphoimidazole linkage.

^{32}P-HPr was hydrolyzed at 100° for 3 hours in 3 N NaOH in order to isolate and further characterize ^{32}P-histidine. Ion-exchange chromatography of the hydrolyzate on Dowex-1-HCO_3 gave the results shown in Figure 19, and the order of elution is similar to that previously reported (1, 44, 46, 52, 53). ^{32}P separated into 3 peaks, and the total ^{32}P recovered was 87% of that placed on the column. The first peak eluted from the column co-migrated with inorganic phosphate on paper electrophoresis. The third peak was pooled and hydrolyzed in 6 N HCl for 12 hours; electrophoresis of the hydrolyzate in pH 6.5 pyridine-acetate buffer showed several ninhydrin-reactive spots. Therefore, the third peak was concluded to be ^{32}P-labeled peptides.

The material from the second peak co-migrated with synthetic 3-P-histidine on paper electrophoresis in ammonium acetate buffer, pH 8.2. In contrast to the previous report (43), the 1- and 3-isomers of P-histidine were not separable, in this system, The ^{32}P-histidine peak also co-migrated with synthetic 3-P-histidine on paper electrophoresis at pH 9.0 and 6.5, and on paper chromatography in 95% EtOH:0.1 M K_2CO_3 (65:35) (65). In addition, the ^{32}P-

FIGURE 19



^{32}P-HPr was hydrolyzed for 3 hours in 3 N NaOH at 100° in a sealed tube. The hydrolyzate was then diluted ten-fold and placed on a column (1 x 7 cm) of Dowex-1-HCO_3 (X-8, 200-400 mesh). A linear gradient of 0 to 1.0 M $KHCO_3$ (total of 900 ml) was used for elution and 7.5 ml fractions collected. Aliquots were assayed for ^{32}P by liquid scintillation methods.

histidine was completely hydrolyzed by treatment for 30 minutes at room temperature in 0.03 M pyridine.

That the ^{32}P-labeled phosphoryl group was linked to the N-1 position of the imidazole ring of histidine in the isolated ^{32}P-histidine, was suggested by the following evidence:

a) The isolated ^{32}P-histidine from ^{32}P-HPr was very much more labile than synthetic 3-P-histidine. At 4°, the ^{32}P-histidine isolated from the ion-exchange column (pH 8.1 solution) was converted to $^{32}P_i$ in one week. Under similar conditions, 3-P-histidine was only slightly hydrolyzed.

b) It was reported that the reagents used in the Martin and Doty inorganic phosphate assay hydrolyzed 1-P-histidine to a large extent at 0°; these conditions hydrolyze little of the 3-isomer (43). The lability of ^{32}P-histidine from ^{32}P-HPr was ascertained as shown in Table 6. Thirty-three percent of the ^{32}P-histidine was hydrolyzed in 5 minutes in the assay procedure, while only 5% of the 3-P-histidine was hydrolyzed.

c) Figure 20 shows the results of a co-chromatography experiment where ^{32}P-histidine and synthetic 3-P-histidine were mixed. The labeled P-histidine is eluted slightly before 3-P-histidine, as expected if it were the 1-isomer (44). The same elution pattern was obtained with a mixture containing the unfractionated hydrolyzate of ^{32}P-HPr and synthetic 3-P-histidine.

d) Figure 21 shows the co-chromatography of a phosphoramidate-histidine reaction mixture and the ^{32}P-histidine. Under the conditions of the reaction, about 50% of the P-histidine is the N-1 isomer. The fractions were assayed for histidine by the method of

TABLE 6

Lability of ^{32}P-Histidine from ^{32}P-HPr

to Martin-Doty Phosphate Reagents*

Substance	Total	Phosphate %Stable	Phosphate %Labile
3-P-Histidine	0.2 umoles	95	5
^{32}P-Histidine	1154 cpm	67	33

*1.5% Ammonium molybdate in 0.35 N H_2SO_4, 0°, 5 minutes.

± 0.05 umole carrier P_i

FIGURE 20

A portion of the ^{32}P-histidine (Figure 19) was diluted 3-fold in water, 5 mg of synthetic 3-P-histidine added, and the mixture immediately adsorbed to a 1 x 7 cm column of Dowex-1-HCO_3. The column was eluted as described in Figure 19.

FIGURE 20

DOWEX-1 CHROMATOGRAPHY OF MIXED P-HISTIDINES:
^{32}P-HISTIDINE + SYNTHETIC 3-P-HISTIDINE

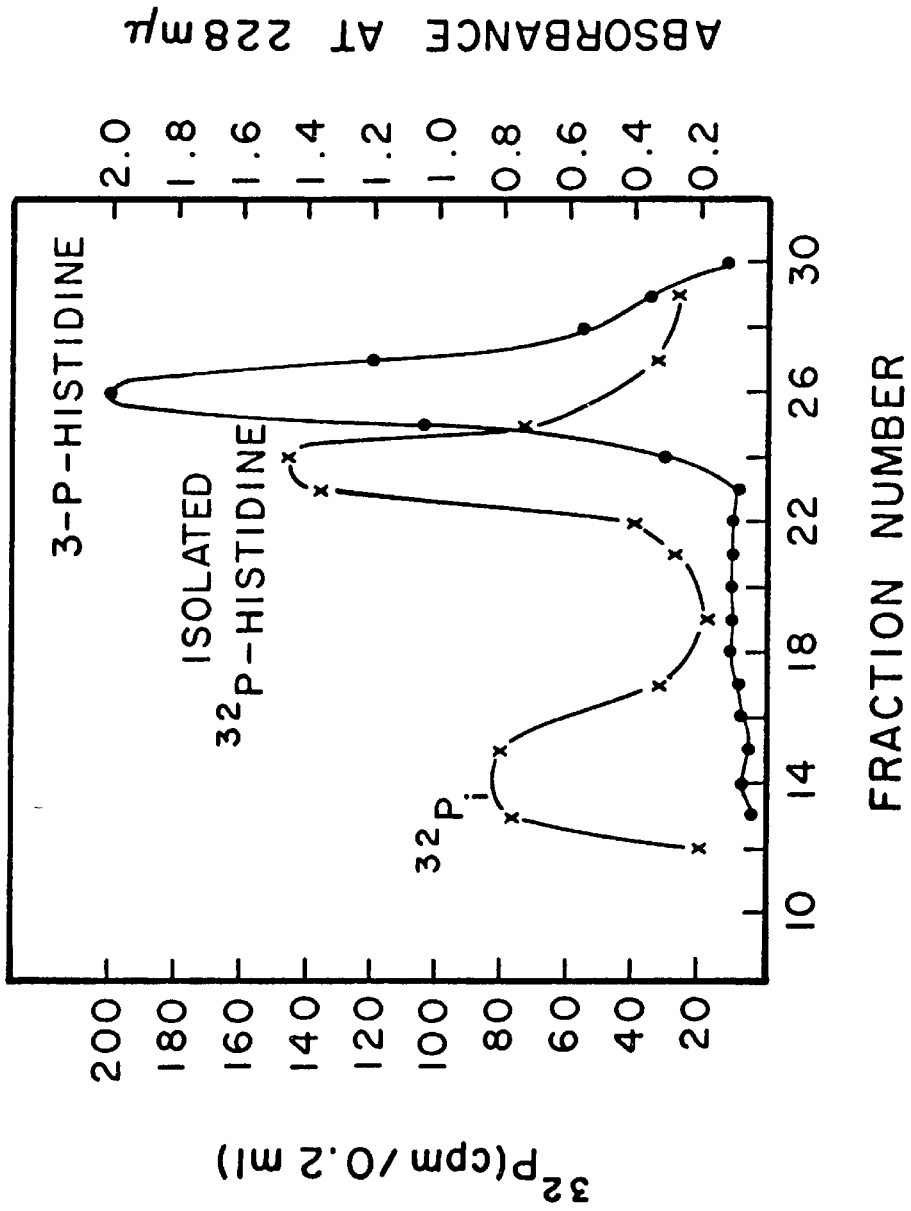
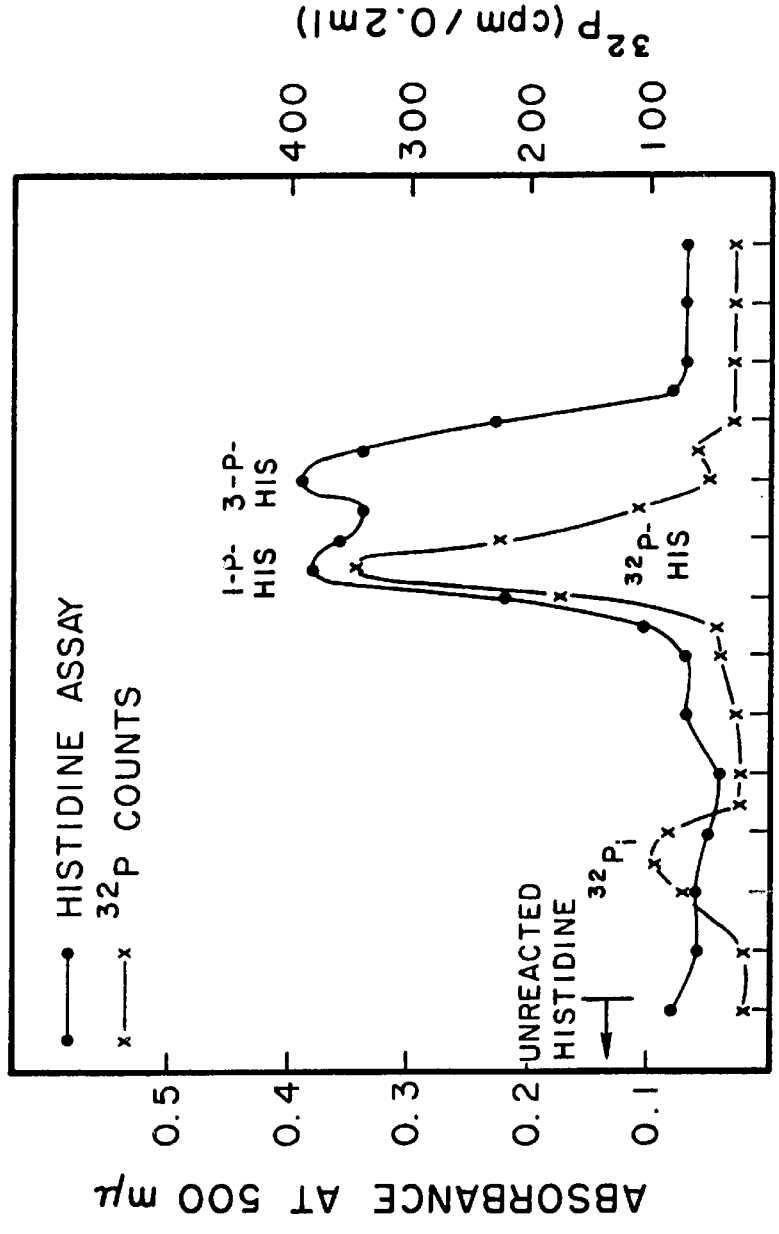


FIGURE 21

^{32}P-histidine was isolated from a base hydrolyzate of ^{32}P-HPr as described in Figure 19. The synthetic P-histidine mixture was prepared by the following method: 100 mg of phosphoramidate and 10 mg of histidine were dissolved in 3 ml of distilled water and allowed to react for 35 minutes at room temperature. The ^{32}P-histidine material from the Dowex-1 column was diluted 3-fold in water, added to the phosphoramidate-histidine reaction mixture, and the mixture immediately adsorbed to a Dowex-1-HCO_3 column (1 x 7 cm.). Elution was performed as described in Figure 19. Aliquots (50 µl) of the fractions were assayed for histidine as described in the text.

FIGURE 21

DOWEX-1 CO-CHROMATOGRAPHY OF MIXED P-HISTIDINES:
^{32}P-HISTIDINE + SYNTHETIC 1-AND 3-P-HISTIDINE



Macpherson (59). The diazotized sulfanilic acid reagent does not react with P-histidine until the N-phosphoryl bond is hydrolyzed. Accordingly, an aliquot of each fraction was hydrolyzed in 0.1 N HCl before adding the other reagents. The fractions of the P-histidine double peak were also assayed by the following modification: An aliquot was adjusted to one ml with water and stored at 0°. The sulphanilic acid plus HCl and the $NaNO_2$ reagents were added and the mixture allowed to react for 10 minutes at 0°. Absorbancies were then determined after adding the Na_2CO_3 reagent. The histidine standard curve was assayed in the same manner. Table 7 shows the amount of histidine assayed at 0°, without prior hydrolysis of the N-P bond, as the percentage of histidine assayed after 0.1 N HCl hydrolysis. This indicates that the first part of the double peak, where the radioactive material was also eluted is the less stable 1-P-histidine as expected (43), while the second peak was 3-P-histidine. In addition, as seen in Figure 20, synthetic 3-P-histidine was eluted in the latter half of the double peak.

All the results cited above, including hydrolysis studies of the intact ^{32}P-HPr, hydrolysis studies of the isolated ^{32}P-histidine, and chromatography of the latter are consistent with the conclusion that the phosphate group in ^{32}P-HPr is linked to N-1 of the imidazole ring of a histidine residue.

TABLE 7

Histidine Assay of Phosphohistidine Peaks of Figure 21

Fraction	Histidine Measured % Labile Without Prior Hydrolysis*
26	98
27	90
28	88
29	60
30	40
31	36

*The results are presented as % of histidine determined at 0° without hydrolysis compared to the quantity measured after hydrolysis with 0.1 N HCl. Experimental details are given in the text.

D. DISCUSSION

The results from this and other laboratories (2, 3, 36-38, 97-99), implicate the phosphotransferase system as a key component of carbohydrate transport systems in bacteria. The present evidence suggests that most sugars are phosphorylated concomitant with their accumulation. This is apparently not true with lactose and its analoques in E. coli, although these compounds are accumulated as phosphate esters in Staphylococcus aureus (22-24). Furthermore, since Enzyme I and HPr mutants of E. coli cannot utilize lactose (or galactose) (93,100), the PTS appears to be involved in the transport of these sugars by this organism. A possible explanation for these apparent discrepant results is that the membrane-bound PTS is coupled to a specific lactose-phosphate phosphatase, yielding free lactose as the product of its transport. To search for such a phosphatase, substrate quantities of the appropriate sugar-P were required, and a method was therefore developed for the synthesis of an analogue of lactose-P, methyl 1-thio-β-D-galactopyranoside 6-phosphate (TMG-6-P).

The synthesis involved condensation of TMG with cyanoethylphosphate followed by base hydrolysis of the cyanoethyl group. The 2, 3, and 4 hydroxyl positions were not blocked since Tener (69) had shown that the rate of phosphorylation at the primary hydroxyl was about ten times greater than the rate at a secondary hydroxyl. Although the synthetic procedure was not studied in sufficient detail to provide optimum conditions, the method in its present form yields adequate quantities of the desired product; the product was shown to be identical to TMG-6-P formed in the

enzymatic reaction, and to the TMG-6-P accumulated by S. aureus.

Preliminary experiments were conducted to determine whether E. coli, induced for the lac operon, contained a specific TMG-6-P phosphatase. Crude extracts and shockates (26) do hydrolyse the phosphate ester, but there is no apparent difference between induced and non-induced cells. A more extensive study will be required to draw any conclusions from these results.

Another important product of the phosphotransferase system is galactose 6-phosphate. This substance is not metabolized through any of the known pathways, and it accumulates in E. coli cells that are transporting this sugar (18). The present studies involve a complete characterization of the enzymatically-synthesized galactose phosphate as the 6-P ester. The ester has recently achieved added significance, since it is formed from lactose-P by S. aureus β-galactosidase (23), and since it, rather than free galactose, is the inducer of the lac operon in this organism (101).

The major problem of this thesis was to isolate and characterize HPr, the phosphate-carrier protein in the phosphotransferase system. Earlier work (1) had shown that the phosphate residue was attached to a histidine moiety in the protein, and had led to the isolation of HPr that was 10-20% pure.

The present studies involved first the isolation of homogeneous HPr from E. coli K235. The purification procedure led to the isolation of two such proteins, separated by chromatography on DEAE-cellulose at the last step in the fractionation; the proteins were designated HPr-1 and HPr-2, and the latter was approximately half as active as the former in the phosphotransferase system.

Both proteins were homogeneous by disc gel electrophoretic analysis at pH's 9.5, 6.6, 4.3, and 2.3. At the two higher pH's, HPr-2 migrated more rapidly than did HPr-1, an expected result in view of the fact that HPr-2 was eluted from the DEAE-cellulose column after HPr-1.

The two proteins were also homogeneous by ultracentrifugal analysis, both in sedimentation velocity and equilibrium experiments. The calculated molecular weights were approximately 9500 g/mole.

The proteins contained no detectable carbohydrate or phosphorus, and amino acid analysis indicated that HPr was unusual in that it contained no half-cystine, tyrosine or tryptophan residues. The protein did contain 2 histidine and 4 phenylalanine residues, and the latter accounts for the absorption spectra exhibited by these proteins. The total amino acid analysis gave a calculated molecular weight that agreed with that found by ultracentrifugation.

On the basis of the amino acid analyses, a total of 10 possible peptides could be formed by trypsin digestion. Actually, 9 such peptides were detected, 2 of which contained histidine according to the Pauly spray test.

A major question in terms of possible biological significance was whether HPr-1 and HPr-2 were both synthesized by E. coli, or whether one was derived from the other during the isolation procedure. The only significant difference between the two proteins was their amide content (Table 2), HPr-2 containing 4 residues less than HPr-1. When HPr-1 was exposed to the first two steps in the isolation procedure, 100° at pH 7.5 followed by acid precipitation in the cold, or when it was simply heated (as in the first step),

it was partially converted to two new proteins. The major degradation product migrated on disc gel electrophoresis with HPr-2, while the minor product migrated more slowly, similar to a minor product detected during the DEAE-cellulose fractionation. From these results, the tentative conclusion was drawn that HPr-2 was an artifact of isolation, formed from HPr-1 by partial hydrolysis of amide groups during the heat step.

The relative lability of the amide groups in protein has been previously reported. Such results have been observed with adrenocorticotropic hormone (76), prolactin and growth hormone (77), insulin (78, 79), and cytochrome c (80-85). Cytochrome c amide hydrolysis has been studied extensively, and was found to occur at mild acid and alkaline (81, 82) conditions, and more important, even at physiological pH, ionic strength, and temperature (84). As with HPr, the cytochrome c experiments showed that the protein decreased in activity as amide groups were lost (85).

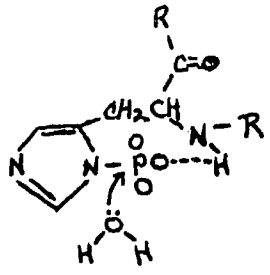
The data summarized above showed that HPr contained two histidine residues. Stoichiometry studies indicated that only one of these accepted a phosphate residue. That is, only 0.82-0.95 mole of phosphate could be incorporated into HPr.

An extensive study on the nature of the phosphoryl linkage to HPr was undertaken since the $-PO_3H_2$ residue can be linked to N-1 or to N-3 of the histidine imidazole ring. Previous work with succinic thiokinase (49-50), and nucleoside diphosphokinase (51), suggested that these phosphohistidine proteins contained the phosphoryl linked to N-3. The present studies lead to the conclusion that P-HPr contains the phosphoryl residue linked at N-1 of the

imidazole ring

This conclusion is based on the following experiments:

a) The first order rate constants for the hydrolysis of P-HPr were studied at two sets of conditions. The constants were 5 times greater than those reported previously (43, 44) for 3-phosphohistidine per se (confirmed in the present experiments), and for the 3-phosphohistidine proteins mentioned above. However, the constants were approximately half the rate constants reported for 1-phosphohistidine (44). This apparent discrepancy is explained by the fact that the free α-amino group of 1-phosphohistidine is in a position to facilitate the hydrolysis of the phosphoimidazole, while in the protein, this amino group is acylated. This explanation would, at first sight, suggest that 1- and 3-phosphohistidine in the proteins should be hydrolyzed at equivalent rates. However, the possibility exists that the remaining H atom on the acylated α-amino group may still participate in the reaction by hydrogen bonding with one of the oxygen atoms attached to the phosphorus. Such hydrogen bonding would induce a greater positive charge on the phosphorus atom thus facilitating the hydrolysis:



Some evidence to substantiate this hypothesis is the fact that the rate constants for the hydrolysis of 1- and 3-phosphohistidine approach the same value when the oxygens attached to the phosphorus become fully protonated as the pH is lowered (44).

b) The phosphohistidine was isolated from ^{32}P-HPr, and characterized by chromatography and electrophoresis. The marked lability of the isolated product was in accord with the conclusion that it was 1-phosphohistidine. More direct evidence was obtained by co-chromatography experiments with the synthetic compounds on ion-exchange resin. An inherent difficulty in these studies was the lability of both P-HPr and the isolated P-histidine, and the small quantities available for characterization. Nevertheless, all of the available data lead to the conclusion that P-HPr contains the phosphoryl linked at N-1 of the imidazole ring of a histidine residue.

It is important to note that if the isolated product was determined to be 3-phosphohistidine, the results would be less convincing. This is because of the much greater probability of transphosphorylation of 1-phosphohistidine to 3-phosphohistidine than the reverse reaction (44). It is therefore very unlikely that phosphoryl migration occurred during either the hydrolysis

of P-HPr, or the isolation of the phosphohistidine*.

The kinetic characteristics of the phosphotransferase system are not easily interpretable and will require further study. That is, HPr and phospho-HPr are substrates for Enzymes I and II, respectively. Unless HPr is present in very large excess, the ratios of HPr to P-HPr may determine the rate of the over-all reaction, i.e., whether Enzyme I or II is the rate-limiting enzyme. A more important difficulty that is encountered is the fact that Enzyme II is membrane-bound, while Enzyme I is soluble. Does HPr "shuttle" between solvent and particle (Enzyme II) carrying the phosphate residue, or is it phosphorylated while bound to Enzyme II? A preliminary kinetic study showed that the specific activity of Enzyme I was increased 10-fold when the activity was measured in the complete system compared with measuring the transfer of phosphate from PEP to HPr. This result is analogous to

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*From the data of Hultquist et al (43), 1-phosphohistidine is formed early in the phosphoramidate-histidine chemical transphosphorylation. As a result of transphosphorylation, and the greater lability of the 1-isomer, longer reaction times yield 3-phosphohistidine as the predominant or exclusive product. Formation of 1-phosphohistidine as the initial product may result from the fact that the nitrogen atom at the 1-position has a higher electron density (75), and because hydrogen bonding between hydrogen atoms attached to the α-amino group and the oxygen atoms attached to the phosphorus of phosphoramidate would both direct the attack at the 1-position (45) and facilitate the reaction by increasing the positive charge on the phosphorus atom.

those observed with E. coli pyruvate decarboxylase, isolated from the pyruvate dehydrogenase complex. The isolated enzyme exhibited a much lower turnover number than in the complex (86). It was suggested that covalent attachment of the subunits in the complex increased the catalytic efficiency of the enzyme. Another example is that of the A- and B-proteins of E. coli tryptophan synthetase. The A-protein alone will catalyze the conversion of indole-glycerol-phosphate to indole and glycerol-phosphate at a rate that is a hundred times less than if B-protein is present. Similarly, the B-protein will synthesize tryptophan from indole and serine at 3% the rate if A-protein is also present in the reaction mixture (87-89). From this point of view, it is of interest to note that while most of the HPr is soluble in crude extracts, the Enzyme II preparations contain always a small amount, and this residual HPr can only be removed by treatment of Enzyme II with charcoal. Similarly, Enzyme I is difficult to completely dissociate from the Enzyme II preparations. It is conceivable that the phosphotransferase system is an easily dissociable enzyme complex, and that Enzyme I and HPr, like Enzyme II, are bound in the cell to the cytoplasmic membrane.

Homogeneous preparations of Enzyme I, II (and probably lipid) will be required to arrive at a complete understanding of the mechanisms involved in the phosphotransferase reactions.

SUMMARY

Two of the products of the phosphoenolpyruvate-hexose phosphotransferase system of Escherichia coli have been isolated and characterized as D-galactose 6-phosphate and methyl 1-thio-β-D-galactopyranoside 6-phosphate. In addition, the chemical synthesis of methyl 1-thio-β-D-galactopyranoside 6-phosphate was described.

The phosphocarrier protein of the phosphotransferase system was purified to homogeneity. Two HPr active fractions (designated HPr-1 and HPr-2) were isolated in the last step of the purification, and both shown to be homogeneous by electrophoresis in polyacrylamide gels and by analysis in the ultracentrifuge. The two proteins were similar in molecular weight (9500 g/mole), sedimentation coefficient (1.6), and amino acid content; the analyses showed two histidine residues (confirmed by peptide mapping), and no half-cystines, tryptophan, or tyrosine. HPr-2 differed from HPr-1 only in having four less amide nitrogens. Thus, HPr-2 is more negatively charged, and its electrophoretic behavior in polyacrylamide gels, and elution pattern from DEAE-cellulose relative to HPr-1 is explained.

HPr-1 showed twice the activity of HPr-2 in the phosphotransferase system, and the ratio of activities was constant with three Enzymes II. HPr-1 was partially converted to a protein showing the same electrophoretic mobility as HPr-2, either by heating alone, or by heating and acid precipitation (the first two steps in the purification). These studies indicate that HPr-2 is an artifact of preparation, probably arising as a result of hydrolysis of amide groups.

It was shown that HPr accepts one mole of phosphate per

mole of protein in the Enzyme I catalyzed phosphoryl transfer. Hydrolysis rate studies on phosphorylated HPr, and characterization of the phosphohistidine isolated from the phosphoprotein, indicate that the phosphoryl bond is at N-1 of the histidine imidazole ring.

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