Journal of Cellular Biochemistry 25:139–159 (1984)

Phosphoproteins and the Phosphoenolpyruvate: Sugar Phosphotransferase System in Salmonella typhimurium and Escherichia coli: Evidence for $III^{Mannose}$, $III^{Fructose}$, $III^{Glucitol}$, and the Phosphorylation of Enzyme $II^{Mannitol}$ and Enzyme $II^{N\text{-}Acetylglucosamine}$

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Phosphoproteins produced by the incubation of crude extracts of Salmonella typhimurium and Escherichia coli with either [^{32}P]phosphoenolpyruvate or [γ^{32}P]ATP have been resolved and detected using sodium dodecyl sulphate polyacrylamide gel electrophoresis and autoradiography. Simple techniques were found such that distinctions could be made between phosphoproteins containing acid-labile or stable phosphoamino acids and between N^1-P-histidine and N^3-P-histidine. Phosphoproteins were found to be primarily formed from phosphoenolpyruvate, but because of an efficient phosphoexchange, ATP also led to the formation of the major phosphoenolpyruvate-dependent phosphoproteins. These proteins had the following apparent subunit molecular weights: 65,000, 65,000, 62,000, 48,000, 40,000, 33,000, 25,000, 20,000, 14,000, 13,000, 9,000, 8,000. Major ATP-dependent phosphoproteins were detected with apparent subunit molecular weights of 75,000, 46,000, 30,000, and 15,000. Other minor phosphoproteins were detected. The phosphorylation of the 48,000- and 25,000-MW proteins by phosphoenolpyruvate was independent of the phosphoenolpyruvate:sugar phosphotransferase system (PTS). The PTS phosphoproteins were identified as enzyme I (soluble; MW = 65,000); enzyme $II^{N\text{-acetylglucosamine}}$ (membrane bound; MW = 65,000); enzyme $II^{mannitol}$ (membrane bound; MW = 62,000); $III^{fructose}$ (soluble; MW = 40,000); $III^{mannose}$ (partially membrane associated; MW = 33,000); $III^{glucose}$ (soluble; MW = 20,000); $III^{glucitol}$ (soluble; MW = 13–14,000); HPr (soluble; MW = 9,000); FPr (fructose induced HPr-like protein (soluble; MW = 8,000). HPr and FPr are phosphorylated on the N-1 position of a histidyl residue while all the others appear to be phosphorylated on an N-3 position of a histidyl residue. These studies identify some previously unknown proteins of the PTS and show the phosphorylation of others, which although previously known, had not been shown to be phosphoproteins.

Key words: PEP: Sugar phosphotransferase, protein kinase, phosphohistidine, enzyme II^{sugar}, factor III^{sugar}

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Received December 27, 1983; accepted February 9, 1984.

In many bacterial species, some sugars are transported into the cell by the phosphoenolpyruvate:sugar phosphotransferase system (PTS). The PTS transports these sugars by a group translocation process resulting in sugar phosphorylation. A number of proteins and enzymes are involved, many of which are soluble. In Salmonella typhimurium and Escherichia coli the proteins of the PTS have been reported to function as shown in Figure 1. Enzyme I is phosphorylated by phosphoenolpyruvate producing P-enzyme I with 3-P-histidine at its active site [1]. P-enzyme I phosphorylates the phosphocarrier protein, HPr, in which 1-P-histidine is formed [2]. P-enzyme I can also phosphorylate a fructose induced HPr-like protein, FPr [3,4]. Both P-HPr and P-FPr can phosphorylate III^{glc} [3] on the N-3 position of a histidyl residue [5]. These soluble phosphocarrier proteins interact with the membrane-bound sugar specific enzyme IIs which phosphorylate and translocate the sugars.

The original description of the constitutive PTS enzyme II is shown in Figure 1A. The enzyme II was reported to consist of three sugar-specific extrinsic membrane proteins (IIA) and a common intrinsic membrane protein (IIB). Reconstitution of this enzyme II required phosphatidylglycerol and Mg^{++} for activity. Fructose, mannose, glucose, and other sugars were thought to be transported and phosphorylated by this enzyme complex [6,7]. The existence of this enzyme complex is now questionable. In addition to this enzyme complex, a glucose-specific phosphotransferase was reported to include an additional phosphocarrier protein, III^{glc} (Fig. 1B). Other sugar-specific phosphotransferases, each possessing a sugar-specific inducible enzyme II exhibiting specificity toward mannitol, glucitol, galactitol [8], β-glucosides [9], and N-acetylglucosamine [10] were presumed to exist as shown in Figure 1C. Lastly, the fructose-specific phosphotransferase (Fig. 1D) has been shown to consist of an inducible soluble phosphocarrier protein [3,4] as well as an inducible enzyme $II^{fructose}$ [11].

Subsequent studies of the in vitro kinetics of the glucose PTS activities have equated the PTS described in Figure 1A as a glucose/mannose enzyme II now called enzyme $II^{mannose}$ which has high affinity for glucose, mannose, and 2-deoxyglucose but poor affinity for fructose and methyl α-glucoside (Km $>$ 20 mM). The enzyme II^{glc}/III^{glc} system has high affinity for both glucose and methyl α-glucoside (Km = 10 μM) [12]. These in vitro results agree with the previously described in vivo properties [13,14].

In addition to its role in sugar transport, the PTS is known to be involved in a number of regulatory phenomena and much of the evidence suggests that III^{glc} plays a pivotal role in these regulatory events [15–20]; Saier has proposed that other phosphoproteins may act as regulators [20]. Such phosphoproteins could be either directly phosphorylated by enzyme I, resulting in a phosphoprotein with a 1-P-

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Abbreviations used: PTS, phosphoenolpyruvate:sugar phosphotransferase system; glc, glucose; man, mannose; frc, fructose; mtl, mannitol; gut, glucitol; nag, N-acetylglucosamine; SDS-PAGE, sodium dodecyl suphate polyacrylamide gel electrophoresis; Hepes, hydroxyethylpiperazine N′-2-ethane sulphonic acid; K, used with molecular weights means 1,000, ie, 9K = 9,000. Nomenclature used for the PTS proteins: HPr, histidine containing phosphocarrier protein; FPr, fructose induced HPr-like phosphocarrier protein; III^{sugar}, a sugar-specific phosphocarrier protein, also known as factor III^{sugar} or enzyme III^{sugar}; enzyme II^{sugar} is a sugar-specific membrane-bound enzyme II of the PTS.

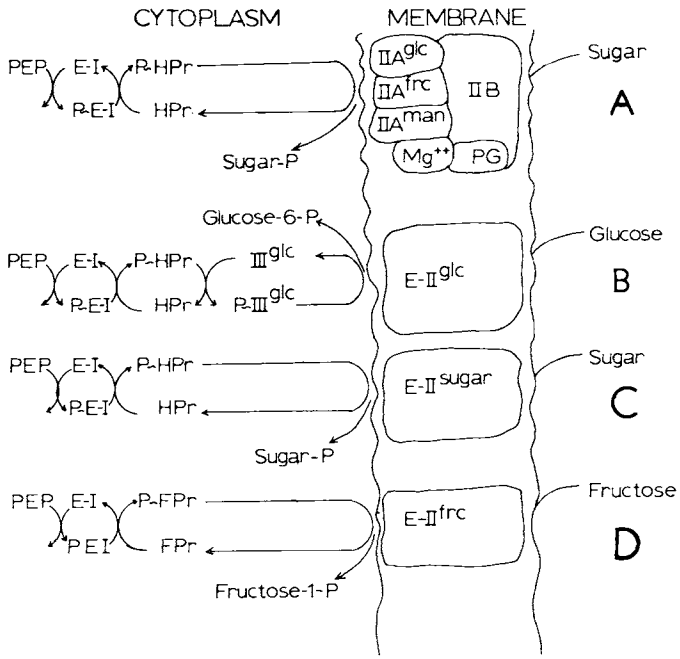

Fig. 1. Models for the PTS. In S typhimurium and E coli a number of different sugar-specific phosphotransferases have been described. Common to all is the soluble protein enzyme I (EI), and common to all except the fructose PTS (1D) is the soluble phosphocarrier protein, HPr. A depicts the PTS that was suggested to be responsible for the transport and phosphorylation of many sugars, in particular glucose, fructose, and mannose [6,7]. A glucose-specific PTS was subsequently described in which an additional phosphocarrier protein (III^{glc}) was described, as shown in B. In addition, phosphotransferases for hexitols and N-acetylglucosamine have been described [8-10] as shown in C, and a fructose-specific system with a fructose-induced HPr-like protein and enzyme II has been described as shown in D [3,4,11].

histidine, or the regulatory proteins could be phosphorylated as III^{glc} from a phosphoprotein substrate of enzyme I such as P-HPr, and would likely contain 3-P-histidine.

Phosphohistidines are acid labile as phosphoamino acids [21]; however, with suitable modifications to the standard procedures it has been possible to routinely detect P-HPr, P-enzyme I, and $P\text{-}III^{glc}$ in crude extracts following resolution by isoelectric focusing gels, and a novel PTS protein was separated [22,23]. By applying the same precautions used for these isoelectric focusing studies to SDS-PAGE, a number of additional phosphoproteins have been detected.

In this paper a method for the detection and the tentative classification of different types of phosphoproteins by SDS-PAGE is described. The occurrence of both phosphoenolpyruvate- and ATP-dependent phosphoproteins in crude extracts of S typhimurium and E coli was investigated and their identification with known phosphoproteins is made where possible. Several novel PTS protein have been detected. A membrane-associated 33K phosphoprotein appears to be $III^{mannose}$ while two of three fructose induced phosphoproteins appear to be $III^{fructose}$ (40K) and FPr

TABLE I. Bacterial Strains: Genotype and Relevant Phenotype

Strain	Genotype	Relevant phenotype	Source	Ref
S typhimurium				
SB1687	ptsM	Lacks enzyme $II^{mannose}$ (derived from LT2)	S. Roseman	[12]
SB3507	trpB223	Wild type	J.C. Cordaro	[27]
SB2226	trpB223 ptsH	Lacks HPr	J.C. Cordaro	[27]
SB2950	trpB223 Δ(trzA-ptsHIcrr49)	Deletion through genes for HPr, enzyme I and factor III^{glc}	J.C. Cordaro	[27]
SB1744	mtlA61	Lacks enzyme $II^{mannitol}$	M.H. Saier, Jr.	[28]
E coli				
1101	thi ptsH	Lacks HPr	A. Peterkofsky	[29]
ZSC17[a]	glk strA	Wild type: lacks glucokinase	W. Epstein	[29]
ZSC103	glk-7 strA ptsG2	Lacks enzyme $II^{glucose}$	W. Epstein	[14]
ZSC112	glk-7 strA ptsG2 ptsM1	Lacks enzyme $II^{mannose}$ and enzyme $II^{glucose}$	W. Epstein	[14]
ZSC114	glk-7 strA ptsM1	Lacks enzyme $II^{mannose}$	W. Epstein	[14]
CSH4	F'trp thi str-r recA	Wild type	J. Weiner	[30]
CSH11	F'trp thi str-r recA	Wild type	J. Weiner	[30]
P650	his^+purC^+ mu-lcts thi/ purC recA56 trp nalA-r str-r mu-lcts61	Diploid for enzyme I HPr and factor III^{glc}	V.N. Gershanovitch	[31]

[a]ZSC17 is equivalent to ZSC13 [14].

(8K); and a $III^{glucitol}$ (13–14K) has been identified. A membrane-bound 62K phosphoprotein was induced by growth on mannitol, and appears to be enzyme $II^{mannitol}$. A similar 65K membrane-bound phosphoprotein was induced by growth on N-acetylglucosamine, and appears to be enzyme $II^{N\text{-acetylglucosamine}}$

METHODS

Materials

[^{32}P]phosphoenolpyruvate was prepared from [γ-^{32}P]ATP [24]. Polyacrylamide from Sigma was recrystallized from chloroform. The PTS proteins enzyme I, HPr, and III^{glc} were prepared from E coli P650 as described elsewhere [22,25,26]. [γ-^{32}P]ATP, 2-deoxy-[1-^{14}C]-glucose, D-[U-^{14}C]glucitol, and methyl α-[U-^{14}C]-glucoside were from New England Nuclear (NEN). D-[U-^{14}C]glucose, D-[U-^{14}C]fructose were from Schwarz-Mann. Egg-white phosphatidylglycerol was from Sigma and was prepared in aqueous form as described by Kundig and Roseman [7].

Cell Strains

The strains used in this study are listed in Table I.

Growth Conditions Investigated

S typhimurium strain SB3507 was grown in a minimal salts medium with glucose, fructose, mannose, glucosamine, N-acetylglucosamine, mannitol, glucitol, salicin, gluconate, citrate, succinate, malate, glycerol, acetate, lactate, ribose, malt-

ose, or melibiose as the carbon source, and the complex medium, nutrient broth. S typhimurium strain SB2950 was grown in minimal salts medium with lactate or in nutrient broth with glucose, fructose, mannitol, N-acetylglucosamine, or glucitol. The *ptsH* strains (Table I) were grown in a minimal salts medium with lactate or fructose as the carbon source. E coli strains have been grown in minimal salts medium with lactate, glucose, fructose, mannitol, or glucitol as the carbon source. For all of these growth conditions, gels similar to those shown in Figure 3 were run following separate incubations with [^{32}P]phosphoenolpyruvate and [γ-^{32}P]ATP.

Cell Growth and Crude Extract Preparation

Cells were grown to mid log phase (OD_{540} = 0.6–0.8) in either minimal salts medium A or nutrient broth as previously described [26]. Carbon sources were used at 0.2% (w/v) except DL-lactate, which was 0.4% (w/v). The cells were harvested by centrifugation at 2°C and subsequently resuspended in 0.01 M potassium phosphate buffer, pH 7.5, 1 mM EDTA, and 0.2 mM dithioerythritol at a ratio of 1 gm wet weight cells to 3 ml buffer. These cells, which had either been frozen (−30°C) or freshly grown were broken by passage through a French pressure cell at 2°C under 10,000 lb/in^2 pressure (1 lb/in^2 = 6.895 KPa). The crude extract was centrifuged (10 min, 12,000 g, 2°C) to remove cell debris, and then centrifuged (2 hr, 200,000g, 2°C) to separate the membranes from soluble proteins. Using dialysis tubing that retained proteins > 3500 MW, the supernatant was then dialysed against 0.01M hydroxyethylpiperazine N′-2-ethane sulphonic acid (Hepes) buffer, pH 7.5, containing 1 mM EDTA and 0.2 mM dithioerythritol. The dialysis was against two lots of a 200-fold excess of buffer for a total of 12–15 hours. In all cases the protein concentration of these dialysates was 10 ± 2 mg/ml.

Soluble Sample Preparation and Incubation

The dialysed samples were usually incubated for 5 min at room temperature at about 7 mg/ml with 0.1 mM [^{32}P]phosphoenolpyruvate (1–10 × 10^5 cpm/nmol), 5 mM $MgCl_2$, and 0.01 M Hepes, pH 7.5. After 5 min, the incubations were diluted twofold into sample buffer (0.0625 M TRIS-HCl, pH 8.0, which contained 2% (w/v) sodium dodecyl sulphate, 10% (v/v) glycerol, 2% (v/v) β-mercaptoethanol and 0.1 ml of 1% (w/v) bromophenol blue per 100 ml). Samples of about 90 μg protein were loaded into alternate sample wells over a 30-min period, during which time the samples were at room temperature. When samples were boiled for 1 min, boiling was performed immediately after the addition of the sample buffer.

Membrane Preparation and Incubation

Membranes were separated from the supernatants as described above. The membranes from 1 gm wet weight of cells were resuspended in 10 ml of 0.01 M Hepes buffer, pH 7.5, containing 1 mM EDTA, 0.2 mM dithioerythritol. They were centrifuged, the supernatants were discarded, and the membranes were resuspended in 10 ml of buffer and centrifuged. The membrane pellet was then resuspended in 0.5 ml of buffer per liter of the original growth media. Incubations with [^{32}P]phosphoenolpyruvate and membrane samples were the same as the soluble protein incubations except that they were for 1 min with approximately 8 mg/ml membrane proteins. The samples applied to the SDS-PAGE gels contained about 80 μg protein.

ATP-Dependent Phosphorylation

Concurrently for both soluble protein and membrane preparation, phosphorylations dependent upon [γ-^{32}P]ATP were investigated. The incubation conditions were the same except that 0.1 mM [γ-^{32}P]ATP was used at the same specific activity as [^{32}P]phosphoenolpyruvate.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Slab gels (16 × 16 cm) were prepared according to Laemmli [32] with polyacrylamide concentrations usually at 12%. However, 7, 10, and 15% gels were used to obtain resolution of some proteins. The gels were 0.74 cm thick and were subsequently run in a Biorad Protean gel electrophoresis apparatus which was precooled to and maintained at 10°C using a circulating water bath. After the samples were loaded, the gels were run at 20 mamp/gel for 4–5 hr. The gels were then frozen and autoradiography was carried out as described previously [22]. Subsequently, acid fixing of frozen gels was carried out by allowing them to thaw at room temperature and then fixing overnight with 10% (v/v) acetic acid and 25% (v/v) isopropanol, staining for 2 hr with the fixing solution with 0.26 gm/liter Coomassie Blue R250, and destaining with 10% (v/v) acetic acid. Unlike the isoelectric focusing gels, the SDS-PAGE gels could not be attached to filter paper [22], and in most cases the gels broke into pieces which had to be sorted out before drying for subsequent autoradiography. The time that a gel was in acid before drying was 24 ± 4 h. If gels were dried before freezing and acid fixation, the acid-fixed gels could not be used as they always broke into many pieces.

Autoradiography

Autoradiographs were taken at −70°C for several time periods on each gel both before and after acid fixing. The time of exposure is thus given as the percent of [^{32}P] decay. The standards, enzyme I, HPr, and III^{glc} were prepared in batches with [^{32}P]phosphoenolpyruvate and used over a period of time during which enzyme I activity as well as the [^{32}P]phosphoenolpyruvate decayed. They were not suitable for a quantitative estimation of the detected phosphoproteins.

Molecular Weight Assignment

Molecular weight standardization of the frozen gel was made using enzyme I, 65K, [25,33], III^{glc}, 20K, [5,34], and HPr, 9K [2,35] as phosphorylated standards. In samples from wild-type cells, these phosphorylated proteins were clearly distinguishable and could be used to standardize each sample. In gels which were acid fixed, stained, and destained, the phosphoproteins were compared to the stained protein standards which were always applied to the SDS-PAGE gels. While acid-fixed gels stretched and were somewhat distorted by the cracks between the reassembled pieces (Fig. 3), the molecular weight estimates from the acid fixed gels and frozen gels were generally in agreement.

PTS Assays

PTS assays were carried out at 37°C as described [3,25]. All assays contained 50 mM Hepes, pH 7.0, 12.5 mM KF, 2.5 mM dithioerythritol, 10 mM phosphoenolpyruvate, and 5 mM $MgCl_2$ in a volume of 0.05 ml. HPr, III^{sugar}, and FPr assay mixtures contained excess enzyme I. The fructose PTS activity was assayed using

membranes (10 μg/0.05 ml assay) from S typhimurium strain SB3507 or strain SB1687 grown on fructose and 1 mM D-[U-^{14}C]-fructose (2,000 cpm/nmole) [3,4]. The glucitol PTS activity was assayed using membranes (10 μg/0.05 ml assay) from S typhimurium strain SB3507 grown on glucitol, 2μM HPr, and 1 mM D-[U-^{14}C]-glucitol (1,000 cpm/nmol). When phosphatidylglycerol was added, 4 μg per assay was used.

Protein Determinations

Protein was determined by the microtuiret method as described [26].

Partial Purification of Fructose and Glucitol Phosphocarrier Proteins

PTS proteins enzyme I, III^{glc}, and HPr can be separated by both DEAE-cellulose chromatography and molecular sieve chromatography [4,25,36]. Fructose grown crude extracts from which the membranes were not removed, were mixed with diethylaminoethyl (DEAE) - cellulose (Whatman DE32) to form a column as previously described [4] and eluted with a 0 to 0.5 M KCl gradient. Membrane-free extracts of glucitol-grown cells were loaded onto a DEAE-cellulose column and similarly eluted. Assays were carried out for HPr, III^{glc}, enzyme I, and for other soluble proteins that stimulated fructose phosphorylation activity and glucitol phosphorylation activity. The stimulators of the fructose and glucitol phosphorylation activities eluted at about 0.15–0.2 M KCl, and the pooled fractions were concentrated (ammonium sulphate precipitation or lyophilization or ultrafiltration). Other fractions without activity were pooled and concentrated. The various fractions were dialysed and then incubated with [^{32}P]phosphoenolpyruvate and enzyme I with and without HPr, and the phosphoproteins were resolved by SDS-PAGE.

The fractions containing the proteins that stimulated fructose and glucitol phosphorylation were then chromatographed on Ultrogel AcA44 (LKB) equilibrated with 0.01 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 0.1 M KCl, and 0.2 mM dithioerythritol. Fractions were assayed for enzyme I, III^{glc}, III^{frc}, and III^{gut}. The fractions containing III^{frc} and III^{gut} were pooled, concentrated, and checked for phosphoproteins.

III^{frc} was purified further by chromatography on phenyl-sepharose (Pharmacia). Both the sample and column were equilibrated with 0.01 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA 0.2 mM dithioerythritol. The column was washed with buffer and III^{frc} was eluted with buffer containing 50% (v/v) ethylene glycol.

RESULTS

ATP or Phosphoenolpyruvate-Dependent Phosphorylation of Proteins

Phosphoproteins formed from ATP and phosphoenolpyruvate in the soluble dialysed crude extracts of S typhimurium and E coli had been investigated by isoelectric focusing and subsequent autoradiography on frozen and acid-fixed gels [22,23]. This work, which forms the basis for the studies reported in this paper, indicated that the most prominent phosphoproteins were phosphorylated at the expense of phosphoenolpyruvate and that the phosphorylation of these proteins was dependent upon the PTS. These phosphoproteins were acid labile. The crude soluble proteins were dialysed to remove sugars and sugar phosphates that might interfere

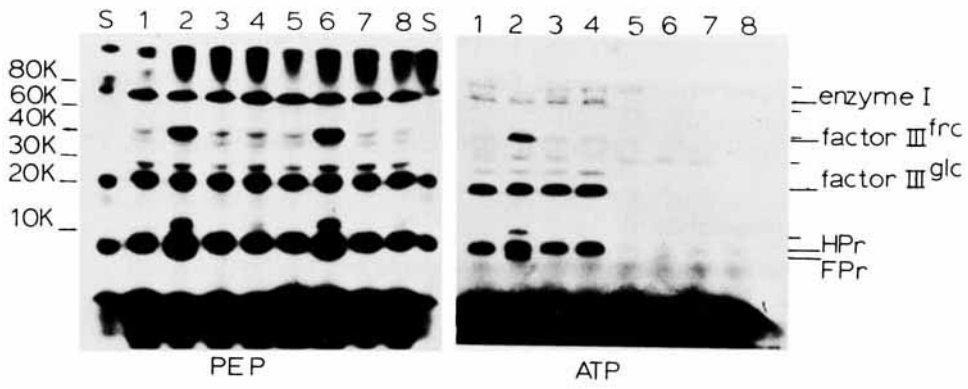

Fig. 2. Resolution by SDS-PAGE of phosphoproteins in S typhimurium strain SB3507. Crude supernatant samples from S typhimurium strain SB3507 grown on minimal salts with glucose (samples 1 and 5), fructose (samples 2 and 6), mannose (samples 3 and 7), and mannitol (samples 4 and 8) were incubated with 0.1 mM [^{32}P]phosphoenolpyruvate (the gel marked PEP), with no added ATP (samples 1–4), and with 1 mM ATP added at the same time as the [^{32}P]phosphoenolpyruvate (samples 5–8); and with 0.1 mM [γ-^{32}P]ATP (the gel marked ATP) with no added phosphoenolpyruvate (samples 1–4) and with 1mM phosphoenolpyruvate added at the same time as the [γ-^{32}P]ATP (samples 5–8). The lane marked S contained the standard preparations enzyme I (65K), factor III^{glc} (20K), and HPr (9K). The samples were incubated and loaded as described in Methods. The 10% acrylamide SDS-PAGE gels were run in the same apparatus with 20 mamp/gel with the temperature maintained at 10°C. The specific activity of both [^{32}P]-labelled substrates was 4 × 10^5 cpm/nmol. Autoradiography of the phosphoenolpyruvate and ATP gels was 24% and 44% of the [^{32}P] decay, respectively.

with the formation of PTS phosphoproteins. Another reason for dialysis of the samples was to remove nucleoside diphosphates and thereby inhibit nucleoside triphosphate formation from phosphoenolpyruvate.

Despite this dialysis, some phosphoexchange between ATP and phosphoenolpyruvate occurs [22]. This phosphoexchange was more clearly seen when the phosphoproteins were resolved by SDS-PAGE. If samples were preincubated with 1 mM ATP before being mixed with 0.1 mM [^{32}P]phosphoenolpyruvate or preincubated with 1 mM phosphoenolpyruvate before addition of 0.1 mM [γ-^{32}P]ATP, all phosphorylations were inhibited (results not shown). In Figure 2, the phosphoproteins produced by [^{32}P]phosphoenolpyruvate incubation in the absence and presence of ATP are shown. The major phosphoenolpyruvate-dependent phosphorylations were not affected by the presence of ATP added at the same time as the [^{32}P]phosphoenolpyruvate. When [γ-^{32}P]ATP was used under identical conditions, most of the phosphorylations produced by [^{32}P]phosphoenolpyruvate (PEP) were detected (Fig. 2) but at much less intensity even after longer autoradiographic exposure. Most of the ATP-dependent phosphorylations were inhibited by phosphoenolpyruvate, but the phosphoproteins found at 75K and 30K MW remained with unaltered intensity. They were therefore presumed to be ATP-specific phosphorylations. One difference between the gel shown in Figure 2 and the others described in this paper is that the ATP/PEP exchange reaction was enhanced by the omission of NaF from the incubation mixtures. When NaF was omitted from the incubations as in Figure 2, a 25K phosphoprotein was detected. When included, as in Figure 3, the

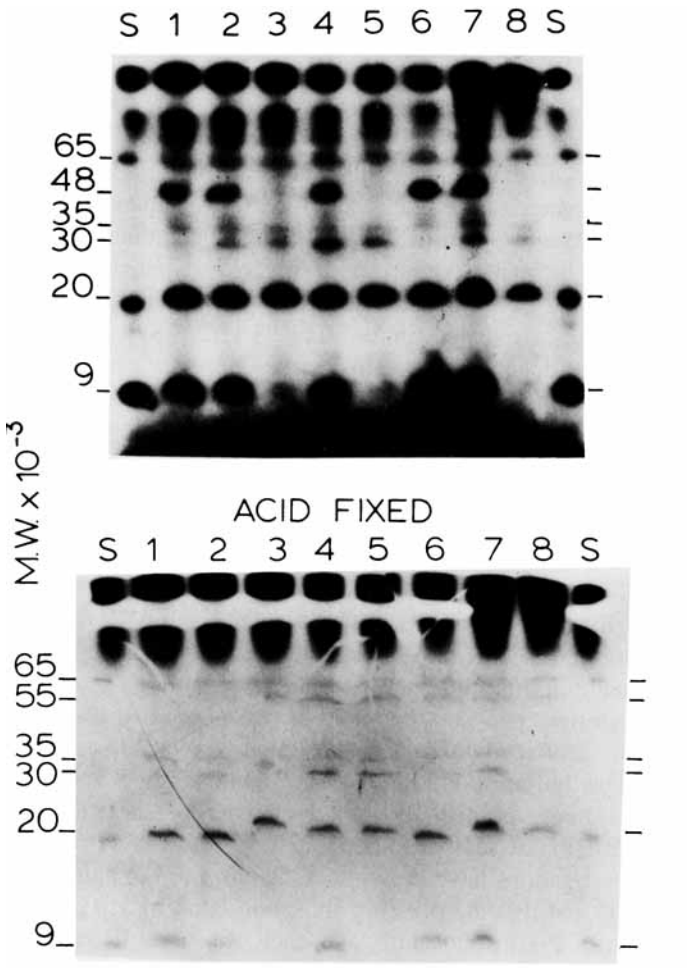

Fig. 3. Phosphoproteins of nutrient broth grown S typhimurium resolved by SDS-PAGE. Comparison of the autoradiograph of the frozen and acid-fixed gels. Crude soluble proteins from nutrient broth grown S typhimurium strain SB3507 were incubated with 0.1 mM [^{32}P]phosphoenolpyruvate (S.A. 5 × 10^5 cpm/nmol) and treated as follows: lane 1) 5-10-sec incubation; lane 2) 5-min incubation; lane 3) 5-min incubation followed by 1-min boiling in SDS-sample buffer; lane 4) 20-min incubation; lane 5) 20-min incubation with 1-min boiling in SDS-sample buffer; lane 6) 5-min incubation with added 1 mM ATP; lane 7) 5-min incubation with added 0.1 mM cAMP; lane 8) as lane 7 but with 1-min boiling in SDS sample buffer. S was the standards, enzyme I (65K), factor III^{glc} (20K), and HPr (9K). The samples were run on a 12% SDS-PAGE gel. The autoradiograph marked frozen was of the frozen gel for 12% of the ^{32}P decay. The lower autoradiograph was acid fixed and dried and was for 27% of the [^{32}P] decay.

25K phosphoprotein is not detected but a 48K phosphoprotein was present; neither the 25K nor the 48K phosphoprotein appear to arise in a reaction dependent on the PTS.

The phosphoproteins identified as enzyme I, III^{glc}, and HPr (Fig. 2) were all easily detectable and present in very large amounts. From the immunological quanti-

tation [26] the amount of HPr in the soluble protein preparations applied to the gels would be expected to be about 80 pmol and for enzyme I (65K) about 8 pmol. III^{glc} would appear to be present at about 40–50 pmol. Fructose-grown cells have three additional major phosphoproteins, 40K, 13K, and 8K.

Assignment of Phosphoamino Acids to Phosphoproteins

The identification of the phosphoamino acid present in each phosphoprotein usually requires characterization of its rate of hydrolysis under a variety of conditions followed by isolation and identification of the phosphoamino acid. However, some generalizations can be made with respect to the phosphoryl linkage. In bacteria, phosphoenolpyruvate-dependent reactions are most likely to give rise to 1-P-histidine or 3-P-histidine if they are dependent on the PTS [1,2,5,37]. In prokaryotes [38–40] and eukaryotes ATP-dependent protein kinases produce phosphoserine and phosphothreonine [41] or phosphotyrosine [42]. Phosphorylated enzyme intermediates usually include 3-P-histidine and acyl phosphate [43–45]. Identification of the phosphoamino acid in the phosphoprotein could be accomplished by determining the stability of the linkage to acid and boiling. It has been previously shown that acid-labile phosphohistidines can be tentatively identified by comparing autoradiographs of frozen and acid-fixed isoelectric focusing (IEF) gels [22]. This procedure was adapted to SDS-PAGE gels. Autoradiographs of frozen gels were obtained, and subsequent acid fixation was carried out as described in Methods. This divided the detected phosphorylation into acid-labile and acid-stable linkages (Figs. 3). No distinctions were made in this study between the acid-stable phosphorylations, so these could be phosphoserine, phosphothreonine, or phosphotyrosine. Acid-labile phosphorylations were divided into two groups: those that were stable to boiling for 1 min in pH 8.0 SDS-sample buffer and those that were labile (Figs. 3 and 5). The former are probably 3-P-histidines, as in P-III^{glc} and P-enzyme I. The latter are probably 1-P-histidines (as in P-HPr) or acylphosphates [22].

Several investigations have shown that the enzyme I of enteric bacteria contains a 3-P-histidine (1) and that the proteins phosphorylated by enzyme I are 1-phosphohistidyl proteins (2). P-HPr phosphorylates each of several IIIs with the formation of 3-phosphohistidyl proteins [5,37]. S typhimurium strain SB2950, which is deleted for the genes which encode enzyme I, HPr, and III^{glc}, was grown on various substrates including nutrient broth plus hexoses or hexitols. These growth conditions induced sugar-specific PTS proteins which were detected by phosphorylation after the addition of pure enzyme I (which revealed the substrates of enzyme I), or the addition of pure enzyme I and HPr (which revealed III^{sugar} or enzyme II phosphoproteins). In all cases, the enzyme I substrates were acid-labile and boil-labile and were classified as containing 1-P-histidine, while those dependent on P-HPr were acid-labile but boil-stable and were classified as 3-P-histidine. It can be seen in Figure 3 that boiling the phosphorylated samples for 1 min in the pH 8.0 SDS-sample buffer hydrolyses the 1-P-histidine in HPr, but does not affect the 3-P-histidine in III^{glc} and enzyme I.

Survey of Growth Conditions and Phosphoproteins Generated After Growth Under Different Conditions

The gel shown in Figure 3 is a standard gel that was used to investigate the soluble phosphoproteins found in S typhimurium strain SB3507 following growth as described in Methods. The different time incubations (5–10 sec, 5 min, and 20 min)

did not reveal phosphatase or protease activity. To check for phosphoryl donor specificity the incubation condition in lane 6 included a tenfold excess of ATP and was changed to contain a tenfold excess of phosphoenolpyruvate when [γ-^{32}P]ATP-dependent phosphorylations were investigated. The possible role of cAMP was investigated but no effects were observed (lane 7 and 8). Comparison of the boiled and nonboiled samples and comparison between the autoradiographs of frozen and the subsequently acid-fixed gels were used to identify the phosphoryl linkage. The autoradiograph of the acid-fixed gel shown in Figure 3 has been photographically enhanced to show the minor bands. In this gel a 55K protein was found that was not detected on the frozen gel, and was therefore classified as acid stable. The 55K protein has been found on gels after incubation with [γ-^{32}P]ATP incubations, but the results shown in Figure 3 indicate that it is [^{32}P]phosphoenolpyruvate dependent since ATP did not inhibit its phosphorylation (compare lane 6 with lane 3). Because of its low level of detection, this contradiction has not been resolved.

Table II summarizes the data that have been obtained. In some cases properties have not been assigned because of insufficient data. There are indications of more protein phosphorylation than listed, but inclusion was precluded due to low copy number. The phosphoamino acid assignments given in Table II were arrived at by their properties in acid or hot pH 8.0 buffer. In the case of the PTS-dependent proteins, these assignments were augmented by either their dependence on just enzyme I (ie, 1-P-histidine) or enzyme I and HPr (ie, 3-P-histidine). The 48K phosphoprotein was more labile than P-HPr, and may be an acyl phosphate [44]. The ATP-dependent phosphorylations at 46K and 15K were not detected when cells were grown on hexoses.

Enzyme I (65K), III^{glc} (20K), and HPr (9K) were readily detectable in wild-type cells regardless of growth conditions (Figs. 2, 3, 5) and missing in S typhimurium strain SB2950. The preparations of standard III^{glc} contained a smaller protein at about 17K which appears to contain a 1-P-histidine. It has been observed at low levels in the crude extracts. The presence of this protein may account for why this III^{glc} preparation can be phosphorylated without added HPr [23]. Other major PTS phosphoproteins were identified as described below, but apart from these proteins no other major phosphoproteins were detected.

The Identification of III^{mannose}

In the crude supernatants of S typhimurium strains SB3507 and SB2950, a 33K phosphoprotein was detected whose phosphorylation was clearly dependent upon enzyme I and HPr. When membrane samples were incubated with [^{32}P]phosphoenolpyruvate, enzyme I, III^{glc}, and HPr, and then boiled for 1 min to denature the protein, the autoradiographs revealed that there was only one major membrane-bound phosphoprotein, 33K MW. Figure 4 shows the results obtained with membranes from glucose grown S typhimurium strain SB3507. Phosphorylation of the 33K protein was dependent upon added enzyme I and HPr, was independent of III^{glc}, and did not occur in the presence of ATP. Its acid lability properties suggested that it contained 3-phosphohistidine (not shown). Nonboiled samples gave identical results except that P-HPr was also detected in those incubations to which it was added. The 33K protein was found in membranes from S typhimurium strain SB3507 grown on glucose, fructose, mannitol, lactate, N-acetylglucosamine, salicin, or mannose. It was also detected in the other S typhimurium strains listed in Table I

TABLE II. Properties of S typhimurium Phosphoproteins Detected by SDS-PAGE and Autoradiography*

Assigned MW	ATP or PEP specific	Hot-SDS-stable	Acid-stable	Phosphoamino acid	Autoradio-graphic intensity	PTS-dependent	Identification
75K	ATP	+	-	3-P-histidine	++	-	PEP synthetase [44]
65K	PEP	+	-	3-P-histidine	+++	+	Enzyme I [1]
65Ka	PEP	+	-	3-P-histidine	+++	+	Enzyme II$^{N\text{-acetylglucosamine}}$
62K[a]	PEP	+	-	3-P-histidine	++++	+	Enzyme II^{mannitol}
57K	PEP	-	ND[b]	1-P-histidine	+	+	
55K	ATP	+	+	P-serine	+	-	Unknown [38]
51K[a]	PEP	ND	ND	ND	+	+	
48K	PEP	-	-	ND	++ -> ++++		
46K	ATP	+	+	P-serine	+	-	Isocitrate dehydrogenase [46]
40K	PEP	+	-	3-P-histidine	++++	+	III^{fructose}
33K[a]	PEP	+	-	3-P-histidine	++++	+	III^{mannose}
30K	ATP	+	-	3-P-histidine	++	-	Succinic thiokinase [43]
25K	PEP	+	-	3-P-histidine	++	-	
20K	PEP	+	-	3-P-histidine	++++	+	III^{glucose} [5]
17K	PEP	-	-	1-P-histidine	+	+	
15K	ATP	-	-	ND	++	-	
14K[c]	PEP	+	-	3-P-histidine	+++	+	III^{glucitol}
13K[d]	PEP	+	-	3-P-histidine	+++	+	III^{glucitol}
9K	PEP	-	-	1-P-histidine	+++++	+	HPr [2]
8K	PEP	-	-	1-P-histidine	+++	+	FPr

*Properties of the phosphoproteins were determined from SDS-PAGE gels of incubations of S typhimurium proteins with [^{32}P]phosphoenolpyruvate and [γ-^{32}P]ATP. Samples of these incubations were denatured at room temperature or with 1-min boiling. Autoradiographs were taken on both frozen and subsequently acid-fixed gels. The MW assigned were the most commonly occurring. Specificity for the phosphoryl donor was determined by the comparison of the autoradiographs of incubations with 0.1 mM [^{32}P]phosphoenolpyruvate in the presence of 1 mM ATP and 0.1 mM [γ-^{32}P]ATP in the presence of 1 mM phosphoenolpyruvate. Stability in hot SDS sample buffer (+) distinguished between 3-P-histidine and 1-P-histidine which was labile (-). When an autoradiographic band increased in intensity or remained the same it was classified as acid-stable (+), as would be phosphoserine, phosphothreonine, and phosphotyrosine. Phosphohistidines were labile (-). Except where references are given, the phosphoamino acid identification means that the phosphoprotein behaves as it has such an amino acid. The intensities were judged by eye. Roughly +++++ corresponds to 300,000 copies per cell and + corresponds to 200 copies per cell as calculated according to Wang and Koshland [39].

[a]Membrane-bound or associated.

[b]ND, no determination.

[c]Found only in E coli.

[d]Found only in S typhimurium.

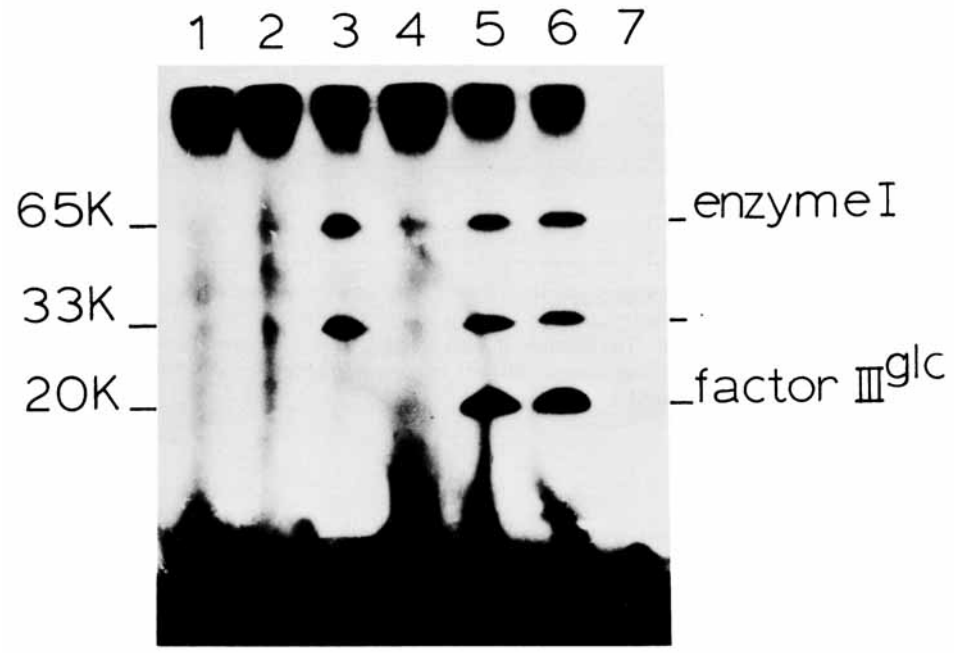

Fig. 4. Phosphoproteins in membranes from glucose grown S typhimurium strain SB3507. Membranes were incubated with 0.1 mM [^{32}P]phosphoenolpyruvate (SA 2 × 10^5 cpm/nmol) as described in Methods. The samples were lane 1, no additions; lane 2, with enzyme I (12 pmol); lane 3, with enzyme I (12 pmol) and HPr (45 pmol); lane 4, with enzyme I (12 pmol) and III^{glc} (24 pmol); lane 5, with enzyme I (12 pmol), HPr (4 pmol), and III^{glc} (24 pmol); lane 6, as in lane 5 but with 1 mM ATP; lane 7 with [γ-^{32}P]ATP (SA 1 × 10^5 cpm/nmol). The samples were run on a 12% SDS-PAGE gel. Autoradiography was for 15% [^{32}P] decay.

regardless of growth conditions. The 33K phosphoprotein was found in S typhimurium strain SB1687, and E coli strains ZSC112 and ZSC114, all of which contain mutants affecting enzyme II^{mannose} activity [12]. The membranes isolated from S typhimurium strain SB2950 appeared to contain more of this 33K phosphoprotein as judged by autoradiography. This strain is known to possess elevated enzyme II^{mannose} activity [3].

Of the various enzyme II^{sugars}, enzyme II^{mannose} most closely resembles the enzyme IIA/IIB complex described by Kundig and Roseman [7]. The finding of a membrane-associated constitutive phosphoprotein invoked comparison to the IIA proteins. The urea/butanol solubilization used to separate the IIA proteins from the membrane [7] was repeated using washed membranes isolated from S typhimurium strain 3507 grown on fructose or glucose and S typhimurium strain 2950 grown on lactate. The urea/butanol treatment destroys the enzyme II^{mannose} activity (Table III), and the 33K phosphoprotein was removed from the insoluble membrane fraction. Enzyme II^{mannose} activity was partially restored by reconstitution (Table III). The reconstituted activity had the reported sugar specificity for enzyme II^{mannose} [12], and not for that described for the IIA/IIB complex [6,7].

These observations and the various biochemical analyses indicate that the 33K protein appears to be part of the constitutive glucose/mannose PTS. Its properties

TABLE III. Enzyme $II^{mannose}$ Activity and Reconstitution.*

S typhimurium strain and growth condition	Untreated membranes	Activity, glucose-6-P produced, nmol/min/mg		
		Urea/butanol pellet	Urea/butanol supernatant	Reconstituted pellet and supernatant
SB2950-lactate	230	12.3	0.24	61.7
SB3507-fructose	35.1	7.1	NA[a]	18.9
SB3507-glucose	29.2	5.0	NA	16.7

*The enzyme $II^{mannose}$ activity measured as described in Methods using 1 mM [U-^{14}C]glucose (1200 cpm/nmol) and 15 μM HPr. The urea/butanol-treated samples were measured as described in the presence of phosphatidylglycerol. The amount of each fraction used for reconstitution was SB2950-lactate, 10-μg pellet and 60-μg supernatant; SB3507-fructose; 11-μg pellet and 50-μg supernatant; SB3507-glucose, 12-μg pellet and 54-μg supernatant.
[a]NA, no activity detectable.

were similar to III^{glc}; phosphorylation was dependent upon both enzyme I and HPr; it behaved as a substrate of an enzyme II (results not shown), and it contains a 3-P-histidine. The 33K protein was membrane associated but was also found in the soluble fraction. It is thus proposed that the 33K phosphoprotein is $III^{mannose}$.

The Fructose PTS

When S typhimurium strain SB3507 was grown on fructose three phosphoproteins (40K, 13K, and 8K) were induced and found in the soluble fraction (Fig. 2). When S typhimurium ptsH mutant, SB2226, was grown on fructose the same proteins were found, but unlike SB3507, some of the 8K protein was associated with the membrane in SB2226. It is shown in the next section that the 13K phosphoprotein is $III^{glucitol}$. The 40K and 8K phosphoproteins appear to be part of the fructose phosphotransferase. They were induced in all S typhimurium and E coli strains grown on fructose, and the 8K phosphoprotein was induced in S typhimurium strain SB2950 grown on nutrient broth with fructose. The phosphorylation of the 8K protein at the expense of phosphoenolpyruvate was dependent upon added enzyme I. The results indicated that there are at least two phosphocarrier proteins involved with the fructose PTS. Because the phosphoamino acid of the 8K protein was identified as 1-P-histidine and that of the other protein (40K) was identified as 3-P-histidine, the former protein is probably FPr, while the latter is $III^{fructose}$.

The III^{frc} was purified free of FPr, III^{glc}, and III^{gut}, as described in Methods. This preparation of the 40K phosphoprotein behaved as a substrate of enzyme $II^{fructose}$ and with saturating concentrations of this phosphocarrier protein, enzyme $II^{fructose}$ had a Km of 15 μM for fructose. Moreover, fructose phosphorylation assayed using 10 μM fructose and the III^{frc} preparation was not inhibited significantly ($< 15\%$) by a 100-fold excess of methyl α-glucoside, methyl β-glucoside, 2-deoxyglucose, glucose, mannose, galactose, glucosamine, N-acetylglucosamine, N-acetylgalactosamine, methyl-β-mannoside, mannitol, arabitol, glucitol, or sorbose. For these studies, membranes from fructose-grown S typhimurium strain SB1687 were used to avoid possible interactions involving enzyme $II^{mannose}$.

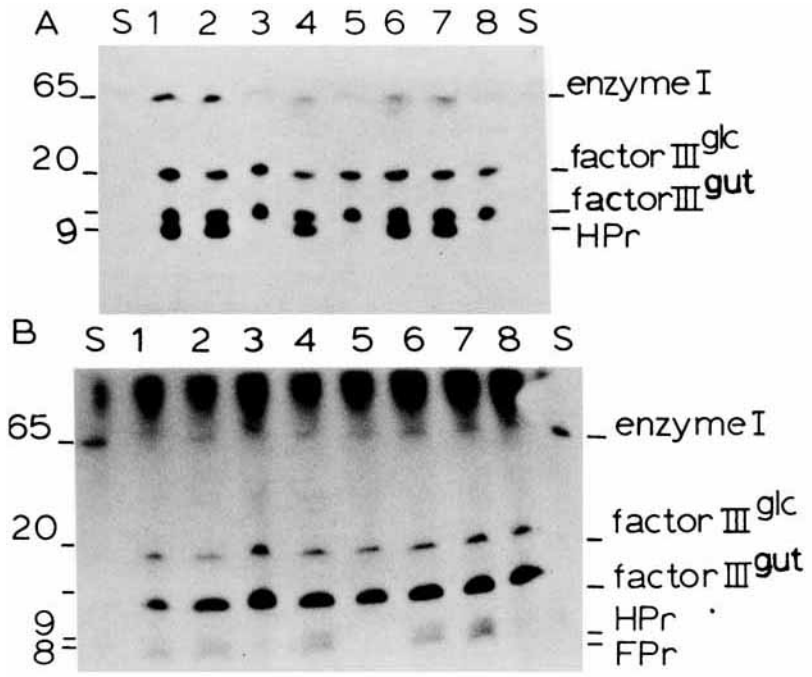

Fig. 5. Phosphoproteins in glucitol grown cells. Crude supernatants from glucitol grown S typhimurium strain SB3507 (A) and E coli 1100 (B) were incubated and run on SDS-PAGE gels as described in Figure 2. Autoradiography was for 20%.

Glucitol Phosphotransferase

When cells from either E coli or S typhimurium were grown in glucitol medium and the phosphoproteins were resolved and detected by SDS-PAGE and autoradiography, a 13K phosphoprotein was found in S typhimurium and a 14K phosphoprotein was found in E coli. As can be seen in Figure 5, this phosphoprotein is as prominent as the other PTS phosphoproteins. In E coli strains 1100 and ZSC17, the 8K protein also was induced to some extent; however, ptsH strains would not grow on or ferment glucitol. This result indicated that the 8K protein, FPr, does not interact effectively with the glucitol phosphotransferase.

When glucitol-grown S typhimurium crude extracts were fractionated as described in Methods, a protein fraction that stimulated glucitol phosphorylation in the presence of HPr was found to coincide with the 13K phosphoprotein. The 13K phosphoprotein has yet to be completely separated from III^{glc}, and thus no sugar specificity studies have been conducted.

Because of the properties of the 13K and 14K phosphoproteins—they appear to contain 3-P-histidine, are induced by growth in the presence of glucitol, and they promote glucitol phosphorylation in vitro—it is concluded that these phosphoproteins are $III^{glucitol}$. Since the completion of this work, we have learned that there is genetic

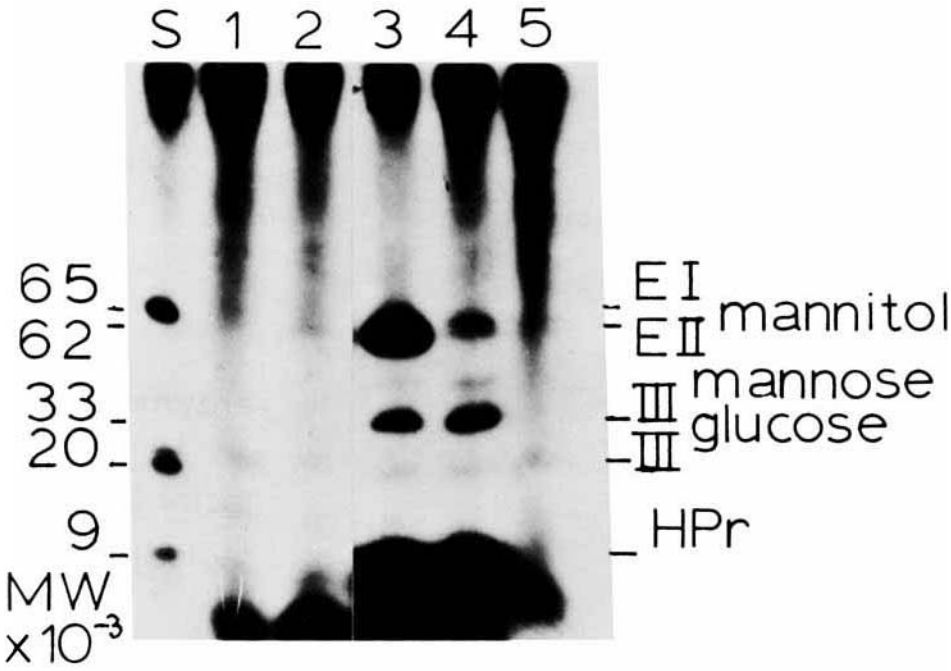

Fig. 6. Phosphoproteins in membranes of mannitol grown S typhimurium strain SB3507. S typhimurium strain SB3507 was grown on mannitol and glucose, and the membranes were prepared as described in Methods. Samples (about 90 μg/ml protein) were incubated with 0.1 mM [^{32}P] phosphoenolpyruvate (1×10^5 cpm/nmol) for 5 min. The samples were loaded onto a 7% acrylamide gel and run for 6 hr; S) standards; 1) no addition; 2) with enzyme I (1 pmol); 3) with enzyme I (1 pmol) and HPr (75 pmol). Samples 1–3 were mannitol-grown membranes. Sample 4 was glucose-grown membranes with enzyme I (1 pmol) and HPr (75 pmol). Sample 5 was the same as sample 3 except [γ-^{32}P]ATP was used. The samples were not boiled, so P-HPr survived (lanes 3 and 4). Autoradiography was for 15%.

and biochemical evidence for a $III^{glucitol}$ [47] and that the $III^{glucitol}$ has been purified to near homogeneity [F. Grenier and M.H. Saier, Jr., unpublished results].

The Mannitol Enzyme II

When membranes from cells grown on mannitol were tested for phosphoproteins, the autoradiographic exposure at the enzyme I position (65K) was often much greater than that accountable by the added enzyme I. The width of the autoradiographic exposure indicated that there was another phosphoprotein present with a slightly lower MW. Membrane samples were investigated using enzyme I levels much lower than those used in Figure 4, and with a longer incubation time (5 min). The results are shown in Figure 6. There was a major phosphoprotein (62K) detected in mannitol membranes, which was dependent upon enzyme I and HPr addition. Other results showed it to be acid-labile, as if it contained 3-P-histidine. This phosphoprotein is missing in enzyme $II^{mannitol}$ mutants grown in nutrient broth and mannitol. The molecular weight and quantity of this protein suggests that it is enzyme $II^{mannitol}$ [48,49].

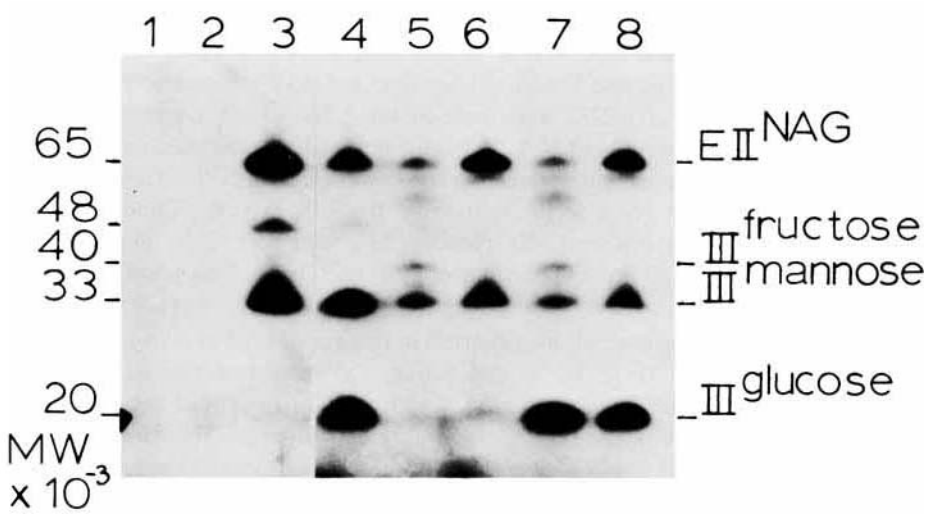

Fig. 7. Phosphorylation in membranes from N-acetylglucosamine grown cells. Membranes were isolated from S typhimurium strain SB3507 grown on N-acetylglucosamine, fructose, and glucosamine as described in Methods. The membranes were incubated with 0.1 mM [^{32}P]phosphoenolpyruvate as follows: lanes 1–4) N-acetylglucosamine membranes; (1) no additions; (2) with enzyme I (1 pmol); (3) with enzyme I (1 pmol) and 75 pmol HPr; (4) with enzyme I (1 pmol), HPr (75 pmol) and factor III^{glucose} (20 pmol); lanes 5 and 7 fructose membranes; lanes 6 and 8, glucosamine membranes; (5) and (6) with enzyme I (1 pmol) and HPr (75 pmol); (7) and (8) with enzyme I (1 pmol), HPr (75 pmol) and factor III^{glucose} (20 pmol). The gel was 7% acrylamide and the HPr ran with the ion front. Sample 4 had been boiled for 1 min.

The N-Acetylglucosamine Enzyme II

Membranes isolated from cells grown on N-acetylglucosamine showed a similar heavy autoradiographic exposure at 65K as did the membranes from mannitol grown cells. It can be seen in Figure 7 that there is an inducible 65K phosphoprotein other than enzyme I in these membranes (compare with fructose membranes). This 65K phosphoprotein was also partially inducible by glucosamine (Fig. 7). The 65K phosphoprotein behaves as a 3-P-histidine and is probably enzyme II$^{N\text{-acetylglucosamine}}$ [10]. No inducible III-like protein was detected in the soluble fraction.

DISCUSSION

A variety of types of phosphoproteins can be detected by SDS-PAGE and autoradiography, and tentative identification of the phosphoamino acid can be made using a variety of simple techniques. The results reported indicate that caution should be used before assuming that acid-fixed phosphoproteins are acid-stable. Further, in bacteria the ATP-dependent phosphorylations must be proved not to be phosphoenolpyruvate-dependent phosphorylations.

Previous reports on the identification of ATP-dependent phosphorylation reactions in E coli and S typhimurium [38–40] have neglected to test the possible involvement of phosphoenolpyruvate. Wang and Koshland [38] have shown that there

is a 53K protein which contains phosphoserine and the 45K protein contains phosphothreonine. They identified a 46K protein as isocitrate dehydrogenase which contains a phosphoserine [39]. Wang and Koshland also detected ATP-dependent phosphoprotein at 72K, 63K, 30K, and 22K, and none of these have been characterized with respect to the phosphoamino acid [J.Y.J. Wang, personal communication]. Because of ATP-phosphoenolpyruvate phosphoexchange demonstrated in this report, consideration must therefore be given to the following: the 75K protein (Table II) may be identical to the previously reported 72K protein [39]; enzyme I, 65K, is possibly the 63K protein detected by ATP phosphorylation [38]; and the III^{glc} is possibly the 22K protein [39].

The novel PTS phosphoproteins reported in this paper lead to a reassessment of the proteins of the PTS (Fig. 1). In this paper, the published procedure for the resolution of enzyme IIA/IIB was repeated, and by use of improved assays [3] only the enzyme $II^{mannose}$ activity can be found. Kundig and Roseman reported [7] almost 100% reconstitution (their Table 4) which was not obtained here. This probably was due to the difference in assay procedures. Considering the findings presented here, it would appear that the IIA proteins previously described [7] are probably identical to the sugar-specific III proteins. In the original report [7] the IIB fraction was not homogeneous and probably contained several different enzyme IIs.

The following sugar-specific phosphotransferases have been characterized in this report: glucose, mannose, and glucitol phosphotransferases exist as III/enzyme II pairs. The inducible fructose PTS consists of FPr, $III^{fructose}$, and enzyme $II^{fructose}$, all of which appear to be specific for fructose. The mannitol and N-acetylglucosamine enzyme IIs appear to function without a III-like protein. However, these phosphotransferases appear to have N-3-histidyl phosphorylated enzyme IIs. And finally, results with E coli, as the S typhimurium strains do not grow on β-glucosides, suggest that there is a $III^{\beta\text{-glucoside}}$ a molecular weight of about 14K (unpublished results).

While the phosphoproteins in E coli were not investigated as thoroughly as those in S typhimurium, they do appear to be identical except for the molecular weight difference between the III^{gut} (Table III, Fig. 5). This difference in mobility was seen when phosphorylated samples of extracts from E coli and S typhimurium grown in glucitol-containing medium were run on the same gel. The mobility of III^{gut} on SDS-PAGE gels was anomalous. Figure 2 would indicate that it had an apparent MW of 11K while Figure 5 was closer to 13K. Samples run on SDS-PAGE gels with higher polyacrylamide concentrations gave the higher value.

Except for the two enzymes IIs for mannitol and N-acetylglucosamine and $III^{mannose}$, no consistent membrane phosphorylations have been detected. However, both kinetic considerations [9,50] and chemical considerations [51] suggest that all the enzyme IIs should be phosphorylated. In addition to the unidentified 48K protein in Figure 7 a membrane-bound 23K phosphoprotein dependent upon enzyme I and HPr has been detected. These phosphoproteins have been detected only in nonboiled incubations of membrane preparations.

There are two genetically determined PTS-associated loci in E coli for which no PTS protein has been assigned. These are iex [52,53] and cif [54]. The former can not be identified with any phosphoprotein [Peri and Waygood, unpublished results]. Because cif maps close to enzyme $II^{fructose}$ and makes the cells insensitive to fructose catabolite inhibition, it may be the gene for $III^{fructose}$. The induction of FPr (8K) has been found under a number of conditions other than fructose growth where $III^{fructose}$

(40K) was not detected. This suggests that the syntheses of $III^{fructose}$ and FPr are not subject to coordinate regulation.

One of the questions that will have to be answered is whether $III^{mannose}$, $III^{fructose}$, and $III^{glucitol}$ have regulatory properties similar to those described for $III^{glucose}$ [14–20]. After all, glucose and methyl α-glucoside are not the only sugars capable of catabolite repression and catabolite inhibition [55]. Perhaps some, or all, of these proteins are involved in the regulation of adenylate cyclase by the PTS [29]. In the course of this investigation, a number of PTS-dependent phosphoproteins have been identified. These minor phosphoproteins (51K, 19K, 17K) have unknown roles. Saier has proposed that regulatory phosphoproteins may function in various aspects of metabolic control [20].

Cyclic AMP has been routinely tested as a potential activator of protein kinases (Figs. 2, 5). No stimulation by cyclic AMP has been found as has been reported by others [38–40]. A cyclic-AMP-activated protein kinase has been reported in E coli [56], but others have not been able to duplicate that report [38-40,57, and Waygood and Sanwal, unpublished results].

A cyclic-AMP-activated protein kinase was previously reported in Streptococcus spp [58], and recently an ATP-dependent protein kinase that phosphorylates a seryl residue in HPr has been identified in Streptococcus pyogenes [59]. Independently, it has been shown that HPr from Streptococcus salivarius is phosphorylated by ATP [Waygood, Mattoo, Vadeboncoeur, unpublished results]. To date, we have not detected this type of phosphorylation in S typhimurium or E coli.

Lastly, the method of phosphoprotein detection reported here should be useful as a screening technique to determine which PTS proteins are missing in mutants without the use of a large number of assays. Preliminary screenings of glucitol-fermentation-negative mutants have given an overproducer of FPr and the 15K protein (Table I) for reasons that are not understood [Peri and Waygood, unpublished]. Clearly, unexpected changes in the detectable phosphoproteins may lead to useful directions of investigations, and to a better understanding of the PTS and its regulatory interactions.

ACKNOWLEDGMENTS

We thank Dr. M.H. Saier, Jr., and Dr. F. Grenier for information about their current work with $III^{glucitol}$. We also thank Dr. M.H. Saier, Jr., Dr. J. Riezer, and Dr. W. Hengstenberg for useful discussions with respect to the work with the ATP-dependent protein kinase in Streptococcus pyogenes. We thank Dr. J.Y.J. Wang for open and useful discussions about the details of her work on ATP-dependent phosphorylations in enteric bacteria. R.L. Mattoo was the recipient of a University of Saskatchewan Postdoctoral fellowship. K.G. Peri was a recipient of a Saskatchewan Health Research Board graduate training fellowship. The technical assistance was provided by Ellen Erickson. This work was supported by the Medical Research Council of Canada.

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NOTE ADDED IN PROOF

The 48K membrane bound phosphoprotein seen in Figure 7 has been identified as enzyme II^{glc} [Peri K, Kornberg HL, and Waygood EB, Febs. Letts. (1984) in press].