THE β-GLUCOSIDE SYSTEM OF ESCHERICHIA COLI III. PROPERTIES OF A P–HPr: β-GLUCOSIDE PHOSPHOTRANSFERASE EXTRACTED FROM MEMBRANES WITH DETERGENT

Steven P. Rose† and C. Fred Fox

*Department of Biochemistry, University of Chicago, Illinois 60637, and the Department of Bacteriology and the Molecular Biology Institute, University of California, Los Angeles, California 90024***

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A P–HPr:β-glucoside phosphotransferase (enzyme II^{bgl}) ‡ has been extracted from membranes of a β-glucoside fermenting strain of *Escherichia coli* K12 using the nonionic detergent Triton X–100. The extracted enzyme was rendered virtually free of both lipid and detergent by chromatography on DEAE-cellulose. At this stage, the partially purified enzyme had negligible activity, but activity was restored effectively by the addition of (1) nonionic detergents of the Tween or Triton series and (2) crude *E. coli* phospholipids or an anionic lipid enriched fraction, but not phosphatidylethanolamine. Detergent activators were most effective at or near the critical micelle concentration, but were inhibitory when added at concentrations above the critical micelle concentration.

In order to obtain maximal initial rates of phosphotransferase activity, it was necessary to incubate the extracted, partially purified enzyme with detergent activator and HPr prior to the addition of the other assay system components. High detergent concentration inhibited the initial rate of phosphorylation by interfering with an essential step (or steps) that occur during this preliminary incubation. The activation occuring during the preliminary incubation was also highly temperature dependent; a precipitous decrease in activation was detected below 16° when Tween 40 was employed as the detergent activator.

Phosphorylation mediated by the membrane associated form of the phosphotransferase was not influenced by the physical state of the lipid components of the membrane. This is in marked contrast to the properties of the phosphorylation reaction mediated by the phosphotransferase in intact cells.

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†Current address, Department of Biological Chemistry, University of California, Los Angeles, California 90024.

**Address reprint request to C. Fred Fox, Department of Bacteriology, University of California, Los Angeles, California 90024.

‡The nomenclature of the enzymes II is that suggested by Lin (1).

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INTRODUCTION

Strains of *E. coli* that ferment β-glucosides are inducible for β-glucoside transport and an aryl-β-glucoside splitting enzyme (2–3). A previous report from this laboratory (4) showed that the initial step in β-glucoside catabolism is phosphorylation by a phosphotransferase system of the type first described by Kundig, Ghosh, and Roseman (5). The sequence of steps involved in β-glucoside catabolism is shown below.

$$\text{P–enolpyruvate} + \text{HPr} \xrightarrow{\text{enzyme I}} \text{P–HPr} + \text{pryuvate}$$

$$\text{P–HPr} + \beta\text{-glucoside} \xrightarrow{\text{enzyme II}^{bgl}} \text{HPr} + \beta\text{-glucoside–6–P}$$

$$\beta\text{-glucoside–6–P} \xrightarrow{\text{phospho-}\beta\text{-glucosidase}} \text{glucose–6–P} + \text{aglycone}$$

Enzyme I and HPr are soluble proteins, and enzyme II^{bgl} activity is membrane associated. The product of the enzyme I mediated reaction, P–HPr, serves as the phosphate donor in phosphorylation of a large number of sugars and sugar alcohols. Sugar specificity resides in the enzymes II, most of which are inducible. Notable exceptions are the constitutive enzymes II of *E. coli* with specificity for D–glucose, D–mannose, and D–fructose. The details of the phosphotransferase system have been described in several reviews (1, 6–7). Kundig and Roseman have characterized the constitutive enzymes II of *E. coli* (8). These have been extracted from membranes, and three separable components were shown to be required for phosphotransferase activity. Two of these were protein fractions (enzymes II–A and II–B) and the third was lipid (phosphatidylglycerol). The enzyme II–A fraction was further resolved into three proteins, each with a specificity for one of the three sugars mentioned above.

Several lines of evidence have implicated the phosphotransferase system in the transport of phosphotransferase system substrates across the cell membrane. The experimental evidence for the involvement of this system in transport has been thoroughly treated in recent reviews (1, 6–7, 9).

We have previously reported that enzyme II^{bgl} activity can be extracted from membranes of *E. coli* K12 by the use of nonionic detergents (10). Presented here is a characterization of the extracted and partially purified enzyme, and a comparison of the properties of the membrane-associated and extracted forms of the enzyme.

MATERIALS AND METHODS

Materials

Phosphoenolpyruvate (crystalline, tricyclohexylammonium salt) and the sodium salt of deoxycholic acid were obtained from Calbiochem; pyridine-free ammonium sulfate, D-glucose and sucrose from Mallinckrodt; methionine, bovine serum albumin, thiamine·HCl, Tween 40, and Tween 80 from Nutritional Biochemicals; sodium dodecyl sulfate from Matheson, Coleman, and Bell; and 2-mercaptoethanol from Eastman Organic Chemicals. Casamino acids were obtained from Difco; Biogel HT (hydroxylapatite) and Dowex-1 (AG1-X2) in the chloride form (50–100 mesh) were obtained from Bio-Rad. DEAE-cellulose DE 23 was obtained from Reeve Angel, 3 MM paper from Whatman, Ficoll and Sephadex G–75 from Pharmacia, dicetylphosphate from Krishell Laboratories, ^{32}P-

inorganic phosphate from New England Nuclear, cis, cis-$\Delta^{9,12}$-octadecadienoic acid and hexadecanoic acid from the Hormel Institute, Austin, Minn., and trans-Δ^{9}-octadecenoic acid from Aldrich. Triton X–100 was obtained from Rohm and Haas. Triton X–45, X–114, X–165, X–305, and X–405 were the gifts of Rohm and Haas; chloramphenicol the gift of Parke Davis and Co.; and streptomycin sulfate the gift of Eli Lilly and Co. The source of ^{14}C-TPG* (thiophenol-labeled specific activity, 2000 cpm per nmole) TPG and PNPG has been described elsewhere (4). Twentyfold and sixtyfold purified phospho-β-glucosidase A preparations were the gift of G. Wilson.

Bacterial Strains

All bacterial strains are derivatives of *E. coli* K12. Strain W1895D*bgl*$^{+}$*c* is constitutive for enzyme II^{bgl} (3, 4). Strain 30E is an unsaturated fatty acid auxotroph that is inducible for enzymes of the β-glucoside system (11). The properties of strain 1100, which is inducible for the enzymes of β-glucoside catabolism and of strain 1005, which is not, are described elsewhere (4, 12).

Growth Conditions

Cells of strains 1100 and 1005 were grown in 10 liter lots at 37°C in medium A (13) containing 20 gm/liter of casamino acids and 5 mg/liter of thiamine·HCl in 15 liter fermenter jars with strong aeration and maximal stirring. At a cell density of 10^9 cells/ml, glucose was added at 20 gm/liter. When the cell density reached 7.5×10^9 cells/ml, chloramphenicol was routinely added at a concentration of 50 mg/liter to arrest growth, and the cells were harvested with a Sharples centrifuge and washed with 500 ml of 0.01 M potassium phosphate buffer of pH 7.6. The cell paste was stored frozen at −20°C.

Cells of strain W1895D1*bgl*$^{+}$*c* were grown at 37°C in 100 liters of medium A (13) containing 10 gm/liter of casamino acids and 5 mg/liter of thiamine·HCl using a New Brunswick Fermacell fermentor. Agitation was at 50 rpm, aeration at 0.75 cubic feet/min and the vessel pressure at 2 psi. At a cell density of 2×10^9 cells/ml, chloramphenicol was added at 50 mg/liter, and the cells were cooled to 20°C and harvested with a Sharples centrifuge. The cells were washed with 2 liters of 0.1 M Tris·HCl buffer of pH 7.6 containing 10 mM 2-mercaptoethanol. The washed cell paste has been stored frozen at −20°C with no apparent loss in the specific activity of enzyme II^{bgl} for periods of over a year.

Phospholipid Purification

Lipids were extracted from *E. coli* K12 cells grown to late logarithmic phase (Miles Laboratory), and the phospholipid fraction was prepared using the procedure of Law and Essen (14). This yields a crude mixture consisting primarily of phosphatidylethanolamine and phosphatidylglycerol. Phosphatidylethanolamine and phosphatidylglycerol were separated by DEAE-cellulose chromatography using Law and Essen's modification of the method of Rouser et al. (15). Phosphatidylserine, phosphatidylethanolamine and cardiolipin were present in the phosphatidylglycerol-enriched fraction below our level of detection (less than 2%). The lipid fractions were evaporated to dryness under nitrogen to remove chloroform, and aqueous suspensions were prepared by subjecting a suspen-

*The abbreviations used are: TPG, phenyl-1-thio-β-D-glucopyranoside; PNPG, *p*-nitrophenyl-β-D-glucopyranoside; *bgl*, inducibility for β-glucoside fermentation; *bgl*$^{+}$*c*, constitutivity for β-glucoside fermentation; P-enolpyruvate, phosphoenolpyruvic acid.

sion of phospholipid (in a solution containing 0.1 M Tris·HCl buffer of pH 7.6 and 10 mM 2-mercaptoethanol) to sonic irradiation for 3 min at ice bath temperature. The sonicated suspensions of phosphatidylglycerol were clear while the suspensions of phosphatidylethanolamine were opalescent.

Protein Determinations

Protein was determined the the method of Lowry et al. (16) except for fractions v and vi of the HPr purification where protein was determined by the biuret method of Chargaff (17). When 2-mercaptoethanol, Tris·HCl, or Tween 40 were present in the proteinaceous supernatant solutions, the 5% trichloracetic acid precipitation procedure described by Lowry et al. was used (16). The supernatant fractions of Triton-extracted membranes and the suspended Triton-treated membrane pellets were subjected to an initial precipitation with cold 80% ethanol prior to the trichloracetic acid step.

Enzyme Assays

Enzyme I and HPr were assayed as previously described (4). Enzyme II^{bgl} was assayed with enzyme I present at a concentration sufficient to maintain essentially all the HPr in the system in the form of P-HPr. HPr was present in sufficient excess so that linear plots of enzyme II concentration vs. activity were obtained. The 0.1 ml standard radioisotopic assay system for enzyme II^{bgl} contained 12.5 mM P-enolpyruvate, 10 mM KF, 50 μM $MgCl_2$, 0.25 mM ^{14}C-TPG, 75 mM Tris·HCl of pH 7.6, 7.5 mM 2-mercaptoethanol, 8.7 μg of 40-fold purified enzyme I (fraction iii), 16 μg of 150-fold purified HPr (fraction v) and enzyme II. The assay system for fraction iii enzyme II^{bgl} also contained 37.5 mM KCl and detergent or phospholipid at the concentrations specified in the text. None of the concentrations of detergent used in this study significantly affected the efficiency of the P-HPr generating system. Unless otherwise noted the assays were performed as follows. Enzyme I, HPr, enzyme II^{bgl}, Tris·HCl, 2-mercaptoethanol and detergent* or phospholipid (where specified) were mixed in a volume of 0.075 ml, to be referred to as the "enzyme mix," and incubated for 10 min at the assay temperature. The "substrate mix" consisting of P-enolpyruvate, $MgCl_2$, ^{14}C-TPG, and KF in a volume of 0.025 ml was added to initiate the reaction. The reaction was terminated by the addition of 1 ml of 1 mM ethylenediamine tetraacetate, and the mixture was applied to a 0.5 X 2.0 cm Dowex-1 column in the formate form. The column was first eluted with 15 ml of water to remove unreacted substrate, and the reaction product was eluted in 15 ml of 0.2 M formic acid–0.5 M ammonium formate solution. A 1.5 ml aliquot of the ammonium formate eluate was mixed with 20 ml of Patterson–Greene scintillation fluid containing Toluene and Triton X–100 in the ratio of 3:1 for liquid scintillation counting (18). In cases where the eluted solution contained low levels of radioactivity, aliquots of 5 or 10 ml were counted in 6.5 or 13 ml, respectively, of Patterson–Greene scintillation fluid containing Toluene and Triton X–100 in the ratio of 7:6. Unless otherwise noted, activity is described as nmoles of P-TPG formed corrected for a control reaction incubated with enzyme II omitted.

The 0.1 ml standard colorimetric assay system for Fraction iii enzyme II^{bgl} contained 12.5 mM P-enolpyruvate, 50 μM $MgCl_2$, 1 mM PNPG, 10 mM KF, 65 mM

*In all studies employing detergents, unless otherwise stated, the detergent concentration given is that of the complete assay system containing both "enzyme mix" and "substrate mix."

Tris-HCl of pH 7.6, 6.5 mM 2-mercaptoethanol, 37.5 mM KCl, 8.7 μg of 40-fold purified enzyme, I, 16 μg of 160-fold purified HPr, 38.6 μg of 20-fold purified phospho-β-glucosidase A, 0.125 mg/ml of Tween 40, and 0.025 ml of an appropriate dilution of Fraction iii enzyme II^{bgl}. After a 10 min incubation of the "enzyme mix" consisting of enzyme I, HPr, detergent, and enzyme II^{bgl} at the assay temperature, the "substrate mix" consisting of P-enolpyruvate, $MgCl_2$, KF, PNPG, and phospho-β-glucosidase A was added in a volume of 0.025 ml to initiate the reaction. Phospho-β-glucosidase A was present in several fold excess, making phosphorylation of substrate rate-determining for its hydrolysis. The reaction was terminated by the addition of 0.9 ml of 95% ethanol:1 M K_2CO_3 (3:7). The absorbance of the p-nitrophenol released was determined at 420 nm and converted to nmoles using the experimentally determined extinction coefficient of 1.38×10^4 M^{-1} cm^{-1}.

Enzyme I Purification

This is an adaptation of the purification procedure described by Kundig and Roseman (19). All operations were done at 0–4°. All buffers were at pH 7.6 and contained 10 mM 2-mercaptoethanol. The purification procedure is summarized in Table I.

Cell Disruption. Strain 1005 frozen cell paste (520 gm) was thawed and suspended in a volume of 2040 ml in 10 mM potassium phosphate buffer. The cells were disrupted by a 4 min sonic irradiation and the cell debris removed by centrifugation at 16,300 × g for 30 min.

Streptomycin Treatment. The sonic extract fraction i was treated with streptomycin sulfate (200 ml of a 10% w/v solution). After 20 min of stirring the suspension was centrifuged at 16,300 × g for 20 min.

Ammonium Sulfate Fractionation. The supernatant solution (2180 ml, 45 gm of protein) obtained from the streptomycin treatment was treated with 455 gm of solid ammonium sulfate, stirred for 30 min and centrifuged at 16,300 × g for 20 min. The ammonium sulfate supernatant solution (2250 ml, 34 gm of protein) was treated with 535 gm of solid ammonium sulfate. The suspension was stirred for 30 min and centrifuged for 20 min at 16,300 × g, and the precipitated material was suspended in a volume of 500 ml with 10 mM potassium phosphate buffer and dialyzed against 10 liters of the same buffer.

TABLE I. Purification of Enzyme I

Fraction	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)
i. Sonic extract	2040	45,000	9,150,000	200
ii. Ammonium sulfate treated, dialyzed material	750	27,000	9,750,000	360
iii. DEAE-cellulose concentrate	38	167	1,270,000	7600

Enzyme I was assayed as described previously (4). One unit of enzyme I activity catalyzes the phosphorylation of 1 nmole of glycoside in 10 min at 28°.

DEAE–Cellulose Chromatography. The dialyzed material (fraction ii) was adjusted to a buffer concentration of 0.2 M potassium phosphate with 0.6 M potassium phosphate buffer, and applied to a 54 cm × 4.90 cm^2 DEAE-cellulose column equilibrated with 0.2 M potassium phosphate buffer. Unbound protein was eluted with 250 ml of equilibrating buffer. Enzyme I was then eluted with 0.34 M potassium phosphate buffer at a flow rate of 60 ml/hr. The high specific activity enzyme I fractions (700 ml, 175 mg of protein) which eluted with the 0.34 M buffer front were concentrated with 330 gm of solid ammonium sulfate. The precipitate was suspended in and dialyzed against 0.05 M Tris·HCl, yielding the DEAE-cellulose concentrate (fraction iii).

Fraction iii enzyme I is free of HPr and enzyme II^{bgl} activities. It has been stored at −20° with frequent freezing and thawing for as long as one year with less than a three-fold decrease in activity.

HPr Purification

All operations were at 0–4°, and all buffers were at pH 7.6. The purification procedure is summarized in Table II.

Cell Disruption. One kilogram of strain 1100 frozen cell paste was thawed and suspended in a volume of 4.6 liters with deionized water. The cells were disrupted by subjecting 100 ml portions to a 4.5 min sonic irradiation.

Streptomycin Treatment. The sonic extract (fraction i) was treated with streptomycin sulfate (460 ml of a 10% w/v solution). After 30 min of stirring, the suspension was centrifuged at 16,300 × g for 20 min and the pellet discarded.

Ammonium Sulfate Concentration. The supernatant solution (4950 ml, 77.5 gm of protein) obtained after streptomycin treatment was treated with 2780 gm of solid ammonium sulfate and stirred overnight. The precipitate was collected by centrifugation

TABLE II. Purification of HPr

Fraction	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)
i. Sonic extract	4600	78,000	5,300,000	68
ii. Acid treatment	1650	18,100	2,700,000	150
iii. First ethanol fractionation				
a. First extract	105	2,840	210,000	74
b. Second-fourth extracts	122	1,470	1,360,000	930
iv. Second ethanol fractionation and re-extraction of IIIa	54	880	204,600	230
v. DEAE combined eluants dialyzed and concentrated	36	116	1,172,000	10,100

HPr was assayed as described previously (4). One unit of HPr activity catalyzes the phosphorylation of 1 nmole of glycoside in 10 min at 28°.

at 16,300 × g for 30 min and suspended in and dialyzed against 10 mM potassium phosphate buffer.

HCl Treatment. The dialyzed material (740 ml) was titrated to pH 4.0 with 1 M HCl, stirred for 20 min and centrifuged at 16,300 × g for 20 min. The pellet was discarded. To the acid-treated supernatant solution (670 ml) was added 980 ml of 0.2 M Tris·HCl to yield a final volume of 1650 ml (fraction ii).

Ethanol Fractionations. Two ethanol precipitation steps were performed. 1) 42.5% ethanol precipitation: 1350 ml of 95% ethanol at −20° was added slowly with mixing to fraction ii and the mixture stirred for 20 min. The precipitate was collected by centrifugation at 16,300 × g for 20 min and discarded. 2) 71% ethanol precipitation: 3390 ml of 95% ethanol at −20° was added slowly with mixing to 2920 ml of the 42.5% ethanol-treated supernatant solution. The mixture was stirred for 20 min and the precipitate collected as above. The 71% ethanol pellet was extracted 4 times with 0.1 M Tris·HCl buffer (100, 60, 30, and 25 ml, respectively), using a motor-driven Potter-Elvejhem assembly, and each extract dialyzed against 1mM Tris·HCl. The second, third, and fourth dialyzed extracts were combined (fraction iiib). The first extract (fraction iiia) obtained from the 71% ethanol precipitate was suspended in 240 ml of water and the ethanol fractionation was repeated. The first three 0.1 M Tris·HCl extracts (25, 20, and 10 ml, respectively) obtained from the second ethanol fractionation were combined and dialyzed against 1 mM Tris·HCl (fraction iv).

DEAE–Cellulose Chromatography. Fractions iiib and iv were combined and applied to a DEAE-cellulose column (60 cm × 4.90 cm^2) which had been equilibrated with 10 mM potassium phosphate buffer. After a column volume elution with equilibrating buffer, the HPr activity was eluted with 50 mM potassium phosphate buffer at a flow rate of 60 ml/hr. The high specific activity HPr fractions (152 ml) which eluted with the 50 mM buffer front were combined, dialyzed against 5 mM Tris·HCl, lyophilized, and adjusted to a final volume of 36 ml and a final buffer concentration of 0.1 M Tris·HCl (fraction v).

HPr fraction v was free of enzyme I and enzyme II^{bgl} activities and lost no activity after storage at −20° for periods exceeding one year. Fraction v HPr was used in all the studies described here.

RESULTS

Requirements for Enzyme II^{bgl} Activity

Sugar phosphorylation mediated by membrane-associated enzyme II^{bgl} requires phosphoenolpyruvate, enzyme I, and HPr (Table III). Fraction iii, a partially purified and essentially phospholipid free enzyme II^{bgl} fraction, requires, in addition, a detergent activator or *E. coli* phospholipid (Table III), and has a Mg^{++} requirement for optimal activity. Other surfactants can replace Tween 40 or *E. coli* phospholipid as activators; the requirements for activation will be discussed in a later section.

Extraction and Partial Purification of Enzyme II^{bgl}

All steps were performed at 0–4°. All buffers were at pH 7.6 and contained 10 mM 2-mercaptoethanol. A representative purification procedure is summarized in Table IV.

Preparation of Washed Membranes. Ten grams of frozen W1895D1*bgl*$^+$*c* cell paste was thawed in 140 ml of 0.1 M Tris·HCl buffer and stirred until the cells were well suspended. The cells were disrupted by sonic irradiation for 3 min, and membranes and debris were sedimented by centrifugation at 156,000 × g for 15 min. The supernatant solution was discarded, and the pellet was suspended in 40 ml of 0.1 M Tris·HCl buffer and centrifuged as above. The sedimented washed membrane fraction was suspended in 30 ml of 0.1 M Tris·HClbuffer and a 4.8 ml portion was removed for assay.

Treatment of Washed Membranes with Detergent. Triton X–100 (2.8 ml of a 50 mg/ml solution) was added to the washed membrane suspension (Fraction i) and the mixture was homogenized for 1 min periods at 0, 5, and 10 min after the addition of detergent using a motor-driven Potter Elvejhem assembly. The Triton-treated membrane suspension was brought to a volume of 30 ml with 0.1 M Tris·HCl buffer and centrifuged at 165,000 × g for 1 hr. The supernatant solution was then decanted (Fraction iia), and the Triton-treated membrane pellet was suspended at a volume of 10 ml with 0.1 M Tris·HCl buffer containing 1 mg/ml of Tween 40 (Fraction iib). A substantial increase in the apparent activity of the Triton supernatant fraction (Fraction iia) over that of the washed membrane suspension (Fraction i) was always observed.

DEAE–Cellulose Chromatography. The Triton supernatant solution (Fraction iia) was applied to a 9 ml DEAE cellulose column (5.1 × 1.77 cm^2) equilibrated with 0.1 M

TABLE III. Requirements for the Assay of Membrane-Associated and Fraction iii Enzyme II^{bgl}

	Phosphoryl-TPG formed	
	Washed membranes (nmoles)	Fraction iii enzyme II^{bgl} (nmoles)
Complete assay system	14.4	11.6
Omit P-enolpyruvate	0.4	< 0.04
Omit Mg++	15.2	6.2
Omit Mg++, add EDTA	0.2	0.1
Omit enzyme I	1.0	< 0.04
Omit HPr	0.6	< 0.04
Omit enzyme ii^{bgl}	< 0.04	< 0.04
Omit Tween 40	14.0	0.4
Omit Tween 40, add *E. coli* phospholipid	n.d.†	4.0
Add *E. coli* phospholipid	n.d.	12.8

Enzyme II^{bgl} activity was determined using the standard radioisotopic assay expanded to a 0.2 ml system. Incubation was for 10 min at 28°. The protein concentration of membrane-bound enzyme II^{bgl} in the assay system was 415 μg/ml and that of Fraction iii enzyme II^{bgl} was 20.5 μg/ml. Tween 40 was included at 125 μg/ml, except where noted. EDTA and *E. coli* phospholipid were added to final concentrations of 10 mM and 1 mg/ml, respectively, where indicated. Activity is expressed as nmoles of P–TPG formed in 10 min. The limit of sensitivity of the assay system was 0.04 nmoles.
†n.d., not determined.

Tris·HCl buffer containing 1 mg/ml of Tween 40. The column was eluted successively with 20 ml of equilibrating buffer, 130 ml of 0.1 M Tris·HCl buffer containing 5 mg of Triton X–100, 50 ml of 0.1 M Tris·HCl containing 1 mg/ml of Tween 40, and 120 ml of 0.1 M Tris·HCl buffer. The column was then eluted with 10 ml of 0.1 M Tris·HCl buffer containing 0.1 M KCl, 10 ml of 0.1 M Tris·HCl buffer containing 0.15 M KCl, and 20 ml of 0.1 M Tris·HCl buffer containing 0.2 M KCl. The enzyme II^{bgl} peak fraction (Fraction iii) eluted with the 0.15 M KCl solvent front. The elution rate was 1 ml/min.

The specific activity of enzyme II^{bgl} in the Triton supernatant fraction (Fraction iia, Table 4) is approximately 20-fold greater than that in the washed membrane fraction (Fraction i). The specific activity of Fraction iii, assayed in the presence of 0.25 mg/ml of Tween 40 is slightly less than the specific activity of Fraction ii. However, when aliquots of the DEAE-cellulose column fractions were added to the assay system containing Fraction iii enzyme II^{bgl}, it was found that the fraction which was eluted with 0.1 M Tris·HCl buffer containing 5 mg/ml of Triton X–100 greatly stimulated the enzyme activity. In contrast, Triton X–100 added to the assay system at an identical concentration completely inhibited Fraction iii enzyme II^{bgl} activity. The search for the identity of this stimulatory factor, subsequently identified as phospholipid, led to the phospholipid and detergent studies which will be described in detail in the following sections.

Properties of Fraction iii Enzyme II^{bgl}. Fraction iii enzyme II^{bgl} activity has a half life of 2 days when stored at 4°, and of 4 months when stored at −20° in 50% (v/v) glycerol. The enzyme is substantially less stable when stored at −20° in the absence of glycerol or in the presence of detergent and glycerol. Freshly prepared Fraction iii (less than 3 days old) was used in the studies described here. Fraction iii was characterized by disc-gel electrophoresis using the procedure described by Weber and Osborn (20). Staining with Coomassie blue revealed 9 protein bands of which 7 were major bands. Fraction iii enzyme II^{bgl} was not contaminated with HPr or enzyme I within the limits of detection of the assay system (Table III).

TABLE IV. Purification of Enzyme II^{bgl}

	Fraction	Volume (ml)	Protein (mg)	Activity (units)	Specific activity† (units/mg)
i.	Washed membrane suspension	25.2	836	141,000	169
iia.	Triton supernatant fraction	27	52	172,000	3,310
b.	Suspended Triton pellet	10	510	40,000	78
iii.	DEAE-cellulose peak fraction	10	2	5,400	2,700

Enzymatic activity was determined using the standard radioisotopic assay at 28°. The detergent concentrations in the assay for Fraction iia are 62.5 μg/ml of Triton X–100 and 250 μg/ml of Tween 40; those in the assay for Fraction iib are 6.25 μg/ml of Triton X–100 and 250 μg/ml of Tween 40; and that for Fraction iii is 250 μg/ml of Tween 40. One unit of enzyme II^{bgl} activity catalyzed the phosphorylation of 1 nmole of TPG in 10 min at 28°.

†The specific activities are only apparent values since P-HPr is not saturating. Kinetic analysis has revealed that the K_m for HPr of Fraction iii enzyme II^{bgl} is approximately one-fourth that of the membrane-associated form of the enzyme.

A rate-zonal sedimentation analysis of Fraction iii enzyme II^{bgl} is given in Fig. 1. Since sucrose inhibits enzyme II^{bgl} activity, the linear gradients were generated with Ficoll. Using the method of Martin and Ames (21), with purified phospho-β-glucosidase A as the molecular weight standard, a value of approximately 190,000 daltons was calculated for partially purified enzyme II^{bgl}. Phospho-β-glucosidase A sedimented at identical rates in the presence or absence of Fraction iii enzyme II^{bgl} activity. The broadness of the activity curve indicates that Fraction iii enzyme II^{bgl} is probably a heterogeneous population of aggregates of the active protein with other proteins in the preparation. Fraction iii has also been subjected to rate-zonal sedimentation in linear Ficoll gradients containing 0.25 mg/ml of Tween 40. The enzyme sedimented more slowly under this condition. This behavior could result either from the dissociation of protein aggregates, or from the sedimentation of the enzyme as a protein–detergent complex. No significant loss of activity occurred during zone sedimentation in the presence or absence of detergent, and no stimulation of activity could be obtained by mixing different fractions of the gradients, indicating that the separation of active subunits, should they exist, did not occur during rate-zonal sedimentation.

Purification of Enzyme II^{bgl} with Respect to Phospholipid. A 750-fold enrichment of enzyme II^{bgl} activity with respect to phospholipid was achieved when enzyme II^{bgl} was purified as described in Table V. The peak DEAE enzyme II^{bgl}fraction (Fraction iiib) is virtually inactive when assayed in the absence of added phospholipid or detergent.

Time Course of the Assay System. Product formation determined in the standard radioisotopic assay for membrane-bound enzyme II^{bgl} at 28° is linear as a function of time for at least 120 min and linear as a function of membrane protein concentration up to an activity of 16 nmoles of TPG phosphorylated in 10 min. Similar results were obtained with Fraction iii enzyme II^{bgl} at optimal detergent concentrations in the standard radioisotopic and colorimetric assay systems at 28°.

pH Optimum. The effect of pH on the rate of formation of phosphoryl TPG mediated by fraction iii enzyme II^{bgl} is shown in Fig. 2. Enzyme I was present at sufficient excess to assure that the pH curve is that for the enzyme II^{bgl}catalyzed reaction, and not for the P-HPr generating system. The enzyme II^{bgl} reaction has a broad optimal pH range of 7.3–8.0. The same optimal pH range has been observed with the membrane-associated form of enzyme II^{bgl}.

Kinetic Constants for HPr and Glycoside Substrates. Double reciprocal plots indicative of a double displacement or "Ping-Pong" kinetic mechanism have been obtained for the enzyme II^{bgl} catalyzed reaction where either the P-HPr or glycoside concentration is held constant, and the concentration of the other substrate is varied (22). We have extended these experiments to compare the kinetic constants for glycoside substrate and HPr for the extracted and membrane-associated forms of enzyme II^{bgl}. The apparent K_m for P-HPr at saturating TPG concentration was 585 μg/ml for the membrane-associated form of enzyme II^{bgl}, and 150 μg/ml for Fraction iii enzyme II^{bgl}, a difference of nearly four-fold (Fig. 3). The difference in apparent K_m for glycoside substrates, on the other hand, is less than two-fold greater for Fraction iii enzyme II^{bgl} than for the membrane-associated form of the enzyme (data not shown) (23).

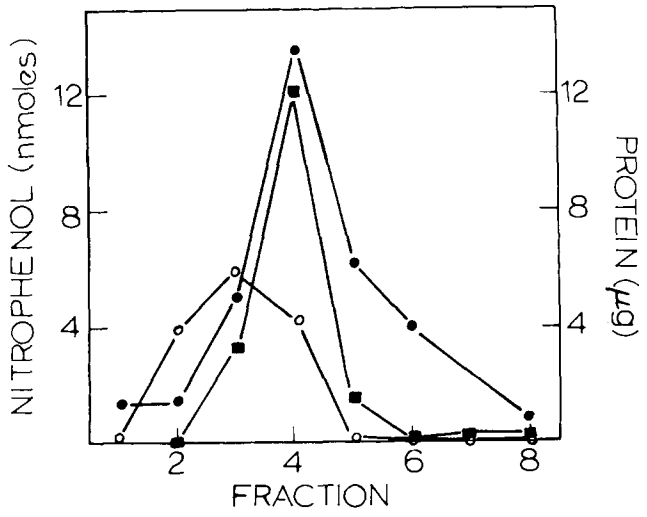

Fig. 1. Rate-zonal sedimentation of Fraction iii enzyme II^{bgl} in the absence of detergent. A mixture of 32.4 μg of Fraction iii enzyme II^{bgl} and 0.05 μg of phospho-β-glucosidase A in a volume of 0.2 ml was layered onto a 5 ml, 14 to 3.5% linear Ficoll gradient containing 0.1 M Tris·HCl buffer of pH 7.6 and 10 mM 2-mercaptoethanol. Centrifugation was for 20 hr at 45,000 rpm and 4° using a Spinco SW 50 rotor. Fractions were collected from the bottom of the gradient (fraction 1), and the activities of both enzymes determined using 0.1 ml colorimetric assay systems at 28°. The phospho-β-glucosidase A assay systems were incubated for 10 min and the enzyme II^{bgl} assay systems (described in Materials and Methods section) for 110 min. The phospho-β-glucosidase A assay system contained 3 mM PNPG–6–P, 70 mM Tris·HCl buffer of pH 7.6, 7 mM 2-mercaptoethanol and enzyme. The phospho-β-glucosidase A assay was initiated by the addition of substrate and terminated by the addition of 0.9 ml of M K_2 CO_3. Product formation was determined as described for the standard colorimetric assay for enzyme II^{bgl}. Protein was determined by the procedure of Lowry et al. (16). Units are μg of protein per gradient fraction (•), and nmoles of nitrophenol formed per 0.025 ml of the gradient fraction during the stated assay time for phospho-β-glucosidase A (▪) and enzyme II^{bgl} (○).

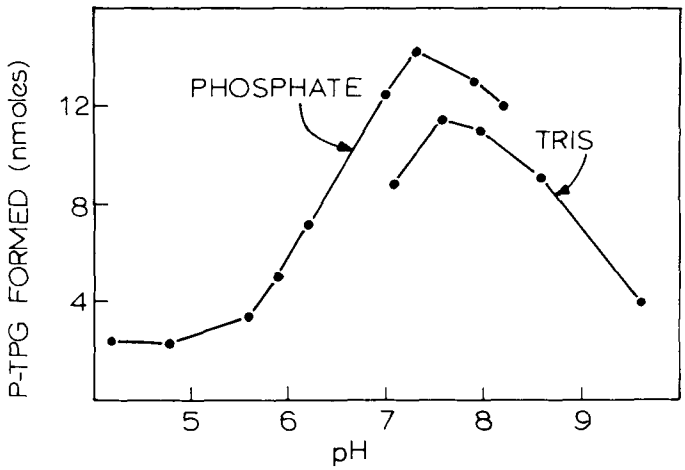

Fig. 2. Enzyme II^{bgl} as a function of pH. Fraction iii enzyme II^{bgl} was assayed for 10 min at 28° in the standard radioisotopic system expanded to a volume of 0.6 ml. Tween 40 was present at 0.25 mg/ml. The concentration of Tris·HCl or potassium phosphate buffer was 40 mM, and the protein concentration of Fraction iii enzyme II^{bgl} was 43 μg/ml. At time 0 and at the end of the reaction a 0.2 ml sample was removed for direct determination of pH using a Radiometer microelectrode system. No pH change was observed during the course of the reaction. After the 10 min reaction period a 0.1 ml aliquot was processed for the determination of product formation by the standard procedure.

Activation of Fraction iii Enzyme II^{bgl} by Phospholipids and Surfactants. Fraction iii enzyme II^{bgl} is virtually inactive in the absence of added phospholipid or detergent. The activation obtained with various phospholipid fractions is described in Fig. 4. At the lower concentrations of added phospholipid the phosphatidylglycerol enriched fraction was a many-fold more effective activator than unfractionated *E. coli* phospholipids. Both the unfractionated *E. coli* phospholipids and the phosphatidylglycerol enriched fraction produced a maximal stimulation of 15-fold in the range of concentrations tested. No activation was observed with phosphatidylethanolamine.

Activation of Fraction iii enzyme II^{bgl} by nonionic detergents of the Triton series is described in Fig. 5. The Triton X–100 concentration required for optimal stimulation was not influenced significantly by the concentration of enzyme protein in the assay system (Fig. 5A). Also, identical detergent concentration optima for Triton X–100 were obtained with three independent preparations of Fraction iii enzyme II^{bgl} (data not shown). The stimulatory effect of various detergents of the Triton X series, which vary in the number of contiguous ethylene oxide units, was studied to see if some chemical or phy-

TABLE V. Purification of Enzyme II^{bgl} with Respect to Phospholipid

Fraction	Volume (ml)	Lipid phosphorus (total counts per minute)	Activity (units)
i. Washed membranes	2	1,170,000	2,140
ii. Triton supernatant fraction	2	325,000	3,320
iii. DEAE-cellulose			
A. Combined detergent washes	80	304,000	0
B. Peak fraction	8	336	460

Two 500 ml cultures of strain W1895D1bgl$^+$c were grown at 37° in 2-liter flasks with rotary agitation at 300 strokes/minute to a cell density of 1.7×10^9 cells/ml in medium containing 1 mg/liter $FeSO_4$, 440 mg/liter Na citrate·$2H_2O$, 100 mg/liter $MgSO_4 \cdot 7H_2O$, 1 gm/liter $(NH_4)_2SO_4$, 3 gm/liter of casamino acids, 5 mg/liter of thiamine·HCl, 4 gm/liter of glycerol, 0.75 mM methionine, and 80 μCi/liter of $^{32}PO_4$. The cells were harvested by centrifugation at 12,100 × g for 10 min, and the pellets were washed twice by suspension in 200 ml of 0.1 M potassium phosphate buffer of pH 7.6 and centrifugation. The cells were suspended at a volume of 30 ml with 0.1 M Tris·HCl of pH 7.6 containing 10 mM 2-mercaptoethanol. Washed membranes were prepared as described for the purification of enzyme II^{bgl} and suspended in 10 ml of 0.1 M Tris·HCl of pH 7.6 containing 10 mM 2-mercaptoethanol. After extraction of the enzyme with Triton X–100 (5 mg/ml), 2 ml of the supernatant solution was chromatographed on a 8 ml DEAE-cellulose column (4.5 cm × 1.77 cm^2). All extraction and purification operations were as described for Table IV, except for the volumes of the eluants for DEAE chromatography. The first and second KCl elutions were with 8 ml volumes, and all other elutions were with one-fourth the volumes indicated for Table IV.

Phospholipids were extracted with chloroform-methanol (2:1, v:v) which was partitioned 3 times with 2 M KCl to remove nonlipid radioactivity. The chloroform extract was pipetted into scintillation vials, evaporated to dryness under a stream of nitrogen, and the radioactivity estimated by scintillation counting. Enzyme II^{bgl} activity was determined using the standard radioisotopic assay at 28°. Enzyme units are defined in Table IV. The triton supernatant fraction (Fraction ii) was assayed at 62.5 μg/ml of Triton X–100 and 250 μg/ml of Tween 40. For the assay of Fraction iii, Fraction ii was diluted 20-fold with a solution which contained 0.1 M Tris·HCl of pH 7.6, 10 mM 2-mercaptoethanol, and 1 mg/ml of Tween 40. This dilution was heated for 10 min at 70° (inactivating enzyme II^{bgl} but not inactivating lipid activators of the enzyme II^{bgl} reaction) and added to the enzyme mix to yield a final assay concentration of 250 μg/ml of Tween 40 and 62.5 μg/ml of Triton X–100.

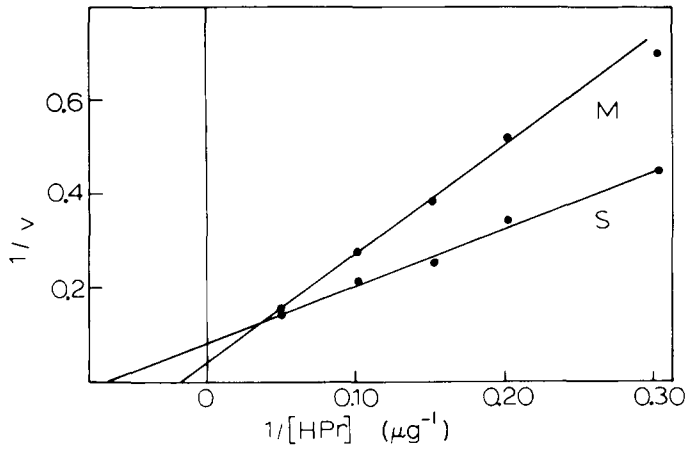

Fig. 3. Double reciprocal plot of HPr concentration vs. reaction velocity for the enzyme II^{bgl} reaction mediated by twice-washed membranes (M) or Fraction iii (S). The standard radioisotopic assay at 28° was used. Twice-washed membranes were present in the assay system at a concentration of 140 μg of protein per ml, and Fraction iii enzyme II^{bgl} at 8 μg of protein per ml. Tween 40 was present in the assay systems at 125 μg/ml. Dilutions of HPr were with a solution containing 0.1 M Tris·HCl of pH 7.6, 10 mM 2-mercaptoethanol, and 1 mg/ml of bovine serum albumin. Units are 1/[HPr], μg^{-1}/0.1 ml system; and 1/v, $nmoles^{-1}$ phosphoryl-TPG formed in 30 min.

sical property of these detergents correlated with their properties as activators. Tritons X–45, X–114, and X–100 were the most effective activators (Fig. 5), Triton X–165 was a less effective activator (data not shown), and no apparent activation was observed with Tritons X–305 and X–405. The above ordering of detergents corresponds to an increase in the length of the Triton polyoxyethylene chain which has been correlated with an increase in the hydrophilic nature of these detergents (24). This suggests that the hydrophile–lipophile balance of the detergent might be important in determining its ability to activate Fraction iii enzyme II^{bgl}. However, limitation of activation efficiency also correlates with the critical micelle concentrations of these detergents (see below). In an attempt to explain the decrease in Fraction iii enzyme II^{bgl} activation in the presence of high detergent concentration, the critical micelle concentrations* for Triton X–100, X–114 and X–45 were determined by a spectrophotometric method based on iodine–micelle complex formation (25) yielding values of 170, 100, and 45 μg/ml, respectively, at 28°. These values are close to the maximal stimulatory concentration ranges for these detergents indicating that the loss of enzyme activation at high detergent concentration is related to the formation of micelles.

As shown in Fig. 6, an activation greater than that expected from additive effects was obtained when Fraction iii enzyme II^{bgl} was assayed in the presence of phosphatidylglycerol and certain concentrations of Triton X–100. In addition, phosphatidylethanolamine, which is itself not an activator, produced the highest stimulation of activity yet observed (greater than 50-fold) in the presence of an inhibitory concentration of Triton X–100 (530 μg/ml).

The activation curves obtained with Tween 40 (Fig. 7) and Tween 80 (not shown) were similar in shape to those obtained with detergents of the Triton X series. Since prolonged incubation of Fraction iii enzyme II^{bgl} with high concentrations of Triton X–100

*The critical micelle concentration is the narrow concentration range in which detergent molecules begin to aggregate and form micelles.

produced significant and irreversible inactivation, we chose to study the Tween 40-enzyme II^{bgl} interactions in detail. As the concentration of Tween 40 was increased, the activation of enzyme II^{bgl} activity increased to a maximum and then decreased (Fig. 7). Maximal activation was achieved at a Tween 40 concentration between 50 and 125 μg/ml. This correlates with a critical micelle concentration of 100 μg/ml which we determined experimentally by iodine–micelle complex formation (25). The decrease in activity encountered at high concentrations is reversible, i.e., when enzyme is incubated with no detergent or high detergent (Tween 40 present at 2 mg/ml) for 20 min, diluted and brought to the same final concentrations of detergent (0.125, 1.0, or 2.0 mg/ml) for assay, the activities of the two samples at each detergent concentration for assay were identical (23).

Activation of Fraction iii enzyme II^{bgl} by Tween 40 was not dependent on a stoichiometric relationship between detergent and enzyme. The formation of P-TPG at 28° was directly proportional to the concentration of Fraction iii enzyme II^{bgl} over a five-fold range of enzyme concentration at 0.125 mg/ml (activation maximum) or 2 mg/ml (inhibitory) Tween 40 concentrations (23). Slight but significant activation has also been achieved with ionic detergents such as sodium dodecyl sulfate, deoxycholate and dicetyl phosphate, and fatty acids such as palmitic and linoleic acid. These compounds yielded activation curves with slopes similar to those obtained with the nonionic detergents (23).

Time Course of the Reaction Catalyzed by Fraction iii Enzyme II^{bgl}. In the standard assay procedure for enzyme II^{bgl}, all proteinaceous components of the assay system and lipid or detergent are incubated together for 10 min at 28° in the absence of phosphoenolpyruvic acid and glycoside substrate. The experiment described in Fig. 8 clearly shows why this preliminary incubation is required in the assay of Fraction iii enzyme II^{bgl}. Unless both HPr and detergent were incubated together with Fraction iii enzyme II^{bgl}, sub-

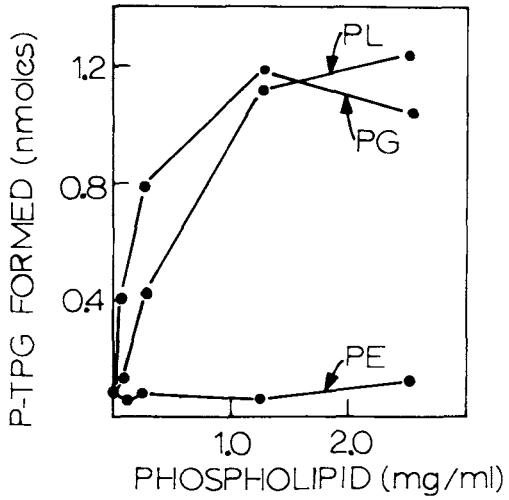

Fig. 4. Activation of Fraction iii enzyme II^{bgl} by *E. coli* phospholipids (PL), a phosphatidylglycerol-enriched fraction (PG), and purified phosphatidylethanolamine (PE). The standard radioisotopic assay system at 28° was used, and the assay time was 10 min. Fraction iii enzyme II^{bgl} was present at a concentration of 8.3 μg/ml.

stantial lags in activity were observed. Incubation of enzyme II^{bgl} with either detergent or HPr alone did not reduce the lag period, and no other component of the assay system substantially decreased the lag period when added together with enzyme II^{bgl}, HPr, and detergent. These data clearly indicate that some interaction between enzyme II^{bgl}, HPr, and detergent is necessary before the catalysis of sugar phosphorylation can occur. This requirement, however, was manifest only with Fraction iii enzyme II^{bgl}, and was not observed with the membrane-associated form of the enzyme.

The data in Fig. 9 suggest that the inhibition of Fraction iii enzyme II^{bgl} activity at high detergent concentration occurs at the stage of the activation process mediated by detergent and HPr. Curve A describes the time course of the reaction where detergent activator was present at optimal concentration, i.e., at approximately the critical micelle concentration. It is apparent that the 10 min preliminary incubation period which preceded substrate addition was sufficient for full activation to be achieved. At detergent concentrations above the optimum, inhibition was observed primarily during the early times of the reaction period. The inhibition observed at high detergent concentration was overcome as the reaction proceeded. These data indicate that interaction of Fraction iii enzyme II^{bgl} with Tween 40 micelles retards interactions with HPr in a fashion that can lead to activation.

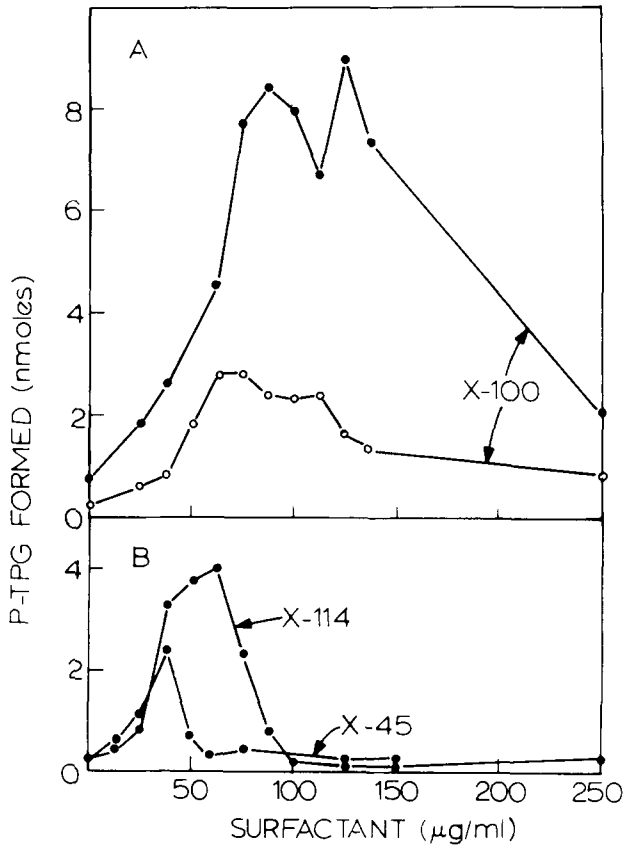

Fig. 5. Activation of Fraction iii enzyme II^{bgl} by detergents of the Triton series. The standard radioisotopic assay at 28° was used, and the assay time was 10 min. Enzyme II^{bgl} concentration of 6.5 μg/ml (○). Enzyme II^{bgl} concentration of 19.5 μg/ml (●).

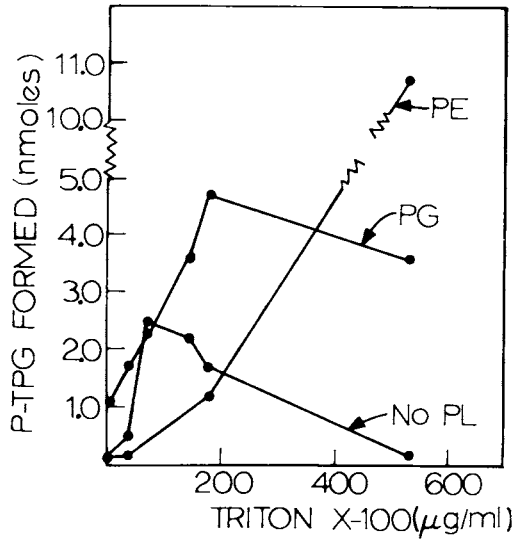

Fig. 6. The effect of Triton X–100 concentration on the activity of Fraction iii enzyme II^{bgl} in the presence of 435 μg/ml of phosphatidylyethanolamine (PE), 435 μg/ml of a phosphatidylglycerol-enriched fraction (PG), and in the absence of added phospholipid (No PL). The standard radioisotopic assay at 28° was used, and the assay time was 10 min. Enzyme II^{bgl} was present at 6 μg/ml.

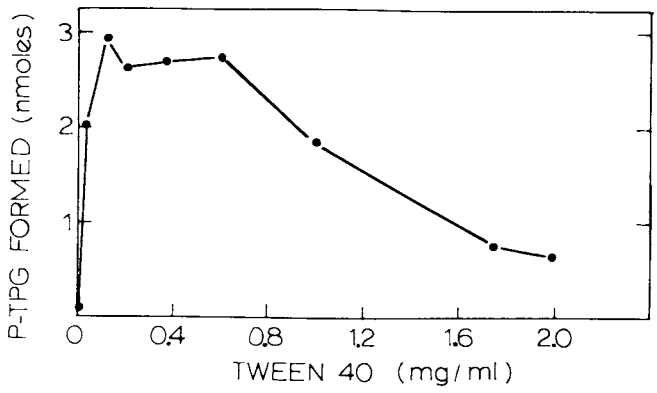

Fig. 7. Activation of Fraction iii enzyme II^{bgl} by the detergent Tween 40. The standard radioisotopic assay at 28° was used, and the assay time was 10 min. Enzyme II^{bgl} was present at 14.4 μg/ml.

The Effects of Temperature on Activation of Fraction iii Enzyme II^{bgl} by Detergent and HPr. A nonlinear Arrhenius plot was obtained when the preliminary incubations of Fraction iii enzyme II^{bgl}, HPr, and Tween 40 were conducted at the indicated assay temperatures before initiating the reactions with substrate. A linear Arrhenius plot was obtained when these components were incubated at 28° (Fig. 10) before initiating the enzyme II^{bgl} assay at the indicated temperatures (plot A). These data indicate that some step during the activation process is exquisitely sensitive to cold. There is no time-dependent activation of activity at 10° following the preliminary incubation at 10° (plot B). The slight activity encountered here was observed to follow a linear time course for 10 hr, and it probably represents the low level of activity observed when Fraction iii enzyme II^{bgl} is assayed in the absence of detergent (Table III). A series of experiments identical to those described in Fig. 10 was conducted with the membrane-associated form

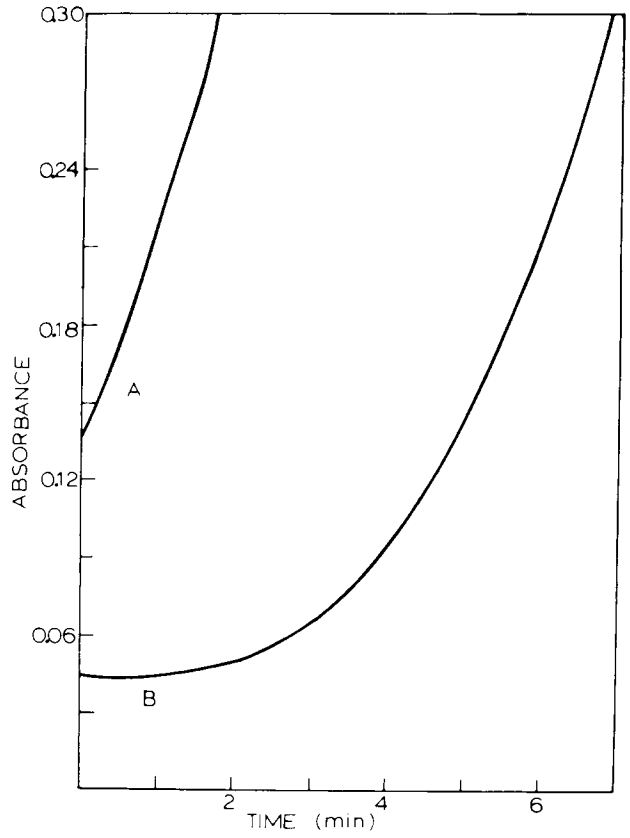

Fig. 8. Time course of the colorimetric assay of Fraction iii enzyme II^{bgl} at 28° showing the rate dependence on the time of HPr addition to the assay system. The colorimetric assay system described in the Materials and Methods section was expanded to 0.7 ml. (A), HPr, enzyme I, enzyme II, and Tween 40 were mixed in a total volume of 0.41 ml and incubated for 10 min at 28°. The reaction was then initiated by the addition of "substrate mix" in a volume of 0.29 ml. For (B), enzyme I, enzyme II, and Tween 40 were mixed in a total volume of 0.41 ml and incubated at 28° for 10 min. HPr and "substrate mix" were then added in a volume of 0.29 ml. The absorbance at 420 nm was followed continuously using a Gilford 2000 recorder and spectrophotometer fitted with a temperature control assembly. The enzyme II^{bgl} concentration was 19 μg/ml.

of the enzyme II^{bgl}. The Arrhenius plots (not shown) were linear and indicated that the membrane-associated form of the enzyme was not influenced by Tween 40 and/or HPr under the conditions of this experiment.

Additional Attempts to Purify Enzyme II^{bgl}. Once the kinetic properties of Fraction iii enzyme II^{bgl} had been fully studied, numerous attempts were made to further purify Fraction iii enzyme II^{bgl} and/or resolve it into two or more protein components. The procedures included chromatography on DEAE, alumina Cγ, hydroxylapatite, affinity columns using *p*-aminophenyl-thio-β-glucoside linked to Agarose, and on unsubstituted Agarose 5M. All these procedures were tested in the absence of detergent or in the presence of Triton X–100 and Tween 40. None of the techniques yielded an enzyme II preparation with a specific activity significantly greater than that of Fraction iii enzyme II^{bgl}. Nor was there more than an additive enhancement of activity when different fractions obtained from a procedure were mixed and tested for activity at previously demonstrated optimal concentrations of detergent (Tween 40) or *E. coli* phospholipid.

In further attempts to resolve enzyme II^{bgl} activity into more than one essential protein fraction, we also treated membranes derived from cells of strain W1895D1bgl^+ *c* with the chaotropic agent lithium 3, 5-diiodosalicylate and by the butanol–urea–water extraction procedure described by Kundig and Roseman (8). These procedures failed to resolve enzyme II^{bgl} into more than one essential protein fraction.

Effects of Temperature and Membrane Lipid Fatty Acid Composition on β-Glucoside Phosphorylation by Intact Cells (Transport) and in Extracts. The rate of β-glucoside transport as a function of temperature has been studied extensively in cells of an unsaturated fatty acid auxotroph of *E. coli* (26–30). Arrhenius plots that describe the log transport rate vs. the reciprocal of the assay temperature (in °K) are multiphasic in slope, intersecting or having discontinuities at characteristic temperatures that define phase boundaries for the onset or completion of freezing of the membrane lipids (28–30).

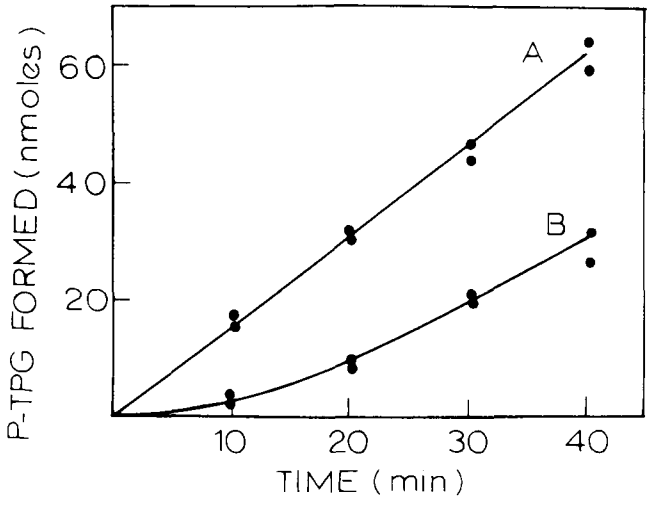

Fig. 9. Time course of the standard radioisotopic assay at 28° for Fraction iii enzyme II^{bgl} in the presence of 0.125 mg/ml Tween 40 (A) and 2 mg/ml Tween 40 (B). An expanded radioisotopic assay system was used, and 0.3 ml aliquots were removed for analysis at the indicated times. Enzyme II^{bgl} concentration was 43 μg/ml.

In a preliminary report, we described the properties of β-glucoside transport (in vivo phosphorylation by enzyme II^{bgl}) and membrane-associated enzyme II^{bgl} activity (in vitro phosphorylation) using cells and washed membrane preparations from an unsaturated fatty acid auxotroph grown and induced for the β-glucoside system in media supplemented either with cis-Δ^9-octadecenoic acid or cis, cis-$\Delta^{9,12}$-octadecadienoic acid (26). The *in vitro* rates of β-glucoside phosphorylation at 5° were identical with membranes prepared from cells grown with either of these unsaturated fatty acid supplements. At this same temperature, however, the *in vivo* rate of phosphorylation, measured by β-glucoside transport, was two-fold greater in cells grown supplemented with cis, cis-$\Delta^{9,12}$-octadecadienoic acid, than in cells grown supplemented with cis-Δ^9-octadecenoic acid. This study has been extended by comparing the *in vivo* and *in vitro* rates of β-glucoside phosphorylation determined for an unsaturated fatty acid auxotroph which was grown and induced for the β-glucoside system in media supplemented with either cis, cis-$\Delta^{9,12}$-octadecadienoic acid or trans-Δ^9-octadecenoic acid (Table VI). Membranes isolated from the cells grown with these fatty acid supplements catalyzed *in vitro* β-glucoside phos-

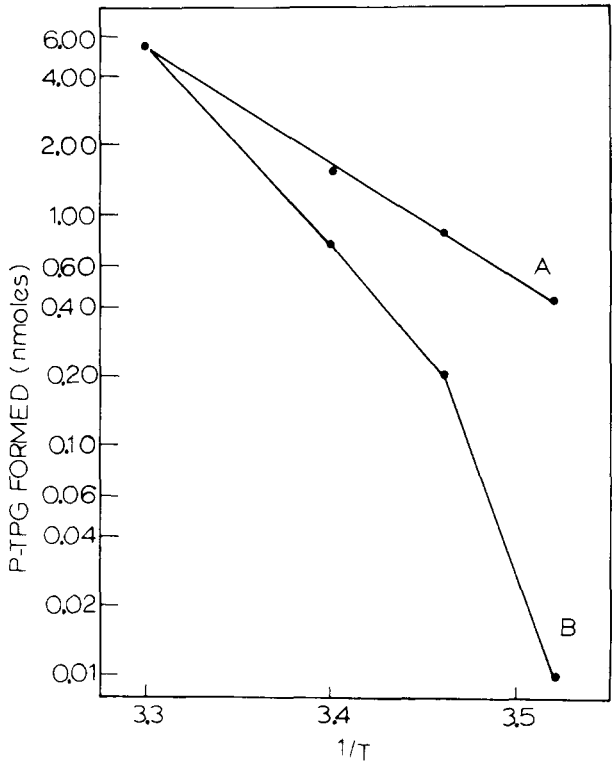

Fig. 10. The effect of temperature on the activity of Fraction iii enzyme II^{bgl}. The units of the abscissa are $(°K)^{-1} \times 10^3$. The standard radioisotopic assay (expanded to a 0.5 ml system) was used with Tween 40 present at 125 μg/ml. In part A the "enzyme mix" was incubated for 20 min at 28° then 20 min at assay temperature for assays done below 28° prior to initiating the reaction with "substrate mix" at the indicated temperatures. In part B, the components of the "enzyme mix" were mixed at 4° and incubated for 20 min at the assay temperature prior to the addition of the "substrate mix". The assay time was 60 min, and the activity is expressed as phosphoryl-TPG formed in 10 min at the given temperature. The concentration of enzyme II^{bgl} in the system was 6.2 μg/ml.

phorylation at identical rates at 28°, 20°, and 10°. The *in vivo* rate of β-glucoside phosphorylation with cells grown with trans-Δ^9-octadecenoic acid supplementation was, on the other hand, only 25% of that observed for cells grown with cis, cis-$\Delta^{9,12}$-octadecadienoic acid when the assays were at 20°, and 5% when the assays were at 10°. These data definitively show that the influence of the temperature-dependent physical properties of membrane lipids on *in vivo* phosphorylation of β-glucosides is lost when the membrane barrier is disrupted. We therefore think it likely that recognition of both HPr and glycoside substrate can take place on the same side of the membrane in broken cell preparations, but must take place on different sides of the barrier during phosphorylation by intact cells. The corollary to this hypothesis states that the events occurring during transport are the ones affected by the physical state of the membrane.

DISCUSSION

The phosphoenolpyruvate-dependent phosphotransferase systems, initially described by Kundig, Ghosh, and Roseman (5), are widely distributed among bacterial genera that are anaerobic or facultative anaerobic (31). Of the systems that have been studied in detail, three classes have been described with respect to the cellular localization or extraction characteristics of the protein component or components which catalyze transfer of phosphate from P-HPr to the sugar substrate. 1) *Systems with soluble and membrane bound components.* The systems for lactose and mannitol phosphorylation in *Staphylococcus aureus* (32–33) and fructose phosphorylation in *Aerobacter aerogenes* (34) are characterized by two inducible sugar specific components, one of which is membrane associated, and the other, soluble. 2) *Systems with two sugar specific, membrane bound*

TABLE VI. Comparison of the Influence of Assay Temperature and Membrane Phospholipid Composition on β-Glucoside Phosphorylation Mediated by Membrane-Associated Enzyme II^{bgl} in Intact Cells (Transport) and in Extracts.[a]

Fatty acid present during growth	Enzyme II^{bgl} activity			Transport activity		
	28°C	20°C	10°C	28°C	20°C	10°C
cis, cis-$\Delta^{9,12}$-octadecadienoic acid	100	57	16	100	58	15
trans-Δ^9-octadecenoic acid	100	63	18	100	14	0.7
Ratio cis, cis-$\Delta^{9,12}$/trans-Δ^9	1	0.9	0.9	1	4	21

[a] A 50 ml culture of strain 30E, an unsaturated fatty acid auxotroph of *E. coli* (Materials and Methods), was grown at 37° in medium A (13) containing 10 gm/liter of casamino acids, 5 mg/liter of thiamine·HCl, 2.5 gm/liter of Triton X–100, and the indicated fatty acid at 0.2 gm/liter. Cultures were inoculated with 5×10^7 cells/ml. Induction with 0.1 mM TPG commenced when the culture density reached 10^8 cells/ml, and growth was continued until the culture density reached 10^9 cells/ml. The cells were harvested and washed twice with medium A containing 40 mg/liter of chloramphenicol, 2.5 gm/liter of Triton X–100, and 0.2 gm/liter of the fatty acid used for growth. Half of the cells were set aside for the assay of transport. The other half were washed once with 0.1 M potassium phosphate buffer of pH 7.6 containing 0.5 mM $MgCl_2$ and suspended in 0.1 M Tris·HCl buffer of pH 7.6 containing 10 mM 2-mercaptoethanol for sonic disruption. The sonic extract was assayed for enzyme II^{bgl} activity at the indicated temperatures using the standard radioisotopic assay procedure. Assays were done in a 0.2 ml system. The concentration of sonic extract protein in the assay system was 530 μg/ml. Assay times were 10, 20, and 60 min for the assays at 28°, 20°, and 10° respectively. The β-glucoside transport assays were done as described previously (26). Enzyme II^{bgl} and transport activities have been arbitrarily assigned values of 100 percent at 28°.

components, one of which can be extracted without detergent treatment. The constitutive phosphotransferase systems for hexose phosphorylation in *E. coli* (see Introduction) have sugar specific, membrane bound components that can be extracted with a butanol–water–urea mixture, and a membrane bound component which resists butanol–water–urea treatment, but can be extracted with detergent (6, 8). 3) *Systems that are not characterized by a sugar specific component that is either soluble or easily resolved from a second, membrane bound component.* The system for β-glucoside phosphorylation described in this report is not characterized by a soluble, sugar specific protein component as are the examples given in the first category above. Furthermore, none of the many procedures tested resolved the β-glucoside phosphotransferase (enzyme II^{bgl}) into two essential protein components. This indicates that enzyme II^{bgl} either differs from the other characterized enzymes II of *E. coli* in that it is composed of but a single protein species, or that it is composed of a number of protein components that are not separated by the procedures that we have applied.

A large number of membrane-associated enzymes have been extracted from membranes, and requirements for lipid activators have been established for many of these. The details surrounding the phenomenology of enzyme activation by lipids have been treated in a number of recent reviews (35–37). Preparations of enzyme II^{bgl} that contain little phospholipid and detergent have a nearly absolute requirement for either phospholipid or detergent activators. Kundig and Roseman have shown that the constitute enzymes II of *E. coli* are activated effectively by phosphatidylglycerol, poorly by phosphatidylserine or cardiolipin, and not at all by phosphatidylglycerol phosphate and phosphatidylethanolamine (8). In this respect, enzyme II^{bgl} is apparently similar to the constitutive enzymes II, in that it is activated by a preparation enriched for phosphatidylglycerol, but not by aqueous dispersions of phosphatidylethanolamine. However, a true comparison of the requirement of the constitutive enzymes II and enzyme II^{bgl} for phospholipid activators cannot be made, since Kundig and Roseman included approximately 60 μg/ml of Triton X–100 in all enzyme assay mixtures (8), and our data in Figs. 4–6 indicate that the properties of lipid–detergent mixtures as activators cannot be predicted from the properties of detergent or lipid acting singly.

The only activation experiments in these two studies that seem truly comparable are activation of the enzymes II by mixtures of Triton X–100 and phosphatidylglycerol. The data in Fig. 6 show that the addition of phosphatidylglycerol to a reaction mixture containing approximately 60 μg/ml of Triton X–100 resulted in no further activation of Fraction iii enzyme II^{bgl}. This is an apparent difference between the properties of our enzyme preparation and the reconstituted constitutive enzyme II preparations of Kundig and Roseman (8). These investigators reported that Triton X–100 at this same concentration was a poor activator when compared with a mixture of Triton X–100 and phosphatidylglycerol. The data in Fig. 6 also indicates that considerable care should be exercised in the interpretation of experiments where mixtures of detergent and phospholipid are used to activate enzymes. Though phosphatidylethanolamine was not itself an activator of Fraction iii enzyme II^{bgl}, it was an effective activator when tested with concentrations of Triton X–100 which were themselves inhibitory. In fact, the most effective activation that we have observed has been with mixtures of phosphatidylethanolamine and Triton X–100. To this effect it is of interest to note that Triton X–100 and phospholipids can form mixed micells (38). It is likely that these would have properties considerably different from those of micelles formed by either phospholipids or detergents alone.

The precise role played by detergents and phospholipids during catalysis mediated by the enzyme studied here has not been defined. It is possible that these amphipathic molecules have permissive effects only, providing a suitable environment for the catalytically active proteins that allows them to assume or maintain an active conformation. A permissive function of this general nature was established for Tween 40 in the HPr-dependent activation of Fraction iii (Figs. 8and 9). These activation experiments also indicate that uncharged HPr interacts directly with Fraction iii enzyme II^{bgl}. It is difficult to assess the composition of the lipid environment of membrane-associated enzyme II^{bgl} from reconstitution experiments such as ours. Though phosphatidylglycerol was a more effective activator at low concentration than total *E. coli* lipid, and phosphatidylethanolamine was ineffective, these results might no more than reflect the fact that phosphatidylglycerol is more water soluble than phosphatidylethanolamine.

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