Biochimica et Biophysica Acta, 443 (1976) 267–283
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BBA 77400

STUDIES ON THE MECHANISM OF PHOSPHORYLATION AND TRANSPORT OF β-GALACTOSIDES BY THE LACTOSE PHOSPHOTRANSFERASE SYSTEM OF *STAPHYLOCOCCUS AUREUS*

KINETIC INVESTIGATIONS USING TOSYL GALACTOSIDES AS REVERSIBLE DEAD-END INHIBITORS

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(Received January 15th, 1976)

SUMMARY

Tosyl galactosides, previously shown to be potent reversible dead-end inhibitors of the membrane-bound Enzyme II^{lac} of the lactose phosphotransferase system of *Staphylococcus aureus*, were used for an investigation of the kinetic mechanism of the sugar phosphorylation/transport reaction catalyzed by this enzyme:

$$\text{phospho-Factor III}^{lac} + \text{sugar} \xrightarrow{\text{Enzyme II}^{lac}} \text{Factor III}^{lac} + \text{sugar phosphate.}$$

Inhibition of Enzyme II^{lac} was studied in three different systems. Washed membranes, and washed membranes in the presence of 0.1 % Triton X-100 were used for phosphorylation experiments, and whole cells were used for transport studies. When washed membranes were used to supply Enzyme II^{lac}, inhibition of phosphorylation by tosyl galactoside was linear non-competitive against both the sugar and phospho-Factor III^{lac} substrates, with an apparent K_i of about 0.5 mM. This K_i decreased with increasing Factor III^{lac} concentration. In the presence of 0.1 % Triton X-100, the phosphorylation reaction was stimulated; under these conditions the inhibition became strictly competitive against sugar, and completely uncompetitive against phospho-Factor III^{lac}. Apparently washed membranes can catalyze phosphorylation both via a reaction sequence in which sugar binds first and via one in which phospho-Factor III^{lac} binds first, but in the presence of 0.1% Triton the reaction does not occur by the former sequence. The inability of bound phospho-Factor III^{lac} to hinder the binding of tosyl galactosides suggests that the initial binding sites of the two substrates of Enzyme II^{lac} are separated by at least the distance of the tosyl moiety. Radioactive methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside was not converted into a phosphorylated product in the reaction mixtures, i.e. it is a true dead-end inhibitor. Inhibition of β-galactoside transport into whole cells by tosyl galactosides was competitive, with an apparent K_i of 5–10 mM, an order of magnitude higher than the K_i for inhibition of phosphorylation by membrane preparations. This result suggests that a significant

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level of unphosphorylated phospho-Factor III^{lac} is present inside the cells, or that cellular levels of this compound are considerably lower than those used for *in vitro* sugar phosphorylation assays. Radioactive tosyl galactoside inhibitor was not transported into whole cells.

INTRODUCTION

Most sugars are accumulated by cells of the gram-positive bacterium *Staphylococcus aureus* as the respective phosphate esters [1]. In particular, lactose, methylthio β-D-galactoside and other β-D-galactopyranosides* (and galactose itself) are phosphorylated at 6-OH of the galactose moiety [2]. The phosphorylation is mediated by a phosphoenolpyruvate-dependent phosphotransferase system, similar in many respects to the systems characterized in the gram-negative organism *Escherichia coli* [3-5] and other bacterial genera [6]. The lactose phosphotransferase system in *S. aureus* mediates a sequence of phosphoryl transfer according to the scheme below**, detailed biochemical evidence for which, obtained from *in vitro* studies, was presented previously [7–9]***.

$$P\text{-enolpyruvate} + \text{histidine protein} \underset{Mg^{2+}}{\overset{\text{Enzyme I}}{\rightleftharpoons}} P\text{-histidine protein} + \text{pyruvate} \quad (1)$$

$$P\text{-histidine protein} + \tfrac{1}{3}\ \text{Factor III}^{lac} \rightleftharpoons \tfrac{1}{3}\ [P_3\text{-Factor III}^{lac}] + \text{histidine protein} \quad (2)$$

$$\tfrac{1}{3}\ [P_3\text{-Factor III}^{lac}] + \text{sugar} \xrightarrow{\text{Enzyme II}^{lac}} \text{sugar-6-}P + \tfrac{1}{3}\ [\text{Factor III}^{lac}] \quad (3)$$

Reaction 1 probably proceeds via a phospho-Enzyme I intermediate, although the precise number of phosphoryl groups in the intermediate is uncertain [10]. Mutant cells lacking phosphotransferase proteins demonstrated corresponding deficiencies in sugar transport, strongly supporting the hypothesis that sugar phosphorylation and transport are concomitantly mediated by this system [11]. Since Reaction 3 involves the final phosphoryl transfer in the sequence, and is mediated by the membrane-bound component of the system, it seems likely that this step is tightly coupled to the membrane transport process. The substrates for this reaction are sugar (β-galactosides) and phospho-Factor III^{lac}, in which the phosphoryl groups are attached to histidine residues of Factor III^{lac} at N-3. Since the standard free energy of hydrolysis of these phospho-histidines is only about 1.5 kcal per mol less negative than of phosphoenolpyruvate itself [9], the equilibrium constant for Reaction 3 must be about 10^8. Thus it is possible that some of the free energy change for this reaction is coupled to the actual membrane translocation process. Information about the order of substrate binding in Reaction 3 and the spatial arrangement of the two substrate sites would therefore provide insight into the nature of the transport process, during which Enzyme II^{lac} must interact both with the outside medium (in order to bind sugar) and

* All sugars are of the D-configuration and glycosides are pyranosides unless otherwise specified.

** Factor III^{lac} and Enzyme II^{lac} are lactose-specific phosphocarrier protein and membrane protein, respectively, of the *S. aureus* lactose phosphotransferase system.

*** The nomenclature for components of the phosphotransferase system has been described previously [3, 7].

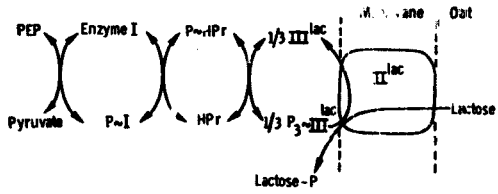


Fig. 1. Schematic representation of the reactions involved in the transfer of phosphate from phosphoenolpyruvate to lactose in the phosphotransferase system of *S. aureus*. HPr, histidine protein; PEP, phosphoenolpyruvate.

with the cell cytoplasm (where Factor III^{lac}, histidine protein and Enzyme I are presumably located). The system is depicted schematically in Fig. 1.

Many of the approaches ordinarily employed in a complete kinetic analysis are unavailable for this reaction. The tightly membrane-bound Enzyme II^{lac} cannot be purified unless it is first "solubilized" by treatment with detergents [12, 9], a procedure which of necessity removes it from its "native" environment. Thus studies of membrane-bound Enzyme II^{lac} are restricted to particulate suspensions fairly low in Enzyme II^{lac} active sites (less than 0.5 μM). These preparations are not suitable for conventional binding experiments, although an ultracentrifuge assay is possible for substrates with very high affinity (K_D less than 1–5 μM) [9]. Product inhibition studies are not feasible since one product, sugar phosphate, does not detectably inhibit the reaction [13] and the other, Factor III^{lac} (a trimeric protein with a molecular weight of 36 000 [8]), is continually reconverted to phospho-Factor III^{lac} under the assay conditions. The feasible kinetic experiments are thus those in which effects of changing substrate and dead-end inhibitor concentrations are studied. The usual substrate-rate experiments, reported previously [7, 9] and repeated during the studies described herein, demonstrate that the mechanism is sequential rather than ping-pong, i.e. no stable phospho-Enzyme II^{lac} intermediate is formed. Inhibitor studies were made possible by the synthesis of 6-*O*-(*p*-toluenesulfonyl) galactose and methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside in this laboratory [14]. These compounds, whose apparent affinities for the enzyme were greater than that of galactose itself, are the only dead-end inhibitors reported for phosphoenolpyruvate-dependent phosphotransferase systems; other C-6-substituted galactose derivatives (including methyl 6-*O*-(*p*-toluenesulfonyl) α-galactoside), did not inhibit Enzyme II^{lac} [14].

We have studied the inhibition of the *in vitro* phosphorylation reaction using "washed membranes" prepared from ruptured cells without chemical treatments, both in aqueous reaction mixtures and in the presence of the non-ionic detergent Triton X-100. The striking differences between the apparent reaction mechanisms observed suggest that the Enzyme II^{lac} conformation is significantly altered in the presence of detergent. The kinetic pattern of inhibition of sugar uptake by whole cells differs further from the *in vitro* results.

MATERIALS AND METHODS

Materials and analytical procedures are described in a previous communication [14].

Preparation of lactose phosphotransferase system components. The preparation of purified fractions of Enzyme II^{lac}, Enzyme I, histidine protein, and Factor III^{lac} has been described in detail [14] and thus is only summarized here. Washed membranes, containing Enzyme II^{lac} free from adsorbed soluble components, were prepared from mechanically disrupted cells of *S. aureus* lactose-constituitive strain C22 [11] by alternate cycles of ultrasonic irradiation, and low-speed and high-speed contrifugation. The three soluble components, Enzyme I, histidine protein, and Factor III^{lac}, were separated completely from one another by gel filtration on Sephadex G-100, and subjected to one or more additional purification and concentration steps. The Factor III^{lac} was purified sufficiently to permit an estimate of its purity (about 70%), and thus a determination of the approximate Factor III^{lac} concentration in the preparation.

Syntheses of inhibitors. The syntheses of unlabeled 6-*O*-(*p*-toluenesulfonyl) galactose and methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside have been described [14]. Radioactive [^{3}H]methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside was prepared as follows: A reaction mixture containing 20 mmol of 2,3,4,6-tetra-*O*-acetyl α-D-galactopyranosyl bromide (Sigma Chemical Co.), 10 mmol of [^{3}H]methanol (specific activity, 2.5 Ci/mol) and 10 mmol of $Hg(CN)_2$ in 50 ml of dry nitromethane/benzene (1 : 1, v/v), was stirred overnight at room temperature [15]. The solid remaining after evaporation of the solvent under reduced pressure was extracted with dry chloroform, and the extracts washed with NaCl solution, and then several times with water. For deacetylation, the syrup resulting from evaporation of the chloroform was dissolved in dry methanol and the apparent pH adjusted to 10–11 (pH paper) using a 0.34 M solution of sodium methoxide in methanol. After several hours at room temperature, the mixture was treated with a 2-fold excess of Dowex 50 (H^+-form) to remove Na^+, and filtered. The syrupy product remaining after evaporation of the methanol was dissolved in water and applied to a Dowex 1-X2 (OH^--form) column. Elution of the column with water yielded a single large peak of coincident radioactivity and carbohydrate. Evaporation of the pooled peak fractions yielded 1 g of crystalline [^{3}H]methyl β-galactoside. This material was subjected to limited tosylation as previously described [14], yielding about 0.5 g [^{3}H]methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside.

Assay of phosphorylation of [^{14}*C*]*methylthio β-galactoside.* The assay procedure has been described in detail [14]. Briefly, ice-cold reaction mixtures were prepared by mixing stock solutions so that the final volumes (usually about 300 μl) contained the following components: phosphoenolpyruvate (3 mM); potassium phosphate buffer (pH 7.5, 10 mM), KF (3 mM); dithiothreitol (2 mM); $MgCl_2$ (4 mM); and appropriate concentrations of [^{14}C]methylthio β-galactoside (New England Nuclear), inhibitors, and Enzyme II^{lac}, Enzyme I, histidine protein and Factor III^{lac}. For inhibition experiments, all of the components (except [^{14}C]sugar and the four protein solutions) were first mixed at room temperature with 15 μl of a dimethyl sulfoxide solution of the inhibitor (or 15 μl of pure dimethyl sulfoxide, in control assays). These mixtures were then chilled and the remaining components added. Unless otherwise indicated, all incubations were for 30 min at 37 °C. After the reaction was stopped by chilling the mixture on ice, the solution was assayed for [^{14}C]sugar phosphate by ion-exchange chromatography as previously described. When [^{3}H]methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside was used instead of [^{14}C]sugar, the ion-exchange chromatography was modified as follows. It was necessary to first elute each column with five

aliquots (8 ml) of 50% methanol (v/v) in order to remove the unreacted tosyl galactosides, which are sparingly soluble in water and adhere tightly to the column. The columns were then washed with 1 M LiCl as described previously to elute sugar phosphate (if any) directly into scintillation vials. In all of the Enzyme II^{lac} kinetic studies, known amounts of Factor III^{lac} and excess Enzyme I and histidine protein were used. Under these conditions the rate of [^{14}C]sugar phosphorylation was proportional to the amount of Enzyme II^{lac} used, and constant for at least 60 min. In these assay mixtures Enzyme II^{lac} was present in catalytic amounts (about 20–100 pmol of [^{14}C] lactose-binding activity), whereas Factor III^{lac} was present in substrate amounts (2–50 nmol). Since Factor III^{lac} was usually present at much less than saturating levels, yet further increases in histidine protein or Enzyme I concentration (or both) did not increase the reaction rate, we conclude that almost all of the Factor III^{lac} remained phosphorylated during the reaction, i.e. the steady-state level of unphosphorylated Factor III^{lac} was negligible. This condition does not necessarily imply that all of the phosphorylated protein is in fact P_3-Factor III^{lac}; the substrate in these reactions is therefore given the (less specific) designation phospho-Factor III^{lac}.

Cell transport experiments. Initial rates of uptake of sugars by whole cells were measured as described previously [14] by quickly filtering and washing aliquots of a mixture of radioactive sugar and cells (previously resuspended in 100 mM potassium buffer, pH 7.2, containing 0.4% (w/v) sodium succinate) at 10-s intervals during the first 1–2 min after mixing. This technique could not be used to test for the uptake of [^{3}H]methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside, since this compound binds strongly to the Millipore filters; we used instead a modification of the centrifugation assay described by Simoni and Roseman [11]. The cells, in potassium phosphate/succinate buffer, were incubated at 25 °C with radioactive substrate, then centrifuged at 20 000 × *g* for 5 min at 4 °C. After the supernatant was carefully removed with a small pipet, the pellet was resuspended in 1 ml water, mixed with scintillation fluid and the amount of radioactive sugar in the cell pellet determined. The observed amounts were corrected for blank values (usually about 30% of observed values), determined from the results of identical experiments in the presence of saturating amounts (100 mM) of unlabeled lactose. Although this procedure is not appropriate for the determination of true initial transport rates, the corrected values for the uptake of [^{14}C]methylthio β-galactoside in the first minute after mixing were proportional to the sugar concentration (at sub-saturating levels). For uptake experiments with [^{3}H]methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside, samples were withdrawn and assayed at various times up to 30 min after mixing.

RESULTS

Previous kinetic studies [5, 6] suggested that the Enzyme II^{lac} reaction did not proceed via a phospho-Enzyme II^{lac} intermediate, since plots of (initial velocity)$^{-1}$ *versus* (variable substrate)$^{-1}$ at fixed levels of other substrates were intersecting, not parallel. The observed binding of lactose to washed membranes, in the absence of added Factor III^{lac} and other phosphotransferase system components [3], was consistent with an ordered mechanism in which sugar is the first substrate to bind. We have confirmed these results with our enzyme preparation, and obtained comparable

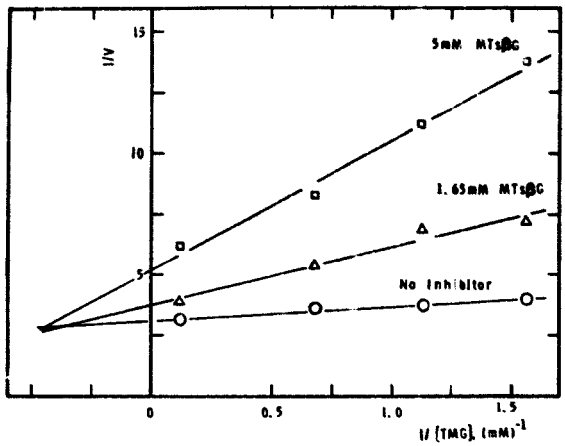


Fig. 2. Effect of inhibitor and sugar concentrations on sugar phosphorylation. Phosphorylation inhibition experiments were performed under the standard assay conditions, as described under Materials and Methods, with 24 μg of Enzyme II^{lac}, 0.13 μM Factor III^{lac}, excess Enzyme I and histidine protein, and [^{14}C]methylthio β-galactoside (TMG) (specific activity, $1.2 \cdot 10^5$ cpm/μmol) and the inhibitor methyl 6-*O*-(*p*-toluenesulfonyl) β-D-galactoside at the indicated concentrations; final volume was 300 μl. The symbol v corresponds to the rate of phosphorylation of [^{14}C]methylthio β-galactoside in units of nmol/min per total reaction mixture. The points correspond to the average of duplicate determinations, and the lines represent the best linear least-squares fit. ○, no inhibitor; △, 1.65 mM inhibitor; □, 5 mM inhibitor. MTsβG, methyl 6-*O*-(*p*-toluenesulfonyl) β-D-galactoside.

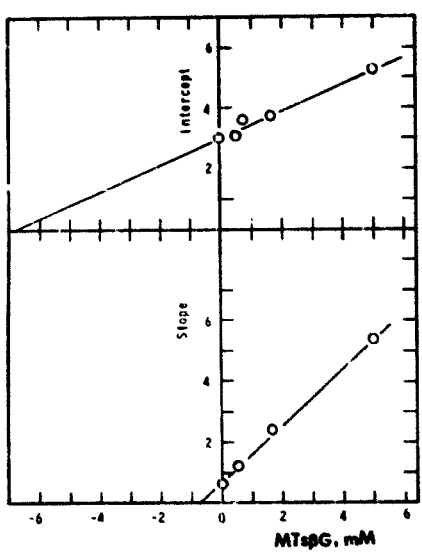


Fig. 3. Replots of slopes and intercepts from phosphorylation inhibition experiment. The slopes and ordinate intercepts from the reciprocal plot in Fig. 2 are replotted against inhibitor concentration. MTsβG, methyl 6-*O*-(*p*-toluenesulfonyl) β-D-galactoside.

values for the kinetic parameters. However, as the inhibition experiments below indicate, the kinetic pattern is more complex than that expected for the simple ordered mechanism with sugar binding first and may to some extent depend on the nature of the membrane preparation containing the Enzyme II^{lac}. In a previous report, we showed the tosyl galactosides were not hydrolyzed (to substrate molecules) during the phoshorylation inhibition experiments [14]. As part of the studies reported here we further determined that they were not phosphorylated by Enzyme II^{lac}. When [^{3}H]methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside, synthesized as described under Materials and Methods (and comparable to the unlabeled compound in its ability to inhibit [^{14}C]methylthio β-galactoside phosphorylation) was used as a substrate in the phosphorylation reaction, a very low amount of phospho-[^{3}H]sugar was detected. This amount could be accounted for by the low level (about 3%) of [^{3}H]methyl β-galactoside impurity in the tosyl galactoside preparation. Thus the tosyl galactosides appear to be true dead-end inhibitors.

Studies on inhibition of phosphorylation by membranes

Although the anomeric specificity exhibited by the dead-end inhibitor methyl 6-*O*-(*p*-toluenesulfonyl) β-D-galactoside suggests that it acts as a substrate analog, double reciprocal plots of the data from phosphorylation experiments yield lines which intersect to the left of the ordinate, suggesting that some inhibition persists at

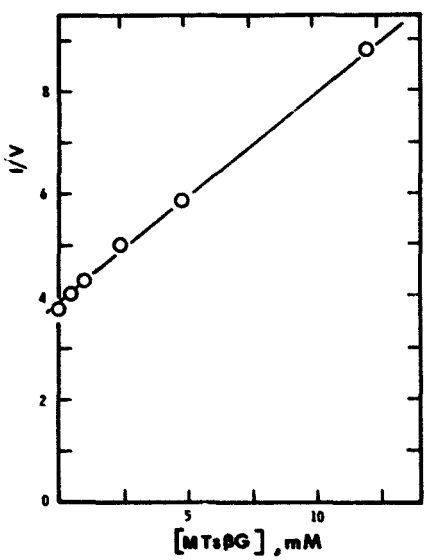


Fig. 4. Effect of inhibitor concentration on rate of sugar phosphorylation. Phosphorylation inhibition experiments were performed as described under Materials and Methods with 48 μg of Enzyme II^{lac}, 0.13 μM of Factor III^{lac}, 2.8 mM [^{14}C]methylthio β-galactoside (TMG) (specific activity $7.87 \cdot 10^5$ cpm/μmol), and methyl-6-*O*-(*p*-toluenesulfonyl) β-galactoside (MTsβG) at the concentrations indicated; final volume was 300 μl. The symbol *v* is defined in the legend for Fig. 2.

saturating substrate concentrations. A typical plot dispalying this non-competitive† inhibition pattern is shown in Fig. 2. The slopes and ordinate intercepts from this figure are replotted against inhibitor concentration in Fig. 3. Both slopes and intercepts obtained from these and similar experiments appear to be linear functions of inhibitor concentration. The linear nature of the inhibition is confirmed by Fig. 4, which displays the results of an experiment in which the methyl 6-*O*-(*p*-toluenesulfonyl) *β*-galactoside concentration was varied over a 25-fold range at a fixed sugar substrate level. Although the pattern can thus be classified as linear non-competitive [7], two apparent inhibition constants are required to account for the data. In general, an intercept effect is obtained if binding by a dead-end inhibitor and the

TABLE I

KINETIC PARAMETERS FOR INHIBITION OF SUGAR PHOSPHORYLATION BY TOSYL GALACTOSIDES WITH SUGAR AS VARIABLE SUBSTRATE

Phosphorylation experiments were performed as described in the legends of Figs. 2 and 6, in the presence of the constant Factor III^{lac} concentrations indicated, varying concentrations of [^{14}C]-methylthio *β*-galactosides (0.2–8.3 mM) and changing fixed levels (0.5–5 mM) of the inhibitors, either 6-*O*-(*p*-toluenesulfonyl)galactose, or methyl 6-*O*-(*p*-toluenesulfonyl) *β*-D-galactoside. The slopes and intercepts from double reciprocal plots (see Figs. 2 and 6) were replotted against inhibitor concentrations (see Fig. 3). The apparent inhibition constants were determined from the replots by assuming that both slopes and intercepts were proportional to $(1+I/K_I)$. The apparent Michaelis constants for [^{14}C]methylthio *β*-galactosides in the absence of inhibitor (at the indicated Factor III^{lac} concentrations) were also determined. The variation is indicated for experiments where duplicate determinations were performed.

Inhibitor	Concentration of Factor III^{lac} (μM)	Apparent Michaelis constant for [^{14}C]methylthio *β*-galactosides (mM)	Apparent K_I	
			Intercepts (mM)	Slopes (mM)
6-*O*-(*p*-Toluenesulfonyl) galactose	0.07	0.17	8.2	1.33
6-*O*-(*p*-Toluenesulfonyl) galactose	0.20	0.13 ± 0.03	6.4 ± 1.4	0.46 ± 0.10
6-*O*-(*p*-Toluenesulfonyl) galactose	0.67	0.16	4.3	0.50
Methyl 6-*O*-(*p*-toluenesulfonyl) *β*-D-galactoside	0.10	0.22 ± 0.03	14 ± 7	0.80 ± 0.04
Methyl 6-*O*-(*p*-toluenesulfonyl) *β*-D-galactoside	0.45	0.18	11	0.34
Methyl 6-*O*-(*p*-toluenesulfonyl) *β*-D-galactoside*	0.24	0.17 ± 0.03	**	0.22 ± 0.04

* Phosphorylation assayed in presence of 0.1 % Triton X-100 at 25 °C (see Fig. 6).

** Ordinate intercepts were the same (within experimental error) at all inhibitor concentrations as in the absence of inhibitor.

† We use the definitions of Cleland [18] for non-competitive (slope and intercept effects) and uncompetitive (intercept effects only) inhibition.

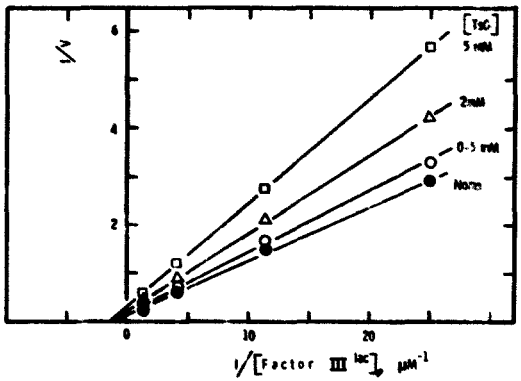


Fig. 5. Effect of inhibitor and Factor III^{lac} concentrations on sugar phosphorylation. Phosphorylation inhibition experiments were performed under the standard assay conditions, as described under Materials and Methods, with 12 μg of Enzyme II^{lac}, 8.3 mM [^{14}C]methylthio β-galactoside (TMG) ($2.4 \cdot 10^5$ cpm/μmol) and Factor III^{lac} and the inhibitor 6-*O*-(*p*-toluenesulfonyl) galactose (TsG) at the indicated concentrations; final volume was 300 μl. The symbol v is defined in the legend of Fig. 2. The points correspond to the average of duplicate determinations, and the lines represent the best linear least-squares fit. ●, no inhibitor, ○, 0.5 mM inhibitor; △, 2.0 mM inhibitor; □, 5 mM inhibitor.

variable substrate are not mutually exclusive events [16]. Such a result is surprising in this case, since the inhibitory tosyl sugars are analogs of the variable substrate. The non-competitive pattern thus suggests that there is a secondary binding of an inhibitor molecule to the enzyme at some point in the reaction sequence after the primary binding to the sugar substrate site. The apparent K_i (slopes) would be expected to be a function of the affinity of the inhibitor for the sugar substrate site, and under certain circumstances would be a direct measure of the thermodynamic dissociation constant for the primary inhibitor-enzyme complex [16]. The K_i (intercepts) would presumably reflect some secondary low-affinity interaction (see Discussion). The observation that K_i (slopes) is an order of magnitude smaller than K_i (intercepts) is consistent with this notion. The apparent inhibition constants obtained in a series of similar experiments are summarized in Table I. The values of K_i (slopes) determined for 6-*O*-(*p*-toluenesulfonyl) galactose and for methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside vary with Factor III^{lac}. Such an effect on the binding of the substrate analogs by prior binding of phospho-Factor III^{lac} is inconsistent with a simple ordered reaction mechanism in which sugar binds first.

Two kinds of phosphorylation inhibition experiments were performed in which fixed amounts of sugar, an excess of Enzyme II and histidine protein and varying concentrations of Factor III^{lac} were used. The concentrations of sugar substrate used were either high enough to saturate the enzyme (5 mM) (Experiment 1) or so high as to exclude inhibitor from any site for which inhibitor and sugar compete, as well as saturating the enzyme (160 mM) (Experiment 2). In Experiment 1, the pattern of the double reciprocal plot, shown in Fig. 5, is non-competitive. The apparent inhibition constants from slope and intercept replots, given in Table II, are both in the order of

TABLE II

KINETIC PARAMETERS FOR INHIBITION OF SUGAR PHOSPHORYLATION BY TOSYL GALACTOSIDES WITH FACTOR III^{lac} AS VARIABLE SUBSTRATE

Phosphorylation experiments were performed as described in the legends of Figs. 5 and 7, using the constant [^{14}C]methylthio β-galactoside concentrations indicated, changing fixed levels (0.5–10 mM) of methyl 6-*O*-(*p*-toluenesulfonyl) β-D-galactoside, and varying Factor III^{lac} concentrations (0.04–4.5 μM). The apparent inhibition constants were determined from replots of the ordinate intercepts and slopes against inhibitor concentration as described in the legend for Table I. The apparent Michaelis constants for phospho-Factor III^{lac} in the absence of inhibitor at the indicated concentrations are also given. The variation is indicated for the experiment in 0.1 % Triton X-100, in which duplicate determinations were performed.

Experiment	Concentration of [^{14}C]methylthio β-galactoside (mM)	Apparent Michaelis constant for phospho-Factor III^{lac} (μM)	Apparent K_i	
			Intercepts (mM)	Slopes (mM)
1	8.3	0.8	5	4
2	160	0.8	15	*
3**	8.3	0.29±0.02	2.2±0.2	*

* The slopes of the double reciprocal plots were the same (within experimental error) at all Factor III^{lac} concentrations as in the absence of inhibitor.

** Experiments in 0.1 % Triton X-100 at 25 °C (see Fig. 7).

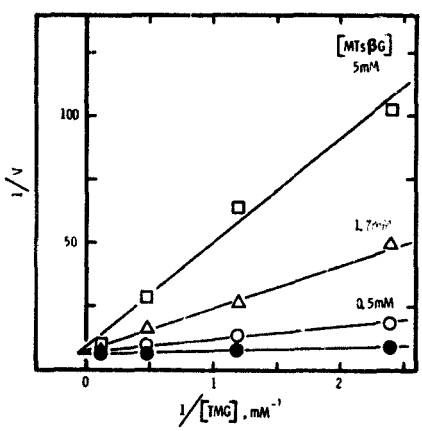


Fig. 6. Effect of inhibitor and sugar concentrations on sugar phosphorylation in the presence of Triton X-100. Phosphorylation experiments were performed in the presence of 0.1 % Triton X-100 at 25 °C, as described under Materials and Methods with 48 μg of Enzyme II^{lac}, 0.8 μM Factor III^{lac}, excess Enzyme I and histidine protein, the indicated concentrations of methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside (MTsβG) and [^{14}C]methylthio β-galactoside (TMG) (specific activity, 2.27 · 10^5 cpm/μmol), and the standard assay components; final volume was 300 μl. The symbol *v* is defined in the legend for Fig. 2. The points correspond to the average of duplicate determinations, and the lines represent the best linear least-squares fit. ●, no inhibitor; ○, 0.5 mM inhibitor; △, 1.7 mM inhibitor; □, 5 mM inhibitor.

5 mM, in contrast to the experiments where sugar substrate was varied (Table I). In Experiment 2, where inhibitor is presumably excluded from the sugar site, the degree of inhibition varied with inhibitor concentration but was the same at all Factor III^{lac} concentrations (16 % inhibition for methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside at a concentration of 2.4 mM). Thus some of the inhibitory effect of the tosyl galactosides on washed membranes was not susceptible to competition by sugar substrate (confirming the result of extrapolating the lines in Fig. 2), or by phospho-Factor III^{lac}.

Effect of Triton X-100 on inhibition of phosphorylation by washed membranes

In agreement with previous reports, we found that low levels of Triton X-100 stimulated the β-galactoside phosphorylation activity of washed membrane preparations. The maximal enhancement (about 2-fold) occurred at a concentration of 0.1 % Triton (v/v), when incubations were performed at 25 °C. (At higher temperatures (30–37 °C) some irreversible inactivation occurs as well.) Its sedimentation characteristics suggest that Enzyme II^{lac} remains particulate at 25 °C in 0.1 % Triton. In detergent-free buffer, almost all of the Enzyme II^{lac} activity was absent from the super-

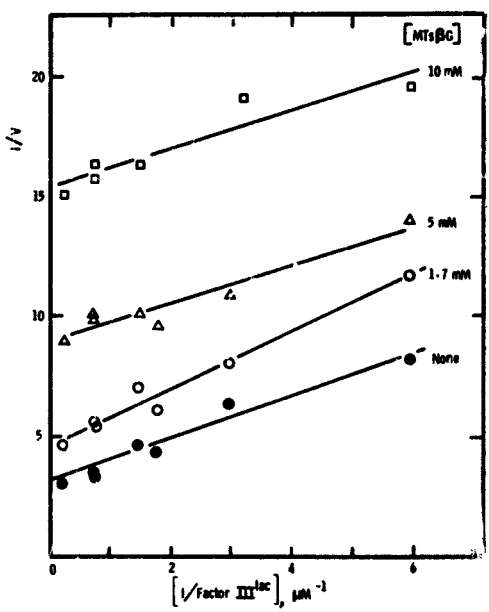


Fig. 7. Effect of inhibitor and phospho-Factor III^{lac} concentration on sugar phosphorylation in the presence of Triton X-100. Phosphorylation inhibition experiments were performed in the presence of 0.1 % Triton X-100 at 25 °C, as described under Materials and Methods, with 40 μg of Enzyme II^{lac}, excess Enzyme I and histidine protein, Factor III^{lac} and methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside (MTsβG) at concentrations indicated, 8 mM [^{14}C]methyl-1-thio β-galactoside (TMG) (specific activity, $1.57 \cdot 10^5$ cpm/μmol), and the standard assay components; final volume was 300 μl. The symbol v is defined in the legend for Fig. 2. The lines represent the best linear least-squares fit. ●, no inhibitor; ○, 1.7 mM inhibitor; △, 5 mM inhibitor; □, 10 mM inhibitor.

natant after the preparation was centrifuged at 25 °C for 90 min at 164 000 × *g*. Of this "sedimentable" activity, 55 % remained in the supernatant after centrifugation in the presence of 0.5 % Triton, but only 10 % after centrifugation in the presence of 0.1 % Triton. The enhancement of Enzyme II^{lac} activity in 0.1 % Triton could be the result of higher affinity for either or both substrates, increased catalytic efficiency, exposure of enzyme molecules previously inaccessible to substrate, alteration of the reaction mechanism, or some combination of these factors. The results of phosphorylation inhibition in Triton experiments with methyl 6-*O*-(*p*-toluenesulfonyl) *β*-galactoside as the inhibitor, at a constant Factor III^{lac} concentration with sugar as the variable substrate, are shown in Fig. 6. In contrast to the results with washed membranes (Fig. 1), the inhibition pattern is almost completely competitive. The kinetic parameters are given in Table I. The K_i (slopes) indicates that binding of the inhibitor is slightly tighter in 0.1 % Triton than in detergent-free buffer.

When phospho-Factor III^{lac} was the variable substrate at constant methylthio *β*-galactoside concentration (8.3 mM), phosphorylation inhibition experiments in 0.1 % Triton resulted in the uncompetitive pattern seen in Fig. 7. The absence of any effect of inhibitor on the slopes is in marked contrast to the results using washed membranes (Fig. 5). The Michaelis constant for phospho-Factor III^{lac} is lower in the presence of Triton (See Table II).

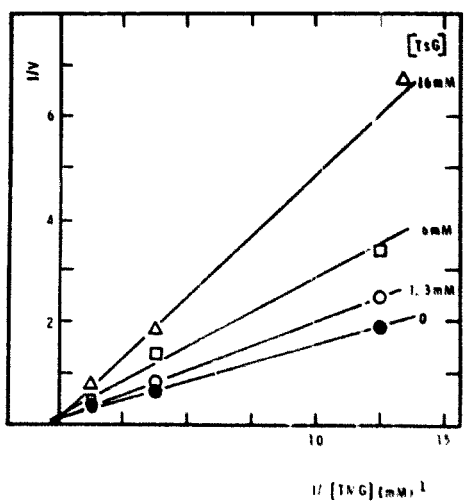


Fig. 8. Effect of inhibitor concentration and sugar concentration on sugar transport. Transport experiments were performed as described under Materials and Methods, at the indicated concentrations of [^{14}C]methylthio *β*-galactoside (TMG) (specific activity, $6.6 \cdot 10^8$ cpm/μmol) and of 6-*O*-(*p*-toluenesulfonyl) galactose (TsG). The symbol *v* corresponds to the initial rate of [^{14}C]methylthio *β*-galactoside uptake, in units of μmol/min per g of cells, dry weight. The lines correspond to the best linear least-squares fit. ●, no inhibitor; ○, 1.3 mM inhibitor; □, 6 mM inhibitor; △, 16 mM inhibitor.

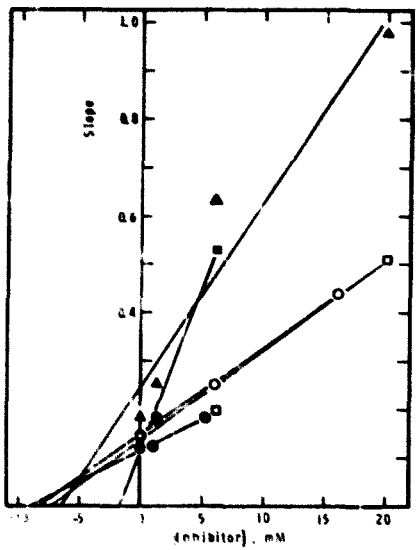


Fig. 9. Replots of slopes from transport inhibition experiments. The slopes of the double reciprocal plots of the data from the experiment shown in Fig. 6 and five similar experiments are replotted against inhibitor concentration. The lines represent the best linear least-squares fit. ○, (experiment of Fig. 8), □, inhibition experiments with 6-*O*-(*p*-toluenesulfonyl) galactose; ●, ■, ▲, experiments with methyl 6-*O*-(*p*-toluenesulfonyl) β-D-galactoside.

Inhibition of transport in whole cells

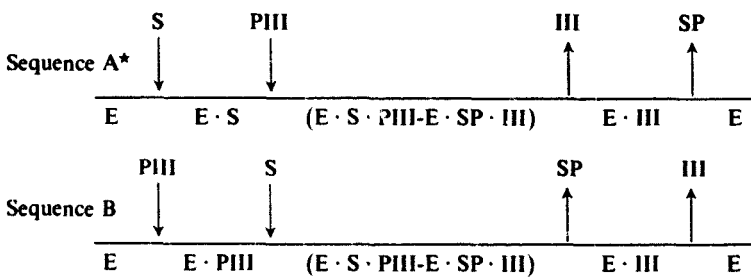
As Fig. 8 indicates, 6-*O*-(*p*-toluenesulfonyl) galactose is a competitive inhibitor of the uptake of methylthio β-galactoside by whole cells. These results are confirmed by the replots of apparent slopes and intercepts from five such experiments. In no case could a systematic dependence of ordinate intercepts on inhibitor concentration be demonstrated, whereas the slopes (Fig. 9) exhibit a dependence on inhibitor concentration which is linear within experimental error. The apparent K_i (slopes) is 5–10 mM, an order of magnitude higher than that determined from the *in vitro* phosphorylation experiments. The inhibitors are apparently not transported into the cells. When uptake experiments were performed using the centrifugation technique (see Materials and Methods) with [^{3}H]methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside as substrate, the radioactivity associated with the cell pellet did not differ significantly from blank values, for aliquots drawn from the cell-sugar mixture over a period of 30 min. However, in similar experiments with [^{14}C]methylthio β-galactoside as substrate, the uptake level was at least three times the blank value within a minute after the initial mixing.

DISCUSSION

An analysis of this heterogeneous multi-enzyme system using the formalism of simple steady-state enzymes kinetics cannot of course be entirely rigorous, and the

absolute values of the kinetic parameters may depend to some extent on undefined interactions among the components of the system. However, the data meet the minimum requirements of reproducibility and linear dependence of phosphorylation rate on Enzyme II^{lac} concentration, and the qualitative features of the inhibition experiments provide important clues to the mechanism of the phosphorylation reaction and thus (indirectly) to the nature of the transport process. The principal observations are: evidence for alternate orders of substrate binding; the persistence of inhibition by the substrate analogs of phosphorylation catalyzed by washed membranes when both substrates are saturating; the marked difference in the apparent reaction mechanism in the presence of detergent; the difference between the apparent K_i values for inhibition of phosphorylation and of transport. There are certain ambiguities inherent in the experimental system. These include the apparent heterogeneity of the membrane preparations, uncertainty as to the relative concentrations of *P*-Factor III^{lac}, P_2-Factor III^{lac}, and P_3-Factor III^{lac}, and the reactivities of these phosphorylated species. Our results, therefore, do not permit us to uniquely determine reaction mechanism(s), but rather to rule out some broad classes of hypotheses. For this latter purpose it is sufficient to restrict the discussion to the simplest possible reaction sequences.

If the Enzyme II^{lac} reaction proceeds by a sequential mechanism, as initial velocity studies suggest [7, 9], then the simplest reaction pathways will be those given below (using the notation of Cleland) and the corresponding kinetic equations will be those given by Cleland for ordered bi-bi reactions [17]. (The order of product release is actually immaterial here).



We can use the rules formulated by Cleland [16] for inhibitor effects to rule out hypothetical reaction sequences. The usual double reciprocal plots of data from experiments performed using different fixed levels of inhibitor will display intercept effects if an infinite concentration of the variable substrate cannot prevent the combination of inhibitor with enzyme. There will be a slope effect in these plots if and only if the dead-end inhibitor (a sugar analog, in these experiments) binds after the variable substrate in the reaction sequence. The phosphorylation inhibition data of Figs. 2 and 6 where sugar is the variable substrate, thus indicate that the substrate analogs (tosyl sugars) bind to the enzyme before sugar (to E in Sequence A or to E · PIII in Sequence

* Definitions of the symbols used: E is Enzyme II^{lac}; S, PIII, III, and SP are reactant sugar, phospho-Factor III^{lac}, Factor III^{lac}, and sugar phosphate, respectively.

B). If Sequence A is the exclusive reaction pathway, the K_i determined from slope replots is the true thermodynamic dissociation constant; if Sequence B is significant, the apparent K_i (slopes) depends on the PIII concentration as well. The data of Table I thus suggest that the phosphorylation cannot occur entirely via Sequence A. Conversely, the slope effects observed in the absence of Triton when phospho-Factor III^{lac} is the variable substrate (Fig. 5) indicate that under these conditions at least some inhibitor binds before phospho-Factor III^{lac}, i.e. the phosphorylation does not occur entirely via Sequence B. This latter result is consistent with previous reports that lactose binds tightly to washed membranes, even in the absence of other phosphotransferase components [9].

In contrast to the data for washed membranes, the phosphorylation experiments in the presence of 0.1 % Triton X-100 suggest that very little of the reaction occurs via Sequence A under these conditions, since there is no slope effect when phospho-Factor III^{lac} is the variable substrate (Fig. 7), and all of the inhibitor must therefore bind after phospho-Factor III^{lac}.

A possible explanation for the apparent change in reaction sequence in the presence of Triton X-100 is a change in the accessibility of Enzyme II^{lac} to phospho-Factor III^{lac}. It seems probable that in the membranes of whole cells Enzyme II^{lac} is oriented with its sugar site accessible to the outside medium, and its phospho-Factor III^{lac} site accessible to the cell cytoplasm. A similar orientation seems likely in at least some of the washed membranes (prepared by sonic irradiation of mechanically disrupted cells without treatment to remove cell wall material), but other orientations are also possible. Some of the Enzyme II^{lac} in these preparations might thus be inaccessible to Factor III^{lac} added to the *in vitro* reaction mixture. The reaction could proceed via a sugar-first mechanism such as Sequence A for "sugar-side out" membranes and via a phospho-Factor III^{lac}-first mechanism such as Sequence B for "phosphoprotein-side out" membranes. In the presence of 0.1 % Triton, accessibility to phospho-Factor III^{lac} is evidently enhanced, since very little reaction occurs via Sequence A. There appears to be no simple way to determine the "natural" sequence *in vivo*.

It is clear from both Figs. 5 and 7 that saturation with phospho-Factor III^{lac} does not prevent inhibitor binding. It thus seems that although the phosphohistidine residue of phospho-Factor III^{lac} must, in order to consumate the phosphoryl transfer, closely approach C-6 of the bound sugar at some time during the phosphorylation/transport reaction, the initial binding of these inhibitory substrate analogs is at a site so far removed from the phospho-Factor III^{lac} site that the bulky tosyl group at C-6 is not significantly hindered by the bound phosphoprotein. Such a separation of binding sites, consistent with the idea that Enzyme II^{lac} must have a sugar site exposed to the outside medium and a phospho-Factor III^{lac} site exposed to the cell cytoplasm, implies that a conformation change is required to bring the bound substrates together for phosphoryl transfer. Such a movement could serve to translocate the sugar across the membrane.

In Fig. 2 inhibitor concentration is seen to affect both the slopes and intercepts of the double reciprocal plot. The inhibitor must therefore bind not only to E and E · PIII in washed membranes, but also to some other enzyme form (with lower affinity). The data can be used to eliminate some of the enzyme forms in Sequence A or B as targets of the low-affinity binding. The low-affinity binding is not to enzyme

forms which also bind the inhibitor at the sugar site (E or E · PIII), since the inhibition is linear (Fig. 4); if an inhibitor, I, can bind to a given enzyme form at two sites, then the rate equation will contain terms in I^2 [18]. The persistence of the inhibition when both sugar and phospho-Factor III^{lac} are saturating (Table II, Experiment 2) indicates that low-affinity binding cannot be to the form E · S. The foregoing implies that the low-affinity binding is to the ternary complex (E · S · III), or to E · SP or E · III. The experimental data do not permit us to distinguish between these possibilities. There is no obvious explanation for the disappearance of the low-affinity inhibition in Triton. The absence of any low-affinity inhibition of transport by whole cells can be explained more readily. The inhibitor, on the outside of the cell, will be unable to bind to enzyme forms such as [E · S · PIII-E · SP · III] or E · III, which most probably exist at the inner surface of the membrane.

The change in apparent K_i (slopes) for tosyl galactosides, from about 0.5 mM for inhibition of *in vitro* phosphorylation to 5–10 mM for inhibition of transport, parallels similar changes in the Michaelis constants for methylthio β-galactoside and other substrates previously reported [13]. These changes may to some extent reflect differences in the conformation of Enzyme II^{lac} in membrane preparations and in whole cells, but kinetic explanations are possible as well. The apparent K_i (slopes) is a true dissociation constant independent of other parameters in these experiments only if the following criteria are satisfied: (1) there is no unphosphorylated Factor III^{lac} present; (2) the reaction proceeds exclusively by Sequence A, or, if it proceeds via Sequence B, then phospho-Factor III^{lac} is at completely saturating concentrations. If these requirements are not met, the apparent K_i (slopes) will be higher than the thermodynamic K_i, due to extra terms in the slope portions of the kinetic equations. Thus the higher K_i (slopes) for inhibition of transport could reflect the presence of intracellular unphosphorylated Factor III^{lac} or of an intracellular level of phospho-Factor III^{lac} significantly lower (relative to its Michaelis constant) than that in the usual *in vitro* phosphorylation mixtures, or both. The notion that the intracellular supply of phospho-Factor III^{lac} is partially limiting in transport experiments is corroborated by the (unpublished) observation in this laboratory that low galactose concentrations actually stimulate methylthio β-galactoside transport, although galactose inhibits the phosphorylation of this compound *in vitro*. Presumably the increased intracellular level of phosphoenolpyruvate formed by galactose metabolism drives the phosphorylation of more Factor III^{lac}.

Dead-end inhibitors are particularly useful probes of reaction mechanism in complex enzyme systems such as the lactose phosphotransferase system, since true inhibition constants are in certain cases actual thermodynamic parameters, whereas Michaelis constants are always complicated functions of the various rate constants and reactant concentrations. Furthermore, the presence or absence of slope effects can be unambiguously interpreted in terms of reaction order. In the experiments described here, the apparent mechanism of sugar phosphorylation catalyzed by membrane preparations was strikingly altered by the presence of the non-ionic detergent Triton X-100 at a low concentration (0.1 %). Further differences between the inhibition of membrane-catalyzed phosphorylation and sugar uptake by whole cells were observed. It would be useful to find dead-end inhibitors of this system other than the arylsulfonyl compounds and use them to verify the generality of the effects observed. The dependence of the apparent mechanism on the nature of the membrane prepara-

tion suggests that future mechanistic studies should employ membranes prepared under well-defined conditions, and thoroughly characterized with respect to the orientation of vesicular structures.

ACKNOWLEDGEMENTS

We are grateful to Professor W. W. Cleland, Department of Biochemistry, University of Wisconsin, who made helpful suggestions concerning the kinetic analyses, and to Professor Y. C. Lee and Mr. Thomas Glass, Department of Biology, The Johns Hopkins University, who provided the [^{3}H]methyl 6-*O*-(*p*-toluenesulfonyl) *β*-galactoside. This work was supported by Grant GM 20211 from the National Institute of General Medical Science, National Institutes of Health.

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