

## Vinylglycolate Resistance in *Escherichia coli*

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*Escherichia coli* K-12 vinylglycolate-resistant mutants have been isolated and characterized. Two of the mutants, JSH 150 and JSH 151, have been determined to be double mutants, lacking both membrane-bound L- and D-lactate dehydrogenases. The lactate transport system is intact in all strains; both radioactive lactate and vinylglycolate are actively taken up. Likewise, the phosphoenolpyruvate-dependent phosphotransferase system for hexose uptake is active. Vinylglycolate, previously shown to inhibit the phosphoenolpyruvate-dependent phosphotransferase system, has very little effect in the double mutants. The extent of vinylglycolate inhibition in other mutants seems directly related to the activity of the lactate dehydrogenases. This indicates that vinylglycolate is oxidized to 2-keto-3-butenate before inactivating the phosphoenolpyruvate-dependent phosphotransferase system. These results were found in whole cells and confirmed in isolated membrane vesicles.

Most but not all bacteria which are facultative anaerobes (6) (and presumably strict anaerobes also [3]) catalyze concentrative uptake of hexoses such as glucose, mannose, and fructose by phosphorylation during passage through the cell membrane. The separate enzymes involved in this combined active transport-phosphorylation sequence of glucose (PTS) have been elegantly characterized by Roseman and colleagues (7) and their physiological role subsequently established (5).

In a previous publication we presented evidence for the specific, irreversible inactivation of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) for hexose uptake in *Escherichia coli* ML 308-225 whole cells and isolated membrane vesicles (12). Vinylglycolic acid (VG; 2-hydroxy-3-butenic acid) specifically and irreversibly inactivates enzyme I of this system. The evidence presented is consistent with the proposal that VG is transported via the lactate transport system (11), and subsequently oxidized to 2-keto-3-butenate by membrane-bound D- and L-lactate dehydrogenases (D- and L-LDH). It was postulated that this conjugated, unsaturated keto acid reacts with a sulfhydryl group(s) in enzyme I to cause inactivation. Evidence was also presented which demonstrates that VG is a potent inhibitor of growth in *E. coli* (11). Some of these properties of VG have allowed the direct demonstration that the great preponderance of *E. coli* mem-

brane vesicles in a given vesicle population catalyze active transport (7a; H. R. Kaback, S. A. Short, G. Kaczorowski, J. Fisher, C. T. Walsh, and S. Silverstein, Abstr. Biochem. Biophys. Meet. 1974, no. 00965).

In this paper, the isolation and characterization of VG-resistant mutants are described. Although the mutants are able to take up VG, they are deficient in membrane-bound dehydrogenases for both D- and L-lactate, and are unable to oxidize VG. Thus, they are resistant to the effects of the unsaturated hydroxy acid with respect to growth and PTS-mediated  $\alpha$ -methylglucoside transport.

### MATERIALS AND METHODS

**Material.** VG was synthesized by earlier procedures (1). [ $1\text{-}^{14}\text{C}$ ]VG (60 mCi/mmol), courtesy of A. Lieberman, was prepared from acrolein and [ $^{14}\text{C}$ ]cyanide on a microscale version of the standard procedure. The radioactive peak was coincident with authentic material by descending paper chromatography in *n*-butanol:water:acetic acid (17:4:1; vol:vol:vol) ( $R_f = 0.4$ ).

**Bacterial strains and isolation of mutants.** All strains used were derived from *E. coli* K-12 and are listed in Table 1. Mutants resistant to VG were isolated as follows. On a minimal glucose agar plate was spread 0.1 ml of an overnight nutrient broth culture of JSH 148, and at the center of the plate approximately 2 mg of vinylglycolate (Li salt) was placed. The plate was incubated in a 37 C incubator

TABLE 1. Bacterial strains derived from *Escherichia coli* K-12 used for this work<sup>a</sup>

Strains	Relevant genotype	Comments
JSH 1	HfrH: <i>thi</i>	From R. F. Schleif. (R.F.S. 1)
JSH 2	HfrH: <i>thi, asn</i>	From JSH 1 by DES <sup>b</sup>
JSH 19	HfrH: <i>thi, asn, dld</i>	From JSH 2 by DES, unable to utilize D-lactate as sole carbon source
JSH 148	HfrH: <i>thi, asn, dld, lld</i>	From JSH 19 by DES, unable to utilize D- or L-lactate as sole carbon source, leaky on L-lactate
JSH 149 to JSH 153	HfrH: <i>thi, asn, dld, lld</i>	From JSH 148 by vinylglycolate resistance, tight on lactic acid as sole carbon source

<sup>a</sup> Growth and isolation of strains are described in Materials and Methods.

<sup>b</sup> DES, Diethylsulfate.

for 24 h. Resistant colonies were picked from the growth inhibition zone and purified.

**Media.** Nutrient broth or minimal salts medium of Vogel and Bonner (9) containing 0.5% carbon source and 40  $\mu$ M thiamin (B<sub>1</sub>) was used. When required, amino acids were added at 0.4 mM. Solid media contained 1.5% agar.

**Growth of cells and preparation of membrane vesicles.** Cells were grown on the minimal media of Davis and Mingioli (2) supplemented by 0.5% glucose or 1.0% sodium succinate (hexahydrate) as sole carbon source as previously described (4). Membrane vesicles were prepared as detailed elsewhere (5a).

**Transport assays.** Methods used to assay proline transport and phosphoenolpyruvate-dependent glucose uptake in isolated membrane vesicles are described in earlier publications (4, 5). Vinylglycolate uptake was measured in the presence of ascorbate-phenazine methosulfate as described earlier (12). The reaction mixtures used in these assays contained 0.11, 0.10, and 0.077 mg of membrane protein for JSH 2, 19, and 151 vesicles, respectively.

Transport assays using whole cells were carried out under similar conditions. Final incubation mixtures of 50  $\mu$ l contained 50 mM potassium phosphate buffer, pH 6.6, 0.215 mg (dry weight) of whole cells, and a radioactive substrate. The reaction was quenched at appropriate times with 3 ml of 0.1 M potassium phosphate buffer, pH 6.6, immediately filtered on a 0.45- $\mu$ m membrane filter, and washed once. The filters were dried and radioactive uptake was determined by dissolving the filters in liquid scintillation cocktail and subsequent analysis (84% efficiency).

Radioactive transport substrates were used in the following specific activities and final concentrations: D,L-[1-<sup>14</sup>C]lactate (45.5 mCi/mmol) at  $1.66 \times 10^{-4}$  M; D,L-[1-<sup>14</sup>C]vinylglycolate (60 mCi/mmol) at  $3.7 \times 10^{-4}$  M;  $\alpha$ -[U-<sup>14</sup>C]methylglucoside (52.2 mCi/mmol)

at  $3.64 \times 10^{-5}$  M; [U-<sup>14</sup>C]glucose (192 mCi/mmol) at  $4.5 \times 10^{-5}$  M; and L-[U-<sup>14</sup>C]proline (255 mCi/mmol) at  $7.6 \times 10^{-5}$  M.

**Oxygen uptake.** Rates of oxygen uptake by various membrane vesicles in response to added electron donors were measured with a Clark-type electrode (YSI model 53 oxygen monitor) as described previously (10).

RESULTS

**Membrane-bound dehydrogenase activities.** Activities of various membrane-bound dehydrogenases from isolated membrane vesicles of the *E. coli* K-12 strains used are presented in Table 2. Several points emerge. Each of the vesicle preparations exhibits minimal rates of oxygen uptake in the absence of added substrate and maximal rates with the addition of succinate. The parent strain, JSH 2, has wild-type levels of D-LDH and L-LDH. JSH 19 vesicles oxidize L-lactate at rates comparable to wild-type vesicles, but do not exhibit any significant activity towards D-lactate. Strains JSH 149 through 153 are derivatives of JSH 148 (a leaky mutant that is not further characterized as yet) which are resistant to bacteriocidal effects of 2 mg of exogenous vinylglycolate on agar plates. As expected none of the VG-resistant mutants oxidize D-lactate appreciably, moreover JSH 150 and 151 have about 13 and 7% of the parent level of L-LDH.

Since VG is a racemic mixture of D- and L-isomers, it ought to be oxidizable by either membraneous lactate dehydrogenase. As evidenced by the data shown in the fourth column of Table 2, VG is efficiently oxidized by JSH 19, 149, 152, and 153 presumable in each case by the L-LDH. Vesicles from JSH 150 and 151 show

TABLE 2. Membrane-bound dehydrogenase content of *Escherichia coli* strains<sup>a</sup>

Substrate (nmol of O <sub>2</sub> /min per mg of protein)					
Strain	No substrate	D-Lactate	L-Lactate	D,L-Vinylglycolate	Succinate
JSH 2	3.4	30.1	70.9	84.6	255.2
JSH 19	4.6	4.6	78.2	100.2	142.5
JSH 149	5.0	5.0	111.2	131.8	254.3
JSH 150	2.0	2.0	13.3	15.4	272.8
JSH 151	2.6	2.9	7.4	10.3	365.7
JSH 152	1.4	1.5	88.7	103.1	191.9
JSH 153	2.4	2.2	89.3	115.3	297.2

<sup>a</sup> In isolated membrane vesicles, dehydrogenase activity was measured by recording the endogenous level of oxygen uptake at 30 C by oxygen electrode. Experimental conditions are as described in Materials and Methods.

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15 and 10% VG-oxidizing activity of JSH 19, which is comparable with the residual L-LDH activities. These data demonstrate that D- and L-LDH are the only membrane-bound dehydrogenases which are able to oxidize VG and suggest that inability to oxidize VG may represent at least one mechanism of VG resistance.

**Active transport of [ $^{14}\text{C}$ ]vinylglycolate by whole cells.** Previous experiments with isolated membrane vesicles have demonstrated that VG is a competitive inhibitor of the lactate transport system with a  $K_i$  that is similar to the  $K_m$  of the system for D-lactate (12). These findings have led to the conclusion that VG gains access to the intravesicular pool via the lactate transport system. The experimental data presented in Fig. 1 suggest that a similar situation exists in whole cells. Thus, VG inhibits the uptake of [ $^{14}\text{C}$ ]lactate by succinate-grown JSH 19 whole cells (Fig. 1A), and lactate inhibits the uptake of [ $^{14}\text{C}$ ]vinylglycolate (Fig. 1B). It should be emphasized that the results presented here are not as clear-cut as those obtained with membrane vesicles presumably because of extensive metabolism of the accumulated substrates. In any case, the results support the previous observations which indicate that VG and lactate share a common transport system.

Figure 2A compares rates of uptake of [ $^{14}\text{C}$ ]vinylglycolate by succinate-grown strains JSH 2, 19, 150, and 151. Although the initial rates of uptake appear to be similar in all four strains (compare 30-s and 1-min points), the extent of vinylglycolate uptake over the 15-min incubation period is some 2.5-fold greater in JSH 2 than in 19 or 150 and 151. Since VG can be oxidized by both D-LDH and L-LDH only in JSH 2 its uptake and metabolism might well be greater in JSH 2 than in the mutants. It is somewhat surprising that the extent of vinylglycolate uptake by JSH 19 is only approximately 25% higher than that observed in JSH 150 and 151, since the latter organisms are markedly deficient in membrane-bound D- and L-LDH activity. It should be emphasized here that the ability to accumulate lactate or VG is apparently normal in all of these strains, and that each strain presumably has the soluble nicotinamide adenine dinucleotide-dependent D-LDH (8) which is also potentially capable of oxidizing VG.

The same general pattern is seen when  $\alpha$ -methylglucoside uptake by the PTS is analyzed in whole cells of JSH 2, 19, 150, and 151 (Fig. 2B). The variations in PTS sugar uptake are not understood but probably are not related to defects in lactate oxidation. Nonetheless, these

data indicate all the cells do contain functional vectorial phosphorylation apparatus for hexose uptake and are testable, therefore, for inhibitory effects of vinylglycolate.

**Effect of VG on  $\alpha$ -methylglucoside uptake in whole cells.** Inactivation of  $\alpha$ -methylglucoside uptake by VG is apparently directly related to the cell's ability to oxidize VG. The data of Fig. 3 indicate the susceptibility of the *E. coli* strains to inactivation of  $\alpha$ -methylglucoside transport by varying amounts of vinylglycolate. In each experiment, whole cells were exposed to the indicated levels of vinylglycolate for a 5-min preincubation, followed by the addition of [ $^{14}\text{C}$ ] $\alpha$ -methylglucoside and then analysis of the sugar transport after 10 min. Under these conditions JSH 2 and 19 show 50% inactivation of uptake at 1 to 2 mM VG. In contrast, the VG-resistant mutants JSH 150 and 151 require 30 to 40 mM and about 80 mM VG, respectively, to achieve a similar degree of inactivation. These results parallel the residual L-LDH activity present in membrane vesicles prepared from these two strains (cf. Table 2).

These relationships are confirmed in the comparison of the behavior of JSH 19 and JSH 151 whole cells for the time dependence of hexose transport blockade. JSH 151 cells were completely unaffected during a 5-min incubation period with 5 mM exogenous vinylglycolate. On the other hand, JSH 19 cells were 50% inactivated within the first minute after  $\alpha$ -methylglucoside was added (data not shown). The eventual inactivation of PTS-mediated sugar uptake in JSH 150 and 151 at extremely high external concentrations of vinylglycolate may be due to participation of the soluble nicotinamide adenine dinucleotide-linked lactate dehydrogenase in VG oxidation. Since this inactivation of sugar uptake requires such high concentrations, this enzyme, if involved at all, cannot be quantitatively important in VG oxidation at low concentrations of added VG.

**Irreversible blockade of hexose-active transport in whole cells.** As shown above (cf. Fig. 2) cells with and without D- and L-LDH activity take up vinylglycolate. However, only those cells which are capable of VG oxidation are sensitive to VG inactivation of  $\alpha$ -methylglucoside transport. The enzymatic product of VG oxidation, 2-keto-3-butenate, would be reactive to Michael-type addition by cellular nucleophiles. These alkylation processes would covalently link radioactive molecules in the cell. No such covalent linkages should occur if VG is not oxidized. The following experiment supported the above postulates. Cells were incu-

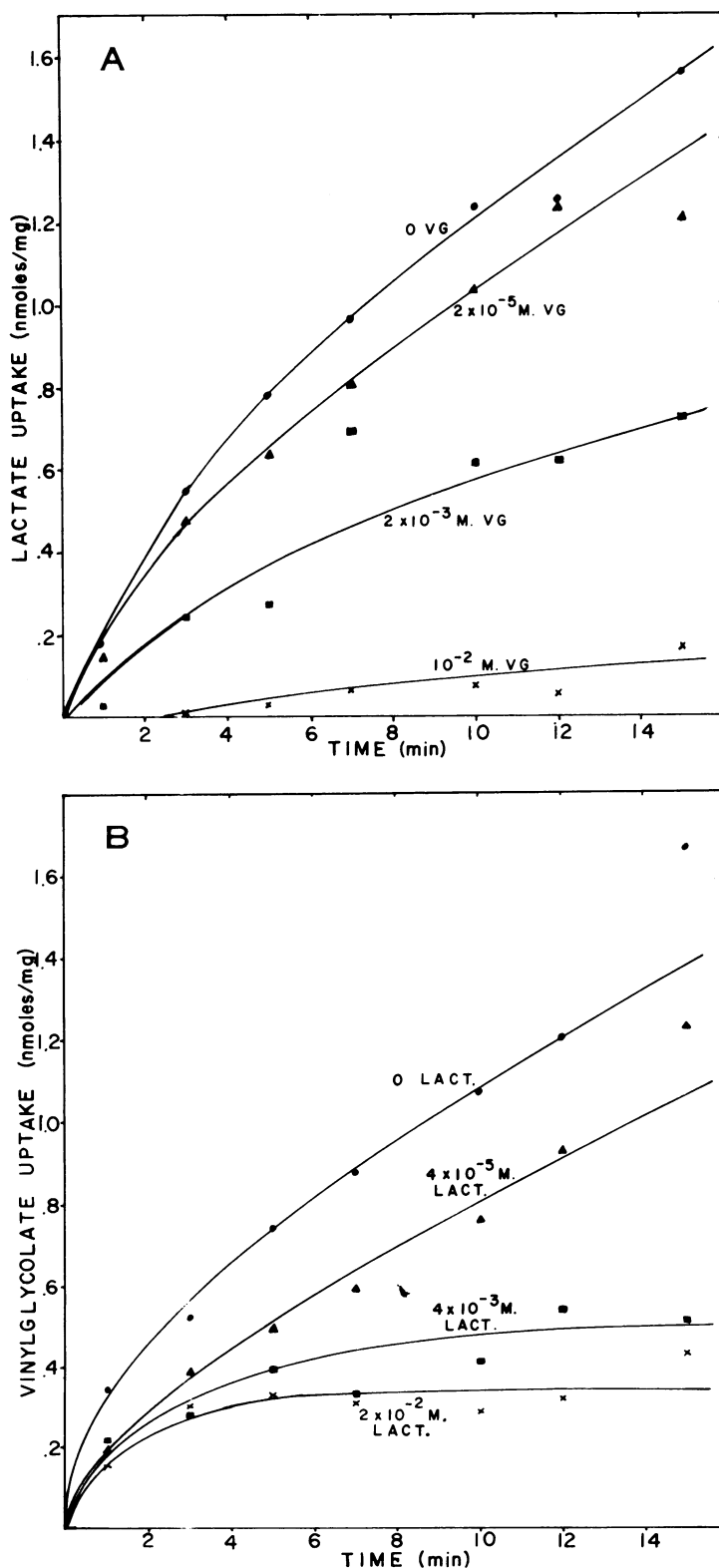


FIG. 1. (A) Vinylglycolate inhibition of [ $^{14}$ C]lactate uptake in JSH 19 whole cells, succinate grown. Experimental conditions are as described in Materials and Methods. Symbols: (●) no cold vinylglycolate; (▲)  $2.0 \times 10^{-5}$  M vinylglycolate; (■)  $2.0 \times 10^{-3}$  M vinylglycolate; (×)  $10^{-2}$  M vinylglycolate. (B) Inhibition of [ $^{14}$ C]vinylglycolate uptake by nonradioactive lactate in JSH 19 whole cells, succinate grown. Experimental conditions are as described in Materials and Methods. Symbols: (●) no cold lactate; (▲)  $4.0 \times 10^{-5}$  M lactate; (■)  $4.0 \times 10^{-3}$  M lactate; (×)  $2.0 \times 10^{-2}$  M lactate.

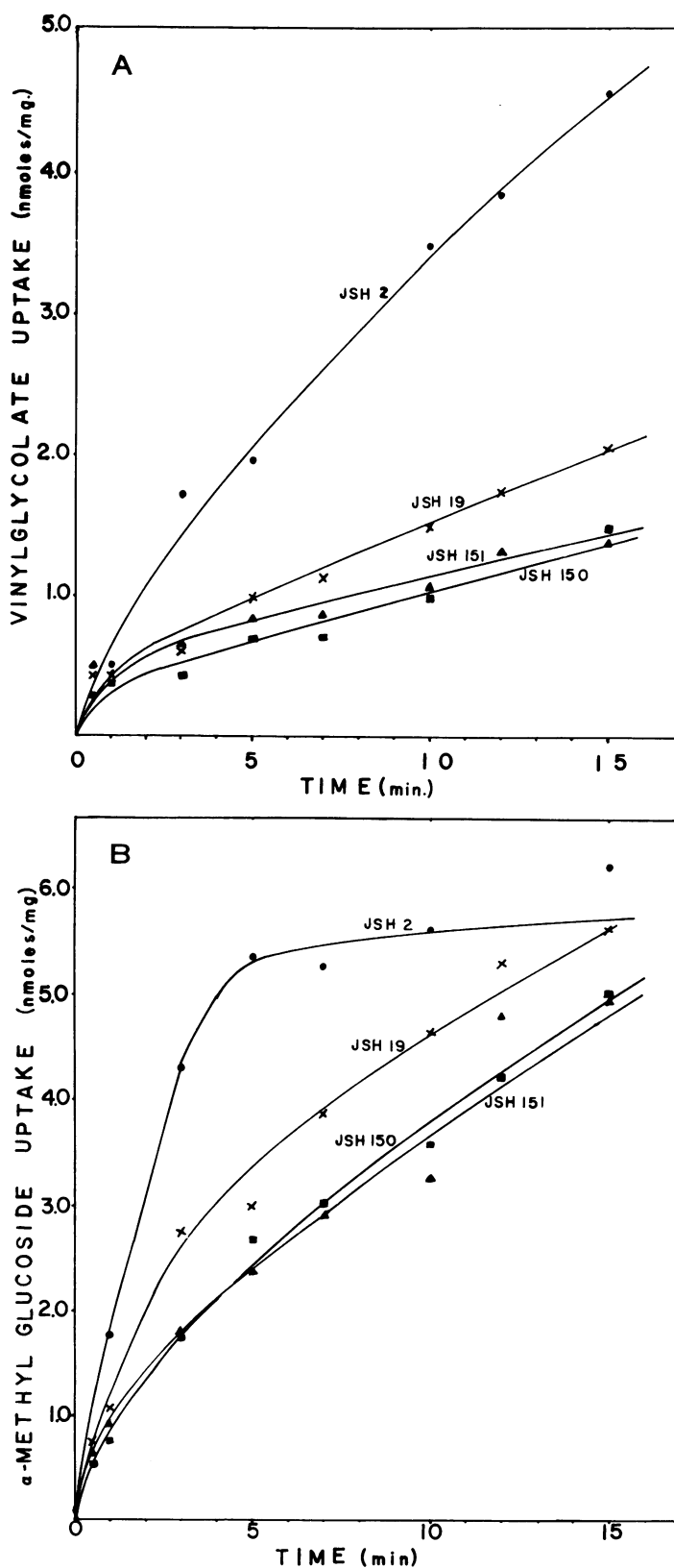


FIG. 2. (A) Rate of  $[^{14}\text{C}]$ vinylglycolate uptake by succinate-grown JSH 2 (●), JSH 19 (×), JSH 150 (■), and JSH 151 (▲) whole cells. Experimental procedure is as described in Materials and Methods. (B) Rate of uptake of  $[^{14}\text{C}]\alpha$ -methylglucoside in whole cells of strains JSH 2 (●), JSH 19 (×), JSH 150 (■), and JSH 151 (▲). Experimental conditions are as described in Materials and Methods.

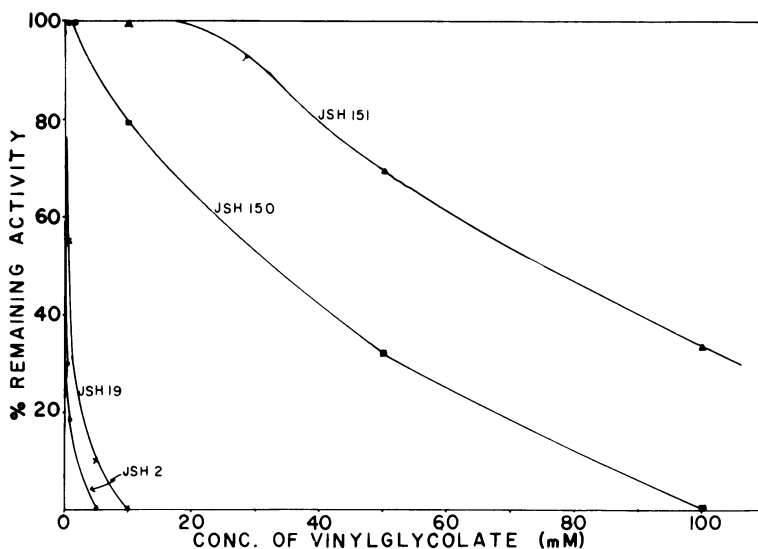


FIG. 3. Inactivation of [ $^{14}\text{C}$ ]  $\alpha$ -methylglucoside transport and phosphorylation by various concentrations of vinylglycolate, as indicated, in whole cells JSH 2 (●), JSH 19 (×), JSH 150 (■) and JSH 151 (▲). Experimental procedure is described in the text.

bated with [ $1\text{-}^{14}\text{C}$ ] vinylglycolate for 10 min, and after terminating the reaction with a 14-fold excess of cold VG, the cells were washed. The cells were resuspended to the initial volume again with the 14-fold excess of nonradioactive VG, as a chase before final resuspension in buffer. Aliquots were removed for analysis of radioactive content as shown in Fig. 4A, and ability to carry out  $\alpha$ -methylglucoside uptake (Fig. 4B). As shown with VG-resistant JSH 151, which contains neither flavin-linked lactate dehydrogenase in active form, essentially all of the radioactivity is easily removed from cells. On the other hand, JSH 2 or JSH 19 retain 30 and 15% (respectively) of the radioactivity. Moreover, as shown in Fig. 4B,  $\alpha$ -methylglucoside uptake is irreversibly inactivated in JSH 2 and 19 but remains unaffected in JSH 151. This inactivation, again corresponds to the activity of the D- and L-LDH in the cells.

**Vinylglycolate uptake and PTS inactivation in membrane vesicles.** When the focus was shifted from whole cells to cytoplasmic membrane vesicles prepared from them, a corresponding pattern emerges. As mentioned before from the data of Table 2, JSH 150 and 151 are totally unable to oxidize D-lactate and severely limited in their ability to oxidize L-lactate and VG. Thus, it is not surprising that D- and L-lactate-dependent proline transport are grossly deficient in JSH 150 and 151 membrane vesicles relative to JSH 2 vesicles (Fig. 5A and

B). In marked contrast, proline transport in the presence of ascorbate-phenazine methosulfate is similar in membrane vesicles prepared from both VG-resistant mutants and JSH 2 (Fig. 5C). Moreover, as shown in Fig. 5D, JSH 150 and 151 vesicles are able to take up VG in the presence of ascorbate-phenazine methosulfate although they are unable to oxidize the compound. The differences between JSH 150 and 151 membrane vesicles and JSH 2 vesicles are probably due to the ability of JSH 2 vesicles to oxidize and thus covalently bind VG (cf. Fig. 4A). Although not shown, it is pertinent that all three preparations also transport D- $[^{14}\text{C}]$ lactate in the presence of the artificial electron donor system, which indicates that none of the mutants are defective in lactate transport.

The data presented in Fig. 6 represent time courses of inactivation of phosphoenolpyruvate-dependent glucose uptake in the presence of 2  $\mu\text{M}$  VG. As shown, striking differential effects occur with vesicles prepared from JSH 2, 19, and 151. With JSH 2 and JSH 19 vesicles, 50% inactivation occurs in 15 to 30 s, and about 50 s, respectively. On the other hand, with JSH 151 vesicles, 50% inactivation requires approximately 4 min. Thus, the inactivation rates observed in this experiment correlate reasonably well with the ability of each vesicle preparation to oxidize VG (cf. Table 2). These results in vesicles are qualitatively similar to those in whole cells (Fig. 3).

## DISCUSSION

We have previously reported that vinylglycolate is a potent irreversible inhibitor of PTS-mediated hexose uptake and phosphorylation in whole cells and isolated cytoplasmic membrane vesicles of *E. coli* ML 308-225 (12). In sharp contrast to the acetylenic analogue, 2-hydroxy-3-butenate which is a suicide substrate for the membrane-bound flavin-dependent D- and L-LDH (10), VG is a noninactivating substrate for the dehydrogenases and undergoes rapid oxidation. The reaction product 2-keto-3-butenate, a reactive electrophilic  $\beta,\gamma$ -unsaturated  $\alpha$ -keto acid, was hypothesized as the actual inactivator of enzyme I of the PTS, perhaps by alkylation of the essential sulfhydryl group.

The evidence presented in this paper substantiates these contentions. Two of the strains used (JSH 150, 151) are double mutants, possessing no detectable D-LDH and only a few percent of wild-type levels of L-LDH. All of the strains can accumulate [ $^{14}\text{C}$ ]vinylglycolate indicating the  $\alpha$ -hydroxy acid transport system is functional in each. Further, all the cells have an operant phosphotransferase system as evidenced by  $\alpha$ -methylglucoside uptake. These data allow unequivocal determination that functional membrane D-LDH and/or L-LDH are essential for vinylglycolate-induced blockade of hexose active transport. Considerably higher concentrations of vinylglycolate are required for  $\alpha$ -methylglucoside blockade in the double mutants. Moreover, the JSH 2 and 19 cells remain irreversibly inactivated, whereas the double mutants are fully active for hexose uptake and phosphorylation. These two points clearly indicate that intracellular oxidation of VG to 2-keto-3-butenate is a prerequisite for PTS inactivation.

In isolated membrane vesicles the same behavior persists. The 150 and 151 vesicles cannot use lactate to drive transport of solutes such as proline. However, [ $^{14}\text{C}$ ]lactate and [ $^{14}\text{C}$ ]vinylglycolate are accumulated in the presence of an artificial electron donor, indicating the carrier system is functional. Blockade of transport of  $\alpha$ -methylglucoside by JSH 151 vesicles is much slower at  $2\ \mu\text{M}$  vinylglycolate than in JSH 2- or JSH 19-derived vesicles.

The evidence from these mutants proves that active transport blockade of  $\alpha$ -methylglucoside both in whole cells and corroborated in membrane vesicles requires prior intracellular or intravesicular accumulation of vinylglycolate followed by its specific oxidation by the two membraneous D- and L-LDH.

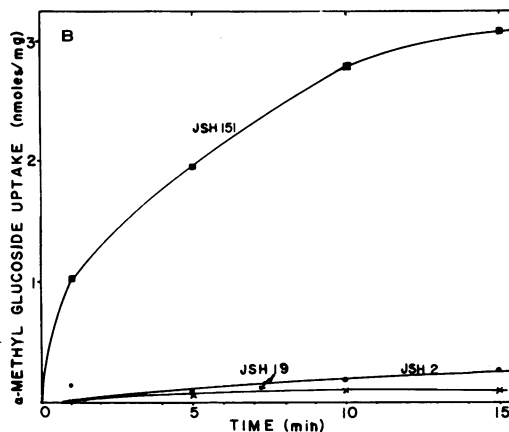
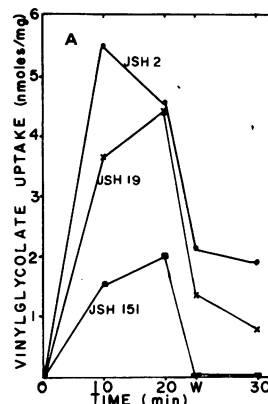


FIG. 4. (A) Comparison of the irreversibility of [ $^{14}\text{C}$ ]vinylglycolate transport in whole cells JSH 2 (●), JSH 19 (×), and JSH 151 (■). Experimental conditions are described in the text. Aliquots were filtered and counted after 10 min of preincubation with [ $^{14}\text{C}$ ]vinylglycolate (10 min); after addition and 10 min of incubation with cold vinylglycolate (20 min); after centrifugation, washing, and resuspension to initial volume (W) and after a second 10 min of preincubation with cold vinylglycolate (30 min). (B) Rate of  $\alpha$ -methylglucoside uptake after exposing whole cells of strains JSH 2 (●), JSH 19 (×) and JSH 151 (■) to vinylglycolate as described in legend of Fig. 4 A. Experimental assay conditions are as described in Materials and Methods.

Finally, it should be emphasized that deficiencies in membrane-bound D- and L-LDH cannot represent the only means by which cells become resistant to VG. Although JSH 149, 152, and 153 are resistant to VG, they exhibit normal oxidase activities with respect to L-lactate and VG. The mechanism of resistance in these organisms is currently under investigation.

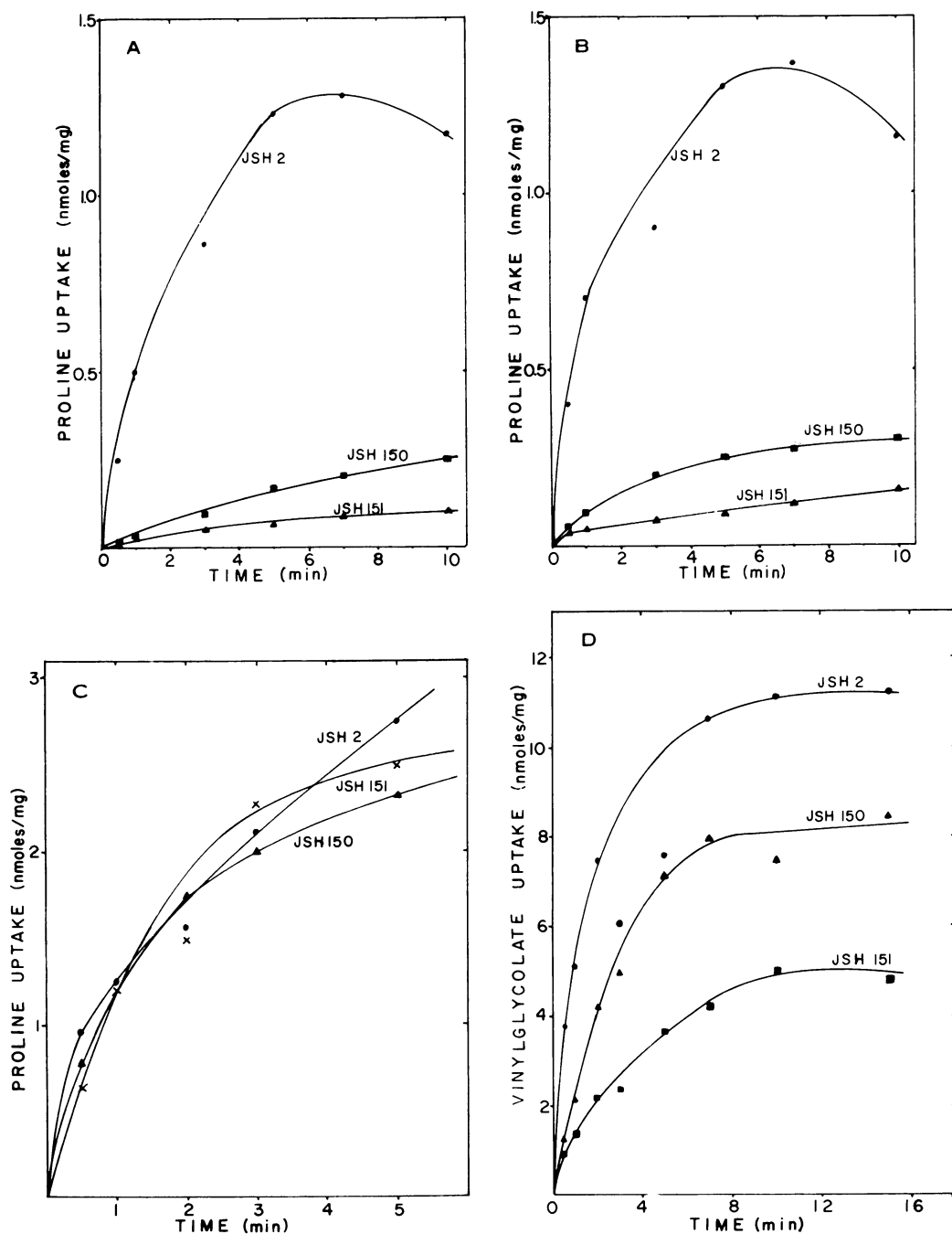


FIG. 5. (A) D-Lactate-driven [ $^{14}$ C]proline uptake in JSH 2 (●), JSH 150 (▲), and JSH 151 (×) isolated membrane vesicles. Experimental conditions are described in the Materials and Methods. Endogenous activity for each of the strains is less than 0.2 nmol of proline/mg of protein after a 5-min incubation with [ $^{14}$ C]proline. (B) L-Lactate-driven [ $^{14}$ C]proline uptake in membrane vesicles of JSH 2 (●), JSH 150 (▲), and JSH 151 (×). Experimental procedure is described in the legend of Figure 5A and the Materials and Methods. (C) Ascorbate-phenazine methosulfate-driven [ $^{14}$ C]proline uptake ( $7.6 \times 10^{-5}$  M, 255 mCi/mmol) in JSH 2 (●), JSH 150 (▲) and JSH 151 (×) membrane vesicles. Vesicles,  $\text{MgSO}_4$  (10 mM) and KPi (pH 6.6) (50 mM) were preincubated under oxygen atmosphere for 5 min. Ascorbate and phenazine methosulfate were then added to final concentrations of  $2 \times 10^{-2}$  M and  $10^{-4}$  M, respectively. The reaction was run under oxygen gas, endogenous activity (not shown) is 2.65, 1.75, and 1.05 nmol/mg of protein after 15-min incubations with JSH 2, JSH 150, and JSH 151, respectively. (D) Ascorbate-phenazine methosulfate-driven [ $^{14}$ C]vinylglycolate uptake ( $1.48 \times 10^{-4}$  M, 60 mCi/mmol) in membrane vesicles of strains JSH 2 (●), JSH 150 (▲), and JSH 151 (×). Experimental conditions are described in the legend of Fig. 5C and the Materials and Methods.



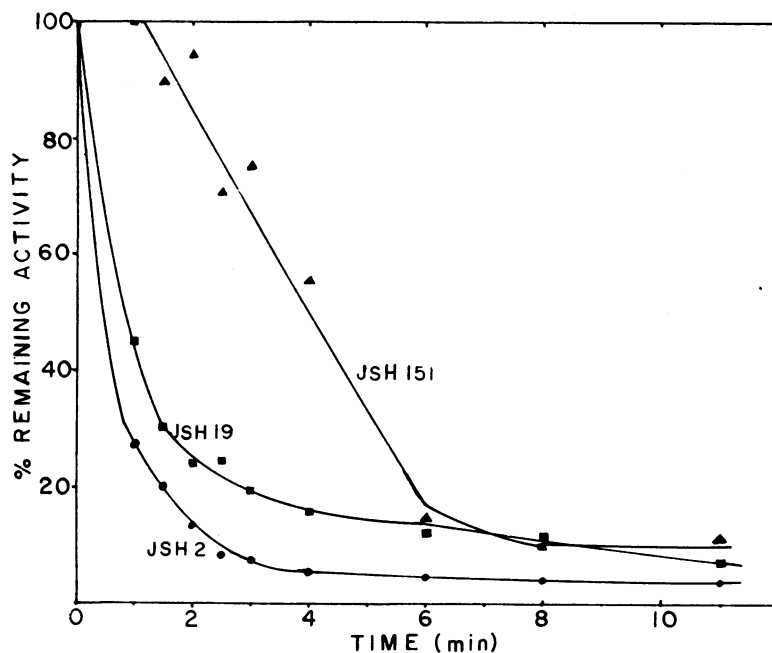


FIG. 6. Remaining capacity of JSH 2 (●), JSH 150 (▲), and JSH 151 (x) membrane vesicles to transport and phosphorylate [ $^{14}\text{C}$ ]glucose ( $4.0 \times 10^{-6}$  M, 192 mCi/mmol) after preincubation with  $2 \mu\text{M}$  vinylglycolate. Times indicated are total VG preincubation including 1-min glucose exposure. Reactions were terminated by a 50-fold dilution and washing with  $0.5 \text{ M LiCl}$  after a 1-min incubation with glucose. Experimental conditions are described in Materials and Methods.

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