Reversible Inactivation of Vectorial Phosphorylation by Hydroxybutynoate in *Escherichia coli* Membrane Vesicles†

Gregory Kaczorowski, H. Ronald Kaback, and Christopher Walsh*

ABSTRACT: The acetylenic hydroxy acid 2-hydroxy-3-butynoate causes irreversible inactivation of the *Escherichia coli* membrane-bound flavoenzyme D-lactic dehydrogenase, and thus blocks D-lactate dependent active transport in isolated membrane vesicles [Walsh, C. T., Abeles, R. H., and Kaback, H. R. (1972), *J. Biol. Chem. 247,* 7858]. The inactivator is a suicide substrate for the dehydrogenase, undergoing a small number of turnovers before partitioning between oxidation and inactivation. It is now demonstrated that reactive product molecules of 2-keto-3-butynoate can diffuse in the membranes to a component of the phosphotransferase system and cause time-dependent and covalent inactivation of phosphoenolpyruvate-dependent hexose uptake. Membrane vesicles from double mutants with low levels of both D- and L-lactic dehydrogenase lose only 30% of their hexose uptake capacity on exposure to hydroxybutynoate under conditions sufficient to fully inactivate hexose transport in wild type vesicles. Transport of 1-[^{14}C]hydroxybutynoate into vesicles is followed by rapid covalent labeling of membrane proteins by the reactive, enzymatically generated keto acid oxidation product. Incubation of hydroxybutynoate-inactivated vesicles (5% residual activity) for 20 min in buffer with 10 m*M* dithiothreitol results in reactivation of 63% of the hexose transport activity, a 12-fold increase in activity. No reactivation occurs if the vesicular phosphotransferase system is inactivated by keto acid derived from membrane oxidation of the olefinic congener 2-hydroxy-3-butenoate. In contrast to thiol reactivation of acetylenic-blocked glucose transport, blockade of D-lactate-stimulated proline uptake is not alleviated, stressing different modes of inactivation of the phosphotransferase system compared to the membranous lactate dehydrogenases.

In recent experiments, we have demonstrated that the acetylenic hydroxy acid, 2-hydroxy-3-butynoate (HBA),[1] functions as a suicide substrate and specific inactivator of a number of flavine-linked hydroxy acid oxidizing enzymes, serving in each case as a covalent titrant for the bound flavine coenzyme (Walsh et al., 1972b; Cromartie and Walsh, 1975). Thus, hydroxybutynoate has been used to probe the functional role of the *Escherichia coli* membrane-bound D-lactate dehydrogenase in active transport (Walsh et al., 1972a). Incubation of isolated cytoplasmic membrane vesicles from *E. coli* with this acetylenic substrate inactivates the dehydrogenase after a small number of turnovers. Concomitant with loss of enzyme activity is a coordinate irreversible blockade of D-lactate dependent lactose, proline, and rubidium transport confirming the causal relationship between the membranous D-lactic dehydrogenase and concentrative solute uptake suggested by a bulk of earlier experiments (Kaback, 1974b). Other membrane flavoprotein dehydrogenases, NADH dehydrogenase and succinate dehydrogenase, remain fully active. Further, all components of the membrane respiratory chain and the transport carrier proteins are still functional, as demonstrated by the lack of inactivation of ascorbate–phenazine methosulfate driven solute transport (Walsh et al., 1972a).

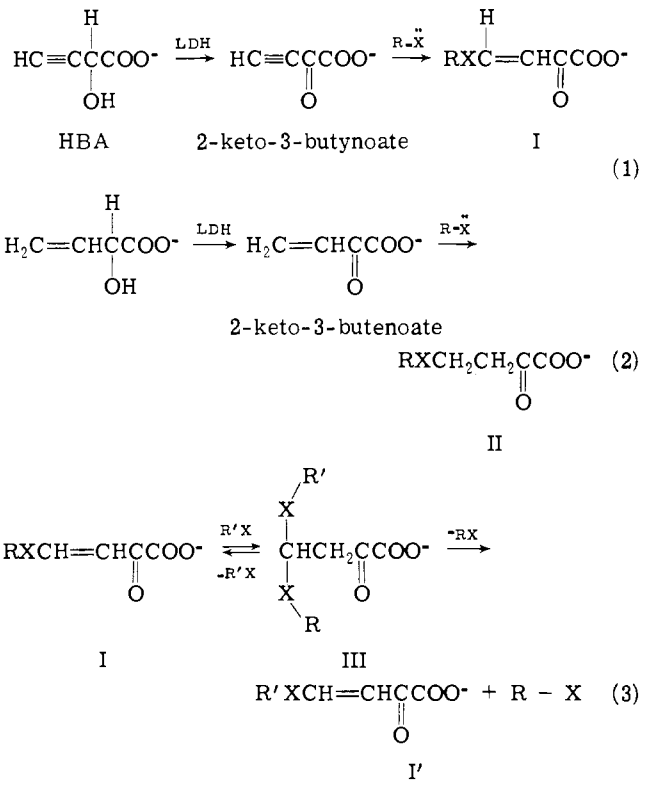
Both the α-hydroxy group and acetylenic functionality are crucial to covalent modification of flavine coenzymes by HBA (Walsh and Kaback, 1974). Thus, butynoic acid has no effect, and the alkenoic analog, 2-hydroxy-3-butenoate (vinylglycolate), is a noninactivating substrate for either D- or L-lactic dehydrogenase in *E. coli* membranes and can function as an electron donor for solute uptake. However, rapid oxidation of vinylglycolate produces thousands of reactive 2-keto-3-butenoate product molecules. This keto acid has an α,β-unsaturated carbonyl system, is susceptible to rapid 1,4 attack by protein nucleophilic groups, and ought to be a reactive alkylating agent in the vesicles. Indeed, vinylglycolate addition to either *E. coli* whole cells or isolated membrane vesicles generates rapid and irreversible inactivation of the second major type of bacterial transport system, the phosphoenolpyruvate-dependent vectorial phosphotransferase system (PTS) for hexose transport and phosphorylation (Walsh and Kaback, 1973). Active transport of glucose, fructose, mannose, and α-methyl glycoside is thus specifically abolished, while D-lactate dehydrogenase coupled transport of proline, lactose, and rubidium remains intact. Experiments with nucleophilic trapping agents, with mutants defective in vinylglycolate oxidation, and PTS enzyme fractionation studies strongly implicate alkylation of the enzyme I component of the PTS by the enzymatic oxidation product 2-keto-3-butenoate (Walsh and Kaback, 1974; Shaw et al., 1975).

Given that hydroxybutynoate partitions between oxidation to product and inactivation of the membrane lactic dehydrogenases, a small number (~25) of molecules of 2-keto-3-butynoate are generated before a dehydrogenase molecule is blocked. The chemical reactivity of this acetylenic keto acid to Michael-type attack by membrane nucleophiles (R–X) should resemble that of 2-keto-3-butenoate (Scheme I). One notable structural difference between Michael adducts I and II is that I still possesses an electrophil-

† From the Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (G.K., C.W.), and the Roche Institute of Molecular Biology, Nutley, New Jersey 07110 (H.R.K.). *Received March 24, 1975.* This work was supported in part by National Science Foundation Grant GB 42031X (C.W.) and also by a grant from the Lilly Research Laboratories (C.W.).

[1] Abbreviations used are: HBA, 2-hydroxy-3-butynoate; VG, 2-hydroxy-3-butenoate (vinylglycolate); PTS, phosphotransferase system; LDH, lactate dehydrogenase.

Scheme I



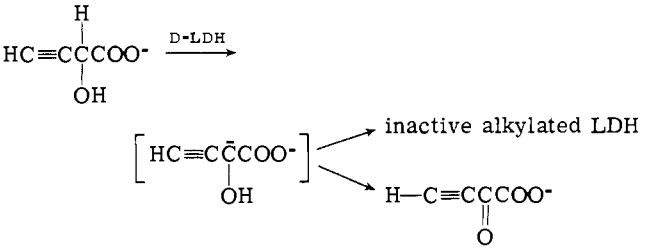
ic α,β-unsaturated carbonyl system while II does not. Adduct I might then undergo a second Michael addition by another nucleophile (R'X) to give structure III (eq 3). However, C-4 of such an adduct III would contain two geminal heteroatoms, and most likely readily eliminate one of these leaving groups. If RX is eliminated rather than R'X, adduct I' would form. Now if the nature of the nucleophiles were controlled such that RX is the thiol group of a membrane protein while R'X is the thiol group of dithiothreitol, conversion of I to I' on addition of dithiothreitol should regenerate the native form of the membrane protein and any function that it possesses. Reactivation from adduct II, under the same conditions, could be considerably less facile, occurring by reversal of the Michael addition reaction (a path also open to I).

In this paper we report experiments demonstrating that hydroxybutynoate can inactivate hexose active transport and phosphorylation in membrane vesicles. We also delineate the functional differences between transport blockade and subsequent reactivation with hydroxybutynoate vs. vinylglycolate as the enzymatic precursor of the inactivating species. While both acetylenic and olefinic molecules block glucose uptake into membrane vesicles, nucleophilic thiols can effect reactivation only when hydroxybutynoate has caused covalent blockade of hexose uptake.

Experimental Section

Materials. Membrane vesicles used in these experiments were prepared from *E. coli* ML 308-225 and two strains of *E. coli* K-12, JSH-2 and JSH-150 (Shaw et al., 1975). L-[^{14}C]Proline (233 mCi/mmol), L-[^{14}C]serine (155 mCi/mmol), and D-[^{14}C]glucose (238 mCi/mmol) were purchased from New England Nuclear. 2-Hydroxy-3-butynoate (HBA) and 2-hydroxy-3-butenoate (VG) were synthesized as described earlier (Cromartie et al., 1974). 1-[^{14}C]-2-Hydroxy-3-butynoate and 1-[^{14}C]-2-hydroxy-3-butenoate were prepared in a microscale synthesis based on the standard procedure using [^{14}C]cyanide by the radiation synthesis section of Hoffmann-La Roche under the direction of Dr. Arnold Liebman. They were prepared at specific activities of 35 and 60 mCi/mmol, respectively. The lithium salt of D-lactate, sodium ascorbate, phenazine methosulfate, phosphoenolpyruvate (trisodium salt), dithiothreitol, and phospholipase A-B were all obtained from Sigma Chemical Co. Membrane filters 0.45 (μ) were from Schleicher and Schuell. All other chemicals were of reagent grade quality and obtained from commercial sources.

Scheme II



Methods. ML 308-225, JSH-2, and JSH-150 membrane vesicles were isolated from cells grown on the minimal medium of Davis and Mingioli supplemented with 1% sodium succinate (Davis and Mingioli, 1950). In all cases, vesicles were prepared as described previously (Kaback, 1971), suspended in 0.1 *M* potassium phosphate (pH 6.6) at protein concentrations of between 5 and 10 mg/ml and stored in liquid nitrogen.

The assay of D-lactate dependent transport of amino acids and phosphoenolpyruvate-dependent uptake of glucose has been described (Kaback, 1974a). Ascorbate-phenazine methosulfate driven uptake of substrates was carried out under an oxygen atmosphere as described previously (Kaback, 1974a). Radioactive substrates were employed at the following final concentrations in transport assays: L-[^{14}C]serine, 17 μM; L-[^{14}C]proline, 15.9 μM; D-[^{14}C]glucose, 21 μM; and [^{14}C]HBA, 1 m*M*. Radioactivity was measured by liquid scintillation counting using ScintiVerse (Fisher Scientific) as cocktail. Counting efficiency was 80%.

Results and Discussion

Hydroxybutynoate Inactivates Phosphotransferase-Mediated Hexose Uptake in Addition to Respiration-Coupled Active Transport. Earlier experiments (Walsh et al., 1972a) have demonstrated that hydroxybutynoate irreversibly inactivates the flavine-linked D- and L-lactate dehydrogenases of *E. coli* membranes, blocking transport of rubidium, proline, and lactose. We have extended this study for the transport of other amino acids, including serine in ML 308-225 membrane vesicles. After a 10-min preincubation of vesicles with 1.0 m*M* D,L-2-hydroxy-3-butynoate, D- or L-lactate-driven active transport of [^{14}C]serine is essentially negligible and the blockade persists even after washing the vesicles free of excess HBA.

We have previously estimated with solubilized, partially purified D-lactate dehydrogenase that about 25 product molecules form before the enzyme is inactivated (Scheme II) (Walsh et al., 1972a). To test whether any of these acetylenic keto acid molecules can diffuse to and alkylate any of the phosphotransferase components in the vesicles, experiments on hexose uptake were performed. In a preliminary experiment on the time course of HBA inactivation of hexose uptake, with 500 μM HBA, 50% inhibition of PEP-de-

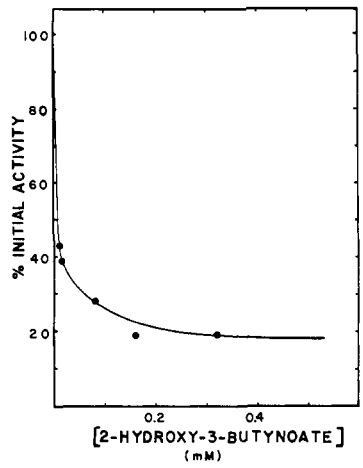


FIGURE 1: Concentration dependence of 2-hydroxy-3-butynoate inactivating [^{14}C]glucose transport in ML 308-225 membrane vesicles. Vesicles (25 μl) (0.125 mg of protein) were incubated at 46° with phosphoenolpyruvate (100 mM), lithium chloride (300 mM), magnesium sulfate (10 mM), various concentrations of HBA, and water to give a final volume of 50 μl. After 10 min of incubation, [^{14}C]glucose was added. Five minutes later, uptake was quenched with 0.5 M lithium chloride and the samples were fast filtered onto membrane filters for counting. The amount of inhibition was determined by comparing glucose uptake in the HBA treated vesicles with 5-min uptake in controls which had not been exposed to the inhibitor.

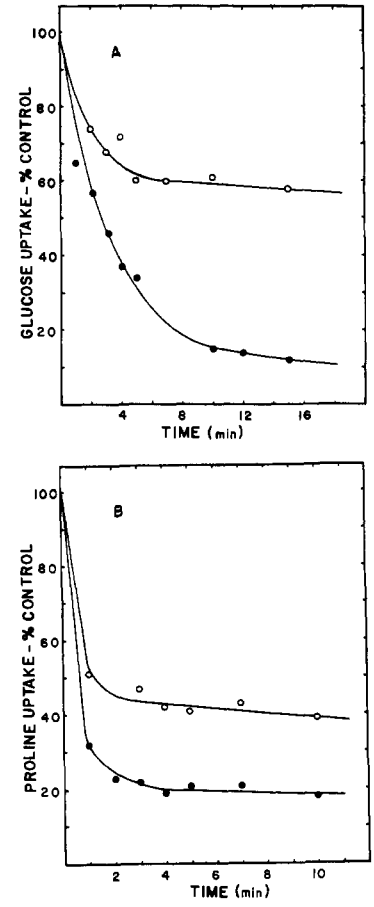


FIGURE 2: The effect of dithiothreitol on the time course of 2-hydroxy-3-butynoate inhibition of glucose and proline uptake. (A) Vesicles (0.25 mg of protein) were exposed to 500 μM HBA for various times during the 10-min incubation with phosphoenolpyruvate and salts at 46°. [^{14}C]Glucose uptake was then measured for 4 min and the amount of inhibition was determined by comparing with untreated controls (●). In a second experiment, the same time course of inactivation was determined except 10 mM dithiothreitol was added 1 min before each addition of HBA (○). (B) Vesicles (0.25 mg of protein) were preincubated with magnesium sulfate (10 mM) and water for 3 min at 25° followed by addition of HBA to 500 μM final concentration. After the appropriate time of exposure to HBA, D-lactate and [^{14}C]proline were added and uptake was quenched after 5 min. Controls were run without exposure to HBA and the amount of inhibition was noted. In a parallel experiment, 10 mM dithiothreitol was added immediately before introduction of HBA and the time course of inactivation was determined as described above.

pendent [^{14}C]glucose transport was achieved in about 2.5 min. Inactivation is irreversible in that it persists after centrifugation and resuspension of vesicles. If the inactivation arises from prior oxidation of HBA to the reactive keto acid by the lactate dehydrogenases, inhibitors of the dehydrogenase activity should diminish the rate of blockade of [^{14}C]glucose transport. Oxamate, a potent competitive inhibitor of D-LDH[1] (K_i = 13 μM) does retard inactivation, slowing down the rate by a factor of 2.4 at saturating concentrations (10 mM). When a concentration dependence for HBA inactivation of PTS-mediated glucose uptake was examined (Figure 1), it was determined that 5 μM external HBA produces 50% loss of PEP-dependent glucose transport. This concentration is comparable to that required for half-maximal blockade of both proline (or serine) transport and D-LDH activity in the vesicles (Walsh et al., 1972a). We have not determined which of the PTS enzyme activities have been inactivated after exposure of the vesicles to HBA, but it is likely that the target is enzyme I, since it is known to contain a reactive sulfhydryl group (Kundig and Roseman, 1971). Furthermore, enzyme I is specifically the activity lost on vinylglycolate-induced blockade of hexose transport (Walsh and Kaback, 1973). Consistent with this is the fact that HBA induces a coordinate rate of transport inactivation for glucose, mannose, and fructose (data not shown), three sugars with a common enzyme I component but separate, specific enzyme II proteins.

We have previously shown that dithiothreitol is an effective protectant against vinylglycolate-generated blockade of PEP-dependent hexose uptake, presumably as a scavenging nucleophile for the reactive ketobutenoate, intercepting it before alkylation reactions with enzyme I and other membrane proteins occurs. The curves of Figure 2A show similar protection by dithiothreitol when hydroxybutynoate is the enzymatic precursor for PTS inactivation. For comparison, Figure 2B shows the effect of dithiothreitol on HBA inactivation of [^{14}C]proline active transport. A degree of protection of about 20% is evident. Relative to Figure 2A, dithiothreitol is comparatively less effective in preventing HBA mediated D- and L-lactate dehydrogenase inactivation and thus offers little protection of amino acid transport capabilities. This distinction is reinforced in reactivation data discussed later and is consistent with the concept that separate mechanisms exist for when HBA produces D-LDH (and respiration-coupled transport) inactivation vs. when HBA induces PTS-mediated hexose transport inactivation. Lactate dehydrogenase inactivation occurs by covalent modification of the FAD at the active site prior to release of inactivator from the enzyme (Short et al., 1974). Thus, external nucleophiles may have limited access to the active site; this lack of access may physically prevent scavenging of the LDH inactivator; conversely, PTS inactivation involves diffusion of keto acid from D-LDH to a nucleophilic target (presumably enzyme I) and can thus be intercepted effectively by high concentrations of low molecular weight thiols.

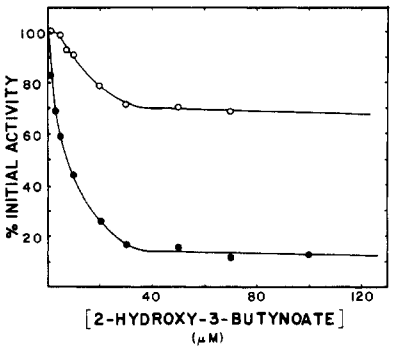


FIGURE 3: Concentration dependence of 2-hydroxy-3-butynoate inactivation of glucose uptake in JSH-2 and JSH-150 membrane vesicles. JSH-2 membranes (0.11 mg of protein) and JSH-150 membranes (0.19 mg of protein) were assayed for 5-min glucose uptake after exposure to various concentrations of HBA for 10 min at 46° as described previously. The inhibition of transport in JSH-2 (●) and JSH-150 (○) was then determined by comparison with glucose uptake in untreated membranes.

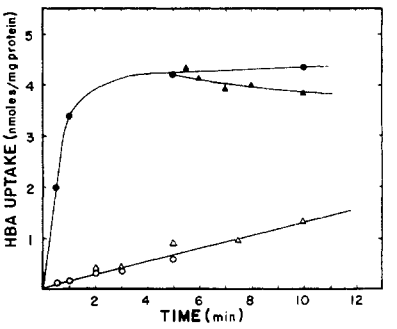


FIGURE 4: The uptake of [^{14}C]-2-hydroxy-3-butynoate by ML 308-225 membrane vesicles. Vesicles (0.12 mg of protein) were incubated with water and magnesium sulfate (10 m*M*) under an oxygen atmosphere for 5 min. Ascorbate and phenazine methosulfate were then added to a final concentration of 20 m*M* and 100 μ*M* followed immediately by the addition of [^{14}C]HBA to give a final total volume of 50 μl. At certain times during the experiment, phospholipase A-B was added at a concentration of 400 μg/ml. The uptake of radioactive substrate was then measured by filter assay in the presence of ascorbate-PMS (●), in vesicles treated with phospholipase 5 min after the addition of energy source and radioactive substrate (▲), after phospholipase treatment prior to the addition of ascorbate-PMS and [^{14}C]HBA (△), and with no energy source (○).

Susceptibility of Glucose Transport to HBA in a Mutant Defective in D- *and* L-*Lactate Dehydrogenases.* We have recently characterized one of several VG-resistant *E. coli* K12 mutants as a mutant defective in membrane bound D- and L-lactate dehydrogenases (Shaw et al., 1975). Isolated membrane vesicles of this mutant, JSH 150, have 2% of the D-LDH and 13% of the L-LDH activity of a parent, JSH 2. As expected, when JSH 2 and 150 membrane vesicles were assayed for sensitivity to blockade of phosphoenolpyruvate-dependent glucose transport by vinylglycolate, at any given concentration of the hydroxy acid the 150 vesicles were more resistant.

When the same experiment was performed with HBA replacing VG, the data of Figure 3 were obtained. In the JSH 2 vesicles, essentially complete blockade of glucose transport can be achieved; 50% inhibition of transport activity is seen with 8 μ*M* HBA. These results are comparable to the data of Figure 1 where ML 308-225 vesicles were used. In JSH 150 vesicles, complete loss of hexose uptake does not occur even at very high HBA concentrations, maximal inhibition remaining constant at 30% loss of activity. This finding implies that insufficient amounts of 2-keto-3-butynoate are generated before the smaller number of functional dehydrogenase molecules in JSH 150 vesicles are inactivated by the suicide substrate.

Active Transport of 1-[^{14}C]HBA and Its Subsequent Fate in Membrane Vesicles. One prediction regarding HBA-induced PTS inactivation is that prior to intravesicular oxidation, the acetylenic hydroxy acid must be transported into vesicles. This view has been substantiated by recent experiments with antibody to homogeneous D-LDH which place the enzyme exclusively on the inner surface of the membrane vesicles (Short et al., 1975).

The transport of 1-[^{14}C]-2-hydroxy-3-butynoate by *E. coli* ML 308-225 vesicles is shown in Figure 4. Uptake can be driven with the artificial electron donor, ascorbate–phenazine methosulfate. One special property of radioactive HBA active transport into vesicles is that unlike normal solutes accumulated, the radioactivity should become covalently incorporated into membrane proteins due to subsequent alkylation by [^{14}C]-2-keto-3-butynoate. Whereas addition of unlabeled proline to vesicles actively transporting [^{14}C]proline will diminish the amount of intravesicular radioactivity due to exchange dilution processes, addition of [^{12}C]HBA does not cause any loss in intravesicular label added as [^{14}C]HBA.

Alternatively, as shown in Figure 4, one can treat vesicles which have accumulated the maximal amount of [^{14}C]HBA with phospholipase A-B which has previously been shown to destroy the membrane diffusion barrier (Kaback, 1972) and cause fast (<1 min) dissipation of normal solute concentration gradients (e.g. proline or glucose 6-phosphate). This treatment does not release more than 10–15% of the intravesicular label (added as [^{14}C]HBA), suggesting the great bulk of radioactivity is covalently bound. If phospholipase treatment of the vesicles precedes [^{14}C]HBA addition, the stimulating effect of ascorbate-phenazine methosulfate is abolished since active transport is no longer possible. The rate of ^{14}C incorporation into membranes under these conditions is controlled by the level of lactate dehydrogenase activity which is unaffected by phospholipase treatment.

As a final index of the covalent labeling which follows 1-[^{14}C]HBA transport and oxidation by the vesicles, the radioactive membranes were dissolved in 1% sodium dodecyl sulfate and placed for 2 min in boiling water; the protein was precipitated with 10% trichloroacetic acid, and then filtered through a Millipore filter. Virtually all of the radioactivity remains with the protein on the filters as indicated in Table I (treatment 1, [^{14}C]HBA labeled membrane).

One might predict that both ketobutynoate and ketobutenoate (derived from HBA and VG dehydrogenation, respectively) alkylate the same population of nucleophilic groups in the membrane. This was corroborated by pretreatment of membrane vesicles with nonradioactive vinylglycolate (4 m*M*), sufficient to titrate PTS inactivation; this treatment reduces the amount of 1-[^{14}C]HBA which can subsequently be incorporated into the membranes by a factor of three (data not shown).

Reactivation of Transport after Vinylglycolate or Hydroxybutynoate Inactivation. We have noted that hydroxybutynoate inactivates both dehydrogenase-coupled active transport and PTS-mediated hexose uptake while vinylglycolate blocks only the latter. Each is a time-dependent, progressive inactivation with covalent modification of membrane proteins. In the introductory statement of this paper

Table I: Stability of Covalent Binding to Sodium Dodecyl Sulfate Solubilized Membranes.[a]

Treatment	[^{14}C]HBA Label Retained (nmol/mg of Protein)	[^{14}C]VG Label Retained (nmol/mg of Protein)
I	4.9	4.5
II	5.1, 4.2	4.8
III	1.1, 1.2	4.7

[a] Membranes (4 mg of membrane protein) were loaded with [^{14}C]-HBA (100 μM) or [^{14}C]VG (100 μM) in the presence of ascorbate–PMS and oxygen for 10 min as described in Figure 5. The uptake was then quenched by diluting the sample with 10 ml of 0.1 M sodium phosphate (pH 6.6) containing 10 mM magnesium sulfate. The vesicles were centrifuged and washed twice with the same buffer. The pellet was resuspended in 0.063 M Tris-HCl (pH 6.8) and sodium dodecyl sulfate was added to a final concentration of 0.2%. The HBA- and VG-labeled vesicles were then subjected to the following treatments: (I) the membrane protein was precipitated with 10% trichloroacetic acid at room temperature and filtered through membrane filters; the filters were washed several times with 10% Cl_3CCOOH; (II) membranes were boiled 2 min prior to the addition of 10% Cl_3CCOOH; they were then subjected to boiling again for 2 min before filtration; (III) membranes were treated with 0.6 M β-mercaptoethanol, boiled for 2 min, treated with 10% Cl_3CCOOH and boiled again for 2 min before filtration. The membrane filters were dried and the amount of ^{14}C labeling was determined by counting the filters.

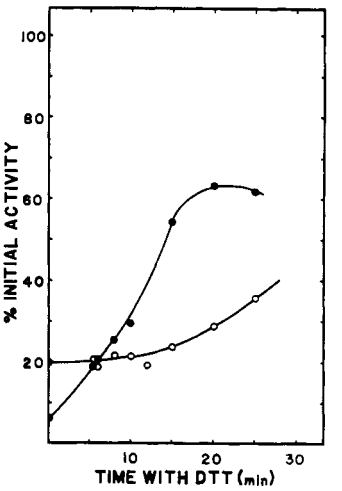


FIGURE 5: Reactivation of HBA and VG inhibited glucose uptake in ML 308-225 vesicles. (A, ●) Vesicles (0.5 ml; 2.5 mg of protein) were incubated with magnesium sulfate (10 mM), HBA (1.5 mM), and water to give a final volume of 1 ml at 46° for 10 min. The membranes were then diluted with 10 ml of cold 0.1 M potassium phosphate (pH 6.6) and centrifuged (4°) at 15,000g for 15 min. The membranes were washed twice by resuspension in phosphate buffer using a wide bore needle and subsequent centrifugation. Finally the membranes were resuspended in 0.1 M potassium phosphate (pH 6.6) to 0.5 ml final volume. Glucose transport was then assayed for a 5-min uptake point as described before. Transport was also assayed after exposing the vesicles to 10 mM dithiothreitol for various times in their incubation medium at 46°. Duplicate samples were assayed, transport measurements averaged, and the percent reactivation was determined by comparing transport with glucose uptake in non-HBA-treated vesicles. (B, ○) In a separate experiment, 0.5 ml of membranes (2.5 mg of protein) was incubated with magnesium sulfate (10 mM), vinylglycolate (25 μM), and water (1 ml total volume) at 25 ° for 10 min. After removing unreacted VG by centrifugation and two washes, the reactivation of glucose uptake in vesicles by 10 mM dithiothreitol was investigated essentially as described above. In these experiments the amount of functional PTS activity remaining after hydroxybutynoate or vinylglycolate treatment was 5 and 20%, respectively, of control, untreated membrane vesicles. The extent of these inactivations is controlled by the concentration of inactivator and time of vesicle exposure in each case.

we noted the structural basis for expecting that different reactivation potentials exist for vesicle transport systems inactivated by HBA vs. VG.

The first experimental index that HBA-induced inactivation might be reversed was derived from attempts at disc gel electrophoresis. Membranes labeled with 1-[^{14}C]HBA were dissolved in sodium dodecyl sulfate and subjected to gel electrophoresis by the method of Ames (1974) for membrane proteins. After radioautography, no radioactive membrane proteins were detected and the label was recovered quantitatively at the dye front. Since the electrophoresis buffers contained β-mercaptoethanol, a good nucleophile, we treated radioactive membranes with 0.6 M β-mercaptoethanol and then followed the protocol of Table I to see how sensitive the covalent membrane label was to a nucleophilic thiol. Line 3 indicates that 75% of the radioactivity is now filterable and indicates that the [^{14}C]HBA-derived covalent membrane label is labile to nucleophiles as proposed in Scheme I. Also in accord with Scheme I is the observation that no such instability is detected when membrane proteins are labeled with 1-[^{14}C]vinylglycolate (Table I).

Next, we determined whether exposure of HBA-inactivated or VG-inactivated vesicles to thiols would regenerate phosphoenolpyruvate-dependent glucose uptake. Figure 5A demonstrates that when HBA-treated membrane vesicles retaining only 5% of control PTS transport capacity were incubated with 10 mM dithiothreitol, the ability to transport glucose was recovered in a time-dependent fashion. A maximum of 63% of the control value was restored within 20 min, representing a 12-fold increase in phosphoenolpyruvate-dependent uptake of glucose. In Figure 5B, this experiment was repeated with vinylglycolate-inactivated vesicles. As shown, incubation with dithiothreitol results in only a small degree of reactivation. When the rate of release of radioactivity from vesicles labeled either from [^{14}C]HBA or [^{14}C]VG is monitored under the experimental conditions of Figure 5, over 50% of the counts from [^{14}C]HBA inactivated vesicles are labilized after 30 min in dithiothreitol while only 17% of the counts from VG-labeled membranes are released. Probably the amount of VG-blocked PTS reactivation and VG-derived radioactivity release observed stems from reverse Michael reaction.

When HBA-inactivated vesicles were examined for reactivation of proline transport in the presence of 10 mM dithiothreitol, no significant reactivation of D-lactate-dependent amino acid uptake was observed. This indicates that thiols cannot release inactivator from the covalent-FAD adduct. Thus, only the PTS transport system can be functionally reactivated after inhibition of D-lactate and phosphoenolpyruvate-dependent transport in bacterial membrane vesicles by hydroxybutynoate.

References

Ames, G. F.-L. (1974), *J. Biol. Chem. 249,* 634.
Cromartie, T., Fisher, J., Kaczorowski, G., Laura, R., Marcotte, P., and Walsh, C. T. (1974), *J. Chem. Soc., Chem. Commun.,* 597.
Cromartie, T., and Walsh, C. T. (1975), *Biochemistry, 14,* 2588.
Davis, B. D., and Mingioli, E. S. (1950), *J. Bacteriol. 60,* 17.
Kaback, H. R. (1971), *Methods Enzymol. 22,* 99.
Kaback, H. R. (1972), *Biochim. Biophys. Acta 265,* 367.
Kaback, H. R. (1974a), *Methods Enzymol. 31,* 698.
Kaback, H. R. (1974b), *Science 186,* 882.
Kohn, L. D., and Kaback, H. R. (1973), *J. Biol. Chem. 248,* 7012.

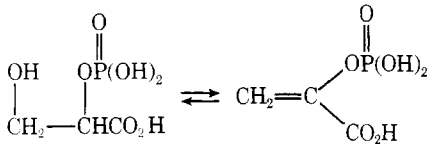
Kundig, E., and Roseman, S. (1971), *J. Biol. Chem. 246,* 1393.
Shaw, L., Grau, F., Kaback, H. R., Hong, J. S., and Walsh, C. T. (1975), *J. Bacteriol. 121,* 1047.
Short, S. A. Kaback, H. R., and Kohn, L. D. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1461.
Short, S. A., Kaback, H. R., and Kohn, L. D. (1975), *J. Biol. Chem. 250,* 4291.
Walsh, C. T., Abeles, R. H., and Kaback, H. R. (1972a), *J. Biol. Chem. 247,* 7858.
Walsh, C. T., and Kaback, H. R. (1973), *J. Biol. Chem. 248,* 5466.
Walsh, C. T., and Kaback, H. R. (1974), *Ann. N.Y. Acad. Sci. 235,* 519.
Walsh, C. T., Schonbrunn, A., Lockridge, O., Massey, V., and Abeles, R. H. (1972b), *J. Biol. Chem. 247,* 6004.

Enolase Catalyzed β,γ-α,β Isomerization of 2-Phospho-3-butenoic Acid to (Z)-Phosphoenol-α-ketobutyrate†

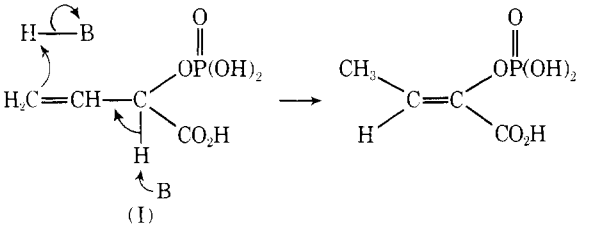
Jonathan Appelbaum and JoAnne Stubbe*

ABSTRACT: 2-Phospho-3-butenoic acid was synthesized and found to be a substrate for both yeast and rabbit muscle enolase (EC 4.2.1.11). Enolase catalyzes the isomerization of 2-phospho-3-butenoic acid to (Z)-phosphoenol-α-ketobutyrate, a β,γ-α,β isomerization. Nuclear magnetic resonance studies on the product indicate only one isomer is formed. This reaction provides indirect evidence in further support of a carbanion intermediate for the enolase reaction. 2-Phospho-3-butenoic acid is also a good competitive inhibitor of both yeast and rabbit muscle pyruvate kinase (EC 2.7.1.40).

Enolase (EC 4.2.1.11, phosphopyruvate hydratase) is a highly specific enzyme catalyzing the dehydration of D-glycerate-2-phosphate (PGA)[1] to phosphoenolpyruvate (PEP):



In connection with our studies on the active site of pyruvate kinase, we prepared a racemic mixture of 2-phospho-3-butenoic acid (I). This compound (I) was found to be a substrate for both yeast and rabbit muscle enolase. Thus enolase catalyzes a β,γ-α,β isomerization: 2-phospho-3-butenoic acid to (Z)-phosphoenol-α-ketobutyrate:



(I)

The equilibrium of this reaction, however, appears to lie very far to the right as no reverse reaction was detectable. Nuclear magnetic resonance (NMR) studies of the product indicate that only one isomer, (Z)-phosphoenol-α-ketobutyrate, is formed during the enolase-catalyzed reaction.

Mechanistic studies to date (Dinovo and Boyer, 1971; Shen and Westhead, 1973; Mildvan et al., 1973) seem to indicate that enolase catalyzes a nonconcerted dehydration reaction. The mechanism probably involves proton abstraction, C-O bond cleavage, and product release, the relative rates being pH dependent.

The mechanism of the β,γ-α,β isomerization probably involves abstraction of the α hydrogen by a base at enolase's active site followed by protonation of the terminal carbon and product release. Thus, our work indirectly supports the partially substantiated hypothesis (Wold, 1971) that there exists a base at enolase's active site which is capable of removing a very nonacidic C-2 proton to form a carbanion intermediate at pH 7.4. The pH studies, furthermore, implicate a histidine as the base which may be involved in proton abstraction.

Material and Methods

Infrared spectra were measured on a Perkin-Elmer infrared spectrophotometer, Model 237. Ultraviolet spectra and kinetic studies were run on a Cary 14. Proton nuclear magnetic resonance spectra were determined on a Perkin-Elmer R-12 using tetramethylsilane or 3-(trimethylsilyl)propanesulfonic acid sodium salt as internal standards. Melting points are uncorrected. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. In studies involving pH changes, measurements were made directly on the reaction mixtures immediately after assay with a Beckman Expandomatic Model SS2 pH meter fitted with an Arthur A. Thomas combined electrode 4094-L15.

† From the Department of Chemistry, Williams College, Williamstown, Massachusetts 01267. *Received March 31, 1975.* This investigation was supported by the National Institutes of Health, General Medical Section, GM 20676-01, and by a grant from the Alfred P. Sloan Foundation to Williams College.

[1] Abbreviations used are: PGA, D-glycerate-2-phosphate; PEP, phosphoenolpyruvate; CH_3-PEP, (Z)-phosphoenol-α-ketobutyrate; CH_2-PEP, α-(dihydroxyphosphinylmethyl)acrylic acid; F-PEP, (Z)-phosphoenol-3-fluoropyruvate; DSS, sodium 3-(trimethylsilyl)propanesulfonic acid.