The Role of Functional Sulfhydryl Groups in Active Transport in *Escherichia coli* Membrane Vesicles†

H. Ronald Kaback* and Lekha Patel

ABSTRACT: Diazinedicarboxylic acid bis(*N,N*-dimethylamide) (diamide) is an inhibitor of lactose and proline transport in membrane vesicles isolated from *Escherichia coli* ML 308-225. However, the compound has no effect on D-lactate or reduced phenazine methosulfate oxidation or on the generation of the proton electrochemical gradient in the presence of these electron donors. *N*-Ethylmaleimide also inactivates lactose and proline transport but, at the same time, blocks D-lactate oxidation and the generation of the proton electrochemical gradient in the presence of this electron donor. Strikingly, however, oxidation of reduced phenazine methosulfate and the generation of the proton electrochemical gradient in the presence of the electron carrier are completely resistant to *N*-ethylmaleimide. These and other findings lead to the following conclusions: (i) there is a sulfhydryl-containing respiratory intermediate involved in D-lactate oxidation that is independent of D-lactate dehydrogenase; (ii) reduced phenazine methosulfate interacts with the respiratory chain at a site which is distal to this sulfhydryl-containing intermediate; and (iii) it is unlikely that sulfhydryl groups play a functional role in the generation of the proton electrochemical gradient. Evidence is presented indicating that diamide affects the lactose and proline porters directly, probably altering the same sulfhydryl groups that are alkylated by *N*-ethylmaleimide. Importantly, however, although *N*-ethylmaleimide inhibits all of the transport systems tested with D-lactate as electron donor, the alkylating agent does not inhibit the transport of glycine, tyrosine, glutamate, lysine, leucine, or succinate with reduced phenazine methosulfate as electron donor. Moreover, these transport systems are not inhibited by diamide in the presence of D-lactate. It is apparent therefore that the catalytic activity of a significant number of porters is not dependent upon functional sulfhydryl groups.

Bacterial cytoplasmic membrane vesicles retain the same polarity as the membrane in the intact cell, are essentially devoid of cytoplasmic constituents, and catalyze active transport of many solutes by a respiration-dependent mechanism that does not involve the generation or utilization of ATP or other high-energy phosphate intermediates (Kaback, 1971, 1972, 1974a, 1976). In vesicles prepared from aerobically-grown *Escherichia coli* and *Salmonella typhimurium*, most active-transport systems are driven by the oxidation of D-lactate or reduced phenazine methosulfate (PMS)[1] via a membrane-bound respiratory chain with oxygen as the terminal acceptor. Alternatively, fumarate or nitrate can function as terminal electron acceptors, provided the vesicles are prepared appropriately from cells grown under specified conditions (Konings and Boonstra, 1977).

In 1971, Kaback and Barnes presented a model for active transport in which the solute-specific components (i.e., the carriers or porters) of a number of transport systems were depicted as electron-transfer intermediates in which reduction of a critical disulfide results in translocation of the carrier-substrate complex to the inner surface of the membrane with a concomitant decrease in the affinity of the carrier for substrate. This model was posed as a hypothesis that could account for essentially two sets of observations: (i) D-lactate oxidation and all translocational properties of certain porters are simultaneously inactivated by various sulfhydryl reagents, and (ii) only those electron-transfer inhibitors that block electron flow at or after cytochrome b_1 cause efflux of accumulated solutes. A very different hypothesis, one that emphasizes the generation of an electrochemical gradient of protons ($\Delta\bar{\mu}_{H^+}$) as the driving force for active transport, was proposed by Mitchell (1961, 1966, 1968, 1973; Harold, 1972). According to this so-called "chemiosmotic hypothesis", $\Delta\bar{\mu}_{H^+}$ is generated through the asymmetric release of protons from the surface of the membrane during substrate oxidation via the membrane-bound respiratory chain or hydrolysis of ATP by the membrane-bound Ca^{2+},Mg^{2+}-stimulated ATPase and is composed of electrical and chemical parameters according to the following relationship:

$$\Delta\bar{\mu}_{H^+} = \Delta\Psi - \left(\frac{2.3RT}{F}\right)\Delta pH \qquad (1)$$

where $\Delta\Psi$ represents the electrical potential across the membrane and ΔpH is the chemical difference in proton concentrations across the membrane ($2.3RT/F$ = 58.8 mV at room temperature). Over the past few years, it has become decisively clear that *E. coli* and *S. typhimurium* membrane vesicles generate a $\Delta\bar{\mu}_{H^+}$ of considerable magnitude during the oxidation of certain substrates and that $\Delta\bar{\mu}_{H^+}$ or one of its components is the immediate driving force for the translocation and accumulation of numerous solutes (Ramos et al., 1976; Ramos and Kaback, 1977a,b,c; Tokuda and Kaback, 1977).

Despite overwhelming experimental support for the role of chemiosmotic phenomena in the energetics and mechanism of active transport, some of the earlier observations which led to the Kaback and Barnes model (1971) have not been completely resolved, primarily because D-lactate oxidation and transport are inactivated concomitantly by most sulfhydryl reagents. In 1973, however, Kaback and Hong discussed preliminary findings which indicated that diazenedicarboxylic

† From the Laboratory of Membrane Biochemistry, Roche Institute of Molecular Biology, Nutley, New Jersey 07110. *Received December 2, 1977.*

[1] Abbreviations used are: PMS, phenazine methosulfate; diamide, diazenedicarboxylic acid bis(*N,N*-dimethylamide); NEM, *N*-ethylmaleimide; *p*-HMB, *p*-hydroxymercuribenzoate; *p*-HMBS, *p*-hydroxymercuribenzenesulfonate.

0006-2960/78/0417-1640$01.00/0

acid bis(*N,N*-dimethylamide) (diamide) inhibits lactose transport without affecting D-lactate oxidation. Diamide was introduced by Kosower et al. (1969) as a reagent which purportedly catalyzes the oxidation of intracellular glutathione specifically. More recent experiments have demonstrated, to the contrary, that the compound is not specific for glutathione and that it catalyzes the oxidation of a variety of natural electron donors (O'Brien et al., 1970; Harris and Biaglow, 1972; Kosower et al., 1972). In this paper, the effects of diamide and other sulfhydryl reagents on D-lactate and reduced PMS oxidation, the generation of $\Delta\bar{\mu}_{H^+}$, and active transport are described.

Experimental Section

Methods

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+z^+$) was grown on minimal medium A (Davis and Mingioli, 1950) containing 1.0% disodium succinate (hexahydrate). Membrane vesicles were prepared from these cells as described previously (Kaback, 1971; Short et al., 1975).

Transport Assays. Transport of radioactive solutes by membrane vesicles was measured by filtration as described elsewhere (Kaback, 1974b). Since reduced PMS is oxidized by diamide, in all experiments where diamide was used in conjunction with this electron donor the vesicles were first treated with diamide, centrifuged, and washed free of excess reagent as described.

Determination of ΔpH. ΔpH was determined by assaying the accumulation of [1,2-^{14}C]acetate using flow dialysis (Ramos et al., 1976, 1978; Ramos and Kaback, 1977a,b,c; Tokuda and Kaback, 1977), and the increase in external pH induced by the oxidation of ascorbate was taken into account in the calculations (Ramos and Kaback, 1977a; Ramos et al., 1978).

Determination of ΔΨ. The electrical potential across the membrane (ΔΨ) was determined by measuring the accumulation of [^{3}H]triphenylmethylphosphonium ($TPMP^+$) (bromide salt) using filtration (Schuldiner and Kaback, 1975) and flow dialysis (Ramos et al., 1976, 1978; Ramos and Kaback, 1977a,b,c; Tokuda and Kaback, 1977).

Oxygen Consumption. Rates of oxygen uptake were measured with a Clark electrode (YSI Model 53 oxygen monitor) as described previously (Barnes and Kaback, 1971).

Materials

Diamide was obtained from Calbiochem, [1-^{14}C]lactose was from Amersham-Searle, and radioactive amino acids and *N*-[^{14}C]ethylmaleimide (NEM) were from New England Nuclear. [^{3}H]Triphenylmethylphosphonium bromide was prepared by the Isotope Synthesis Group at Hoffmann-La Roche, Inc., under the direction of Dr. Arnold Liebman as described (Schuldiner and Kaback, 1975). All other materials were reagent grade obtained from commercial sources.

Results

Effect of Diamide on Lactose and Proline Transport. Diamide is a potent inhibitor of lactose transport with D-lactate as electron donor (Figure 1A). In the experiment shown, vesicles were incubated with given concentrations of diamide for 15 min and then assayed for lactose uptake. Under these conditions, half-maximal inhibition is observed at approximately 1.0 mM diamide, and inhibition is essentially complete at 10 mM and above. Similar results were obtained when vesicles

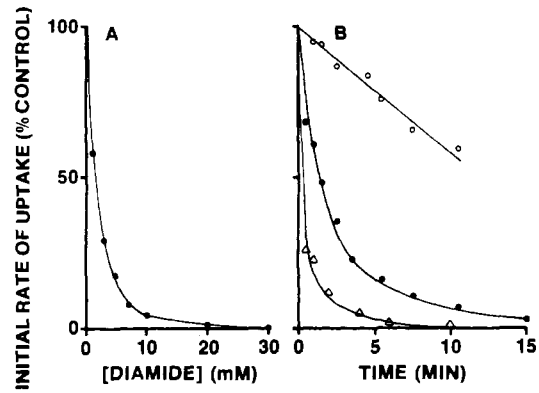


FIGURE 1: Inhibition of transport by diamide. (A) Initial rate of lactose transport vs. diamide concentration. Aliquots (25 μL) of membrane vesicles containing about 0.1 mg of membrane protein were diluted to a final volume of 50 μL containing, in final concentrations, 0.05 M potassium phosphate (pH 6.6), 0.01 M magnesium sulfate, and given concentrations of diamide. The samples were incubated at 25 °C for 15 min when lithium D-lactate (20 mM, final concentration) was added, followed immediately thereafter by addition of [1-^{14}C]lactose (14.9 mCi/mmol) to a final concentration of 0.4 mM. After 30 s, the incubations were terminated and samples assayed as described (Kaback, 1974b). Results are presented as a percentage of control values obtained with samples incubated in the absence of diamide. The control value was 26.7 nmol (mg of membrane protein)$^{-1}$ 30 s^{-1}. (B) Time course of diamide inhibition of lactose and proline transport. Aliquots (25 μL) of membrane vesicles containing about 0.1 mg of membrane protein were diluted to a final volume of 50 μL containing, in final concentrations, 0.05 M potassium phosphate (pH 6.6), 0.01 M magnesium sulfate, and 10 (Δ) or 20 mM (○,●) diamide. The samples were incubated at 0 or 25 °C for the times shown and lithium D-lactate and [1-^{14}C]lactose (14.9 mCi/mmol) or L-[U-^{14}C]proline (214 mCi/mmol) were added to final concentrations of 20 and 0.4 mM or 9.4 μM, respectively (samples incubated at 0 °C were transferred to 25 °C immediately after addition of D-lactate and [1-^{14}C]lactose). Incubations were continued at 25 °C for 30 s and terminated, and the samples were assayed as described (Kaback, 1974b). Results are presented as a percentage of control values obtained with samples incubated in the absence of diamide. The control values were [in nmol (mg of membrane protein)$^{-1}$ 30 s^{-1}] 13.2 (lactose uptake by control sample incubated at 0 °C), 21.5 (lactose uptake by control sample incubated at 25 °C), and 1.83 (proline uptake by control sample incubated at 25 °C). (○–○) Lactose transport after incubation with 20 mM diamide at 0 °C; (●–●) lactose transport after incubation with 20 mM diamide at 25 °C; (Δ–Δ) proline transport after incubation with 10 mM diamide at 25 °C.

were treated with various concentrations of diamide, washed, and assayed with ascorbate and PMS as the electron donor system (data not shown). It is also noteworthy that the compound is effective at considerably lower concentrations when the vesicles are incubated with diamide for longer periods of time (cf. Figure 3).

At 25 °C in the presence of 20 mM diamide, 50% inactivation of lactose transport is observed in about 1.5 min and over 95% inactivation in 15 min (Figure 1B). On the other hand, when incubation with diamide is carried out at 0 °C, the rate of inactivation is markedly diminished. In contrast to the *lac* transport system, proline transport is significantly more sensitive to diamide, as evidenced by the observation that transport of this amino acid is inactivated by 50% on exposure to 10 mM diamide for less than 30 s.

In contrast to sulfhydryl reagents such as NEM, *p*-hydroxymercuribenzoate (*p*-HMB), and *p*-hydroxymercuribenzenesulfonate (*p*-HMBS) which inactivate both D-lactate oxidation and transport (Barnes and Kaback, 1971; Kaback and Barnes, 1971; Kerwar et al., 1972; Gordon et al., 1972; Lombardi and Kaback, 1972), diamide has essentially no effect on D-lactate oxidation or on the ability of NEM (Table I) or *p*-HMBS (not shown) to block D-lactate oxidation. Importantly, moreover, neither NEM nor *p*-HMBS inhibits the

TABLE I: Effect of Diamide and NEM on D-Lactate Oxidation.

Additions	Oxygen uptake[a] [ng of atoms min^{-1} (mg of protein)$^{-1}$]
None	198
10 mM Diamide[b]	183 (92%)
1 mM NEM[b]	49 (25%)
10 mM Diamide[b] + 1 mM NEM[b]	46 (25%)

[a] Oxygen uptake was measured with a Clark oxygen electrode as described previously (Barnes and Kaback, 1971). [b] Vesicles were incubated with inhibitors for 15 min prior to measurement of oxygen uptake. Values given in parentheses represent the percentage of the appropriate control value. Although not shown, similar results were obtained when the conversion of D-[U-^{14}C]lactate to pyruvate was measured by thin-layer chromatography as described by Kaback and Milner (1970).

TABLE II: Effect of Diamide and NEM on $\Delta\bar{\mu}_{H^+}$ Generated in the Presence of D-Lactate or Reduced PMS.

Electron donor	Additions	ΔpH[a] (mV)	$\Delta\Psi$[b] (mV)
D-Lactate	None	Nd	−72
D-Lactate	20 mM Diamide[c]	Nd	−80
D-Lactate	1 mM NEM[c]	Nd	−40
Reduced PMS	None	−82	−105
Reduced PMS	Diamide treated[d]	−72	−107
Reduced PMS	Diamide treated[d] + 1 mM NEM[c]	−72	−97

[a] ΔpH was determined from acetate distribution measurements at pH 6.6 using flow dialysis as described under Methods. [b] $\Delta\Psi$ was determined from $TPMP^+$ distribution measurements at pH 6.6 using flow dialysis and filtration as described under Methods. [c] The vesicles were incubated with the inhibitor for 15 min prior to the measurements. [d] Vesicles were treated with 20 mM diamide, centrifuged, washed, and resuspended as described in Figure 4. [e] Nd, not determined.

oxidation of reduced PMS to any extent whatsoever (data not shown). These observations are consistent with the suggestions that there is a sulfhydryl group(s) required for D-lactate oxidation which is blocked by NEM and *p*-HMBS but not by diamide and that reduced PMS interacts with the respiratory chain at a site distal to this sulfhydryl group(s). It is also clear from the data presented in Table II that diamide does not impair the generation of $\Delta\bar{\mu}_{H^+}$ in the presence of reduced PMS or the generation of $\Delta\Psi$ in the presence of D-lactate. On the other hand, NEM inhibits the generation of $\Delta\Psi$ in the presence of D-lactate,[2] but has no significant effect in the presence of reduced PMS. The results taken as a whole indicate that diamide inhibits lactose and proline transport by inactivating solute translocation without altering respiration or the generation of $\Delta\bar{\mu}_{H^+}$. Further evidence supporting this contention will be presented. In addition, the data indicate that the sulfhydryl group(s) involved in D-lactate oxidation is proximal to the site in the respiratory chain at which $\Delta\bar{\mu}_{H^+}$ is generated.

TDG Protection against Diamide Inhibition. β-D-Galactosyl-1-thio-β-D-galactopyranoside (TDG) and melibiose partially protect the *lac* carrier protein (i.e., M protein) from inactivation by NEM, and this property of the system has allowed the labeling, solubilization, and partial purification of the *lac y* gene product (Fox and Kennedy, 1965; Kennedy, 1970). As shown by the experiments presented in Figure 2, TDG protects the *lac* transport system against diamide inactivation at least as well as it protects against NEM inactivation. These findings provide direct evidence for the argument that diamide inactivates lactose transport at the level of the *lac* carrier protein. Although not shown, inactivation of proline transport by NEM or diamide is not affected by the presence of substrate.

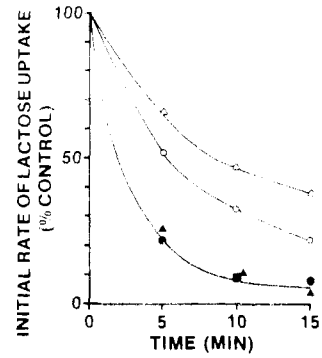


FIGURE 2: TDG protection against NEM and diamide inactivation of lactose transport. Aliquots (100 μL) of membrane vesicles containing approximately 0.8 mg of membrane protein were diluted to a final volume of 200 μL containing, in final concentrations, 0.05 M potassium phosphate (pH 6.6) and 0.01 M magnesium sulfate. In addition, designated samples contained the following additions (in final concentrations): (●–●) 0.5 mM NEM; (○–○) 0.5 mM NEM plus 0.25 mM TDG; (▲–▲) 20 mM diamide; (△–△) 20 mM diamide plus 0.25 mM TDG. These samples and control samples to which NEM, diamide, and TDG were not added were incubated at 25 °C for given periods of time when 5 mL of cold 0.05 M potassium phosphate (pH 6.6) containing 0.01 M magnesium sulfate was added. The samples were then centrifuged at about 40 000*g* for 30 min and the pellets resuspended in 180 μL of 0.05 M potassium phosphate (pH 6.6) containing 0.01 M magnesium sulfate. Initial rates of lactose transport were then assayed with ascorbate and phenazine methosulfate as electron donor as described in Figure 1 and Methods. Control samples incubated without diamide or NEM for 5, 10, and 15 min, respectively, took up 23.3, 23.3, and 26.0 nmol of lactose per mg of membrane protein. Results are presented as a percentage of the control values.

Effect of Diamide on NEM Reactivity. In the experiments shown in Figure 3, vesicles were treated with diamide for approximately 14 h in the cold and then assayed for reactivity with [^{14}C]NEM. Under these conditions, the ability of the sulfhydryl reagent to alkylate the vesicles decreases sharply at diamide concentrations ranging from 0.01 to about 0.1 mM, where 65–70% inhibition is observed. At higher diamide concentrations, inhibition of NEM reactivity becomes progressively more severe, but it is apparent that diamide concentrations in excess of 1.0 mM are required for complete inhibition. Although lactose transport responds similarly to diamide under these conditions, transport is less sensitive to diamide inactivation over the lower range of concentrations tested. It should be noted that under these conditions 50% inactivation of transport is achieved at about 0.1 mM diamide rather than 1.0 mM as obtained during short-term treatment with the inactivator (cf. Figure 1A).

Reversal of Diamide Inactivation. When membrane vesicles are treated with 10 mM diamide and washed free of excess reagent, both lactose transport (Figure 4A) and NEM reactivity (Figure 4B) are dramatically diminished relative to untreated control samples. If the diamide-inactivated samples are then exposed to dithiothreitol, lactose transport is reactivated to about 50–60% of the control level (Figure 4A) and almost twice as much NEM reactivity is observed relative to control vesicles which were not treated with diamide (Figure

[2] The decrease in $\Delta\Psi$ from −72 to −40 mV in the presence of NEM represents a 3.4-fold decrease in the concentration gradient observed with $TPMP^+$ (i.e., from 17.4 in the control to 5.1 in the NEM-treated sample).

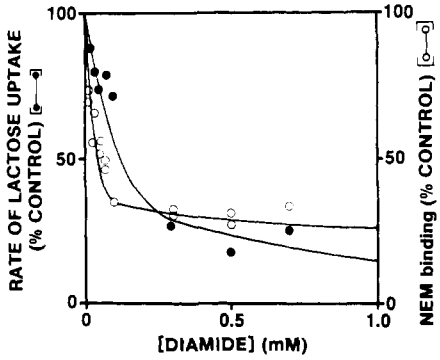


FIGURE 3: Effect of diamide on lactose transport and NEM reactivity. Aliquots (25 μL) of membrane vesicles containing about 0.2 mg of membrane protein were diluted to a final volume of 50 μL containing, in final concentrations, 0.05 M potassium phosphate (pH 6.6), 0.01 M magnesium sulfate, and given concentrations of diamide. The samples were then incubated at 4 °C for approximately 14 h. At this time, samples were transferred to 25 °C and assayed for lactose transport in the presence of D-lactate as described in Figure 1 (●-●). Alternatively, samples were incubated with [^{14}C]NEM (1.84 mCi/mmol) at a final concentration of 1.0 mM for 15 min (○-○) as described by Kaback et al. (1974). Data are presented as a percentage of control values obtained with samples incubated in the absence of diamide. The control values were (in nmol per mg of membrane protein) 18.6 for lactose transport and 10.8 for NEM binding.

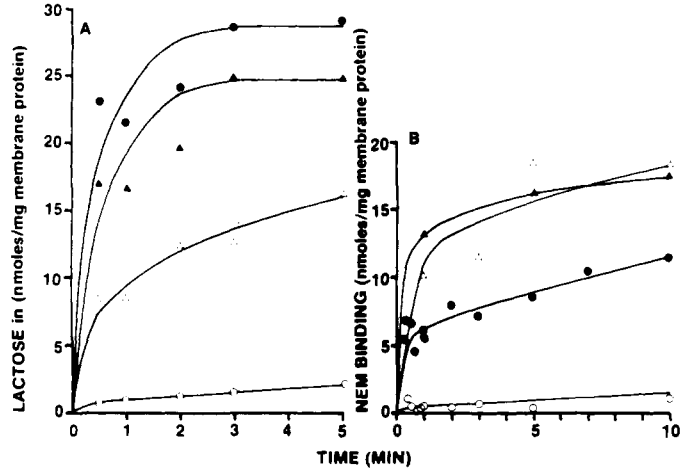


FIGURE 4: Effect of dithiothreitol on lactose transport and NEM reactivity in diamide-treated vesicles. Two aliquots (1.0 mL each) of membrane vesicles containing about 8.0 mg of membrane protein were diluted to a final volume of 2.0 mL containing, in final concentrations, 0.05 M potassium phosphate (pH 6.6) and 0.01 M magnesium sulfate. Diamide was added to one sample to a final concentration of 10 mM, and both samples were incubated at 25 °C for 15 min. Ten milliliters of cold 0.05 M potassium phosphate (pH 6.6) containing 0.01 M magnesium sulfate was added and the samples were centrifuged at about 40 000g for 30 min. The pellets were resuspended and washed once in 0.05 M potassium phosphate (pH 6.6) containing 0.01 M magnesium sulfate and resuspended in the same salts to a final concentration of approximately 6.0 mg of membrane protein/mL. Samples (50 μL) were then assayed for lactose uptake with D-lactate as electron donor (A) and [^{14}C]NEM reactivity (B) as described in Figures 1 and 3, respectively. Dithiothreitol was added to the remaining control and diamide-treated membrane suspensions to a final concentration of 10 mM, and they were incubated at 25 °C for 15 min. The suspensions were diluted fivefold with cold 0.05 M potassium phosphate (pH 6.6) containing 0.01 M magnesium sulfate, centrifuged at 40 000g for 30 min; the pellets were washed once and resuspended in the same salts to a concentration of about 6.0 mg of protein/mL. Samples (50 μL) of each suspension were assayed for lactose uptake with D-lactate as electron donor (A) and [^{14}C]NEM reactivity (B) as described in Figures 1 and 3, respectively. (●-●) Untreated control samples; (▲-▲) samples treated with dithiothreitol; (○-○) samples treated with diamide; (△-△) samples treated with diamide and then dithiothreitol.

4B). Interestingly, exposure of untreated vesicles to dithiothreitol also results in a significant increase in NEM reactivity (Figure 4B), suggesting that some of the sulfhydryl groups in the native vesicles are not available for alkylation because they are presumably in the disulfide configuration. In any case, these cryptic reactive groups are probably unrelated to lactose transport because treatment of the control vesicles with dithiothreitol does not stimulate either the rate or extent of lactose transport (Figure 4A). To the contrary, slight inhibition is observed.

Diamide Protection against NEM Inactivation. If diamide reversibly blocks the same sulfhydryl group(s) in the lactose and proline porters which react irreversibly with NEM, diamide treatment should protect these porters against NEM inactivation. When vesicles are treated with NEM alone, both lactose (Figure 5A) and proline transport (Figure 5B) are severely inhibited (bar 1) and subsequent treatment with dithiothreitol produces insignificant reactivation (bar 2). In confirmation of previous findings (cf. Figures 1 and 4), diamide treatment also markedly inhibits lactose (A) and proline transport (B), and the effect is largely reversed by subsequent exposure to dithiothreitol (bars 3 and 4, respectively). It is significant in this regard that proline transport is reactivated to approximately 90% of the control value (B), while lactose transport is reactivated to only about 55% of the control (A; cf. Figure 4 in addition). As expected, treatment of the vesicles with diamide and then NEM virtually abolishes transport (bar 5 in A and B). However, when these vesicles are exposed to dithiothreitol, lactose (A) and proline transport (B) are reactivated to 20% and 83% of the control values, respectively (bar 6). Thus, although the results are more clear-cut with proline than lactose, it seems reasonable to conclude that diamide and NEM modify the same reactive sites in these porters.

NEM Inactivation of Active Transport. Although D-lactate-dependent accumulation of many solutes is inhibited by NEM, *p*-HMB, and *p*-HMBS (Kaback and Barnes, 1971; Kerwar et al., 1972; Gordon et al., 1972; Lombardi and Kaback, 1972), as noted by Kaback and Barnes (1971), this observation does not provide unequivocal evidence that the porters require functional sulfhydryl groups, because D-lactate oxidation is also inhibited by these reagents. Moreover, in light of some of the observations presented above, the possibility arises that inhibition of some of these transport systems may have been due primarily to inhibition of D-lactate oxidation rather than solute translocation. In the experiments presented in Table III, vesicles were incubated with NEM and then assayed for transport of a number of solutes in the presence of D-lactate or reduced PMS. It is evident that the transport systems tested fall into two groups: (i) those that are inhibited by NEM regardless of the electron donor utilized (i.e., lactose, proline, tyrosine, and serine), and (ii) those that are inhibited by NEM in the presence of D-lactate but not in the presence of reduced PMS (i.e., glycine, glutamate, lysine, leucine, and succinate). In addition, it is noteworthy that transport of the latter group of solutes is not inhibited by diamide when D-lactate is used as electron donor (data not shown). The results provide a strong indication that not all porters are dependent upon functional sulfhydryl groups for activity.

Discussion

The findings presented here are consistent with the simplistic diagram presented in Figure 6. Although obviously incomplete, the respiratory chain is depicted as a series of coupled reactions that lead to the generation of $\Delta\bar{\mu}_{H^+}$ in accordance with the chemiosmotic hypothesis (Mitchell, 1961, 1966, 1968, 1973;

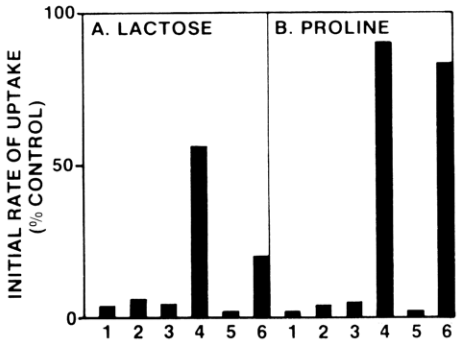


FIGURE 5: Diamide protection of lactose (A) and proline (B) transport against NEM inactivation. Aliquots (250 μL) of membrane vesicles containing approximately 1.0 mg of membrane protein were diluted to a final volume of 500 μL containing, in final concentrations, 0.05 M potassium phosphate (pH 6.6) and 0.01 M magnesium sulfate. As indicated below, diamide was added to some of the samples to a final concentration of 20 mM. The samples were incubated to 25 °C for 15 min when 10 mL of cold 0.05 M potassium phosphate (pH 6.6) containing 0.01 M magnesium sulfate was added. The suspensions were then centrifuged at about 40 000*g* for 30 min and the pellets resuspended in 500 μL of the same salts. As indicated below, NEM was added to some of the samples to a final concentration of 1.0 mM, and the samples were incubated at 25 °C for 15 min. At this time, dithiothreitol was added to some of the samples to a final concentration of 20 mM, and incubation was continued at 25 °C for another 15 min. The samples were then diluted with 10 mL of cold 0.05 M potassium phosphate (pH 6.6) containing 0.01 M magnesium sulfate and centrifuged at 40 000*g* for 30 min, and the pellets were resuspended in 450 μL of the same salt solution. Initial rates of [1-^{14}C]lactose and [U-^{14}C]proline uptake were assayed at 30 s with ascorbate and phenazine methosulfate as electron donor as described in Figure 1. 1, samples incubated with NEM alone; 2, samples incubated with NEM and subsequently treated with dithiothreitol; 3, samples incubated with diamide alone; 4, samples incubated with diamide and subsequently treated with dithiothreitol; 5, samples incubated with diamide and subsequently with NEM; 6, samples incubated with diamide and subsequently with NEM and then dithiothreitol. Data are presented as percentages of control samples which were treated in identical fashion as the experimental samples, except for the omission of NEM, diamide, and dithiothreitol. Control values were 53.0 and 2.8 nmol (mg of membrane protein)$^{-1}$ 30 s^{-1} for lactose and proline, respectively.

TABLE III: NEM Inhibition of Transport with D-Lactate or Reduced PMS as Electron Donors.[b]

Transport substrate	NEM inhibition (% control)	
	D-Lactate	Reduced PMS
Lactose	0	0
Proline	2	1
Serine[a]	47	30
Glycine	37	104
Tyrosine	5	13
Glutamate	43	108
Lysine	45	96
Leucine	46	97
Succinate	34	100

[a] Samples tested for serine transport were incubated with NEM for 30 min prior to assay. [b] Aliquots (25 μL) of membrane vesicles containing about 0.1 mg of membrane protein were diluted to a final volume of 50 μL containing, in final concentrations, 0.05 M potassium phosphate (pH 6.6) and 0.01 M magnesium sulfate. The samples were incubated at 25 °C for 1–2 min, at which time NEM was added to a final concentration of 1.0 mM. Incubation was continued for 1, 3, 5, 10, and 15 min when D-lactate or ascorbate and PMS were added to final concentrations of 20, 20, and 0.1 mM, respectively. Immediately after the addition of electron donor, radioactive transport substrates were added at the following specific activities and final concentrations: [1-^{14}C]lactose (20 mCi/mmol) at 0.4 mM; [U-^{14}C]proline (250 mCi/mmol) at 8 μM; [U-^{14}C]serine (156 mCi/mmol) at 13 μM; [U-^{14}C]glycine (42 mCi/mmol) at 22 μM; [U-^{14}C]tyrosine (404 mCi/mmol) at 3.6 μM; [U-^{14}C]glutamate (229 mCi/mmol) at 17 μM; [U-^{14}C]lysine (318 mCi/mmol) at 6 μM; [U-^{14}C]leucine (285 mCi/mmol) at 14 μM; and [2,3-^{14}C]succinate (5.2 mCi/mmol) at 0.1 mM. After 30 s, the reactions were terminated and the samples assayed as described (Kaback, 1974b). Results obtained after a 15-min incubation with NEM are given as a percentage of control samples incubated under identical conditions, except for the omission of NEM. Control values were as follows [in nmol (mg of membrane protein)$^{-1}$ 30 s^{-1} in the presence of D-lactate and reduced PMS, respectively]: lactose, 11.5 and 21.8; proline, 0.6 and 1.6; serine, 1.0 and 2.2; glycine, 1.2 and 2.9; tyrosine, 0.4 and 1.0; glutamate, 0.2 and 0.6; lysine, 0.6 and 0.8; leucine, 0.2 and 0.3; and succinate, 0.5 and 5.8.

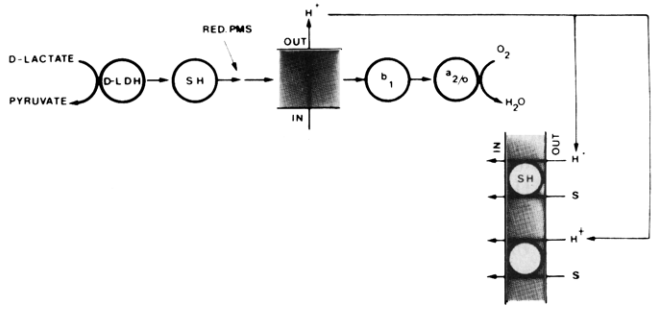


FIGURE 6: Schematic representation of the sequence of electron transfer from D-lactate to oxygen showing the site of interaction of reduced PMS (RED. PMS) with the respiratory chain and the site at which $\Delta\bar{\mu}_{H^+}$ is generated. Abbreviations used are: D-LDH, D-lactate dehydrogenase; SH, functional sulfhydryl group; b_1, cytochrome b_1; a_2/o, cytochromes a_2 and o. Substrate-specific porters which catalyze active transport in response to $\Delta\bar{\mu}_{H^+}$ (lower right) are spatially separate from the respiratory chain and may or may not require functional sulfhydryl groups (see text): S, substrate.

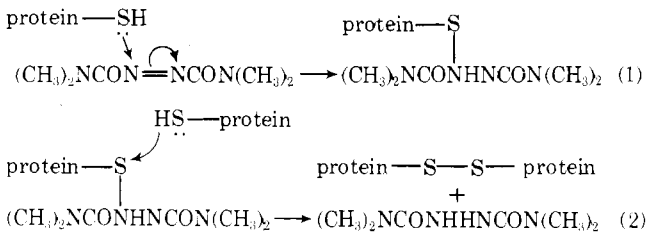
Harold, 1972). In addition, as opposed to an earlier model proposed by Kaback and Barnes (1971), the solute-specific porters which catalyze active transport in response to $\Delta\bar{\mu}_{H^+}$ are spatially separate from the respiratory chain, a contention that is consistent with certain direct observations (Short et al., 1974). Despite a lack of precise information regarding the chemical nature, number, order, and topographical arrangement of the respiratory intermediates within the matrix of the membrane, to say nothing of the mechanism of proton extrusion, certain noteworthy points have been documented elsewhere: (i) oxidation of D-lactate or reduced PMS from either side of the membrane is able to generate a $\Delta\bar{\mu}_{H^+}$ of the same polarity (interior negative and alkaline) (Short et al., 1975; S. Ramos and H. R. Kaback, unpublished information); (ii) although ubiquinone-8 is an important respiratory intermediate in *E. coli*, it is probably not completely mobile within the membrane (Stroobant and Kaback, 1975; P. Stroobant and H. R. Kaback, in preparation); and (iii) the site at which $\Delta\bar{\mu}_{H^+}$ is generated in vesicles prepared from aerobically grown *E. coli* is located prior to the cytochromes (Barnes and Kaback, 1971; Kaback and Barnes, 1971; Stroobant and Kaback, 1975).

When considered in conjunction with some of these points, the results presented here lead to important conclusions regarding the role of functional sulfhydryl groups in respiration, the generation of $\Delta\bar{\mu}_{H^+}$, and carrier function. Although D-lactate oxidation is inhibited by various sulfhydryl reagents, the activity of purified homogeneous D-lactate dehydrogenase is unaffected by these reagents (Kohn and Kaback, 1973). Moreover, treatment of the solubilized, purified enzyme with NEM does not affect its ability to reconstitute vesicles prepared from a mutant devoid of D-lactate dehydrogenase activity (G. Kaczorowski and H. R. Kaback, unpublished information). Thus, a sulfhydryl-containing respiratory component that is independent of the primary dehydrogenase is involved in D-

lactate oxidation. Since oxidation of reduced PMS is totally unaffected by sulfhydryl reagents, this exogenous electron carrier must interact with the respiratory chain at a site that is distal to the sulfhydryl-containing component involved in D-lactate oxidation. In addition, it has been demonstrated that generation of $\Delta\Psi$ during D-lactate oxidation is inhibited by NEM and *p*-HMBS but that these reagents have no effect on the generation of $\Delta\bar{\mu}_{H^+}$ in the presence of reduced PMS. It seems unlikely therefore that sulfhydryl groups play a functional role in the mechanism by which $\Delta\bar{\mu}_{H^+}$ is generated.

Diamide does not block D-lactate or reduced PMS oxidation or the generation of $\Delta\bar{\mu}_{H^+}$ in the presence of these electron donors; however, it is a potent inhibitor of lactose and proline transport. Various lines of evidence indicate that diamide affects the lactose and proline porters directly, probably altering the same sulfhydryl group(s) that are alkylated by NEM: (i) TDG, a high-affinity substrate for the *lac* transport system, protects the *lac* carrier protein against inactivation by diamide in much the same way that it protects against NEM inactivation; (ii) diamide treatment protects the lactose and proline transport systems against inactivation by NEM; and (iii) inhibition of transport by diamide and its reversal by dithiothreitol correlate reasonably well with the availability of NEM reactive sites in the vesicles. Importantly, however, diamide does not inactivate a number of other transport systems in the vesicles, suggesting that functional sulfhydryl groups are not required for the activity of all porters, as implied by the findings of Kaback and Barnes (1971). This conclusion receives strong support from studies of NEM inactivation of various transport systems with D-lactate or reduced PMS as electron donors. In each case, those transport systems which are resistant to diamide are also impervious to NEM when assayed with reduced PMS as electron donor but sensitive when D-lactate is the electron donor. Clearly, the latter effect is due to NEM inhibition of D-lactate oxidation and the generation of $\Delta\bar{\mu}_{H^+}$ and not to an effect of the alkylating agent on these porters.

Diamide purportedly catalyzes sulfhydryl oxidation in the manner shown in eq 1 and 2 (Kosower and Kanety-Londner,



1976). If this mechanism is correct, however, it is apparent that diamide could inactivate sulfhydryl groups in either of two ways: (i) by forming a stable adduct with a single sulfhydryl (i.e., reaction 1), or (ii) by catalyzing the oxidation of two sulfhydryls to form a disulfide (i.e., reaction 1 + reaction 2). In a heterogeneous system such as bacterial membrane vesicles, moreover, it is possible that both modes of inactivation occur. Although precise distinctions between these alternatives cannot be made from the data at hand, it can be calculated from the titration experiments presented in Figure 3 that approximately 2.5 nmol of diamide is required to block 1 nmol of NEM reactive sites in the vesicles. Since this relationship is fivefold higher than the stoichiometry predicted from the overall reaction given above (i.e., 1 mol of diamide should oxidize 2 mol of NEM reactive sites, yielding a stoichiometry of 0.5), it seems unlikely that diamide functions solely as a sulfhydryl oxidant in this case. On the other hand, the results presented here suggest that this reagent may offer a degree of selectivity in certain systems that is not obtained with a number of other sulfhydryl reagents.

References

Barnes, E. M., Jr., and Kaback, H. R. (1971), *J. Biol. Chem. 246,* 5518.

Davis, B. D., and Mingioli, E. S. (1950), *J. Bacteriol. 60,* 17.

Fox, C. F., and Kennedy, E. P. (1965), *Proc. Natl. Acad. Sci. U.S.A. 54,* 891.

Gordon, A. S., Lombardi, F. J., and Kaback, H. R. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 358.

Harold, F. M. (1972), *Bacteriol. Rev. 36,* 172.

Harris, J. W., and Biaglow, J. E. (1972), *Biochem. Biophys. Res. Commun. 46,* 1743.

Kaback, H. R. (1971), *Methods Enzymol. 22,* 99.

Kaback, H. R. (1972), *Biochim. Biophys. Acta 265,* 367.

Kaback, H. R. (1974a), *Science 186,* 882.

Kaback, H. R. (1974b), *Methods Enzymol. 31,* 698.

Kaback, H. R. (1976), *J. Cell. Physiol. 89,* 575.

Kaback, H. R., and Barnes, E. M., Jr. (1971), *J. Biol. Chem. 246,* 5523.

Kaback, H. R., and Hong, J.-s. (1973), *Crit. Rev. Microbiol. 2,* 333.

Kaback, H. R., and Milner, L. S. (1970), *Proc. Natl. Acad. Sci. U.S.A. 66,* 1008.

Kaback, H. R., Reeves, J. P., Short, S. A., and Lombardi, F. J. (1974), *Arch. Biochem. Biophys. 160,* 215.

Kennedy, E. P. (1970), in The Lactose Operon, Beckwith, J. R., and Zipser, D., Ed., Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory, p 49.

Kerwar, G. K., Gordon, A. S., and Kaback, H. R. (1972), *J. Biol. Chem. 247,* 291.

Kohn, L. D., and Kaback, H. R. (1973), *J. Biol. Chem. 248,* 7012.

Konings, W. N., and Boonstra, J. (1977), *Curr. Top. Membr. Transp. 9,* 177.

Kosower, E. M., Correa, W., Kinon, B. J., and Kosower, N. S. (1972), *Biochim. Biophys. Acta 264,* 39.

Kosower, E. M., and Kanety-Londner, H. (1976), *J. Am. Chem. Soc. 98,* 3001.

Kosower, N. S., Kosower, E. M., and Wertheim, B. (1969), *Biochem. Biophys. Res. Commun. 37,* 593.

Lombardi, F. J., and Kaback, H. R. (1972) *J. Biol. Chem. 247,* 7844.

Mitchell, P. (1961), *Nature (London) 191,* 144.

Mitchell, P. (1966), *Biol. Rev. Cambridge Philos. Soc. 41,* 445.

Mitchell, P. (1968), in Chemiosmotic Coupling and Energy Transduction, Bodmin, England, Glynn Research Ltd.

Mitchell, P. (1973), *J. Bioenerg. 4,* 63.

O'Brien, R. W., Weitzman, P. D. J., and Morris, J. G. (1970), *FEBS Lett. 10,* 343.

Ramos, S., and Kaback, H. R. (1977a), *Biochemistry 16,* 848.

Ramos, S., and Kaback, H. R. (1977b), *Biochemistry 16,* 854.

Ramos, S., and Kaback, H. R. (1977c), *Biochemistry 16,* 4271.

Ramos, S., Schuldiner, S., and Kaback, H. R. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1892

Ramos, S., Schuldiner, S., and Kaback, H. R. (1978), *Methods Enzymol.,* in press.

Schuldiner, S., and Kaback, H. R. (1975), *Biochemistry 14,* 5451.

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.
Stroobant, P., and Kaback, H. R. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3970.
Tokuda, H., and Kaback, H. R. (1977), *Biochemistry 16,* 2130.

Bimodal Substrate Inhibition of Lactate Dehydrogenase. Factors Affecting the Enzyme in Vivo†

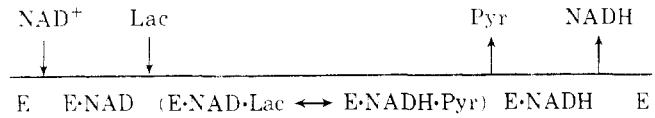
J. W. Burgner II,* G. R. Ainslie, Jr.,‡ W. W. Cleland, and W. J. Ray, Jr.

ABSTRACT: In the presence of NAD, pyruvate inhibits various isozymes of lactate dehydrogenase via (1) the rapidly reversible formation of a dead-end, (abortive) ternary complex, E·NAD·pyruvate, and (2) the slowly reversible formation of a binary enzyme·inhibitor complex in which the inhibitor is the adduct of pyruvate and NAD, NAD-Pyr. Thus, pyruvate-induced inhibition patterns obtained from *initial* velocity studies of the normal enzymic reaction, NADH + pyruvate → NAD + lactate, are caused solely by the dead-end ternary complex (E·NAD·pyruvate). Because of weak binding, it is unlikely that this complex is important in vivo in mammalian systems. The curvature of the product–time plots obtained with saturating substrate concentrations and product concentrations far from equilibrium is produced by the slow formation of the binary E·NAD-Pyr complex ($t_{1/2}$ = 5 min at 15 °C for dogfish A_4 enzyme). In the *limiting steady state,* at pH 7, and at *saturating NADH* concentration formation of the adduct complex reduces the fraction of active enzyme only by about 50%. But as the system approaches equilibrium a much greater reduction in active enzyme can be observed. In addition, the decomposition of the adduct complex is slow ($t_{1/2}$ = 1 to 5 min under pseudophysiological conditions). However, significant amounts of the adduct complex cannot be detected in extracts of rat heart that were rapidly prepared under conditions where the adduct complex is relatively stable. Hence, the adduct complex, as well, is unlikely to be important, physiologically, in mammalian systems.

The NAD^+-dependent lactate dehydrogenases (LDH)[1] of vertibrates are present in vivo largely in two isomeric forms: the A isozyme, which is obtained from skeletal muscle, and the B isozyme, which is isolated from cardiac muscle. (There also are three additional isomeric forms that are present at substantially lower concentrations; see Masters & Holmes, 1974.) This paper deals only with isozymes that have a uniform subunit composition: A_4 and B_4.

The biological significance of the A and B isozymes and their distribution in various tissues often is correlated with the oxidative capacity of the tissues (cf., Everse & Kaplan, 1973, 1975). Thus, the B form, found principally in "oxidative" tissue, may be primarily a lactate oxidase, because it has a smaller Michaelis constant for lactate than the A form; conversely, the A form may be primarily a pyruvate reductase, because it is found principally in "glycolytic" tissue and because of the presumed inability of voluntary muscle to oxidize lactate. In addition, metabolic control of the B form of the enzyme might be exerted by pyruvate in the Pyr → Lac reaction, since this form of the enzyme is subject to pyruvate inhibition at significantly lower concentrations than the A form (see below). However, comparisons between tissue levels of pyruvate and its inhibitor constant do not lend support to the latter suggestion and, as an alternative, Stambaugh & Post (1966) suggest that "product inhibition" may be an important factor. Furthermore, Rose & Rose (1969) suggest that such metabolic control of the B form is unlikely, since perfusion experiments with rat hearts (Williamson, 1965) indicate that the lactate/pyruvate ratio is at its equilibrium value, except when abnormally high pyruvate concentrations (10 mM) are used in the perfusate.

The results of in vitro studies involving initial velocity, product inhibition, isotope exchange, and rapid mixing experiments with isozymes from several different organisms (Hakala et al., 1965; Zewe & Fromm, 1962, 1965; Anderson et al., 1964; Silverstein & Boyer, 1964; Stambaugh & Post, 1966; Urban, 1969; Holbrook & Gutfreund, 1973; Boland & Gutfreund, 1975) are consistent with an ordered sequential process (both substrates add before release of either product):



(1)

Substrate inhibition of either the forward or reverse reactions by lactate or pyruvate, respectively, where these are present at concentrations greater than their respective Michaelis constants, also is well documented (see Everse & Kaplan,

† From the Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907 (J.W.B. and W.J.R), and the Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706 (G.R.A. and W.W.C.). *Received August 30, 1977.* This work is supported by grants from the National Science Foundation to W.J.R. (GB7500480) and to W.W.G. (GB8396).

‡ A portion of this work is abstracted from the Ph.D. dissertation of G. R. Ainslie, Jr., University of Wisconsin, 1970.

[1] Abbreviations used: LDH, lactate dehydrogenase; E, a subunit of the LDH tetramer; Pyr, pyruvate; Pyr_K and Pyr_E, the keto and enol pyruvate forms, respectively; Lac, lactate; APAD and APADH, the oxidized and reduced forms of 3-acetylpyridine analogue of NAD, respectively; NAD-Pyr and APAD-Pyr or adduct, the covalent addition product of the oxidized coenzyme and pyruvate produced by LDH.

0006-2960/78/0417-1646$01.00/0