Substrate Kinetic Isotope Effects in Dehydrogenase Coupled Active Transport in Membrane Vesicles of *Escherichia coli*†

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ABSTRACT: Succinate dehydrogenase in isolated membrane vesicles of *E. coli* ML 308-225, and the inducible D-alanine dehydrogenase in vesicles of *E. coli* B, oxidize perdeuterio succinate and D-[2-^{2}H]alanine with isotope effects at V_{max} of 2.3 and 1.9, respectively. These kinetic isotope effects have been used to determine how changes in substrate oxidation rates affect various transport parameters. We have made the following observations: (1) The isotope effects persist both in dye-coupled dehydrogenase assays and in rates of oxygen uptake, showing C–^{2}H cleavage during oxidation of these substrates is a slow step relative to subsequent electron flow through membrane respiratory chain components. (2) The initial rates of amino acid or lactose active transport into vesicles energized by succinate or D-alanine oxidation show a carry-over (either partial or complete) of the dehydrogenase kinetic isotope effects to the maximum velocity of solute influx under initial conditions (V_i). (3) Either ^{1}H or ^{2}H energy sources stimulate the same steady-state level of soluble uptake for several different transport systems examined, despite the twofold differences in V_{max} rates of oxidation. (4) Similarly, the total driving force for transport ($\Delta\bar{\mu}_{H^+}$) was the same during protio or deuterio substrate oxidation as determined by measuring both the transmembrane electrical potential ($\Delta\Psi$) and the chemical potential of the proton gradient (ΔpH) in respiring vesicles. (5) By manipulating the size of $\Delta\bar{\mu}_{H^+}$ and measuring the initial rate of solute transport, it was found that initial rates of carrier-mediated influx are a function of $\Delta\Psi$ (the parameter measured), suggesting that the V_{max} of each solute carrier system increases with increasing values of $\Delta\bar{\mu}_{H^+}$. (6) Preincubation of vesicles with an energy source (either succinate or D-lactate) for various lengths of time before addition of transport solute results in a progressive increase in the initial rate of solute influx; for the case of [^{1}H]- and [^{2}H]-succinate, the V_i for proline accumulation reaches the same final value and the kinetic isotope effect during substrate oxidation is no longer manifested in differences in the initial rate of transport. These results suggest that differences in initial rates of solute uptake (V_i) stimulated by ^{1}H and ^{2}H energy sources are not due to different levels of $\Delta\bar{\mu}_{H^+}$ formed, but rather to the slow formation of the transmembrane electrochemical potential.

Cytoplasmic membrane vesicles of bacteria can be isolated which remain competent to accumulate solutes against a concentration gradient, provided an appropriate energy source is present (Hong and Kaback, 1973; Kaback 1974a). Kaback's laboratory has been foremost in determining the nature of substrates which can be oxidized by membrane-bound dehydrogenases and thus provide the driving force for active transport (Barnes and Kaback, 1970). A variety of experiments, performed initially by Harold and co-workers (Hirata et al., 1973; Altendorf et al., 1975), have shown that a transmembrane potential, net negative in the interior, is generated across vesicle membranes during substrate oxidation (Schuldiner and Kaback, 1975). For a proton-impermeable membrane, this electrochemical potential is comprised of the chemical potential for the proton, ΔpH, and the electrical potential across the membrane, $\Delta\Psi$. The generation of a membrane potential by an artificially induced potassium diffusion gradient has been shown to drive transient solute accumulation by membrane vesicles (Hirata et al., 1974; Altendorf et al., 1974). Furthermore, recent work from Kaback's laboratory has demonstrated the existence of the ΔpH component of the transmembrane electrochemical potential in the vesicle system; quantitative measurements of both $\Delta\Psi$ and ΔpH indicate that the magnitude of $\Delta\bar{\mu}_{H^+}$ is sufficient to account for the energy required for solute active transport (Ramos et al., 1976). These and other experiments (Kashket and Wilson, 1974) corroborate the view that some form of the chemiosmotic hypothesis may adequately describe the energization of bacterial membranes both in isolated vesicles and in whole cells.

With the idea that the transmembrane electrochemical potential for a proton-impermeable membrane is a key force in active transport, we have examined isolated membrane vesicles to see how controlled changes in V_{max} values for substrate oxidation would be reflected on the initial rates and on the steady-state levels of solute accumulation and concomitantly on the components of $\Delta\bar{\mu}_{H^+}$. To this end we have considered the following questions: do some membrane-bound dehydrogenases oxidize deuterated substrates with a detectable isotope effect; does the V_{max} kinetic isotope effect on substrate oxidation become translated into a rate difference on the initial rates of solute active transport and/or the steady-state levels of accumulation achieved; is the rate at which a membrane carrier transports solute a function of the size of the electrochemical potential; can anything be inferred about the rate of formation of $\Delta\bar{\mu}_{H^+}$ on addition of an oxidizable substrate to membranes. The demonstration of detectable isotope effects at V_{max} for succinate oxidation in membranes from *E. coli* ML308-225 and for D-alanine oxidation in vesicles of induced *E. coli* B have allowed us to answer these questions.

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Experimental Procedure

Materials

All ^{14}C-labeled amino acids, [^{14}C]lactose, [^{14}C]-5,5-dimethyloxazolidine-2,4-dione, [^{3}H]acetate, and $^{86}RbCl$ used in these experiments were supplied either by New England Nuclear or Amersham/Searle. [2,3-$^{2}H_4$]Succinic acid and [2,3-$^{2}H_2$]fumaric acid were from Merck Sharp and Dohme. D-[2-^{1}H]Alanine and valinomycin were obtained from Sigma Chemical Co., while carbonyl cyanide *m*-chlorophenylhydrazone was purchased from Calbiochem. [^{3}H]Triphenylmethylphosphonium bromide, prepared by the Roche Radiochemical Synthetic Group under the direction of Dr. A. Lieberman, was the generous gift of Dr. H. R. Kaback of the Roche Institute of Molecular Biology, Nutley, N.J. The D-[2-^{2}H]lactate (lithium salt) used in these experiments was kindly provided by S. Ghisla, Biology Department, University of Konstanz, Konstanz, Germany. All other chemicals used were of reagent grade quality and obtained from commercial sources.

Methods

Membrane Preparation and Transport Assays. Isolated cytoplasmic membrane vesicles were prepared from *E. coli* B and *E. coli* ML 308-225 as described previously (Kaczorowski et al., 1975a; Kaback, 1971). *Azotobacter vinelandii* membranes were prepared according to Barnes's procedures (Barnes, 1972). In all cases, vesicles were prepared and then stored at liquid nitrogen temperatures in 0.1 M potassium phosphate (*E. coli* B, pH 7.5; *A. vinelandii*, pH 7.0; and *E. coli* ML 308-225, pH 6.6) until further use.

Dehydrogenase-coupled uptakes of radioactive amino acids and sugars were assayed by the methods of Kaback (Kaback, 1974b). Valinomycin-mediated rubidium uptake and triphenylmethylphosphonium ion ($TPMP^+$)[1] accumulation were monitored by methods previously described (Lombardi et al., 1973; Kaczorowski and Walsh, 1975). The specific activities and final saturating concentrations of ^{14}C-labeled substrates used in the transport assays are as follows: L-proline (290 mCi/mmol), 17.2×10^{-6} M; L-serine (155 mCi/mmol), 17×10^{-6} M; L-glutamate (214 mCi/mmol), 23.4×10^{-6} M; L-lysine (330 mCi/mmol), 15.2×10^{-6} M; L-phenylalanine (495 mCi/mmol), 10.1×10^{-6} M; glycine (80 mCi/mmol), 50×10^{-6} M; L-tyrosine (483 mCi/mmol), 10.4×10^{-6} M; and lactose (11 mCi/mmol), 4×10^{-4} M. In addition, $^{86}RbCl$ (5-15 mCi/mmol), 4×10^{-3} M, and [^{3}H]$TPMP^+Br^-$ (1.33 Ci/mmol), 24×10^{-6} M, were used in experiments to measure membrane potential. Sodium [^{3}H]acetate (685 mCi/mmol), 18.1×10^{-6} M, and [^{14}C]-5,5-dimethyloxazolidine-2,4-dione (DMO) (46.6 mCi/mmol), 2.1×10^{-4} M, were employed for the ΔpH measurements.

Determination of Enzyme Activities. D-Alanine, succinate, D- and L-lactate, malate, and NADH dehydrogenase activities as well as alanine racemase activity were all measured by following oxygen consumption by membrane vesicles with a Clark-type electrode (YSI Model 53 oxygen monitor) as explained previously (Kaczorowski et al., 1975a; Barnes, 1973). D-Alanine and succinate dehydrogenase activities were also assayed by monitoring spectrophotometrically the reduction of an acceptor dye, dichloroindophenol (DCIP), at 600 nm (Kaczorowski et al., 1975b; Ruiz-Herrera and Ramirez, 1973).

ΔpH Measurements. The internal pH of membrane vesicles was measured during respiration by following the accumulation of the weak acids acetate and DMO as described by Ramos, Schuldiner, and Kaback (Ramos et al., 1976). The flow dialysis apparatus employed was identical with the one used in that paper. After having determined the internal and external pH values, the chemical potential of the proton gradient was calculated from the difference in proton concentrations across the membrane ($-2.3(RT/F)\Delta pH$).

Chemical Syntheses. D,L-[2-^{2}H]Alanine was prepared by exchanging the α proton of D,L-alanine in $^{2}H_2O$, catalyzed by Cu^{II} ion and salicylaldehyde (Ikawa and Snell, 1954). Typically, 1 g of D,L-alanine was dissolved in 3 mL of $^{2}H_2O$ and lyophilized. The solid was redissolved in 5 mL of $^{2}H_2O$, and then 100 mg of salicylaldehyde and 100 mg of anhydrous $CuSO_4$ were added. The solution was heated in a sealed tube at 140–150 °C for 3 h. After cooling, the solution was filtered and the filtrate was dried in vacuo. The solid was washed two times with boiling anhydrous ethanol and then recrystallized in aqueous ethanol to yield a white crystalline solid. Analysis by NMR showed at least 95% isotopic purity. Typical yield was 60–70%.

Resolution of D,L-[2-^{2}H]alanine was performed by the standard method of acylase I catalyzed hydrolysis of the *N*-chloroacetyl derivative (Greenstein and Winitz, 1961). The recovered D- and L-[2-^{2}H]alanine were again recrystallized from aqueous ethanol. NMR analysis showed no loss of isotopic purity. Optical purity was determined by subjecting a sample to D-amino acid oxidase coupled to lactic dehydrogenase. The samples were at least 95% optically pure.

2*S*,3*S*-[2,3-$^{2}H_2$]Malic acid was prepared by the fumarase-catalyzed hydration of [$^{2}H_2$]fumaric acid. [$^{2}H_2$]Fumaric acid (100 mg) was dissolved in 10 mL of 0.1 M sodium phosphate buffer, pH 7, which was again adjusted to pH 7.1 with NaOH. Fumarase (0.4 mg) was added, and the solution was left standing at room temperature for 24 h. A trace of [2-^{14}C]malate (Cheung et al., 1975) was added. The solution was passed through a Dowex 1, Cl^- form, column (~8 mL). After thorough washing, the column was developed with 25 mM HCl, and fractions of 3 mL each were collected. Malic acid was located by the ^{14}C tracer and fumaric acid by absorption at 250 nm. They were shown to be well separated. The radioactive fractions were pooled, and solvent was removed by evaporation in vacuo. The residue was taken up in 5 mL of ether, filtered, and the volume reduced to 0.5 mL. After storage in the freezer overnight, 50 mg of a white crystalline solid was obtained (mp 101–103 °C).

4*R*-[^{2}H]NADH and 4*S*-[^{2}H]NADH were both prepared in this laboratory by Dr. J. Fisher. The 4*R*-[^{2}H]NADH was made using [^{2}H]ethanol and alcohol dehydrogenase, while 4*S*-[^{2}H]NADH was prepared with lipoamide, dithioerythritol, and lipoamide dehydrogenase in D_2O (Oppenheimer et al., 1971).

D,L-[2-^{2}H]Lactate was chemically synthesized from pyruvate by sodium borodeuteride reduction.

Results

Kinetic Isotope Effects in Membrane Dehydrogenase Action. Initial experiments were performed to ascertain whether any of the membrane-bound dehydrogenases known to be coupled functionally to solute active transport in bacterial membrane vesicles displayed a lower maximal velocity when oxidizing deuterated substrates. Such a decrease in V_{max} would

[1] Abbreviations used are: DCIP, dichloroindophenol; $TPMP^+$, triphenylmethylphosphonium ion; DMO, 5,5-dimethyloxazolidine-2,4-dione; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone, PMS, phenazine methosulfate; NMR, nuclear magnetic resonance.

TABLE I: Measurement of ^1H- and ^2H-Labeled Substrate Oxidation Rates by Membrane Bound Enzymes in Bacterial Vesicles.[a]

Substrate	V_{max} for ^1H form (nmol of O_2 min^{-1} (mg of protein)$^{-1}$)	V_{max} for ^2H form (nmol of O_2 min^{-1} (mg of protein)$^{-1}$)	$k_{^1H}/k_{^2H}$	Source of membrane vesicles
D-[2-^{2}H]Alanine	27.4	14.3	1.9	*E. coli* B
L-[2-^{2}H]Alanine	3.9	3.8	1.0	*E. coli* B
[2,3-$^2H_4$]Succinate	43.1	19.7	2.2	*E. coli* ML 308-225
2*S*,3*S*-[2,3-$^2H_2$]Malate	101.6	91.5	1.1	*A. vinelandii*
4*R*-[^{2}H]NADH	92.4	105.0	0.9	*E. coli* ML 308-225
4*S*-[^{2}H]NADH	92.4	82.2	1.1	*E. coli* ML 308-225
D,L-[2-^{2}H]Lactate	21.1	18.5	1.1	*E. coli* ML 308-225
D-[2-^{2}H]Lactate	20.0	16.1	1.2	*E. coli* ML 308-225

[a] One hundred microliters of *E. coli* B membranes (0.8 mg of protein), 100 μL of 0.1 M potassium phosphate, pH 7.5, 10 mM magnesium sulfate, and water were placed in the chamber of an oxygen electrode to give a final volume of 0.4 mL. After 5 min of preincubation at 25 °C, ^1H- or ^2H-labeled substrate was added to a final concentration of 20 mM and the rate of oxygen usage by the membranes was recorded. This same procedure was repeated using *E. coli* ML 308-225 membrane vesicles (1.0 mg of protein) buffered at pH 6.6. All substrates were employed at 20 mM except NADH, which was added at 10 mM final concentration. The assays of oxygen uptake in *Azotobacter vinelandii* vesicles contained 100 μL of membranes (0.5 mg of protein), 50 mM potassium phosphate, pH 7.0, 2 mM magnesium sulfate, 0.5 mM calcium chloride, and 50 μM flavin adenine dinucleotide in a final volume of 0.4 mL. Malate oxidation was measured at 20 mM substrate concentrations.

indicate a kinetic isotope effect during catalysis and imply that breakage of the carbon–deuterium bond was rate determining in the oxidation (fully or partially, depending on the size of the isotope effect). Thus, substrates were used which were specifically deuterated at the oxidizable carbons, as indicated in Table I.

Kaback and colleagues (Kaback, 1974a; Stroobant and Kaback, 1975) have demonstrated that D-lactate, L-lactate, and NADH dehydrogenase are involved in the active transport of solutes in *E. coli* ML308-225 membrane vesicles. However, none of these enzymes show a reduction in V_{max} when deuterated substrates were used in place of their protio counterparts. Similarly, 2*S*,3*S*-[$^2H_2$]malate, the energy source which energizes active transport in *Azotobacter vinelandii* membranes (Barnes, 1972), is oxidized by malate dehydrogenase without any change in the rate of cleavage of the C_2–^{2}H bond. In none of these four flavin-linked dehydrogenases, then, can the initial cleavage of the C–H bond at the oxidizable locus be a slow step in catalysis, under the assay conditions (V_{max}).

However, two of the membrane dehydrogenases listed in Table I do show kinetic isotope effects. The inducible D-alanine dehydrogenase (Kaczorowski et al., 1975a; Raunio and Jenkins, 1973; Raunio et al., 1973) oxidized D-[2-^{2}H]alanine 1.8-fold slower than D-[2-^{1}H]alanine at saturating levels of each substrate. Also, when succinate dehydrogenase is assayed with perdeuterio succinate, fumarate production is reduced 2.3-fold at V_{max}. In contrast to oxidation of the D-[2-^{2}H]alanine, processing of the enantiomeric L-[2-^{2}H]alanine by the vesicles shows no reduction in the rate of catalysis.

It has recently been shown that oxidation of L-alanine to pyruvate and ammonia by *E. coli* membrane vesicles occurs solely by the sequential action of alanine racemase and D-alanine dehydrogenase, i.e., L-alanine → D-alanine → pyruvate (Kaczorowski et al., 1975a; Raunio and Jenkins, 1973; Raunio et al., 1973). Since the racemization step is slow relative to subsequent dehydrogenation (Table I), a decrease in the V_{max} of this reaction would necessarily show up in a diminished rate of pyruvate formation. This is not observed and indicates that the membrane-associated alanine racemase catalyzes the racemization of L-[2-^{2}H]alanine without isotope effect at V_{max}.

All of the assays of the membrane-bound enzymes in Table I utilize the rate of reduction of molecular oxygen to water by the vesicles as a measure of the rate of substrate oxidation. This assay is clearly not a simple measure of the specific dehydrogenase for it includes all subsequent electron transfers through the cytochrome chain. The actual isotope effect in dehydrogenase catalysis could therefore be larger; if one of the subsequent electron transfer processes goes at a rate equivalent to that of C–H cleavage, the full effect will not be manifested. Thus, even those dehydrogenases showing no isotope effect by oxygen utilization assay could have a rate-determining step in dehydrogenation masked.

One could check these possibilities in two ways. First, the membraneous D-lactate dehydrogenase has been assayed after its solubilization and purification to homogeneity (Kohn and Kaback, 1973).[2] The purified flavoenzyme still shows no isotope effect with saturating levels of D-[2-^{2}H]lactate. None of the other membrane enzymes of Table I are available in purified form. However, D-alanine dehydrogenase and succinate dehydrogenase have been solubilized from membrane vesicles in our laboratory.[3] In both cases, the enzymes in the solubilized preparations exhibit the same magnitude of isotope effect upon oxidation as found during catalysis by the corresponding membrane-associated forms (data not shown).

A second method is to assay the dehydrogenases in the membrane vesicles with an alternate electron acceptor. Dichloroindophenol (DCIP) is a dye which has a redox potential more negative than O_2 so that it can accept electrons at some point more proximal to the flavoenzymes than the cytochrome oxidase. After adding cyanide to inhibit electron flow to oxygen, we and others (Ruiz-Herrera and Ramirez, 1973) found that the combination of DCIP and phenazine methosulfate provides good rates of electron transfer from the membrane dehydrogenases with alanine and succinate as substrates. Double-reciprocal plots for D-[2-^{2}H]alanine and D-[2-^{1}H]-alanine and for [2,3-$^2H_4$]succinate and [2,3-$^1H_4$]succinate were then made. The data from these experiments emphasize two points. In each instance, the K_m values for protio and deuterio substrates are identical (D-alanine, 8.2 mM; succinate, 1.3 mM), while the V_{max} values are different. This indicates that both D-alanine and succinate dehydrogenase catalyses

[2] S. A. Short, H. R. Kaback, and C. Walsh, unpublished observations.

[3] G. Kaczorowski, P. Olsiewski, and C. Walsh, unpublished results.

TABLE II: Effect of ^1H and ^2H Energy Sources on the Initial Rates of Solute Uptake in *E. coli* Membrane Vesicles.[a]

Dehydrogenase substrate	Transport substrate	V_i for ^1H form (nmol min^{-1} (mg of protein)$^{-1}$)	V_i for ^2H form (nmol min^{-1} (mg of protein)$^{-1}$)	$\frac{k_{1H}}{k_{2H}}$	Source of membrane vesicles
D,L-[2-^{2}H]Alanine	Pro	0.29	0.15	1.9	*E. coli* B
	Ser	0.26	0.14	1.9	
D-[2-^{2}H]Alanine	Pro	0.65	0.39	1.7	*E. coli* B
	Lys	0.59	0.38	1.6	
	TPMP$^+$	2.89	2.19	1.3	
	Rb$^+$/valinomycin[b]	17.0	13.5	1.2	
[2,3-$^2H_4$]Succinate	Pro	1.60	0.84	1.9	*E. coli* ML 308-225
	Ser	1.46	0.82	1.8	
	Gly	0.23	0.14	1.6	
	Lactose	19.0	12.4	1.5	
	Tyr	0.42	0.30	1.4	
	Glutamate	0.32	0.22	1.4	
	Lys	0.14	0.12	1.1	
	Phe	0.24	0.22	1.1	
	TPMP$^+$	0.45	0.35	1.3	
	Rb$^+$/valinomycin	4.55	4.0	1.1	
L-[2-^{2}H]Alanine	Pro	0.11	0.11	1.0	*E. coli* B
	Rb$^+$/valinomycin	5.70	5.70	1.0	
D,L-[2-^{2}H]Lactate	Pro	0.44	0.44	1.0	*E. coli* ML 308-225
D-[2-^{2}H]Lactate	Glutamate	0.32	0.32	1.0	*E. coli* B

[a] Twenty-five microliters of *E. coli* B membrane vesicles (0.2 mg of protein), water, and 10 mM magnesium sulfate were incubated together in a final volume of 50 μL for 3 min at 25 °C. An energy source (20 mM) was then added followed immediately by radioactive substrate and solute uptake was monitored. A complete time course of transport was recorded, stimulated by either [^{1}H]- or [^{2}H]dehydrogenase substrates. The initial uptake rates were determined from time points taken during the first minute of the experiment. For all transport substrates several measurements were made at each time point and values averaged to determine the transport profiles. Identical assays were performed with *E. coli* ML 308-225 membrane vesicles except that 0.125 mg of protein was used for each assay. In the case of valinomycin-dependent $^{86}Rb^+$ uptake, potassium ions were removed by washing and resuspending the membranes in 0.1 M sodium phosphate at the appropriate pH as described before (Lombardi et al., 1973; Kaczorowski and Walsh, 1975). [b] Rb$^+$ assayed in the presence of 40 mM D-[2-^{1}H]- and -[2-^{2}H]alanine.

exhibit purely V_{max} kinetic isotope effects. Secondly, the isotope effects at infinite substrate concentrations are 1.9 for D-alanine and 2.3 for succinate, values identical with those obtained by the oxygen utilization assay of Table I. This identity proves that, for both D-alanine and succinate dehydrogenase, all steps in the respiratory chain after the point where DCIP accepts electrons are faster than the substrate oxidation step.

Deuterated Substrates as Energy Sources for Solute Active Transport. Effect on Initial Rates. Since kinetic isotope effects on oxidation of D-[2-^{2}H]alanine and [2,3-$^2H_4$]succinate by membrane vesicles were observed, the effect on the initial rate of active transport of various solutes energized by dehydrogenation of these substrates was next examined. It is possible to study both carrier-mediated uptake of amino acids and sugars, and the uptake of lipophilic cations such as [^{3}H]triphenylmethylphosphonium ion (TPMP$^+$) or cations with the appropriate ionophore (^{86}rubidium/valinomycin) which, although actively concentrated, do not utilize specific carrier proteins to traverse the vesicle membrane.

The first experiments were performed with *E. coli* B membrane vesicles using D-[2-^{1}H]alanine and D-[2-^{2}H]alanine as energy sources. As shown in Table II, D-[2-^{1}H]alanine stimulates faster initial accumulation of proline, serine, lysine, TPMP$^+$, and Rb$^+$ than does D-[2-^{2}H]alanine, although the difference in stimulation of initial rates of uptake by the two energy sources varies depending on the transport solute investigated. Using either the racemic mixture or the separated D isomer of [2-^{2}H]alanine, the initial rates of concentrative uptake of proline and serine are 1.7- to 1.9-fold slower than when the corresponding [2-^{1}H]alanine is oxidized. This difference in initial rates mirrors the isotope effect seen in D-alanine dehydrogenase catalysis (Table I). On the other hand, the difference in lysine uptake stimulated by the two energy sources is slightly less than the isotope effect seen on oxidation, while cation accumulation driven by the two types of D-alanine shows an even smaller initial rate difference.

In order to convince ourselves that the change in initial rates of solute active transport seen in the above experiments was real and correlatable to kinetic isotope effects on substrate oxidation, transport was assayed in the presence of ^{1}H and ^{2}H substrates for enzymes which exhibit no kinetic isotope effect on oxidation. The L isomers of [2-^{1}H]- and [2-^{2}H]alanine were used to stimulate active transport of both proline and rubidium. Since there is no isotope effect in L-alanine oxidation on deuterium substitution (Table I), there should be no change in the initial rates of solute uptake driven by these energy sources. The results in Table II confirm that [2-^{1}H]- and [2-^{2}H]-L-alanine drive both proline (carrier-mediated) and rubidium (valinomycin-mediated) uptake at identical rates. Similarly, Table II includes the expected observations of no isotope effect when [2-^{1}H]- or [2-^{2}H]lactate is employed as an energy source for either proline uptake in membranes from *E. coli* ML 308-225 or glutamate transport in *E. coli* B membrane vesicles.

Since succinate dehydrogenase yields a kinetic isotope effect of 2.2 during catalysis, and since the characteristics of the lactose and amino acid carriers in ML 308-225 membrane

TABLE III: Effect of ^1H and ^2H Energy Sources on the Initial Rates of Solute Transport in *E. coli* ML 308-225 Membrane Vesicles at pH 7.5.[a]

Transport substrate	V_i for ^1H form (nmol min^{-1} (mg of protein)$^{-1}$)	V_i for ^2H form (nmol min^{-1} (mg of protein)$^{-1}$)	$\frac{k_{1H}}{k_{2H}}$	$\frac{V_i \text{ (pH 7.5)}^b}{V_i \text{ (pH 6.6)}}$
Pro	0.58	0.50	1.2	0.36
Ser	0.35	0.35	1.0	0.24
Gly	0.12	0.10	1.2	0.52
Lactose	4.8	4.4	1.1	0.25
Tyr	0.20	0.18	1.1	0.48
Glutamate	0.21	0.14	1.5	0.66
Lys	0.09	0.09	1.0	0.64
Phe	0.17	0.17	1.0	0.71

[a] *E. coli* ML 308-225 membrane vesicles (in 0.1 M potassium phosphate, pH 6.6) were diluted tenfold into 0.1 M potassium phosphate, pH 7.5, and pelleted by centrifugation at 45 kG for 30 min. After resuspension, the membranes were washed twice by this procedure and finally diluted to a concentration of 5 mg of protein/mL in this buffer. Twenty-five microliters of vesicles (0.125 mg of protein) were incubated with water and 10 mM magnesium sulfate in a final volume of 50 μL at 25 °C for 3 min. [2,3-^1H]- or [2,3-^2H]-succinate, 20 mM (neutralized to pH 7.5), was then added along with radioactive substrate and the initial rate of transport measured for several different solutes. [b] V_i's for transport driven by [2,3-$^1H_4$]succinate.

vesicles have been well defined (Lombardi and Kaback, 1972), complementary experiments to those performed with D-alanine were attempted with [2,3-$^1H_4$]- and [2,3-$^2H_4$]succinate. Figure 1 shows typical results of proline (A), serine (B), lactose (C), and phenylalanine (D) transport stimulated by the two forms of potassium succinate at pH 6.6. Again, as observed with D-alanine, [2,3-$^1H_4$]succinate stimulates a greater initial rate of solute transport than does [2,3-$^2H_4$]succinate. Indeed, this general pattern is carried over to glycine, glutamate, lysine, tyrosine, $TPMP^+$, and Rb^+ accumulation, as shown in Table II. However, the kinetic isotope effect of 2.2 for succinate oxidation appears to carry over only partially to the initial rates of active transport of the seven amino acids, one disaccharide, and two cations examined; the rate ratios for [2,3-$^1H_4$]succinate to [2,3-$^2H_4$]succinate as energy source range from a maximum of 1.9–1.8 (for proline and serine) to a minimum of 1.1 (for lysine, phenylalanine, and rubidium), with the other transport solutes giving intermediate values.

To ensure that the rate ratios presented in Table II do represent a full range from 1.9 to 1.1, initial rate studies have been performed numerous times under identical conditions at pH 6.6, with both transport solute and energy source at saturating concentrations and both in the presence and absence of an oxygen atmosphere, to minimize artifacts arising during the determination. The values are reproducible and in the case of proline, for example, more than 300 time points confirm the observed rate ratios.

The pH profile for succinate dehydrogenase activity in *E. coli* ML 308-225 membrane vesicles has been determined, and the pH optimum was found to be at pH 8.0 (data not shown). When membranes are suspended in 0.1 M potassium phosphate, pH 7.5, the membrane-bound enzyme turns over twice as fast as it does at pH 6.6 under the same conditions. At this pH value, the magnitude of the kinetic isotope effect also increases to 3.0. It was therefore of interest to determine the effects of energizing transport with [2,3-$^1H_4$]- and [2,3-$^2H_4$]succinate at pH 7.5 to see if the threefold kinetic isotope effect on oxidation carries over to the rate ratios of solute uptake.

Quite the opposite effect is seen, however, as shown in Table III. The initial rate of all eight carrier-mediated transport systems is decreased with [^1H]succinate at pH 7.5 as energy source compared with similar determinations made with

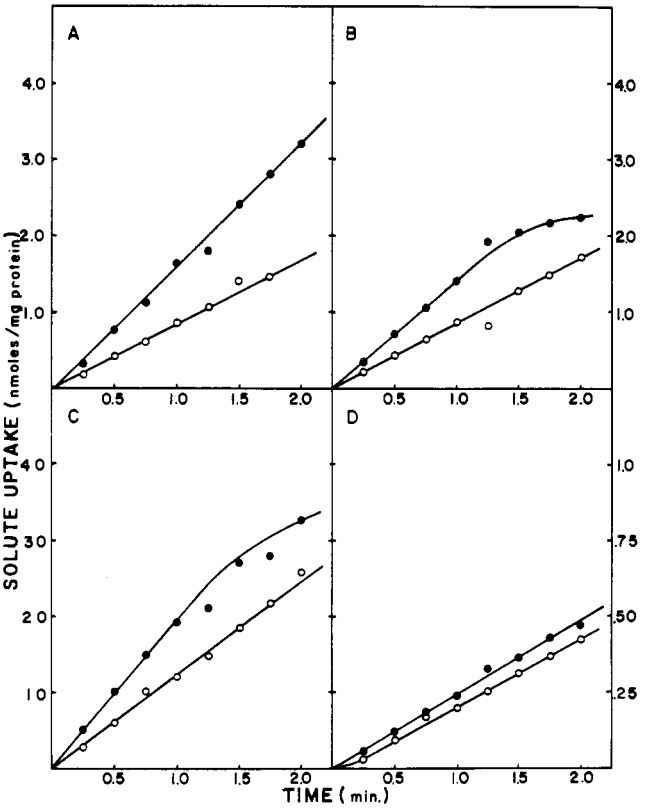

FIGURE 1: Initial rates of amino acid and sugar transport stimulated by succinate oxidation in *E. coli* ML 308-225 membrane vesicles. Twenty-five microliters of *E. coli* ML 308-225 membranes (0.125 mg of protein) was added to water and 10 mM magnesium sulfate to give an assay mixture containing 50 mM potassium phosphate (pH 6.6) in 50 μL final volume. After incubation at 25 °C for 2 min, 20 mM [2,3-$^1H_4$]succinate (●—●) or [2,3-$^2H_4$]succinate (○—○) (neutralized to pH 6.6) and radioactive substrate were added. Solute transport was assayed for (A) L-proline; (B) L-serine; (C) lactose; and (D) L-phenylalanine.

[^1H]succinate at pH 6.6 (column 4, Table III). This is probably due to the fact that most of the membrane-bound carriers exhibit maximal activity around pH 6.5 and are less active at pH 7.5 (Lombardi and Kaback, 1972). The magnitude of the isotope effect on initial rates ([^1H]succinate/[^2H]succinate stimulated) also decreases in seven out of the eight cases, with

TABLE IV: Determination of Steady-State Concentration of Transport Solutes by Membrane Vesicles in the Presence of ^1H and ^2H Energy Sources.[a]

	E. coli B vesicles				*E. coli* ML 308-225 vesicles					
	D-[2-^{1}H]Alanine nmol		D-[2-^{2}H]Alanine nmol		[2,3-$^1H_4$]Succinate nmol		[2,3-$^2H_4$]Succinate nmol		D-Alanine ^{1}H in/out	Succinate ^{1}H in/out
Solute	mg of prot.	In/out	mg of prot.	In/out	mg of prot.	In/out	mg of prot.	In/out	^{2}H in/out	^{2}H in/out
Pro	2.53	165	2.49	158	3.75	252	3.75	252	1.04	1.00
Ser	1.99	102	2.00	103	3.70	254	3.70	254	1.00	1.00
Gly	0.34	3	0.33	3	1.00	44.4	1.05	47.3	1.00	0.94
Lactose	18.58	20	18.00	19.49	40.0	48.6	38.9	46.9	1.03	1.04
Tyr					1.40	101	1.40	101		1.00
Glutamate	1.47	40	1.47	40	1.60	39.9	1.58	39.3	1.00	1.02
Lys	2.32	182	2.30	177	1.00	38	0.99	37.6	1.03	1.01
Phe	0.82	56	0.85	59	0.63	35.7	0.63	35.7	0.95	1.00
Rb^+/valinomycin	76.0[b]	9.15	70.0	8.37					1.09	
$TPMP^+$ (pH 6.6)					0.64	12.86	0.61	12.20		1.05
$TPMP^+$ (pH 7.5)					0.89	18.47	0.89	18.47		1.00

[a] Twenty-five microliters of *E. coli* B (0.2 mg of protein) or *E. coli* ML 308-225 (0.125 mg of protein) membrane vesicles was added to water and 10 mM magnesium sulfate to give 50 μL. After 3 min, an energy source and labeled substrate were added and a complete time course of transport was determined as described earlier. The steady-state value of substrate accumulated in nmol/mg of membrane protein was then ascertained from the transport profile. The concentration gradient for each solute ($solute_{in}/solute_{out}$) was then calculated assuming 2.2 μL/mg of protein as the internal volume of the membrane vesicles (Schuldiner and Kaback, 1975; Kaczorowski et al., 1975a). The steady-state accumulation of some solutes was also determined using flow dialysis (Colowick and Womack, 1969). In all cases the steady-state concentration of amino acids measured was the same as determined by filtration assay. [b] Assay performed with 40 mM D-alanine as substrate.

only the rate ratio for glutamate transport remaining unchanged. Proline and serine transport, which showed maximal rate ratios at pH 6.6 of 1.9 and 1.8, now exhibit ratios of 1.2 and 1.0, respectively. Thus, at pH 7.5, the initial rate data indicate that the transport carriers are not only less active, but also that the manifestation of the enzymatic kinetic isotope effect on initial rate of solute accumulation is greatly decreased.

Relationship between the Steady-State Levels of Carrier-Mediated Solute Transport and the Isotope Effect on Substrate Oxidation. Next it was determined whether the difference in oxidation rates of ^1H- and ^2H-labeled substrates affects the steady-state levels of solute accumulation by membrane vesicles. In *E. coli* B membranes, D-[2-^{1}H]alanine and D-[2-^{2}H]alanine were used as energy sources and the steady-state levels of various solutes actively accumulated were determined from the plateau region of the transport profile. This plateau was reached in all cases approximately 12 min after initiation of the experiment and remained constant for another 8 min. The data from these profiles is presented in Table IV both as nmol of transport solute accumulated/mg of protein and as concentration gradients (in/out ratios) maintained with the protio and deuterio energy sources. The ratio of concentration gradients supported by D-[2-^{1}H]alanine vs. D-[2-^{2}H]alanine is shown in the last column of Table IV. These ratios quite remarkably are all approximately 1.0, despite a 1.8-fold difference in substrate oxidation rates.

The steady-state accumulation of solutes was similarly monitored in membrane vesicles from *E. coli* ML 308-225 at pH 6.6 using either filtration assay or flow dialysis (Colowick and Womack, 1969) with [^{1}H]succinate and [^{2}H]succinate as energy sources. The data in Table IV indicate that all solutes are accumulated at the steady-state equally well, stimulated by the two different energy sources. When this same type of experiment was performed with [2,3-$^1H_4$]- and [2,3-$^2H_4$]-succinate at pH 7.5 and proline accumulation monitored, there was no difference in the final steady-state level of amino acid concentration achieved (data not shown). These results argue that, although an isotope effect may exist on enzyme turnover, the total driving force established by D-alanine or succinate dehydrogenase catalysis is the same regardless of the change in rate of oxidation.

The Relationship between Kinetic Isotope Effects on Substrate Oxidation and the Total Driving Force for Active Transport in Membrane Vesicles. According to the chemiosmotic hypothesis, the driving force for active transport is established by the vectorial expulsion of protons from the membrane, resulting in the formation of an electrochemical potential ($\Delta\bar{\mu}_{H^+}$) across the membrane. $\Delta\bar{\mu}_{H^+}$ is then the energy derived from the transmembrane electrical potential due to charge separation ($\Delta\Psi$) and the chemical potential due to the concentration gradient of protons ($-2.3(RT/F)\Delta pH$) maintained across the membrane. Thus

$$\Delta\bar{\mu}_{H^+} = \Delta\Psi - 59\Delta pH$$

(at room temperature). We therefore examined the effects which a change in the rate of substrate oxidation might have on each component of $\Delta\bar{\mu}_{H^+}$ to understand the initial rate and steady-state observations listed above.

Using the steady-state concentration gradient of $TPMP^+$ or Rb^+, cations which passively enter respiring vesicles (Schuldiner and Kaback, 1975), the transmembrane potential can be calculated by an application of the Nernst equation (Harold and Altendorf, 1974). Figure 2A shows the uptake of $TPMP^+$ at pH 6.6 in the presence of either [2,3-$^1H_4$]- or [2,3-$^2H_4$]succinate. Although initially there is a slight difference in the rate at which this lipophilic cation crosses the membrane in the presence of the two energy sources (Table II), the steady-state accumulation reached and sustained after 5 min of uptake is nearly identical in both cases. When the concentration gradient representing this accumulation (Table III) is used to calculate $\Delta\Psi$ by the Nernst equation, −64 to −66 mV of membrane potential is obtained, in good agreement with the value reported by Kaback and co-workers (Ramos et al., 1976).

It should be emphasized that probes such as $TPMP^+$ appear

only to measure the final level of membrane potential, and not the rate of $\Delta\Psi$ formation. As shown in Figure 2B, preincubation of membranes with each energy source 5 min before $TPMP^+$ addition still results in steady-state cation accumulation requiring 4–5 min for completion. These results indicate that the rate-limiting step in the determination of $\Delta\Psi$ is the diffusion of $TPMP^+$ across the vesicle membrane. Therefore, although the 2.3-fold difference in the rate of oxidation of [^{1}H]- and [^{2}H]succinate is not manifested in different steady-state values of $\Delta\Psi$, the kinetic isotope effect on dehydrogenase catalysis may still affect the rate of $\Delta\Psi$ formation.

Experiments performed with [^{1}H]- and [^{2}H]succinate at pH 7.5 also indicate that the steady-state levels of $TPMP^+$ accumulation are not affected by the threefold difference in the rate of substrate oxidation at this pH (Table IV). The level of membrane potential formed, however, is approximately −75 mV, slightly greater than that formed at pH 6.6. Similarly, when [^{1}H]- and [^{2}H]-D-alanine were employed as energy sources in *E. coli* B membrane vesicles and Rb^+ accumulation was monitored at the steady-state, the isotope effect on oxidation is not translated into the final $\Delta\Psi$ achieved. Table IV shows that [^{1}H]- and [^{2}H]-D-alanine result in approximately the same concentration of Rb^+, each giving a membrane potential between −55 and −57 mV after 10 min of uptake.

Recently, determination of the ΔpH component of $\Delta\bar{\mu}_{H^+}$ in membrane vesicles and bacterial cells has become possible (Ramos et al., 1976; Padan et al., 1976). Kaback's laboratory has used the technique of flow dialysis to follow the distribution of weak acids across vesicle membranes in the presence of an energy source, from which ΔpH can be calculated. They have found the magnitude of the proton gradient to be dependent on the pH of the external medium, varying from −110 mV at pH 5.5 to 0 mV at pH 7.5 in *E. coli* ML 308-225 (Ramos et al., 1976).

In order to see if changes in substrate oxidation rates affect the formation of the proton gradient, this technique has been used with both acetate and DMO as probes to measure ΔpH in the presence of ^{1}H- and ^{2}H-labeled substrates. Although the observed changes in the external concentration of weak acids are small and variation in the calculated ΔpH values can arise due to the imprecision of the flow dialysis technique at conditions where less than the maximum ΔpH is expressed, these determinations have been performed numerous times with both probes to convince ourselves of their validity. With either [^{1}H]- or [^{2}H]succinate at pH 6.6, the pH of the vesicle lumen increases to between 7.4 and 7.5 upon energization of the membrane in good agreement with published data for ML 308-225 (Ramos et al., 1976). Unfortunately, this method of ΔpH determination yields essentially no information about the kinetics of proton gradient establishment with the protio or deuterio substrate. However, there appears to be no significant difference in the intravesicular pH generated by either energy source. The corresponding ΔpH values yield a chemical potential of −48 to −55 mV for the proton gradient formed.

When D-[^{1}H]- and D-[^{2}H]alanine are oxidized by *E. coli* B membrane vesicles at pH 7.5, a ΔpH is also formed. Unlike *E. coli* ML 308-225 vesicles, which maintain an internal pH of 7.5 during respiration, *E. coli* B membranes establish a higher internal pH; membrane vesicles oxidizing either form of D-alanine generate a pH of 8.35–8.4 inside, corresponding to a chemical potential of −50 to −52 mV. Again, as with succinate oxidation, there is no difference in the concentration gradient of protons established when D-[^{1}H]- or D-[^{2}H]alanine is employed as an energy source.

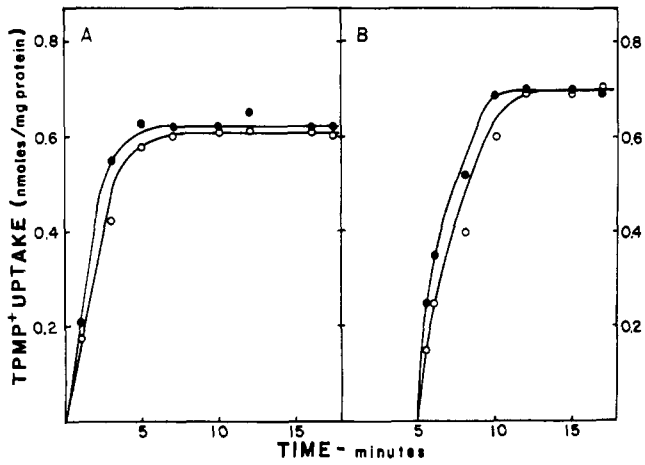

FIGURE 2: Uptake of triphenylmethylphosphonium ion stimulated by succinate oxidation in *E. coli* ML 308-225 membrane vesicles. (A) Twenty-five microliters of *E. coli* ML 308-225 membranes (0.125 mg of protein) were added to 10 mM magnesium sulfate and water to give 50 μL of solution. After incubation for 3 min at 25 °C, 20 mM [2,3-$^1H_4$]-succinate (●—●) or [2,3-$^2H_4$]succinate (○—○) (each buffered at pH 6.6) was added along with [^{3}H]triphenylmethylphosphonium bromide and uptake followed by filtration onto cellulose acetate filters. (B) In a similar experiment to that described in A, membranes were preincubated with succinate for 5 min before $TPMP^+$ addition.

The above results indicate that, despite the kinetic isotope effect seen on succinate and D-alanine oxidation, there is no difference in the total driving force ($\Delta\bar{\mu}_{H^+}$) formed, which is responsible for the concentration of transport solutes. This is consistent with the observations of the last section where deuterated substrates stimulate the same final concentration gradients for amino acid and sugar transport as their protio counterparts.

Factors Affecting the Initial Rate of Carrier-Mediated Transport in E. coli ML 308-225 Membrane Vesicles. In order to determine how the kinetic isotope effects from succinate and D-alanine oxidation are exerted on initial rates of solute uptake, experiments were designed to examine the correlation between initial rate of transport and the rate of formation of the electrochemical potential.

First, the relationship between the initial rates of solute accumulation and $\Delta\bar{\mu}_{H^+}$ was determined, whether the V_{max} for carrier-mediated transport increases as $\Delta\bar{\mu}_{H^+}$ increases. In order to do this, the size of $\Delta\bar{\mu}_{H^+}$ must be varied in a controlled way. Schuldiner and Kaback have recently shown that the amount of D-lactate-supported $TPMP^+$ accumulation at the steady-state by membrane vesicles can be manipulated by addition of minute amounts of uncouplers, among them carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Schuldiner and Kaback, 1975). Both $\Delta\Psi$ and ΔpH are titrated in parallel fashion by this uncoupler so that $\Delta\bar{\mu}_{H^+}$ can be varied from 0 to its maximum value in the absence of CCCP (Ramos et al., 1976).

When respiring vesicles oxidizing D-lactate are incubated with various concentrations of CCCP (10^{-8} to 10^{-6} M), and V_i for proline transport is measured in a 10–60-s interval, a range of initial rates are obtained (Figure 3A). In parallel simultaneous experiments, the vesicles were allowed to accumulate [^{3}H]$TPMP^+$ to the steady state to measure the magnitude of the effective membrane potential at each CCCP concentration. Using the Nernst equation to calculate $\Delta\Psi$ from the observed $TPMP^+$ concentration gradients, the plot shown in Figure 3B can be made of V_i for proline transport vs. $\Delta\Psi$ (in

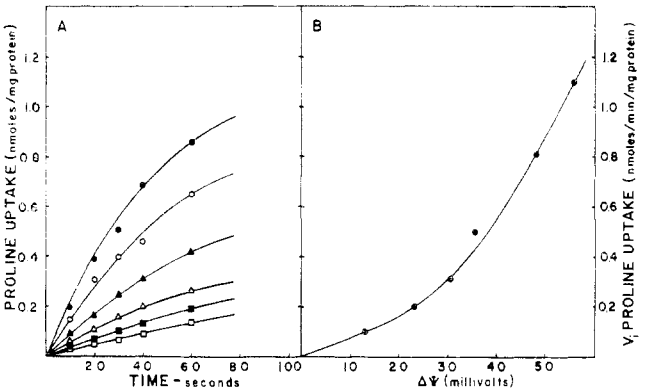

FIGURE 3: The effect of membrane potential on the initial rate of proline transport in *E. coli* ML 308-225 membrane vesicles. (A) The effect of various concentrations of carbonyl cyanide *m*-chlorophenylhydrazone on the initial rates of D-lactate stimulated proline uptake in *E. coli* ML 308-225 membrane vesicles was investigated. Twenty-five microliters of membranes (0.25 mg of protein) was preincubated with 10 mM magnesium sulfate, water, 20 mM D-lactate, and various concentrations of CCCP at 25 °C for 5 min (50 μL final volume). L-[^{14}C]Proline was then added and uptake monitored at several time points during the first minute of transport. The CCCP concentrations employed were: no CCCP (●—●); 2.5×10^{-8} M CCCP (○—○); 5×10^{-8} M CCCP (▲—▲); 1.5×10^{-7} M CCCP (△—△); 3×10^{-7} M CCCP (■—■); 4.5×10^{-7} M CCCP (□—□). (B) The initial rates of proline uptake were measured from the transport profiles in A. In identical experiments performed at the same time, $TPMP^+$ accumulation was monitored at 5, 7, 10, and 15 min. The steady-state level of cation uptake was estimated from these values, and the magnitude of membrane potential in millivolts was calculated by the Nerst equation. The initial rates of proline transport were then plotted against the corresponding ΔΨ values.

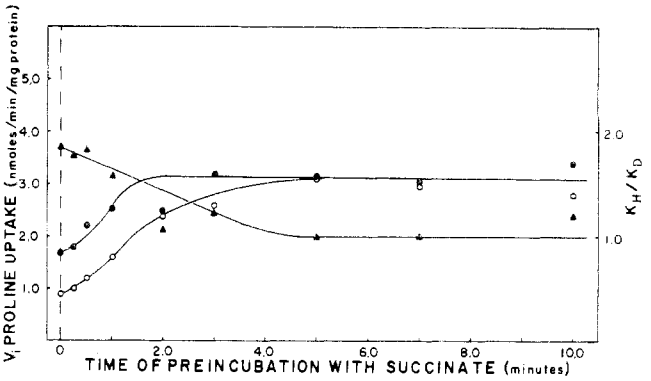

FIGURE 4: The effect of preincubation with energy source on the initial rate of proline transport in *E. coli* ML 308-225 membrane vesicles. Twenty-five microliters of *E. coli* ML 308-225 membranes (0.125 mg of protein) was mixed with 10 mM magnesium sulfate and water to give 50 μL (final concentration, 50 mM potassium phosphate, pH 6.6). [2,3-$^1H_4$]Succinate (●—●) or [2,3-$^2H_4$]succinate (○—○), 20 mM, was added followed by [^{14}C]proline after different lengths of preincubation and transport monitored for 30 s. The initial rates of proline uptake observed were then plotted against time of preincubation with succinate. Also represented is the ratio of rates of transport stimulated by [^{1}H]succinate and [^{2}H]succinate (▲—▲) at each of the various preincubation times (k_H/k_D).

millivolts). Examination of these data show an initial curvature for solute velocity at low values of ΔΨ followed by apparent linear dependence of initial rates at higher ΔΨ levels.[4] Since proline is above saturating concentration for its respective carrier system in all of the experiments (confirmed by explicitly measuring K_m values for proline transport at different ΔΨ values (data not shown)) and D-lactate is also at saturating levels for the D-lactate dehydrogenase, each initial rate measured is for the proline carrier functioning at V_{max} for a given value of membrane potential. It is therefore the V_{max} value for proline accumulation which increases with increasing ΔΨ. It should be noted that in these experiments, membranes were preincubated with energy source for 5 min to ensure the complete formation of $\Delta\bar{\mu}_{H^+}$ before addition of transport solute (see Discussion below).

Although the ΔpH component of $\Delta\bar{\mu}_{H^+}$ was not measured explicitly in the above experiment due to the difficulty in measuring small ΔpH values by the current techniques, presumably the relationship between initial rates of transport and the electrochemical potential would be similar to the ΔΨ dependence observed. This predicts that, as $\Delta\bar{\mu}_{H^+}$ increases to its maximum value, the initial rates of solute transport should also increase. In an initial test of this hypothesis, *E. coli* ML 308-225 membranes were assayed for proline transport immediately after the addition of D-lactate and also after a 5-min preincubation with the energy source. It was found that the initial rate of proline accumulation increased 1.3-fold, from 1.6 nmol min^{-1} (mg of protein)$^{-1}$ to 2.1 nmol min^{-1} (mg of protein)$^{-1}$, after the 5-min preincubation (data not shown).

Experiments were next performed to see what effect a preincubation with [^{1}H]- and [^{2}H]succinate would have on initial rates of proline transport at pH 6.6. Upon a 1.5-min preincubation with energy source, the rate of proline accumulation stimulated by [^{1}H]succinate increased from 1.65 to 2.5 nmol min^{-1} (mg of protein)$^{-1}$, while that energized by [^{2}H]succinate also increased from 0.85 to 1.6 nmol min^{-1} (mg of protein)$^{-1}$, as determined from time points taken during the first 30 s of solute transport. Quite interestingly, the difference in solute influx stimulated by [^{1}H]- and [^{2}H]succinate decreases from 1.9 to 1.6 because of the 1.5-min preincubation. Figure 4 shows a complete time course of various preincubation times with [^{1}H]- and [^{2}H]succinate and the corresponding V_i's for proline uptake. In both cases, [^{1}H]- and [^{2}H]succinate eventually stimulated the same maximal value of 3.1 nmol min^{-1} (mg of protein)$^{-1}$ for the initial rate of proline accumulation. This rate is achieved in the case of the protio-substrate after approximately 2 min, while the deuterated compound requires 4 min of oxidation in order to stimulate proline uptake at this rate. Also plotted in Figure 4 are the rate ratios for transport, which decrease from 1.9 to 1.0 during increasing preincubation times with energy source.

It has been reported that the activity of the mammalian succinate dehydrogenase can be stimulated by preincubation with succinate (Hatefi and Stiggall, 1976). In order to ensure that the stimulation in transport rates are not simply a function of increasing enzyme activity, membranes were preincubated with substrate for various times (0–5 min) and the rate of dye reduction was monitored. In all cases the rate of enzyme turnover remained constant (data not shown).

When the corresponding preincubation experiments were performed with [^{1}H]- and [^{2}H]succinate at pH 7.5, a similar type of relationship was noted. The small difference in initial rates disappears as membranes are preincubated with either form of succinate for 2 min. The V_i's also increase from 0.57 and 0.51, respectively, to 0.92 nmol min^{-1} (mg protein)$^{-1}$, which is the maximal rate of proline accumulation seen at pH

[4] The initial curvature of Figure 3B may be due to some threshold value of ΔΨ (and $\Delta\bar{\mu}_{H^+}$) required to make the proline transport system functional. The need for such a phenomenon is totally unproven, but there is a comparision with the observation of a clear step function for ATP synthesis driven by an electrochemical potential in in vitro experiments with mitochondria (Thayer and Hinkle, 1975a,b).

7.5. The preincubation experiments at this pH indicate that the difference in initial rates stimulated by [^{1}H]- and [^{2}H]-succinate is eliminated more rapidly upon preincubation with energy source than was the case at pH 6.6 (data not shown).

The experiments presented above suggests two conclusions: that the V_i for solute transport is a function of $\Delta\bar{\mu}_{H^+}$ and that the electrochemical potential (or at least one of its components) requires a rather long time (minutes instead of seconds) to reach its maximal level. It would appear that the substrate kinetic isotope effect is mirrored by the initial rates of proline transport at early times (either fully or partially depending on pH) because $\Delta\bar{\mu}_{H^+}$ formation is slowed by the oxidation of [^{2}H]succinate. If $\Delta\bar{\mu}_{H^+}$ is allowed to reach its final steady-state size before transport is initiated and, as we have shown above, the magnitude of $\Delta\bar{\mu}_{H^+}$ is the same for [^{1}H]- and [^{2}H]succinate oxidation, then the initial rate of proline accumulation should be the same in the presence of the two forms of succinate and the kinetic isotope effect on transport should disappear. This is precisely what is observed.

Discussion

Using the presence of isotope effects when D-2-[^{2}H]alanine and 2,3-[$^2H_4$]succinate are oxidized by membrane-bound enzymes, we have probed the coupling of substrate oxidation to both initial rates and steady-state levels of solute active transport, and to the generation of the electrochemical potential by membrane vesicles. By employing protio and deuterio isomers of the same energy source, identical dehydrogenase and respiratory chain components are used, yet the rate of electron flow down the cytochrome chain is controlled (without the use of inhibitory agents) so that this flux is the only variable. In this way, various parameters of the transport process, especially the kinetics of formation of the energized membrane state, can be analyzed.

The substrate kinetic isotope effect on dehydrogenase action is manifested only on the initial rate of solute accumulation in membrane vesicles not on the steady-state levels of transport. This observed rate difference in solute uptake could arise in two ways. If the electrochemical potential is generated slowly, at rates controlled by oxidation of protio and deuterio substrates, this slow buildup could control the initial rate of carrier-mediated active transport. A kinetic isotope effect, then, in substrate oxidation could translate to one on $\Delta\bar{\mu}_{H^+}$ formation and consequently affect rates of solute uptake. The second possibility is that $\Delta\bar{\mu}_{H^+}$ is established very rapidly and the distinct turnover numbers for protio and deuterio substrates then yield different values for the steady-state electrochemical potential, higher with the more rapidly oxidized protio-energy source. At the higher value of $\Delta\bar{\mu}_{H^+}$, the rate of carrier-mediated active transport is faster. Since the steady-state level of $\Delta\bar{\mu}_{H^+}$ formed by protio and deuterio substrate oxidation is the same, this second possibility can be eliminated. Unsolved, however, is the mechanism by which a twofold difference in the rate of dehydrogenase turnover can result in the same final level of $\Delta\bar{\mu}_{H^+}$ formation.

Although it has not been possible in the *E. coli* membrane vesicle system to measure the kinetics of $\Delta\Psi$ and ΔpH formation with the current techniques available, literature precedents favor rapid membrane potential formation in some other systems. In mitochondria (Laris et al., 1975) and in chloroplasts (Bulychev et al., 1976), the rise in $\Delta\Psi$ has been measured with fluorescent dyes and microcapillary glass electrodes, respectively. It was found that membrane potential formation was complete within 1 s in the case of chloroplasts and in less than 10 s with mitochondria. It has been argued that, in whole bacterial cells, $\Delta\Psi$ formation precedes the complete formation of the proton concentration gradient and calculations show that, under certain conditions, a cell must pump 10^3 more protons to achieve a ΔpH of -60 mV than is required for a $\Delta\Psi$ of -60 mV[5] (Mitchell, 1966). If this is true, the ΔpH component of the electrochemical potential may be slow to form in membrane vesicles.

If $\Delta\bar{\mu}_{H^+}$ does rise slowly and, as shown in the Results section, initial rates of transport do vary with $\Delta\bar{\mu}_{H^+}$, then one would predict that preincubation of vesicles for various times with an energy source before addition of a transport solute should produce a concomitant increase in V_i for solute influx. This has been observed for both D-lactate- and succinate-stimulated proline uptake. More importantly, the carryover of the kinetic isotope effect for [2,3-$^2H_4$]succinate oxidation to the initial rate of proline accumulation disappears as the velocity of proline influx becomes maximal. One explanation of these results is that, if proline transport requires the cotransport of a proton (i.e., symport) (Mitchell, 1970, 1973), the effect of preincubation with an energy source is to raise the concentration of hydrogen ions available with time, due to proton extrusion into the external medium by the vesicles, allowing V_i's to correspondingly increase. Since the oxidation of ^{1}H and ^{2}H substrates eventually gives the same final ΔpH, the ultimate maximal velocity of influx possible must be the same with the two energy sources.

It should be noted that Lanyi and co-workers see a similar slow time course for the production of an energized state which can be used to stimulate glutamate transport in *H. halobium* cell envelope vesicles (Lanyi et al., 1976a,b). In this system, light is the stimulus for transport. When these vesicles are illuminated with light for various lengths of time, there is a concomitant increase in the initial rate of glutamate accumulation. In this case, the proton motive force $\Delta\bar{\mu}_{H^+}$ was shown to be achieved very rapidly, while the formation of a transmembrane Na^+ gradient ($Na^+_{out}/Na^+_{in} \gg 1$) requires more time, and it is this secondary Na ion gradient which is the driving force for glutamate transport. The existence of an identical mechanism for proline transport in *E. coli* ML 308-225 membrane vesicles is unlikely since there is no Na^+ present in the assay systems; however, the possibility of slow formation of some other secondary ion gradient (i.e., $K^+_{out}/K^+_{in} > 1$) has not been excluded and would be consistent with the experimental results of this paper.

Finally, in order to explain the range of transport rate ratios seen with the protio and deuterio forms of succinate and D-alanine as energy sources, the nature of the transport solutes should be divided into two categories: those which cross the membrane bound to carrier proteins, and those which must rely on passive diffusion or ionophores for permeability. In general, for the case of the carrier-bound solutes, if response of the membrane carrier to $\Delta\bar{\mu}_{H^+}$ and subsequent translocation into the vesicle lumen is fast relative to the formation of the driving force, then the full magnitude of the kinetic isotope effect on oxidation should be mirrored by initial rates of transport. This is seen for proline and serine transport stimulated by either D-alanine or succinate. However, if carrier response is of equivalent rate or slow relative to $\Delta\bar{\mu}_{H^+}$ formation, then the apparent isotope effect can decrease in magnitude, k_H/k_D going to 1.0. The issue is further complicated by the observation that certain bacterial membrane carrier systems respond primarily to $\Delta\Psi$, ΔpH, or $\Delta\bar{\mu}_{H^+}$ (Ramos and Kaback, 1977).

[5] M. Weiss, unpublished calculations.

Therefore, the rate of formation of each of these components considered along with the intrinsic rate of an individual carrier response can provide the variability in isotope effect ratios observed with the different solutes.

For transport stimulated by succinate at pH 7.5, the rate ratios in seven out of eight cases are decreased to near unity. This is probably due to two facts: that the carriers are intrinsically less active at this pH (the initial rates of uptake are all decreased from that at pH 6.6), and $\Delta\Psi$ (there is no net ΔpH component at pH 7.5) builds up more rapidly because the succinate dehydrogenase turns over two times faster at this pH. The net result may be that carrier response to $\Delta\Psi$ becomes slower than C–H bond cleavage in succinate oxidation, resulting in the disappearance of the threefold kinetic isotope effect when solute influx is measured.

As for non-carrier-mediated cation accumulation (Rb^+, $TPMP^+$), we have pointed out above the shortcomings of using these probes to measure the kinetics of membrane potential formation. Because diffusion across the vesicle membrane appears slow, there should necessarily be a lessening in the rate difference seen for cation uptake stimulated by protio and deuterio energy sources. This prediction is borne out by the data of Table II and reinforces our argument that slow response to the electrochemical potential formation during protio and deuterio substrate oxidation will result in diminished rate differences in the initial rate of solute active transport.

Acknowledgments

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