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Enthalpy changes in the formation of the proton electrochemical potential and its components

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Abstract

Enthalpy changes in the formation of a proton electrochemical potential ($\Delta\bar{\mu}_{H^+}$) and its components, ΔpH (proton gradient) and $\Delta\psi$ (electrical potential), across two types of *E. coli* membrane vesicles were investigated. Flow dialysis experiments showed that in 0.1 M KP_i, pH 6.6, *E. coli* GR19N membrane vesicles coupled with D-lactate exhibited 57 mV for ΔpH, 70 mV for $\Delta\psi$, and 127 mV for $\Delta\bar{\mu}_{H^+}$. Microcalorimetric measurements revealed that the corresponding enthalpy changes (ΔH_{pH}, ΔH_{ψ} and ΔH_m) were 3.5, 3.3 and 6.9 kcal/mole, respectively. Moreover, in *E. coli* ML 308-225 membrane vesicles across which 120 mV of $\Delta\bar{\mu}_{H^+}$ was generated, values of ΔH_{pH} and ΔH_{ψ} were determined as 7.0 and 6.6 kcal/mole, as compared with the previously reported 14.1 kcal/mole for ΔH_m. Comparisons of these enthalpy data revealed that component enthalpies (ΔH_{pH} and ΔH_{ψ}) essentially added up to the total enthalpy (ΔH_m), providing a self-consistent test for the obtained data. In both membranes, the ratio of ΔH_{ψ} to $\Delta\psi$ was comparable to that of ΔH_{pH} to ΔpH in the formation of $\Delta\bar{\mu}_{H^+}$. These observations indicated that the process of the movement of H^+ across the membranes was the major contributor to the observed energetic changes. Moreover, the enthalpy change in the formation of $\Delta\bar{\mu}_{H^+}$ was compared with the membranes derived from GR19N and ML 308-225 and coupled with NADH and D-lactate. The results were discussed in terms of trans-membrane phenomena.

Keywords: Membrane potential; Microcalorimetry; Thermodynamics

1. Introduction

Thermochemical methods are useful in the studies of molecular interactions and their related energetics in biological systems [1–6]. Nevertheless, such methods have not been generally applied to directly measure the energetics of trans-membrane phenomena [7–10]. The proton electrochemical potential ($\Delta\bar{\mu}_{H^+}$) across biological membranes is an important driving force for the accumulation of nutrients such as β-galactosides [11–15]. Owing to the crucial role of $\Delta\bar{\mu}_{H^+}$ in many living systems [16–18], information on its energetics and the use of this knowledge in a practical way have been important to biophysical chemists who are interested in trans-membrane phenomena.

In our previous work [7–10], we applied thermochemical methods to examine $\Delta\bar{\mu}_{H^+}$ across ML *E. coli* 308-225 membranes and to confirm its forma-

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tion from a thermochemical point of view. In the presence of an enzyme-associated (D-lactate) or an artificial electron donor system (phenazine methosulfate–ascorbate) to provide the energy needed to generate $\Delta\bar{\mu}_{H^+}$, it was found that the enthalpy change (ΔH_m) in the formation of $\Delta\bar{\mu}_{H^+}$ was 10 to 14 kcal/mole, when 110 mV of $\Delta\bar{\mu}_{H^+}$ was generated across the membranes. This magnitude of enthalpy change was just about one quarter to one third of that released from the D-lactate or ascorbate oxidation reaction, suggesting that some of the excess energy might be available to facilitate other membrane-related events.

To further examine the enthalpy change in the formation of $\Delta\bar{\mu}_{H^+}$ across the membranes, it is important to determine the enthalpy changes in the formation of the components of $\Delta\bar{\mu}_{H^+}$, the proton gradient (ΔpH) and the electrical potential ($\Delta\psi$). Such an investigation will enable us to elucidate the relationship among enthalpy changes in the formation of $\Delta\bar{\mu}_{H^+}$, ΔpH and $\Delta\psi$, and to provide a self-consistency test for these directly determined energetic data. A self-consistency test is not feasible for a fluorescence quenching or a flow dialysis method in its determination of $\Delta\bar{\mu}_{H^+}$, ΔpH and $\Delta\psi$, because in either method $\Delta\bar{\mu}_{H^+}$ is obtained from the total of the measured ΔpH and $\Delta\psi$, and is not determined directly.

In extension of our previous studies [7–10], we have further applied thermochemical methods to investigate the enthalpy changes (ΔH_m, ΔH_{pH} and ΔH_ψ) in the formation of $\Delta\bar{\mu}_{H^+}$, ΔpH and $\Delta\psi$ across two types of *E. coli* (GR19N and ML 308-225) membrane vesicles. The obtained results were compared with the magnitudes of $\Delta\bar{\mu}_{H^+}$, ΔpH and $\Delta\psi$ as determined by a flow dialysis method. The substrate oxidation reaction needed to provide an energy source for the formation of membrane potentials was assayed by an oxygen electrode. These observations were analyzed to determine the component enthalpy changes in relation to the total enthalpy change, and to obtain other thermodynamic information. The obtained information was further scrutinized to elucidate the contributions of H^+ and other ions such as K^+ to the energetics of the formation of membrane potentials. Moreover, comparative studies were performed on the membranes derived from GR19N and 308-225 and coupled with the substrates, NADH and D-lactate, to examine enthalpy data in terms of trans-membrane phenomena.

2. Experiments

2.1. Membrane vesicles

The cultures of *E. coli* were generously provided by Dr. R.H. Kaback, the University of California at Los Angeles. *E. coli* GR19N was grown aerobically into late logarithmic phase in minimal medium A containing 0.50% sodium lactate and 0.15% casamino acids [19]. *E. coli* ML 308-225 was grown in minimal medium A containing 1.0% disodium succinate [20]. Cells were harvested by centrifugation. Right-side-out membrane vesicles were prepared according to known procedures [21], and suspended in 0.10 M KP_i buffer solution (pH 6.6).

2.2. Chemicals

Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was obtained from Calbiochem (San Diego, CA). D-Lactate and NADH were purchased from Sigma Chemical Co., as were valinomycin and nigericin. All other chemicals were of reagent grade purchased from commercial sources.

2.3. Oxygen uptake measurements

The oxidation reaction of D-lactate or NADH was assayed by monitoring the oxygen uptake rate in the membranes using a Clark oxygen electrode (Gilson Medical Electronics) connected to a YSI biological oxygen monitor (Yellow Springs, Inc.) [7,10]. Assay mixtures were prepared under the same experimental conditions as for the calorimetric measurements. The assay was also performed in the presence of an appropriate low concentration of uncoupler such as CCCP, nigericin or valinomycin.

2.4. Flow dialysis measurements

Flow dialysis experiments were carried out to measure ΔpH and $\Delta\psi$ [15,22], the two components of $\Delta\bar{\mu}_{H^+}$ across the membranes. The procedures have previously been described [10]. Right-side-out

membranes exhibited $\Delta\bar{\mu}_{H^+}$ with the interior negative and basic [7,8,11,15]. The uptake of [^{14}C]-acetic acid and [^{3}H]-tetraphenylphosphonium ([^{3}H]-TPP$^+$) by membrane vesicles were therefore measured to determine ΔpH and $\Delta\psi$, respectively. The flow dialysis cell consisted of two chambers separated by a synthetic membrane. The top chamber contained membrane vesicles plus [^{14}C]-acetic acid or [^{3}H]-TPP$^+$. The buffer medium solution was pumped from the bottom chamber. To initiate the formation of $\Delta\bar{\mu}_{H^+}$ across the membranes, D-lactate or NADH was added. The presence of $\Delta\bar{\mu}_{H^+}$ drove the uptake of [^{14}C]-acetic acid or [^{3}H]-TPP$^+$ in the membranes, which then distributed itself between inside and outside of the membranes. At a later stage of the measurements, CCCP was added to the top chamber to abolish $\Delta\bar{\mu}_{H^+}$ [23,24]. The fraction in the outer medium of membrane vesicles containing [^{14}C]-acetic acid or [^{3}H]-TPP$^+$ was collected, and was measured by a Beckman scintillation counter. The ratio of [^{14}C]-acetic acid or [^{3}H]-TPP$^+$ inside to outside of the membranes was determined to calculate ΔpH and $\Delta\psi$, respectively, according to the Nernst equation. The value of $\Delta\bar{\mu}_{H^+}$ was then determined from the total of ΔpH and $\Delta\psi$.

2.5. Calorimetry

The enthalpy change (ΔH_m, ΔH_{pH}, or ΔH_ψ) in the formation of $\Delta\bar{\mu}_{H^+}$, ΔpH, or $\Delta\psi$ across the membranes was determined by a twin-reaction-cell batch microcalorimeter according to our previous procedures [7–10,25,26]. The calorimeter contained sample and reference cells, which were made of 18-karat gold. Each cell was divided into two compartments with capacities of about 1.6 and 1.2 ml. Both indirect and direct calorimetric schemes were employed to test self-consistency of the measurements.

In the indirect microcalorimetric scheme [7–9], ΔH_m, ΔH_{pH}, or ΔH_ψ was measured by two separate experiments: (a) a mixing reaction accompanied by the formation of $\Delta\bar{\mu}_{H^+}$, and (b) a mixing reaction in which the formation of $\Delta\bar{\mu}_{H^+}$, ΔpH, or $\Delta\psi$ was prevented. In experiment (a), the mixing reaction of membrane vesicles with D-lactate or NADH consisted of the substrate oxidation reaction and the formation of $\Delta\bar{\mu}_{H^+}$ across the membranes. In contrast, in a separate experiment (b), the mixing reaction was performed in the presence of CCCP (6 μM), nigericin (0.10 μM), or valinomycin (2 μM), which was used to abolish the formation of $\Delta\bar{\mu}_{H^+}$, ΔpH, or $\Delta\psi$ across the membranes, respectively. Consequently, ΔH_m, ΔH_ψ, or ΔH_{pH} was determined from the data obtained with experiments (a) and (b) (see Results Section).

In the direct microcalorimetric scheme [10], the above experiments (a) and (b) were performed in a single calorimetric mixing: mixing of experiment (a) in the sample cell and that in experiment (b) in the reference cell. To do so, one compartment of the sample cell contained the substrate (D-lactate or NADH), and the other contained the membranes. Meanwhile, one compartment of the reference cell contained D-lactate or NADH, and the other contained the membranes in the presence of CCCP, nigericin, or valinomycin.

The observed heat changes obtained in the indirect scheme were compared with those in the direct scheme to assure the self-consistency of measurements. From the amount of D-lactate or NADH oxidized (determined by oxygen uptake measurements), the enthalpy change in the formation of $\Delta\bar{\mu}_{H^+}$, ΔpH, or $\Delta\psi$ across the membranes was determined.

3. Results

Typical flow dialysis measurements on the proton gradient (ΔpH) and the electrical potential ($\Delta\psi$) across *E. coli* membrane vesicles are shown in Figs. 1 and 2, respectively. The addition of D-lactate to the membranes generates $\Delta\bar{\mu}_{H^+}$ across the membranes, causing the uptake of [^{14}C]-acetic acid or [^{3}H]-TPP$^+$ in the membranes. As a result of this, the amount of [^{14}C]-acetic acid or [^{3}H]-TPP$^+$ in the outer medium is decreased and the curve shifts downward. In contrast, upon the addition of CCCP, $\Delta\bar{\mu}_{H^+}$ across the membranes is abolished. This results in the release of [^{14}C]-acetic acid or [^{3}H]-TPP$^+$ from the membranes to the outer medium and the curve shifts upward. From the measured ratio of [^{14}C]-acetic acid or [^{3}H]-TPP$^+$ inside to outside of the membranes, ΔpH or $\Delta\psi$ can be determined, respectively, according to the Nernst equation. The obtained ΔpH or $\Delta\psi$ is a

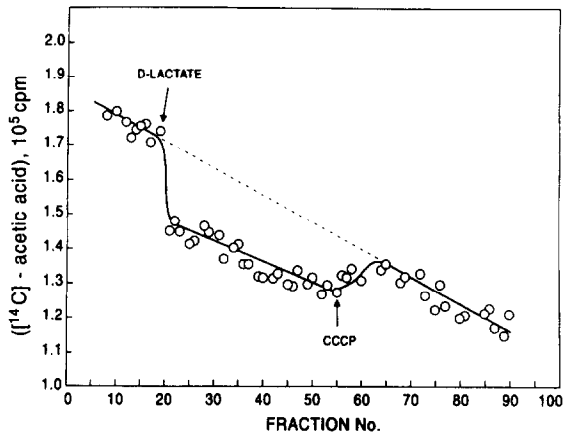

Fig. 1. Flow dialysis measurement of [^{14}C]-acetic acid uptake by *E. coli* membrane vesicles in the determination of ΔpH. Cpm of [^{14}C]-acetic acid refers to the outer medium. D-Lactate and CCCP were added to the membranes at the stages indicated by arrows. Final concentrations of the membranes, D-lactate, CCCP and [^{14}C]-acetic acid were 14 mg/ml, 19 mM, 20 μM, and 0.07 μM, respectively. All solutions were in 0.10 M KP_i, pH 6.6 buffer containing 10 mM $MgSO_4$.

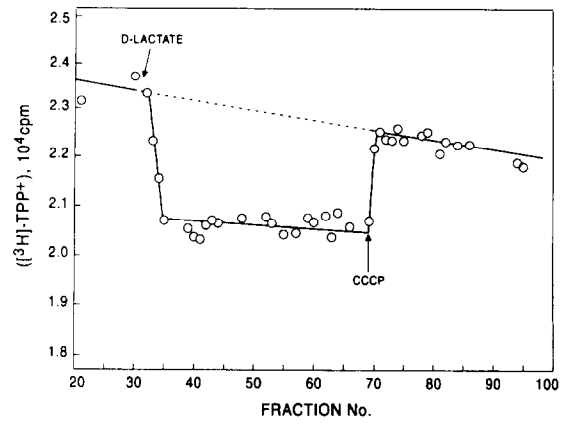

Fig. 2. Flow dialysis measurement of [^{3}H]-tetraphenylphosphonium ([^{3}H]-TPP^+) uptake by *E. coli* membrane vesicles in the determination of $\Delta\psi$. Cpm of [^{3}H]-TTP^+ refers to the outer medium. D-Lactate and CCCP were added into the membranes at stages as indicated by arrows. Final concentrations of the membranes, D-lactate, CCCP, and [^{3}H]-TPP^+ are 7.7 mg/ml, 20 mM, 20 μM and 0.9 μM, respectively. All solutions were in 0.10 M KP_i pH 6.6 buffer containing 10 mM $MgSO_4$.

steady-state potential which is imposed across the membranes in the presence of D-lactate oxidation reaction. The sum of ΔpH and $\Delta\psi$ is equal to $\Delta\bar{\mu}_{H^+}$.

Table 1 indicates that, when the medium is 0.10 M KP_i pH 6.6 and the electron donor is D-lactate, the values of ΔpH, $\Delta\psi$, and $\Delta\bar{\mu}_{H^+}$ across *E. coli* GR19N are 57 ± 0.3, 70 ± 0.6 and 127 ± 0.7 mV, respectively. For comparison purposes, Table 1 also includes the previously reported values of ΔpH (63 mV), $\Delta\psi$ (57 mV), and $\Delta\bar{\mu}_{H^+}$ (120 mV) across *E. coli* 308-225 membrane vesicles under the same experimental conditions [15]. The results showed that, when the substrate is D-lactate, the magnitude of $\Delta\bar{\mu}_{H^+}$ is comparable across these two types of membranes. In contrast, when the electron donor is NADH, Table 2 shows that their values are 76 ± 0.6, 81.0 ± 1.0, and 157 ± 1.3 mV, respectively. Comparison of these results with those obtained with D-lactate reveals that the magnitude of $\Delta\bar{\mu}_{H^+}$ across the membranes coupled with NADH is significantly higher than that with D-lactate. Tables 1 and 2 show that the reproducibility of our obtained flow dialysis results is very good and is within ± 1 mV.

Table 1
Enthalpy changes in the formation of ΔH_{pH}, ΔH_ψ and ΔH_m across membrane vesicles vesicles prepared from *E. coli* GR19N and ML 308-225 [a]

E. Coli membranes [b]	ΔpH (mV)	$\Delta\psi$ (mV)	$\Delta\bar{\mu}_{H^+}$ (mV)	ΔH_{pH} (kcal/mole) [c]	ΔH_ψ (kcal/mole)	ΔH_m (kcal/mole)
GR19N	57 ± 0.3	70 ± 0.6	127 ± 0.7	3.5 ± 0.2	3.3 ± 0.2	6.9 ± 0.9
ML 308-225	63 [d]	57 [d]	120 [d]	7.0 ± 0.6	6.6 ± 0.6	14.1 ± 0.8 [e]

[a] The value of ΔH_{pH}, ΔH_ψ or ΔH_m is an average of 4–5 measurements. The value of $\Delta\bar{\mu}_{H^+}$ is the sum of ΔpH and $\Delta\psi$ and is not independently measured. The value of ΔpH, $\Delta\psi$ or $\Delta\bar{\mu}_{H^+}$ is an average of 3–4 measurements.
[b] External medium solution was 0.10 M pK_i, pH 6.6.
[c] Enthalpy data are in the unit of kcal per mole of D-lactate.
[d] Taken from Ref. [15].
[e] Taken from Ref. [7].

Table 2
Comparison of the enthalpy changes in the formation of $\Delta\bar{\mu}_{H^+}$ across *E. coli* GR19N membrane vesicles coupled with D-lactate and NADH [a]

Source of substrate	ΔpH (mV)	$\Delta\psi$ (mV)	$\Delta\bar{\mu}_{H^+}$ (mV)	ΔH_m (kcal/mole)
D-lactate [b]	57 ± 0.3	70 ± 0.9	127 ± 1.0	6.9 ± 0.9 [d]
NADH [c]	76 ± 0.6	81 ± 1.0	157 ± 1.3	10.0 ± 0.8 [e]

[a] See footnote a in Table 1.
[b] External medium solution was 0.10 M pK_i pH 6.6.
[c] External medium solution was 0.10 M pK_i pH 7.5, since NADH was almost instantly decomposed in acid solution and was relatively stable in pH 7.5 or higher.
[d] kcal/mole of D-lactate.
[e] kcal/mole of NADH.

In microcalorimetric measurements, the heat change (ΔQ_a) in the mixing of membrane vesicles coupled with D-lactate or NADH was denoted as

$$\Delta Q_a = \Delta Q_{ox} + \Delta Q_m \quad (1)$$

$$= \Delta Q_{ox} + \Delta Q_{pH} + \Delta Q_\psi \quad (2)$$

$$\Delta Q_m = \Delta Q_{pH} + \Delta Q_\psi \quad (3)$$

In these equations, ΔQ_{ox} was the heat of oxidation reaction, and ΔQ_m, ΔQ_{pH}, and ΔQ_ψ were the heat changes in the formation of $\Delta\bar{\mu}_{H^+}$, ΔpH, and $\Delta\psi$, respectively.

It has been known that nigericin is an ionophore that catalyzes electrical exchange of protons for K^+, thereby collapsing ΔpH [15,24,27,28]. In contrast, valinomycin abolishes $\Delta\psi$ in the presence of K^+ [15,29]. CCCP can collapse $\Delta\bar{\mu}_{H^+}$ since it functions as a proton carrier which transports protons across the membranes [23,24]. In the presence of the appropriate concentration of CCCP, nigericin, or valinomycin, the mixing reaction therefore proceeded with: the oxidation reaction alone, the oxidation reaction plus the formation of $\Delta\psi$, or the oxidation reaction plus the formation of ΔpH, respectively. The corresponding heat change (ΔQ_b, ΔQ_c or ΔQ_d) was represented by

$$\Delta Q_b = \Delta Q_{ox} \quad (4)$$

$$\Delta Q_c = \Delta Q_{ox} + \Delta Q_\psi \quad (5)$$

$$\Delta Q_d = \Delta Q_{ox} + \Delta Q_{pH} \quad (6)$$

In Eqs. (5) and (2), ΔQ_ψ was taken as equal, presuming that the compensated $\Delta\psi$ resulting from the movement of K^+ due to the presence of nigericin did not significantly contribute to the total heat change (ΔQ_ψ) in the formation of $\Delta\psi$ across the membranes. This assumption will be justified in the Conclusions.

From Eqs. (1–6), the magnitude of ΔQ_m, ΔQ_{pH}, or ΔQ_ψ was determined from Eq. (1)–Eq. (4), Eq. (2)–Eq. (5), and Eq. (2)–Eq. (6), respectively. These relationships were described as follows:

$$\Delta Q_m = \Delta Q_a - \Delta Q_b \quad (7)$$

$$\Delta Q_{pH} = \Delta Q_a - \Delta Q_c \quad (8)$$

$$\Delta Q_\psi = \Delta Q_a - \Delta Q_d \quad (9)$$

From the obtained ΔQ_m, ΔQ_{pH}, and ΔQ_ψ and the known amount of D-lactate or NADH oxidized, the corresponding enthalpy change (ΔH_m, ΔH_{pH}, or ΔH_ψ) in the formation of $\Delta\bar{\mu}_{H^+}$, ΔpH, or $\Delta\psi$ across the membranes could be determined.

Alternatively, ΔQ_{pH} and ΔQ_ψ could also be obtained by the following relationships:

$$\Delta Q_{pH} = \Delta Q_d - \Delta Q_b \quad (10)$$

$$\Delta Q_\psi = \Delta Q_c - \Delta Q_b \quad (11)$$

Self-consistency of the results could be tested by comparing the determined ΔQ_{pH} from Eq. (8) with that from Eq. (10), and the determined ΔQ_ψ from Eq. (9) with that from Eq. (11).

An appropriate low-concentration of CCCP (6 μM), nigericin (0.1 μM) or valinomycin (2 μM) was employed to abolish $\Delta\bar{\mu}_{H^+}$, ΔpH or $\Delta\psi$ across membrane vesicles in microcalorimetric measurements. Uncoupler-concentration-dependent experiments were performed to assure that the designated membrane potential was abolished under such conditions. In addition, it was found that such concentrations of uncouplers cause only a slight decrease in the oxygen uptake rate in the membranes. For instance, Clark oxygen electrode measurements showed that the oxygen uptake rates were $(4.4 \pm 0.4) \times 10^{-8}$ and $(2.6 \pm 0.2) \times 10^{-7}$ mole/(min mg protein), when the substrates were D-lactate and NADH, respectively. In the presence of 6 μM CCCP, the oxygen uptake rates were $(4.2 \pm 0.2) \times 10^{-8}$ and $(2.4 \pm 0.2) \times 10^{-7}$ mole/(min · mg protein), respectively. This slight decrease in the oxygen uptake rate was compensated by a slight increase in the reaction

time (see below). Consequently, the total amount of oxygen consumed (a product of the rate and the duration of the reaction) was essentially unchanged in the presence of such concentrations of uncouplers.

The above amount of D-lactate or NADH oxidized was determined by the amount of oxygen uptaken in the membranes. As D-lactate was coupled with the membranes, the substrate oxidation reaction proceeded according to Eq. (12).

$$\text{D-lactate} + \tfrac{1}{2}O_2 \rightarrow \text{pyruvate} + H_2O \qquad (12)$$

Eq. (12) was catalyzed by D-lactate dehydrogenase in the membranes [30,31]. The amount of D-lactate oxidized was twice that of the consumption of oxygen. Similarly, when NADH was added to the membranes, the substrate oxidation reaction proceeded according to Eq. (12).

$$NADH + \tfrac{1}{2}O_2 + H^+ \rightarrow NAD^+ + H_2O \qquad (13)$$

According to Eq. (13), the amount of NADH oxidized was also twice that of the consumption of oxygen by the membranes.

Fig. 3 shows typical indirect microcalorimetric determination of ΔH_{pH} across *E. coli* GR19N membrane vesicles. The mixing reaction was involved with membrane vesicles coupled with D-lactate. Curve a in the figure represents the mixing reaction in the absence of nigericin, where $\Delta Q_a = -15.9$ mcal and the total amount of D-lactate oxidized = 5.79×10^{-7} mole, and $\Delta H_a = -27.5$ kcal/mole. A reliable determination of ΔQ from the area under curve a or b where the level of the final baseline is higher than that in the initial baseline has been described previously [7]. The validity of the extrapolation of the postreaction baseline back through the reaction was confirmed by the self-consistency between the indirect and direct measurements in the energetic studies in inverted membrane vesicles [10], and further confirmed in the present study (see below). Curve b denotes the mixing reaction in the presence of 0.1 μM nigericin, where $\Delta Q_c = -18.0$ mcal and the total amount of D-lactate oxidized = 5.80×10^{-7} mole, and $\Delta H_c = -31.0$ kcal/mole. According to Eq. (8), ΔH_{pH} was determined by the difference between ΔH_a and ΔH_c, and was found to be 3.5 kcal/mole. A good reproducibility of the determined ΔH_{pH} in five measurements (± 0.2 kcal/mole or $\pm 5\%$ (see Table 1)) confirmed the reliability of subtracting the enthalpy value in curve a from that in curve b.

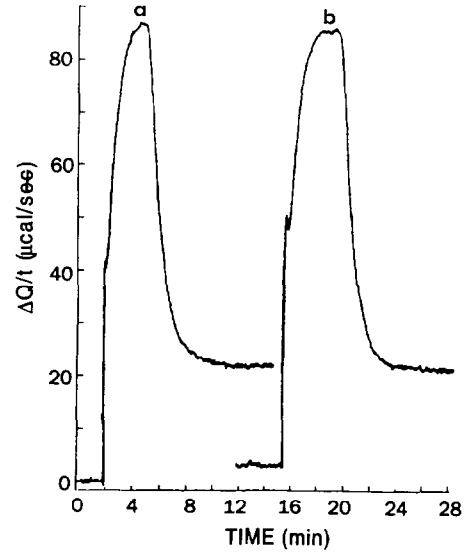

Fig. 3. Typical calorimetric measurements of the mixing of *E. coli* GR19N membrane vesicles with D-lactate: (a) in the absence of nigericin and (b) in the presence of nigericin. The concentrations of membrane vesicles, D-lactate, and nigericin were 3 mg/ml, 5 mM, and 0.1 μM, respectively. All solutions were in a 0.10 M KP_i, pH 6.6 buffer containing 10 mM $MgSO_4$.

In a similar indirect microcalorimetric measurement involving the uncoupler CCCP (figure not shown), it was found that the mixing of the membranes with NADH was exothermal, where ΔQ_a was -17.6 ± 0.2 mcal, the total amount of NADH oxidized was 3.32×10^{-7} mole, and the enthalpy change ΔH_a was -53 ± 2 kcal/mole. In the presence of 6 μM CCCP, ΔQ_b was determined as -20.6 ± 0.2 mcal, the total amount of NADH oxidized was 3.18 $\times 10^{-7}$ mole, and the enthalpy change ΔH_b was -64 ± 2 kcal/mole. According to Eq. (7), the enthalpy change (ΔH_m) in the formation of $\Delta\bar{\mu}_H$ was determined as 11 ± 2 kcal/mole. Moreover, in a direct microcalorimetric scheme, it was found that ΔH_m exhibited a value of 10 ± 1 kcal/mole, consistent with 11 ± 2 kcal/mole obtained in an indirect microcalorimetric scheme. Self-consistency between the results obtained with direct and indirect mi-

crocalorimetric schemes assured not only the reliability of our measurements, but also the validity of the baseline determination in the indirect measurement as described above.

Table 1 lists the values of ΔH_{pH}, ΔH_{ψ}, and ΔH_m as 3.5 ± 0.2, 3.3 ± 0.2 and 6.9 ± 0.9 kcal/mole, respectively, in GR19N membrane vesicles coupled with D-lactate. These data show that the total of ΔH_{pH} and ΔH_{ψ} is essentially equal to ΔH_m. Moreover, in 308-225 membrane vesicles coupled with the same substrate, the values of ΔH_{pH} and ΔH_{ψ} are 7.0 ± 0.6 and 6.6 ± 0.6 kcal/mole, respectively. The total of these two values is comparable to our previously reported 14.1 ± 0.8 kcal/mole for ΔH_m [7]. These results demonstrate that the sum of the component enthalpy changes (ΔH_{pH} and ΔH_{ψ}) is equivalent to the total enthalpy change (ΔH_m).

Table 2 shows that, in GR19N membrane vesicles coupled with NADH, the value of ΔH_m is 10 ± 0.8 kcal/mole. This value is significantly higher than that in the membrane vesicles coupled with D-lactate (6.9 ± 0.9 kcal/mole). In parallel, the magnitude of $\Delta\bar{\mu}_{H^+}$ obtained with NADH (157 mV) is also significantly higher than that with D-lactate (127 mV).

4. Conclusion

$\Delta\bar{\mu}_{H^+}$ across the membranes is composed of two components, ΔpH and $\Delta\psi$. A respiratory chain is involved in the movement of protons across *E. coli* membranes leading to the formation of $\Delta\bar{\mu}_{H^+}$. Examination of the data of $\Delta\bar{\mu}_{H^+}$, ΔH_{pH} and ΔH_{ψ} as shown in Table 1 reveal that, in the two types of membrane vesicles under study, the total of ΔH_{pH} and ΔH_{ψ} is essentially equal to ΔH_m. These results suggested that, of the ions in the medium, H^+ was not only the primary ion which moved across the membranes in the formation of ΔH_m and its components, but also the major contributor to the enthalpy changes. This, in turn, supported the validity of these energetic parameters for the formation of membrane potentials.

The above additivity of enthalpy changes in the formation of membrane potentials also justified the assumption that ΔQ_{ψ} was taken as equal in Eq. (2) and (5) (see the Results Section). Nigericin simultaneously abolishes ΔpH across the membranes and compensates $\Delta\psi$, resulting in an increase in the magnitude of $\Delta\psi$ [15,24,27,28]. If ΔQ_{ψ} in Eq. (2) were not equal to that in Eq. (5), Eq. (8) does not hold and the sum of ΔH_{pH} and ΔH_{ψ} would not be equal to the total ΔH_m. Furthermore, a compensated increase in the magnitude of $\Delta\psi$ due to the presence of nigericin, occurs as H^+ and K^+ move across the membranes in an opposite directions. The additivity of enthalpy changes implies that the movement of K^+ across the membranes makes a minor contribution to the enthalpy changes, as compared with H^+. The observation of a significant enthalpy change in the process of the movement of H^+ across the membranes leading to the formation of $\Delta\bar{\mu}_{H^+}$ is not unexpected, due to complex respiratory chain mechanisms involved in the process.

Table 1 reveals that in each of the two membrane vesicles the ratio ΔH_{ψ} to $\Delta\psi$ is comparable to that of ΔH_{pH} to ΔpH. The latter ratio is attributed to H^+ exclusively, while the former ratio is attributed to H^+ in addition to other ions in the medium. The observed comparable values between the two ratios suggested that the translocation of H^+ across the membrane was the major contribution to the enthalpy change in the formation of an electrical potential ($\Delta\psi$), while the redistribution of ions other than H^+ on the membrane was less important in contributing to ΔH_{ψ}.

Table 1 also shows that, when the membranes couple with the same electron donor (D-lactate), $\Delta\bar{\mu}_{H^+}$ exhibits a comparable value across membrane vesicles derived from *E. coli* GR19N and *E. coli* 308-225 (127 versus 120 mV). In contrast, the magnitude of ΔH_m for the strain GR19N (6.9 kcal/mole) is significantly lower than that for the strain 308-225 (14.1 kcal/mole), revealing a lower ratio of $\Delta H_m/\Delta\bar{\mu}_{H^+}$ for GR19N membranes. These observations could be related to the characteristics of GR19N which is a cytochrome *d*-deficient mutant with a relatively simple, linear system [19,32,33].

The enthalpy changes in the formation of $\Delta\bar{\mu}_{H^+}$ derived from the two substrates, D-lactate and NADH, are compared in Table 2, where the magnitude of $\Delta\bar{\mu}_{H^+}$ generated across the membranes coupled with NADH (157 mV) is significantly larger than that with D-lactate (127 mV). These determinations were carried out at pH 7.5 for NADH and pH 6.6 for D-lactate. The table also shows that the value of

ΔH_m for NADH is larger than that for D-lactate (10 versus 7 kcal/mole). It was reported that two membrane coupling sites are involved with NADH, as compared with one for D-lactate [34]. The presently observed higher ΔH_m for NADH may be associated with this trans-membrane coupling event.

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