Reconstitution of Bovine Heart Mitochondrial Transhydrogenase: A Reversible Proton Pump†

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ABSTRACT: Homogeneous bovine heart mitochondrial transhydrogenase has been reconstituted into proteoliposomes by using a cholate dialysis procedure. Transhydrogenase activity decreased with increasing phosphatidylcholine/protein ratios, although addition of protonophore stimulated both forward and reverse reactions in all preparations to an activity comparable to that of unreconstituted enzyme. This respiratory control was highest in proteoliposomes containing phosphatidylcholine, while phosphatidylethanolamine gave about equal activity but poor coupling. Proteoliposomes formed from acidic phospholipids or from phosphatidylcholine and dicetyl phosphate gave low but coupled transhydrogenase activities, whereas positively charged phosphatidylcholine liposomes containing stearylamine were coupled and most active. Fractionation of proteoliposomes on Sepharose 4B yields large multilamellar structures containing inactive enzyme and smaller, primarily unilamellar structures containing active transhydrogenase. Lysis of proteoliposomes with the detergents Triton X-100 and lysophosphatidylcholine abolishes respiratory control but does not release any latent transhydrogenase activity, suggesting that the enzyme is unidirectionally incorporated in the membrane. Cholate dilution was as effective a method as cholate dialysis in reconstituting transhydrogenase, but neither sonication in the presence of phospholipids nor addition of the enzyme to preformed liposomes resulted in substantial functional incorporation. During transhydrogenation between NADPH and NAD^+, a pH gradient is established across the proteoliposome membrane ($pH_{in} < pH_{out}$), as indicated by the quenching of the fluorescence of either 9-aminoacridine or intravesicular fluorescein isothiocyanate labeled dextran. Creation of pH gradients, either acidic or basic inside the vesicles, by addition of nigericin to proteoliposomes prepared with appropriate potassium gradients, had little effect on transhydrogenase rate in either direction. However, valinomycin-dependent movement of potassium ions, in a direction opposite to proposed transhydrogenase-coupled proton movements, stimulated the rate of transhydrogenation markedly.

A fundamental tenet of the chemiosmotic mechanism for oxidative phosphorylation is that oxidation–reduction reactions at energy-coupling sites of the respiratory chain are linked directly to the translocation of protons across a membrane that is inherently impermeable to protons (Mitchell, 1966). Several laboratories have recently provided evidence supportive of this proposal by reconstituting the energy-coupling sites of the respiratory chain. Electron transport through reconstituted NADH–coenzyme Q reductase (complex I) and reduced coenzyme Q–cytochrome *c* reductase (complex III) has been reported to be coupled to proton translocation, giving $H^+/2$ electron ratios of 1.4 and 1.9, respectively (Ragan & Hinkle, 1975; Guerrieri & Nelson, 1975; Leung & Hinkle, 1975). Other evidence suggests that cytochrome *c* oxidase (Wikström & Saari, 1977; Krab & Wikström, 1978; Casey et al., 1979) and pyridine dinucleotide transhydrogenase (Höjeberg & Rydström, 1977; Earle et al., 1978a) are proton pumps, although proton translocation at the cytochrome *c* oxidase site has been challenged (Moyle & Mitchell, 1978; Lorusso et al., 1979). Transhydrogenase, an integral protein of the inner mitochondrial membrane, catalyzes a direct and reversible transfer of a hydride ion equivalent between oxidized and reduced forms of matrix NAD and NADP (Lee & Ernster, 1966; Rydström, 1977). The reduction of NAD^+ by NADPH is coupled to the uptake of protons (Mitchell & Moyle, 1965), lipophilic anions (Dontsov et al., 1972), and ATP synthesis (Van de Stadt et al., 1971) by bovine heart submitochondrial particles. Similarly, purified transhydrogenase, composed of two 120 000 molecular weight subunits (Anderson & Fisher, 1978), when reconstituted into dioleoylphosphatidylcholine liposomes promotes, coincident with NAD^+ reduction, the uncoupler sensitive uptake of tetraphenylboron anion (Höjeberg & Rydström, 1977) and acidification of the intravesicular space as indicated by 9-aminoacridine fluorescence quenching (Earle et al., 1978a). Reconstituted transhydrogenation is stimulated several-fold by uncouplers in the forward (Earle et al., 1978a) and reverse directions (Höjeberg & Rydström, 1977; Earle et al., 1978a), suggesting that an electrochemical hydrogen ion gradient generated by transhydrogenase-coupled proton translocation inhibits the reactions.

In this paper we report studies on the optimization of mitochondrial transhydrogenase reconstitution into proteoliposomes. Direct evidence is provided, demonstrating that reduction of NAD^+ by NADPH is coupled to the transport of protons to the intravesicular space. The influence of valinomycin and nigericin, in the presence of potassium ions, on transhydrogenation in Na^+- and K^+-loaded proteoliposomes suggests that the rate of the coupled reaction, under the conditions studied, is controlled primarily by the membrane electrical potential ($\Delta\Psi$), with the ΔpH across the membrane contributing to a lesser extent.

Materials and Methods

Materials. Pyridine dinucleotides, with the exception of $AcPyAD^+$,[1] were products of P-L Biochemicals. $AcPyAD^+$

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[1] Abbreviations used: $AcPyAD^+$, oxidized 3-acetylpyridine adenine dinucleotide; 9AA, 9-aminoacridine; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; FITC-dextran, fluorescein isothiocyanate labeled dextran; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; $NaDodSO_4$, sodium dodecyl sulfate; thio-$NADP^+$, oxidized thionicotinamide adenine dinucleotide.

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was prepared as described by Kaplan & Ciotti (1954). Nigericin was a generous gift of Dr. David Wong of Eli Lilly Labs. Valinomycin, FITC-dextran (M_r 20000), 9-aminoacridine, Sepharose 4B, dicetyl phosphate, stearylamine, egg yolk phosphatidylcholine (type V-E), dioleoylphosphatidylcholine, and bovine brain phosphatidylserine were from Sigma Chemical Co. Bovine heart phosphatidylcholine and soybean phosphatidylcholine were products of Avanti Biochemicals, Inc. All other phospholipids were obtained from Serdary Research Labs. FCCP was supplied by Pierce Chemical Co. Aldrich Chemical Co. supplied 1,6-diphenylhexatriene. All enzymes and substrates were obtained from Sigma Chemical Co.

Transhydrogenase Purification. Bovine heart mitochondrial transhydrogenase was purified to homogeneity as before (Anderson & Fisher, 1978) except that Lubrol WX was exchanged for cholate, and the enzyme was concentrated, by initial adsorption to a minimal amount of calcium phosphate gel, followed by elution with a minimal volume (3–5 mL) of 100 mM sodium phosphate (pH 7.5) containing 5 mM dithiothreitol and 0.05% sodium cholate.

Transhydrogenase Assays. The forward transhydrogenase reaction was assayed by the reduction of thio-$NADP^+$ by NADH (Fisher & Kaplan, 1973). The reaction mixtures (3 mL) at 25 °C contained 80 mM potassium phosphate at the indicated pH, 175 μM NADH, and 117 μM thio-$NADP^+$. The reverse reaction was assayed by the reduction of Ac-$PyAD^+$ by NADPH as described (Blazyk et al., 1976). For the assay of the reconstituted enzyme in either direction, an initial coupled rate was obtained, followed by determination of the rate in the presence of 1.3 μM FCCP.

Transhydrogenase Reconstitution. For most experiments transhydrogenase was reconstituted essentially as described before (Earle et al., 1978a), with the exception that 10–15 μg of purified transhydrogenase was reconstituted with 4.4 mg of the designated phospholipid. For preparation of K^+-loaded proteoliposomes for the ionophore experiments, 4.4 mg of egg yolk phosphatidylcholine in chloroform was taken to dryness under N_2, and the residue was resuspended in 0.12 mL of 0.1 M potassium phosphate, pH 6.3, containing 5% potassium cholate. The solution was sonicated for 10 min in a bath-type sonicator and 13 μg of transhydrogenase was added. Cold H_2O and 0.2 M potassium phosphate, pH 6.3, were added to bring the volume to 0.5 mL and the potassium phosphate to 0.1 M. The mixture was dialyzed for 20 h against 65 mL of 0.1 M potassium phosphate, pH 6.3, containing 1 mM dithiothreitol and 0.2 mM EDTA. The buffer was changed once after the initial 4 h. The Na^+-loaded proteoliposomes were prepared in an analogous manner.

FITC-dextran loaded proteoliposomes were prepared exactly like K^+-loaded vesicles, with the inclusion of 5 mg of FITC-dextran per mL.

Sepharose 4B Chromatography. One milliliter of dialyzed proteoliposomes (8.8 mg of egg yolk phosphatidylcholine, 26.6 μg of transhydrogenase) was applied to a Sepharose 4B column (41 × 1.8 cm) equilibrated at 22 °C with 0.01 M Tricine–KOH, pH 7.5, containing 0.1 M NaCl and 0.2 mM EDTA and eluted with the same buffer. Of the 1-mL fractions collected, 100-μL aliquots were assayed for transhydrogenase activity, and 50-μL aliquots were analyzed for phosphorus as described (London & Feigenson, 1978). Fractions 20–28, 31–39, and 40–48 were pooled and centrifuged at 105000*g* for 3.5 h to sediment vesicles. Pellets were resuspended in 0.03 M sodium phosphate, pH 7, containing 1% $NaDodSO_4$, 1% β-mercaptoethanol, and 10% glycerol, and $NaDodSO_4$–polyacrylamide gel electrophoresis was performed as described before (Anderson & Fisher, 1978). In a parallel experiment, the pellets were resuspended in column buffer and negatively stained with phosphotungstic acid for evaluation by electron microscopy.

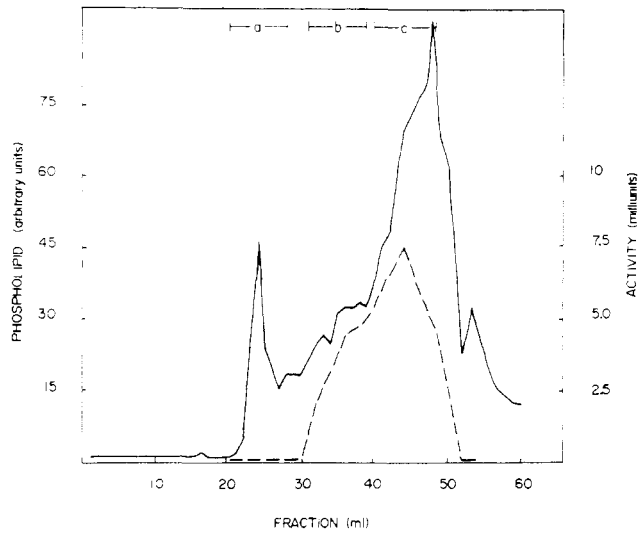

FIGURE 1: Sepharose 4B column chromatography of proteoliposomes prepared by cholate dialysis. Transhydrogenase was reconstituted into egg yolk phosphatidylcholine liposomes and fractionated as described under Materials and Methods. Fractions were assayed for transhydrogenation between NADPH and $AcPyAD^+$ in the presence of 1.3 μM FCCP (---) and phospholipid content (—).

Results

Transhydrogenase Reconstitution by Cholate Dialysis. Purified bovine heart transhydrogenase was reconstituted into egg yolk phosphatidylcholine liposomes by the cholate dialysis method (Kagawa & Racker, 1971). After dialysis, the size distribution of the proteolipsomes was assessed by Sepharose 4B column chromatography. As can be seen in Figure 1, proteoliposomes were separated into two major fractions. Electron microscopic examination of the fractions revealed large multilamellar vesicles eluting at the void volume and a smaller, primarily unilamellar vesicle fraction. In the absence of the uncoupler, FCCP, no transhydrogenase activity was observed in any column fraction. In the presence of uncoupler, transhydrogenase activity was apparent at the leading edge of the small vesicle fraction. As much as 95% of the transhydrogenase activity applied to the column was recovered. It was somewhat surprising to find a selective incorporation of the enzyme into the small vesicles. For determination of whether transhydrogenase in an inactive form was being incorporated into multilamellar vesicles, column fractions 20–28 (a), 31–39 (b), and 40–48 (c) were pooled. The liposomes were sedimented by centrifugation, and the presence of transhydrogenase was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Inactive transhydrogenase was eluted at the column void volume. The amount of enzyme in fraction a, based on the intensity of staining with Coomassie Blue, was about 20% of that in the small vesicle containing fractions.

For determination of the amount of phospholipid required for optimal reconstitution, transhydrogenase was mixed with varying amounts of dioleoylphosphatidylcholine in a standard cholate dialysis reconstitution mixture. The resulting proteoliposomes were assayed for activity in the presence and absence of uncoupler. The ratio of these two activities is expressed as the respiratory control ratio. As can be seen in Figure 2, preparations containing the lowest amounts of

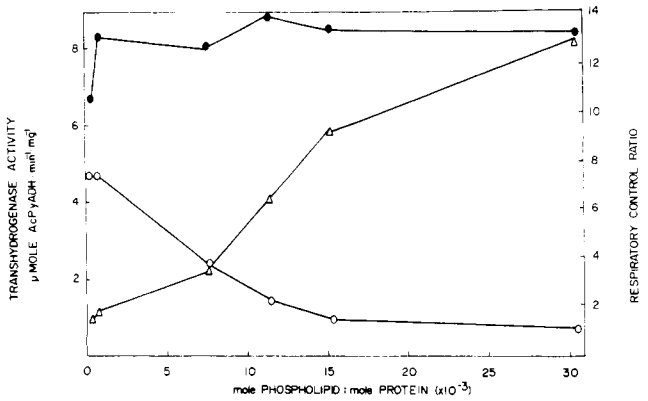

FIGURE 2: Influence of the phospholipid/protein ratio on the coupled and uncoupled activity of reconstituted transhydrogenase. Transhydrogenase (23 μg, 1.92×10^{-10} mol) was reconstituted with various amounts of dioleoylphosphatidylcholine and assayed for the reduction of $AcPyAD^+$ by NADPH in the presence (●) and absence (○) of FCCP as described under Materials and Methods. The uncoupled (+FCCP) transhydrogenase rate divided by the coupled rate is given as the respiratory control ratio (Δ).

phospholipid gave the highest activities in the absence of uncoupler, resulting in low respiratory control ratios. As the phospholipid/protein ratio (mole/mole) was increased, the coupled activity decreased and the respiratory control ratio increased. Transhydrogenase activity in the presence of uncoupler remained relatively constant at all phospholipid/protein ratios tested. In subsequent reconstitutions a phospholipid/protein ratio in the range of $(5–7) \times 10^4$ was employed to ensure optimum functional (coupled) incorporation of transhydrogenase.

Effect of Lipids on Reconstitution. Bovine heart mitochondrial transhydrogenase is active as a lipoprotein complex. The activity of homogeneous enzyme was enhanced twofold on incubation with phosphatidylcholine or mixed mitochondrial lipids (Anderson & Fisher, 1978). Addition of cardiolipin, phosphatidylcholine, or phosphatidylethanolamine reactivated partially purified and delipidated preparations of bovine heart (Rydström et al., 1976) and *Escherichia coli* (Houghton et al., 1976) transhydrogenases. Rydström et al. (1975) found further that phosphatidylcholine, but not phosphatidylethanolamine, was effective for reconstituting the impure transhydrogenase into proteoliposomes capable of lipophilic anion uptake during reverse transhydrogenation.

Purified transhydrogenase was reconstituted by cholate dialysis with several different classes of phospholipids. Only two lipids, phosphatidylcholine and phosphatidylethanolamine, yielded active enzyme after dialysis, with phosphatidylcholine showing the highest respiratory control ratio (Table I, experiment 1). Phosphatidylethanolamine provided about the same uncoupled activity as phosphatidylcholine but little respiratory control. Phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, and cardiolipin failed to yield active vesicles. Phosphatidylcholines from a variety of sources were about equally effective in reconstitution (Table I, experiment 2), indicating that the variability of the constitutent fatty acids of the various lipids is not a significant factor for optimal reconstitution. Inclusion of 5 mol % of cardiolipin, phosphatidylinositol, or phosphatidylglycerol or 20 mol % of phosphatidylserine with phosphatidylcholine inhibited substantially the reconstitution (Table I, experiment 3). Phosphatidylcholine and phosphatidylethanolamine mixed in any proportion allowed the recovery of transhydrogenase activities comparable to that with phosphatidylcholine alone, but respiratory control ratios were generally low at PC/PE ratios

Table I: Reconstitution with Various Phospholipids

liposome composition[a]	transhydrogenase act.[b] −FCCP	transhydrogenase act.[b] +FCCP	respiratory control ratio[c]
Experiment 1: Phospholipid			
dioleoyl-PC	1.15	8.67	7.54
dioleoyl-PG	0	0	
dioleoyl-PE	6.42	9.88	1.54
pig liver PI	0	0	
bovine brain PS	0	0	
cardiolipin	0	0	
Experiment 2: Phosphatidylcholine			
dioleoyl-PC	1.53	15.1	9.87
egg yolk PC	2.15	22.9	10.6
bovine heart PC	1.70	15.2	8.94
soybean PC	1.13	13.0	7.1
Experiment 3: Mixed Phospholipids			
phosphatidylcholine	0.47	5.70	12.2
PC plus cardiolipin (95:5)[d]	0	0.79	
PC plus PE (95:5)	0.54	6.24	11.5
PC plus PI (95:5)	0.18	3.57	19.8
PC plus PG (95:5)	0	1.12	
PC plus PS (95:5)	0.61	6.28	10.2
PC plus PS (70:30)	0	0.14	
PC plus PE plus PS (60:20:20)	0	0.79	
Experiment 4: Charged Liposomes			
dioleoyl-PC	1.13	13.8	12.2
dioleoyl-PC plus stearylamine (95:5)	3.56	23.8	6.69
dioleoyl-PC plus dicetyl phosphate (95:5)	0.62	8.14	13.1

[a] Proteoliposomes were formed by the cholate dialysis method as described under Materials and Methods. [b] Reverse transhydrogenase activity, assayed at pH 6.3, is expressed as micromoles of AcPyADH per minute per milligram of protein. [c] Respiratory control ratio is calculated by dividing the transhydrogenase rate in the presence of FCCP by that rate in the absence of uncoupler. [d] The mole fraction ratio of lipids used in reconstitution.

of less than 5. The combination of PC/PE/PS (60:20:20), potentially useful in liposome fusion experiments (Miller & Racker, 1976; Miller et al., 1976), yielded inactive preparations. Table I, experiment 4, demonstrates that reconstituted phosphatidylcholine vesicles with a net negative charge provided by the inclusion of 5 mol % dicetyl phosphate had less transhydrogenase activity but similar respiratory control ratios when compared to control proteoliposomes. Positively charged phosphatidylcholine vesicles containing 5 mol % stearylamine showed increased coupled and uncoupled transhydrogenase rates, with lowered respiratory control ratios.

pH Profile of Reconstituted Transhydrogenase. Figure 3 shows that when assayed at pH 7.4 identical amounts of uncoupled reconstituted and unreconstituted transhydrogenase had similar activities in the reverse direction, but in the forward direction the reconstituted activity was considerably less than that of the soluble enzyme. This discrepancy can be attributed to an alteration in the pH dependency of the forward, but not the reverse, transhydrogenase reaction occurring on reconstitution. As can be seen in Figure 4A, the pH profiles for the soluble and uncoupled reconstituted enzymes are similar in the reverse direction, with pH optima at about 6.25. These results are comparable to those of the submitochondrial particle enzyme (Earle et al., 1978b). However, the pH optima for forward transhydrogenation with soluble enzyme and submitochondrial particles are 5.5 (Figure 4B) and 6–6.5 (Earle et al., 1978b), respectively, whereas the rate of the uncoupled

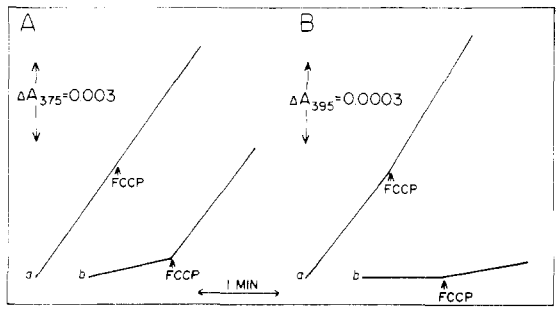

FIGURE 3: Effect of reconstitution and FCCP on reverse and forward transhydrogenase activities. The influence of uncoupler on soluble and reconstituted transhydrogenase activities at pH 7.4 was studied. Reconstitution of transhydrogenase (13 μg) with dioleoylphosphatidylcholine and assays were performed as described under Materials and Methods. Experiment A compares the rates of AcPyAD$^+$ reduction catalyzed by either (a) 1.3 μg of unreconstituted transhydrogenase or (b) 1.3 μg of reconstituted transhydrogenase. Experiment B compares the rates of thio-NADP$^+$ reduction catalyzed by either (a) 2.6 μg of unreconstituted transhydrogenase or (b) 2.6 μg of reconstituted transhydrogenase. FCCP (1.3 μM) was added where indicated.

Table II: Respiratory Control Ratio as a Function of pH

assay pH	respiratory control ratio,[a] transhydrogenase reaction: reverse (NADPH → AcPyAD$^+$)	forward (NADH → thio-NADP$^+$)
5.0	8.0	2.6
5.5	13.6	3.8
6.0	13.4	4.8
6.25	12.1	*b*
6.5	12.4	
7.0	43.0	
7.5	29.0	n.d.[c]
8.0		n.d.
8.5		n.d.
9.0		n.d.

[a] Respiratory control ratio as defined under Table I was calculated from the data presented in Figure 4. [b] When the coupled transhydrogenase rate was absent, but the uncoupled rate was measurable, no respiratory control ratio was calculated. [c] n.d. indicates that the uncoupled transhydrogenase rate was too low to measure.

reconstituted reaction continued to increase to the lowest pH tested (5.0). Although the soluble transhydrogenase is active in the forward reaction up to pH 9.0, reconstituted enzyme is inactive above pH 7.5. The pH profiles of coupled transhydrogenation, while reflecting lower activities, showed the same pH dependence in both directions as the uncoupled reconstituted enzyme.

The respiratory control of reconstituted forward and reverse transhydrogenation is given in Table II as a function of pH. As can be seen, there was a general increase in respiratory control ratio for both forward and reverse reactions as the pH increased, until the coupled rate became zero. At this point, no respiratory control ratio could be calculated even though significant uncoupled transhydrogenase activity remained. The magnitude of the respiratory control ratio of the reverse reaction was invariably larger than that of the forward reaction.

Transhydrogenase Orientation. Conceivably reconstitution might result in the random orientation of transhydrogenase, such that active sites would be distributed on both the external and internal surfaces of the proteoliposomes. In this case, since the intact liposomes are impermeant to substrates, disruption of the lipid bilayer by the addition of detergent should release latent transhydrogenase activity. Alternatively, the enzyme might be unidirectionally oriented as seen with cytochrome *c* oxidase (Racker, 1972). Since lysolecithin is effective in solubilizing transhydrogenase from submitochondrial particles and reportedly does not appreciably inhibit the enzyme at higher concentrations (Rydström et al., 1974; Anderson & Fisher, 1978), it was initially chosen to lyse proteoliposomes, but, as shown in Table III, it proved unsuitable. Not only did lysolecithin fail to uncouple reconstituted transhydrogenase

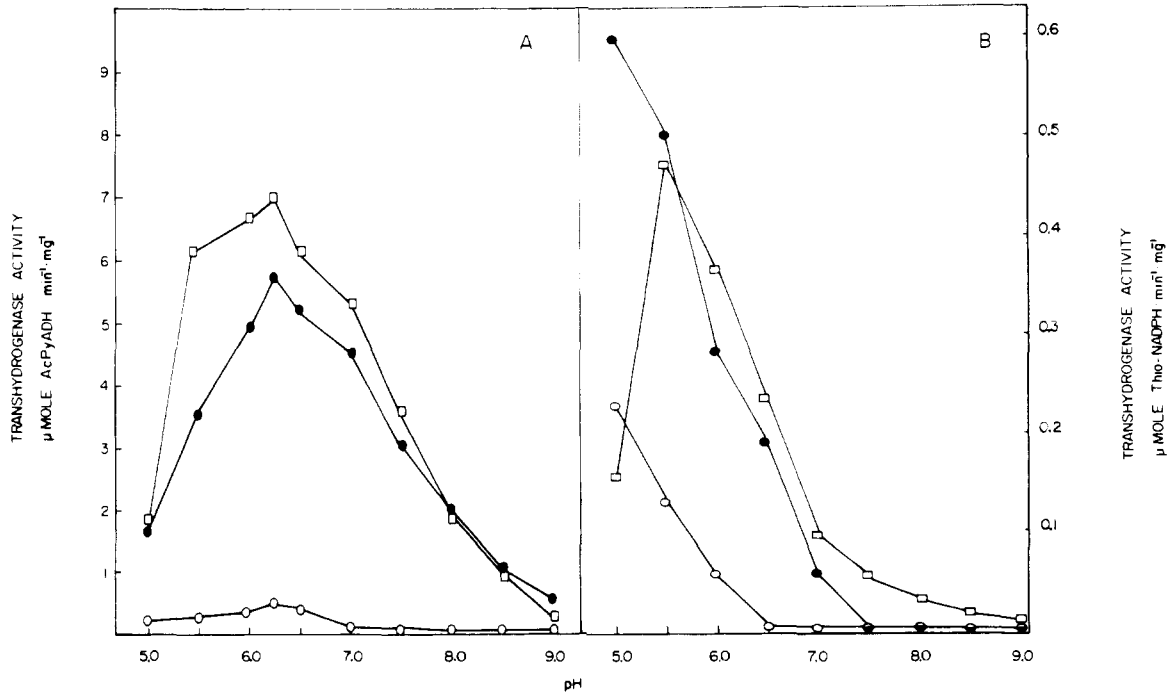

FIGURE 4: pH profiles for reverse and forward transhydrogenation. Conditions were those described for Figure 3 with the pH of the assay medium varied as indicated. The rates for soluble (□), coupled (−FCCP) reconstituted (○), and uncoupled (+FCCP) reconstituted (●) transhydrogenases in the reverse (NADPH → AcPyAD$^+$) and forward (NADH → thio-NADP$^+$) reactions are shown in experiments A and B, respectively.

Table III: Effect of Detergents on Solubilized and Reconstituted Transhydrogenase

addition[b]	transhydrogenase act.,[a] enzyme prepn		
		reconstituted[c]	
	soluble	−FCCP	+FCCP
none	12.3	2.58	11.2
LPC	18.6	2.18	7.67
Triton X-100	6.50	2.58	9.63
LPC plus Triton X-100	17.9	10.3	10.7

[a] Reverse transhydrogenase, assayed at pH 6.3, is expressed as micromoles of AcPyADH per minute per milligram. [b] One microgram of transhydrogenase, either solubilized or reconstituted, was incubated in a medium (2.5 mL) at 22 °C containing 0.1 M potassium phosphate, pH 6.3. Substrates were added after 1 min to initiate the assay. When included, lysophosphatidylcholine (0.0075%) was added at zero time and Triton X-100 (0.0073%) was added after 1 min, immediately prior to assay. [c] Transhydrogenase (10 μg) was reconstituted with dioleoylphosphatidylcholine.

but it also apparently inhibited the uncoupled enzyme. In addition, lysolecithin caused an ~50% stimulation of soluble enzyme activity. Triton X-100 proved inhibitory to the soluble enzyme, but not to the reconstituted enzyme, and also failed to uncouple proteoliposomes. However, incubation of proteoliposomes with lysolecithin, followed by addition of Triton X-100, stimulated the transhydrogenase rate to that seen in the presence of uncoupler. This detergent combination did not inhibit soluble transhydrogenase. Thus, under conditions where detergents are not inhibitory to the unreconstituted enzyme, they release no latent transhydrogenase activity from the proteoliposomes. These data indicate that reconstituted transhydrogenase has the same orientation as in submitochondrial particles, with all or nearly all of its active sites available to the external medium. The data in Figure 3A also argue against a random orientation of transhydrogenase, in that identical quantities of soluble and reconstituted enzyme have comparable activities.

Other Reconstitution Procedures. Dilution of a small aliquot of the cholate dialysis mixture, immediately prior to dialysis, into the transhydrogenase assay medium revealed that the enzyme was coupled, giving respiratory control ratios as high as those obtained after 20 h of dialysis (Table IV, experiment A). The specific activity of the transhydrogenase reconstituted by the dilution method (Racker et al., 1975) was higher than that of the enzyme reconstituted by the dialysis method, although identical amounts of enzyme, phosphatidylcholine, and detergent were used.

Attempts were made to reconstitute transhydrogenase by sonication with phosphatidylcholine in the absence of added detergents. This procedure has been used successfully for the reconstitution of bacteriorhodopsin as well as other ion pumps (Racker, 1973). After several minutes of sonication, a slight increase in the respiratory control ratio was accompanied by a substantial decrease in transhydrogenase activity (Table IV, experiment B). Another reconstitution method that is especially effective with cytochrome *c* oxidase is the addition of enzymes to preformed liposomes (Eytan et al., 1976). As shown in Table IV, experiment C, addition of transhydrogenase to phosphatidylcholine liposomes stimulates activity by nearly twofold, but only low respiratory control ratios were obtained. After an initial period of stability, enzyme activity rapidly decreased. This contrasts with the cholate dialysis preparation that is stable and coupled for several weeks when stored at 4 °C.

Table IV: Alternative Reconstitution Methods

	transhydrogenase act.[a]		respiratory control ratio[b]
	−FCCP	+FCCP	
Experiment A: Cholate Dilution Method[c]			
dialysis time (h)			
0	1.59	12.0	7.55
20	0.94	7.35	7.82
unreconstituted	6.23	5.18	0.83
Experiment B: Sonication Method[d]			
sonication time (min)			
3	5.81	7.95	1.37
6	4.65	6.86	1.48
9	1.63	3.49	2.13
12	1.13	2.58	2.29
15	0.73	1.82	2.50
unreconstituted	5.08	5.66	1.11
Experiment C: Addition to Phosphatidylcholine Liposomes[e]			
incubation time (min)			
1	8.46	11.8	1.39
7	8.24	10.9	1.32
20	5.74	7.92	1.38
30	2.65	4.14	1.56
unreconstituted	5.08	5.66	1.11

[a] Reverse transhydrogenase activity, assayed at pH 6.3, is expressed as micromoles of AcPyADH per minute per milligram. [b] Respiratory control ratio is described under Table I. [c] Either immediately prior to dialysis or after 20 h of dialysis, a 50-μL aliquot of a standard cholate dialysis reconstitution mixture containing 1 μg of transhydrogenase and 0.44 mg of egg yolk phosphatidylcholine was diluted into the reverse assay medium and assayed immediately. The activity of 1 μg of undialyzed, unreconstituted soluble transhydrogenase is included for comparison. [d] Transhydrogenase (16 μg) was added to a solution (0.46 mL) containing 10 mM Tricine–KOH, pH 8.0, 1 mM dithiothreitol, 25 mM sucrose, 0.15 M KCl, and 4.4 mg of egg yolk phosphatidylcholine and sonicated in an ice-cooled bath-type sonicator (Earle et al., 1978a). At the indicated times, 50-μL aliquots of the sonicate were assayed. Unreconstituted transhydrogenase was not sonicated. [e] Preformed liposomes were prepared by sonication for 20 min in an ice-cooled bath-type sonicator of 4.4 mg of dioleoylphosphatidylcholine in a medium (0.46 mL) containing 10 mM Tricine–KOH, pH 8.0, 1 mM dithiothreitol, 25 mM sucrose, and 0.15 M KCl. Transhydrogenase (40 μL containing 16 μg of protein) was added to the liposomes. After incubation on ice for the indicated times, 50-μL aliquots were assayed.

Proteoliposome Energization. We have previously employed 9-aminoacridine to monitor membrane energization of transhydrogenase-containing liposomes (Earle et al., 1978a). Quenching of 9AA fluorescence, associated with the reduction of NAD^+, indicates an acidification of the intravesicular space (Deamer et al., 1972). Figure 5A illustrates 9AA fluorescence quenching during reverse transhydrogenation and its reversal by the uncoupler, FCCP. The increase in transhydrogenase rate, and presumably transhydrogenase-coupled proton influx, resulting from the addition of uncoupler, would partially counterbalance the uncoupler-dependent efflux of protons, thus accounting for the slow restoration of 9AA fluorescence. Addition of valinomycin in the presence of K^+ did not affect 9AA fluorescence, although subsequent addition of nigericin to totally uncouple transhydrogenation resulted in an instantaneous return of fluorescence to initial levels (Figure 5B). Nigericin alone restored initial fluorescence, indicating that the ionophore acts by exchanging protons pumped into the vesicles for external potassium ions (Figure 5C).

Since the accuracy of ΔpH measurements with 9AA has been challenged (Kraayenhof, 1977), FITC-dextran was incorporated into the proteoliposomes as an internal pH indicator (Ohkuma & Poole, 1978). Proteoliposomes were prepared in

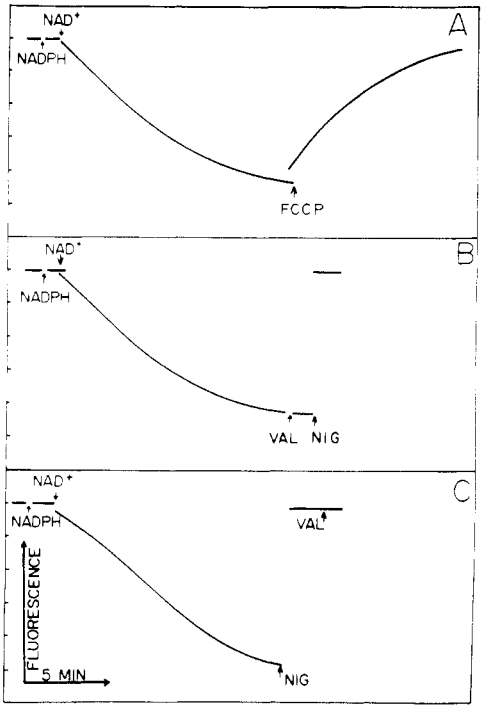

FIGURE 5: Effect of ionophores on quenching of 9-aminoacridine fluorescence during transhydrogenation. The fluorescence of 9AA was monitored during the reduction of NAD^+ by NADPH, with excitation at 420 nm and emission determined at 500 nm. The reaction mixtures (1.04 mL) at pH 7.4 contained 50 μL of proteoliposomes (0.44 mg of egg yolk phosphatidylcholine and 1.3 μg of transhydrogenase), 84 mM choline chloride, 1.25 mM Tricine–KOH, 9.3 mM pyruvate, 0.44 mM glucose 6-phosphate, 9.7 units of lactate dehydrogenase, 11 units of glucose-6-phosphate dehydrogenase, and 1.6 μM 9AA. Where indicated, 45 μM NADPH, 45 μM NAD^+, 1.3 μM FCCP, 1 μM valinomycin, and 0.1 μM nigericin were added.

the presence of FITC-dextran by the cholate dialysis method, and external FITC-dextran was removed by passing the mixture through a Sepharose 4B column (41 × 1.8 cm) equilibrated with 0.1 M KP_i, pH 6.3, containing 0.2 mM EDTA. Proteoliposome-containing fractions with transhydrogenase activity were pooled and concentrated by centrifugation at 105000g for 5 h and then resuspended in 80 μL of column buffer and scanned fluorometrically to confirm the incorporation of FITC-dextran. A decrease in the fluorescence of internal FITC-dextran was observed during the reduction of $AcPyAD^+$ by NADPH, indicating a decrease in internal pH, that was reversed by the addition of uncoupler (Figure 6). The fluorescence change corresponded to the creation of an apparent ΔpH of about 0.05 unit. It is of interest to note that the development of a ΔpH is rather slow in both the 9AA and FITC-dextran experiments. This may result from a substantial proton efflux rate compared to transhydrogenase-coupled proton uptake. Thus, considerable time is required to attain a steady-state pH gradient.

Effect of Ionophores. Since uncouplers stimulate reconstituted transhydrogenation in both directions (Earle et al., 1978a; Figure 3), we have proposed that the respiratory control seen in transhydrogenase-containing proteoliposomes could result from a balancing of the substrate oxidation–reduction potential against the electrochemical hydrogen ion gradient formed across the membrane. It was of interest to determine if control of transhydrogenation might be explained in terms of simple mass-action principles, i.e., the proton gradient, wherein an increase of internal protons would decrease the reverse reaction rate and a depletion of internal protons would

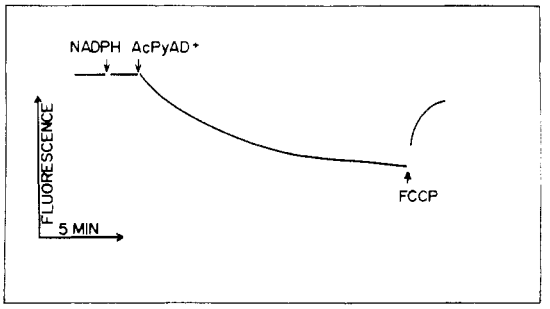

FIGURE 6: Quenching of FITC-dextran fluorescence during reconstituted transhydrogenation. The acidification of the intravesicular space was monitored by a decrease in the fluorescence of internal FITC-dextran. Proteoliposomes were loaded with FITC-dextran as described under Materials and Methods. Resuspended vesicles (15 μL) were assayed in 0.1 M potassium phosphate, pH 6.3, containing 175 μM NADPH and 175 μM $AcPyAD^+$. Excitation was at 490 nm and fluorescence emission was monitored at 515 nm. FCCP (1.3 μM) was added where indicated.

limit the forward reaction as described by the equation:

$$n\mathrm{H}^+_{\mathrm{in}} + \mathrm{NADH} + \mathrm{NADP}^+ \rightleftarrows \mathrm{NAD}^+ + \mathrm{NADPH} + n\mathrm{H}^+_{\mathrm{out}}$$

Alternatively, transhydrogenation might be controlled primarily by the membrane potential ($\Delta\Psi$) or by a combination of ΔpH and $\Delta\Psi$. Kaback and co-workers (Kaback, 1977) have demonstrated that certain active proton-symport transport systems of *E. coli* vesicles respond to the $\Delta\Psi$, whereas others are driven exclusively by proton gradients across the membrane. For investigation of the roles of ΔpH and $\Delta\Psi$ on reconstituted transhydrogenation, the effects of the ionophores, valinomycin and nigericin, on the forward and reverse reactions were studied. Proteoliposomes were prepared in 0.1 M potassium phosphate or sodium phosphate and assayed in 0.1 M sodium phosphate and potassium phosphate, respectively. During the forward reaction it is postulated that protons are pumped out of the proteoliposomes. Hence, for Na^+-loaded vesicles, valinomycin-mediated uptake of K^+ would counter proton movements and reduce or eliminate the formation of a membrane potential. As can be seen in Figure 7A, transhydrogenation was not observed in the absence of uncoupler. Valinomycin addition elicited an even greater initial transhydrogenase rate than FCCP. Valinomycin-dependent transhydrogenation was nonlinear, possibly because of either a concentration of K^+ in the vesicles or a depletion of the internal proton pool. Further addition of nigericin allowed for complete uncoupling and stimulation of transhydrogenation. Nigericin, which should promote the neutral exchange of external K^+ for internal protons (Pressman, 1976), would increase the intravesicular pH without altering the $\Delta\Psi$. As shown in Figure 7A, nigericin only slightly stimulated forward transhydrogenation, which is further stimulated by valinomycin to about the same rate as observed with FCCP. In the corollary experiment with K^+-loaded vesicles, valinomycin addition as expected did not affect the coupled transhydrogenase rate (Figure 7B). Presumably, valinomycin-dependent K^+ efflux generates a membrane potential of the opposite polarity (–inside) required to dissipate the potential developed during transhydrogenation. Addition of nigericin, which couples the uptake of protons to the efflux of K^+ in liposomes, would be expected to stimulate forward transhydrogenation if the reaction were even partially influenced by ΔpH. That nigericin alone did not affect the reaction (Figure 7B) suggests that the major controlling parameter is a rapidly developed $\Delta\Psi$, which is not collapsed in the absence of valinomycin. Parts C and D of Figure 7 represent an analogous experiment to that in

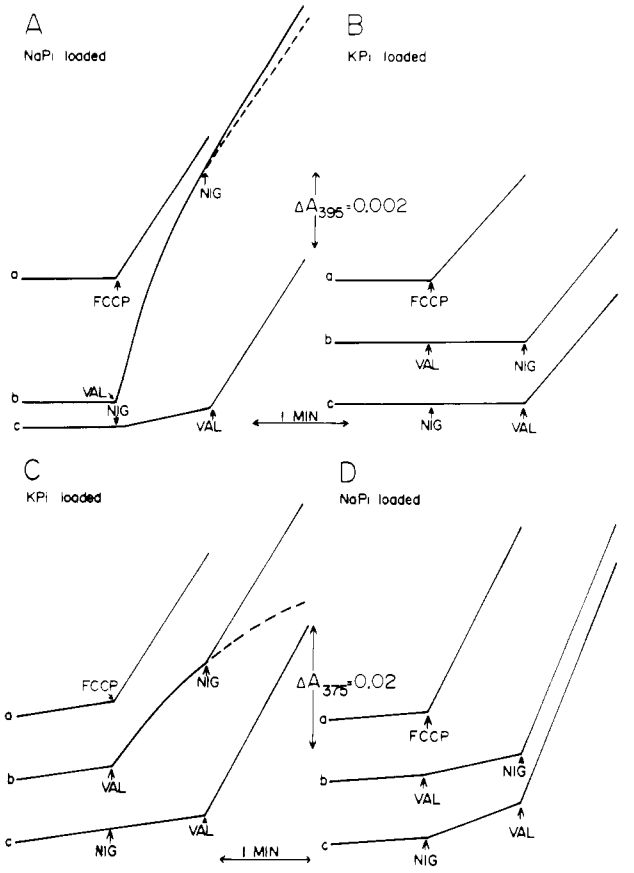

FIGURE 7: Effects of ionophores on forward and reverse transhydrogenation in K^+- and Na^+-loaded proteoliposomes. Proteoliposomes were loaded with either Na^+ (experiments A and D) or K^+ (experiments B and C) as described under Materials and Methods and were assayed in either 0.1 M potassium phosphate or sodium phosphate, pH 6.3, respectively. Experiments A and B show the effects of the addition of valinomycin (1 μM), nigericin (0.1 μM), and FCCP (1.3 μM) on the reduction of thio-$NADP^+$ by NADH in Na^+- and K^+-loaded vesicles, respectively. Experiments C and D show the effects of the ionophores on the reduction of $AcPyAD^+$ by NADPH in K^+- and Na^+-loaded vesicles, respectively. In part A, trace b, and part C, trace b, the dotted lines indicate activity in the absence of added nigericin.

parts A and B of Figure 7, in which the reverse reaction, during which it is proposed that protons are pumped into the proteolipsomes, was monitored. Valinomycin stimulated initially the reverse transhydrogenase reaction when added to K^+-loaded liposomes (Figure 7C), probably because K^+ efflux creates a membrane potential (–inside) that can be countered by an influx of protons coupled to NAD^+ reduction by NADPH. As the internal K^+ is depleted, the membrane potential is reduced and the transhydrogenase rate approaches that seen in the absence of valinomycin. Subsequent addition of nigericin stimulates the reaction to a rate similar to that with FCCP. Addition of nigericin to the vesicles in the absence of valinomycin had little effect on transhydrogenase activity, although the resulting acidification of the vesicle interior would be expected to inhibit the reaction by mass action, provided the initial coupled transhydrogenase rate reflects that of functionally reconstituted enzyme (cf. Figure 3 and 4B). This is another indication that reverse transhydrogenation is controlled to a greater extent by $\Delta\Psi$ than by ΔpH. The effects of valinomycin and nigericin on reverse transhydrogenation in Na^+-loaded vesicles are shown in Figure 7D. As can be seen, valinomycin only slightly stimulated the coupled activity. The somewhat greater, but still small, stimulation of transhydrogenation by nigericin may be explained if the reaction is regulated to a limited extent by ΔpH and the ionophore dissipates a developing proton gradient.

Discussion

Reconstitution of transhydrogenase with both phosphatidylcholine and phosphatidylethanolamine gave preparations with high rates of transhydrogenation in the presence of uncoupler, but only phosphatidylcholine formed tightly coupled vesicles displaying high respiratory control ratios. Rydström et al. (1975) have previously shown that partially purified transhydrogenase reconstituted with phosphatidylcholine, but not with phosphatidylethanolamine, promotes the uptake of the tetraphenylboron anion into the vesicles coupled to NAD^+ reduction by NADPH, presumably in response to a transhydrogenase-generated membrane potential (+inside). These results may be explained by the fact that phosphatidylethanolamine does not form vesicular structures (Papahadjopoulos & Miller, 1967). Dialysis of purified transhydrogenase in the absence of phospholipids under conditions employed to form proteoliposomes inactivates the enzyme totally. Activity, in the presence or absence of uncoupler, was also lost during proteoliposome formation from acidic phospholipids, including phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, and cardiolipin. This is consistent with the observation that cardiolipin inactivates lipid-depleted transhydrogenase subsequent to enzyme activation at low concentrations (Rydström et al., 1976) and may represent a general effect of negatively charged phospholipids. Acidic phospholipids, when added in 5 mol % fractions to liposomes formed predominantly from phosphatidylcholine, yielded partially inactivated proteoliposomes. Transhydrogenase was most inhibited in vesicles containing cardiolipin and least inhibited in vesicles containing phosphatidylserine, although higher amounts of phosphatidylserine led to substantial inactivation. Inhibition by other acidic phospholipids ranged between these two extremes. The low transhydrogenase activity of negatively charged proteoliposomes responded to uncoupler, giving high respiratory control ratios, indicating that although the enzyme is partially inactivated, the membranes are apparently as impermeable to protons as phosphatidylcholine vesicles. Since transhydrogenase activity in phosphatidylcholine vesicles containing dicetyl phosphate was affected similarly to that of phosphatidylcholine vesicles containing acidic phospholipids, it is concluded that surface charge may contribute to the lower activities seen with acidic phospholipid vesicles.

Reconstituted transhydrogenase typically displays a low activity in the absence of uncoupler, when assayed at pH 6.3 (cf. Figure 4). This activity may represent enzyme adhering to the surface of the liposomes, but not functionally incorporated, or transhydrogenase that is functionally incorporated but not completely inhibited by the membrane electrochemical potential. As reported above, the pH profiles for forward transhydrogenation catalyzed by the soluble and reconstituted enzymes in the presence of uncoupler differ, although that of the soluble enzyme closely resembles that of the native membrane. In the absence of uncoupler, the forward pH profile of the reconstituted enzyme does not mimic that of the soluble enzyme; rather, it is comparable to uncoupled reconstituted transhydrogenase. This suggests that the coupled activity represents functionally reconstituted transhydrogenase. If the respiratory control ratio represents a measure of the proton permeability of the proteoliposomes, this may be reflected in the coupled rates of forward and reverse transhydrogenation. Thus, the low respiratory control ratios for the forward reaction (Table II) indicate that the rate of transhydrogenase-coupled

proton translocation may be only slightly greater than that of the passive proton efflux from the vesicles. However, for the reverse reaction a greater electrochemical potential can be developed across the membrane because the rate of transhydrogenation, and, hence, proton translocation, is about 20-fold faster than the forward reaction. Since the membrane potential formed inhibits transhydrogenation, the greater potential formed during the reverse reaction (+inside) would limit that reaction to a greater extent than a lower electrochemical potential (−inside) developed during the forward reaction.

As first detected through studies on 9-aminoacridine fluorescence quenching (Earle et al., 1978a), the development of a pH gradient during transhydrogenation between NADPH and NAD^+ was confirmed by the quenching of the fluorescence of intravesicular FITC-dextran. The FITC-dextran fluorescence allows a continuous pH measurement of the intravesicular space and the direct evaluation of a pH gradient. A ΔpH of 0.05 unit was observed during reverse transhydrogenation. This pH gradient must be regarded as a minimum value, since it is unlikely that all liposomes contain functionally incorporated transhydrogenase. However, it is probable that every liposome contains internal FITC-dextran. An undetermined fraction of FITC-dextran molecules contained in liposomes without reconstituted transhydrogenase would be in an environment not influenced by the enzyme. Another explanation for the apparently low ΔpH observed is the relatively low amount of redox energy available and the slow rate of the transhydrogenase reaction, both of which are manifested in submitochondrial particles by the development of a much lower membrane potential during transhydrogenation than during succinate oxidation or ATP hydrolysis (Dontsov et al., 1972). Similarly, the low transhydrogenase-generated membrane potential provides only a marginal amount of energy for ATP synthesis (Dontsov et al., 1972).

Experiments with nigericin and valinomycin suggest that the low rate of coupled reconstituted transhydrogenation in both directions results primarily from the influence of $\Delta\Psi$, with only a limited contribution from ΔpH on the reaction. An indication that ΔpH is relatively unimportant in controlling reconstituted transhydrogenase rates is that initial coupled activities are linear rather than showing high initial rates followed by inhibition in response to the formation of a pH gradient. The response of FITC-dextran fluorescence to transhydrogenation illustrates that formation of a pH gradient of maximum value takes several minutes. In addition, nigericin failed to stimulate forward transhydrogenation in K^+-loaded vesicles. Even though this would increase the proton concentration of the intravesicular space, the $\Delta\Psi$ would remain unaltered. Collapse of the reverse transhydrogenase-generated proton gradient while maintaining $\Delta\Psi$, on addition of nigericin to Na^+-loaded proteoliposomes, also had little effect on the reaction rate. The role of $\Delta\Psi$ in regulating transhydrogenase activity can also be inferred from the observations that valinomycin-dependent movement of potassium ions, in a direction opposite to proposed transhydrogenase-coupled proton movements (cf. Figure 7), stimulated the rate of transhydrogenation to at least that seen with uncoupler. Hence, transhydrogenation would not be impeded when, during the forward reaction, valinomycin-mediated influx of potassium ions prevents the formation of a $\Delta\Psi$ associated with the efflux of protons. Similarly, in the reverse reaction, the influx of protons may be compensated for by the efflux of potassium ions in the presence of valinomycin. The use of valinomycin to allow rapid rates of coupled transhydrogenation should allow the determination of the stoichiometry of proton translocation to hydride ion transfer.

References

Anderson, W. M., & Fisher, R. R. (1978) *Arch. Biochem. Biophys. 187*, 180–190.

Blazyk, J. F., Lam, D., & Fisher, R. R. (1976) *Biochemistry 15*, 2843–2848.

Casey, R. P., Chappell, J. B., & Azzi, A. (1979) *Biochem. J. 182*, 149–156.

Deamer, D. W., Prince, R. C., & Crofts, A. R. (1972) *Biochim. Biophys. Acta 274*, 323–335.

Dontsov, A. B., Grinius, L. L., Jasaites, A. A., Severina, I. I., & Skulachev, V. P. (1972) *J. Bioenerg. 3*, 277–303.

Earle, S. R., Anderson, W. M., & Fisher, R. R. (1978a) *FEBS Lett. 91*, 21–24.

Earle, S. R., O'Neal, S. G., & Fisher, R. R. (1978b) *Biochemistry 17*, 4683–4690.

Eytan, G. D., Matheson, M. J., & Racker, E. (1976) *J. Biol. Chem. 251*, 6831–6837.

Fisher, R. R., & Kaplan, N. O. (1973) *Biochemistry 12*, 1182–1188.

Guerrieri, F., & Nelson, B. D. (1975) *FEBS Lett. 54*, 339–342.

Höjeberg, B., & Rydström, J. (1977) *Biochem. Biophys. Res. Commun. 78*, 1183–1190.

Houghton, R. L., Fisher, R. J., & Sanadi, D. R. (1976) *Biochem. Biophys. Res. Commun. 73*, 751–757.

Kaback, H. R. (1977) *Biochemistry of Membrane Transport, 1976*, 598–625.

Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 246*, 5477–5487.

Kaplan, N. O., & Ciotti, M. M. (1954) *J. Am. Chem. Soc. 76*, 1713–1714.

Kraayenhof, R. (1977) *Struct. Funct. Energy-Transducing Membranes, Proc. Symp., 1977*, 223–236.

Krab, K., & Wikström, M. (1978) *Biochim. Biophys. Acta 504*, 200–214.

Lee, C.-P., & Ernster, L. (1966) *Regul. Metab. Processes Mitochondria, Proc. Symp., 1965*, 218–234.

Leung, K. H., & Hinkle, P. C. (1975) *J. Biol. Chem. 250*, 8467–8471.

London, E., & Feigenson, G. W. (1978) *Anal. Biochem. 88*, 203–211.

Lorusso, M., Capuano, F., Buffoli, D., Stefanelli, R., & Papa, S. (1979) *Biochem. J. 182*, 133–147.

Miller, C., & Racker, E. (1976) *J. Membr. Biol. 30*, 283–300.

Miller, C., Arvan, P., Telford, J. N., & Racker, E. (1976) *J. Membr. Biol. 30*, 271–282.

Mitchell, P. (1966) *Biol. Rev. Cambridge Philos. Soc. 41*, 445–502.

Mitchell, P., & Moyle, J. (1965) *Nature (London) 208*, 1205–1206.

Moyle, J., & Mitchell, P. (1978) *FEBS Lett. 88*, 268–272.

Ohkuma, S., & Poole, B. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3327–3331.

Papahadjopoulas, D., & Miller, N. (1967) *Biochim. Biophys. Acta 135*, 624–638.

Pressman, B. C. (1976) *Annu. Rev. Biochem. 45*, 501–530.

Racker, E. (1972) *J. Membr. Biol. 10*, 221–235.

Racker, E. (1973) *Biochem. Biophys. Res. Commun. 55*, 224–230.

Racker, E., Chien, T.-F., & Kandrach, A. (1975) *FEBS Lett. 57*, 14–18.

Ragan, C. I., & Hinkle, P. C. (1975) *J. Biol. Chem. 250*, 8472–8476.

Rydström, J. (1977) *Biochim. Biophys. Acta 463*, 155–184.

Rydström, J., Hoek, J. B., & Hundal, T. (1974) *Biochem. Biophys. Res. Commun. 60*, 448–455.
Rydström, J., Kanner, N., & Racker, E. (1975) *Biochem. Biophys. Res. Commun. 67*, 831–839.
Rydström, J., Hoek, J. B., Ericson, B. G., & Hundal, T. (1976) *Biochim. Biophys. Acta 430*, 419–425.
Van de Stadt, R. J., Nieuwenhuis, F. J. R. M., & Van Dam, K. (1971) *Biochim. Biophys. Acta 234*, 173–176.
Wikström, M. K. F., & Sarri, H. T. (1977) *Biochim. Biophys. Acta 462*, 347–361.

Dynamic Properties of Binary Mixtures of Phosphatidylcholines and Cholesterol†

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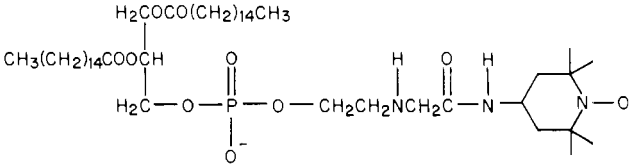
ABSTRACT: We have observed the paramagnetic resonance spectra of a head group spin-labeled phosphatidylethanolamine (L-α-dipalmitoylphosphatidyl-*N*-ethanolamine) as a function of temperature and cholesterol concentration in binary mixtures of cholesterol and dimyristoylphosphatidylcholine. These spectra bear on two interrelated topics involving mixtures of phosphatidylcholine and cholesterol: (1) lipid phase equilibria and the lateral ordering and diffusion of lipid molecules and (2) model membrane immunochemistry using spin-label lipid haptens.

There are three interesting, interrelated problems involving model membranes composed primarily of binary mixtures of phosphatidylcholine and cholesterol. One problem concerns the lipid phase equilibria and the lateral ordering and motion of lipid molecules in the plane of the membrane. The second problem concerns the lateral motion and distribution of membrane proteins included in these binary mixtures. The third problem concerns the immunochemical (or, more generally, biochemical) properties of these model membranes. In the present paper we describe the paramagnetic resonance spectra of the head group spin-label I in binary mixtures of



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cholesterol and dimyristoylphosphatidylcholine as a function of composition and temperature. We show how these spectral data are related to the first and last of the three problems mentioned above and how they are related to earlier studies that bear on these three problems. The effects of lipid composition on the distribution and motion of membrane proteins have been described earlier (Kleemann & McConnell, 1976; Grant & McConnell, 1974). The effects of lipid composition on protein diffusion will be described in a subsequent publication.

Materials and Methods

Lipids. Dimyristoylphosphatidylcholine was obtained from Sigma and stored in ethanol. Concentrations were determined by phosphate assay (McClare, 1971). Cholesterol was recrystallized twice from ethanol and stored in an ethanolic solution under argon. Concentrations were determined as described by Solow & Freeman (1970). All lipids were stored at −20 °C.

Spin-Labeled Lipids. Spin-labeled dipalmitoylphosphatidylethanolamine (I) was prepared by the method of Brûlet & McConnell (1976a,b). A cholestane spin-label was synthesized as described by Keana & Dinerstein (1971).

Liposomes. Liposomes were formed by mixing 5 μmol of various combinations of dimyristoylphosphatidylcholine and cholesterol with 0.005 μmol of spin-labeled lipid. These samples were rotoevaporated to remove the ethanol solvent and resuspended in 0.1 mL of redistilled chloroform. This solution was rotoevaporated to form a thin homogeneous film. The samples were then placed in a vacuum desiccator for >8 h to remove any residual organic solvent. A 0.5-mL amount of 0.010 M $NaPO_4$ and 0.15 M NaCl, pH 7.0 (PBS),[1] degassed and stored under argon, was added to the lipid film. The samples were then incubated at 50 °C for 2 min and vigorously vortexed for 10 s. A 1.5-mL amount of PBS was added to the samples, which were transferred to centrifuge tubes. The liposomes were collected as a pellet, after spinning at 500*g* for 5 min, and transferred to a 50-μL Corning glass micropipet. The pipet was sealed at one end by melting the glass and sealed at the other end with a Teflon septum cap. Attempts were made to keep the volume of the resuspended pellet as low as 25 μL to obtain as many of the liposomes as possible in the ESR cavity.

Electron Spin Resonance. Spectra of the spin-labeled phospholipid and steroid were recorded on a Varian E-12 spectrometer with temperature control to ±0.5 °C. The spectra were stored in a computer where they were integrated and normalized to the same number of spins. Typical spectra of the spin-labeled phospholipid in mixtures containing phosphatidylcholine and cholesterol are shown in Brûlet & McConnell (1976a).

Peak heights in normalized derivative curve paramagnetic resonance spectra are quite sensitive to line shapes; peak heights are inversely proportional to the square of the line width for signals of a specific form (i.e., Gaussian, Lorentzian, etc.). The particular base line correction used for the present

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[1] Abbreviation used: PBS, 0.5 mol of 0.010 M $NaPO_4$ and 0.15 M NaCl, pH 7.0.

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