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Electron-Transferring Enzymes in the Plasma Membrane of the Ehrlich Ascites Tumor Cell†

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ABSTRACT: The plasma membrane of the Ehrlich ascites tumor cell contains an NADH dehydrogenase. This activity was shown not to be due to contamination by other subcellular membranes. A variety of electron acceptors have been compared as to rate with the following result: ferricyanide ≫ cytochrome *c* > cytochrome b_5 > glyoxylate > dichlorophenolindophenol. Oxygen acceptance could not be detected. The optimum assay temperature and pH ranges were 30–40 °C and pH 6–8, respectively. With respect to either NADH or ferricyanide, the kinetics yielded linear double-reciprocal plots. Inhibition of the enzyme by sulfhydryl reagents could be blocked by excess NADH. Detergents such as Triton X-100 or cholate resulted in solubilization of the enzymatic activity, but phospholipase A_2 did not. The activity differed from that of the mitochondria in that it was not inhibited by rotenone or antimycin A. The possible involvement of NADH oxidation in the energetics of plasma membrane transport is discussed.

In bacteria the plasma membrane is well-known to be the location of electron transport. In contrast, the corresponding functions of eukaryotic cells have been assumed to be localized in the endoplasmic reticulum and mitochondrial membranes. Only recently has attention returned to possible electron transport in the plasma membranes of animal cells. Although either NADH or NADPH oxidoreductase activities have been shown in almost every isolated plasma membrane preparation tested, they have usually been considered to be due to contamination by other subcellular membranes. Recent evidence indicates, however, that one or both of these enzymes may be intrinsic to the plasma membrane. This topic has been reviewed by Löw & Crane (1978).

Recently, several investigations have indicated that neither cellular ATP nor alkali-ion gradients can fully explain energization of amino acid transport across plasma membranes (Christensen et al., 1973; Schafer et al., 1977; Banay-Schwartz et al., 1974). We have considered the possibility that the plasma membrane NADH oxidation may play a role in energizing membrane transport. Inhibition of amino acid uptake by the flavin antagonist quinacrine and restoration of transport in energy-depleted cells by pyruvate or phenazine methosulfate indicated that perhaps a mitochondrial energy source other than ATP may drive plasma membrane transport (Garcia-Sancho et al., 1977). The implications of this proposal have led us to an investigation of the NADH dehydrogenase in membrane vesicles from the Ehrlich cell. The present report provides evidence that the NADH oxidizing activity is due to a plasma-membrane-bound enzyme and demonstrates some of its characteristics.

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Experimental Procedures

Isolation of Plasma Membrane Vesicles. Ehrlich ascites tumor cells were maintained by intraperitoneal injection of Swiss mice and harvested between 8 and 10 days after transfer. The cells were filtered through cheesecloth during collection in an ice-cold beaker. All procedures were performed at 4 °C unless specified otherwise in the text. The cells were washed free of red blood cells in 0.9% saline by centrifuging at 900*g* for 30 s. This step was followed by two washings in 15 mM Tris, pH 7.5, 15 mM NaCl, and 1 mM $MgCl_2$ (buffer A) with centrifugation at 2000*g* for 3 min. After suspension in an equal volume of buffer A, the cells were kept on ice for a minimum of 20 min. The swollen cells were then homogenized in a glass Dounce homogenizing apparatus fitted with a tight pestle. Complete cellular disruption was ensured by checking the homogenate with a light microscope. The homogenate was centrifuged at 650*g* for 10 min and the cloudy supernatant was discarded. From here on, the procedure differs from that used earlier (Im et al., 1976). The pellet was resuspended in buffer A with a glass rod and centrifuged at 500*g* for 5 min. The resulting pellet was taken up in buffer A and dispersed with two to three gentle strokes in the Dounce homogenizer. The homogenate was then diluted and centrifuged for 5 min at 60*g*, followed by 3 min at 500*g*. The crude plasma membrane fraction floats as a snow-white layer over a pinkish tan pellet. The supernatant may contain significant amounts of plasma membrane if homogenization was too vigorous. The white layer was carefully removed, diluted with buffer A, and washed free of any residual nuclei by centrifuging at 60*g* for 5 min. The supernatant was then centrifuged at 12000*g* for 10 min. The resulting pellet was mixed with 94.3% (w/v) sucrose in a ratio of 3:7 (10 mL of total volume) and overlayered in turn with 10 mL of 54.1% (w/v) sucrose, 10 mL of 48.5% (w/v) sucrose, and 8 mL of 39.0% (w/v) sucrose. These gradients were centrifuged for 2 h at 100000*g*. The layer of membranes at the 39.0–48.5% interface was collected

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Table I: Enzymatic Activities Present in the Homogenate and the Purified Plasma Membranes of the Ehrlich Cell[a]

subcellular marker	homogenate	plasma membrane	rel. sp act.
NADH:cytochrome *c* reductase	30.2 ± 1.7	58.1 ± 1.9	1.92
NADH:ferricyanide dehydrogenase	24.0 ± 0.9	855 ± 39	35.6
NADPH:cytochrome *c* reductase	10.3 ± 0.2	13.9 ± 0.8	1.35
Mg^{2+}-ATPase	6.42 ± 0.07	4.90 ± 0.21	0.76
Na^+K^+-ATPase	0.97	26.2	27.0
monoamine oxidase	8.70 ± 0.22	1.12 ± 0.46	0.13
succinate:cytochrome *c* reductase	8.13 ± 0.39	1.49 ± 0.30	0.18
cytochrome *c* oxidase	7.9	1.0	0.13
glucose 6-phosphatase	1.01 ± 0.03	0.12 ± 0.04	0.12
phosphorylcholine diglyceride transferase	1.68 ± 0.16	0.42 ± 0.04	0.25
RNA	171 ± 5	64.5 ± 4.8	0.38

[a] All of the assays were performed at 37 °C as described under Experimental Procedures. The activities of Mg^{2+}-ATPase, Na^+K^+-ATPase, and glucose 6-phosphatase are given as micromoles per hour per milligram of protein, while RNA is reported as micrograms of RNA per milligram of protein. All other activities are reported as nanomoles per minute per milligram of protein. The results shown are the average of at least three determinations, and standard deviations are given except in those cases where the activity is based on a difference.

with a syringe and diluted 1:6 with 10 mM Tris, pH 7.4, 50 mM NaCl, and 3 mM $MgCl_2$ (buffer B). After centrifuging this suspension at 27000*g* for 10 min, we washed the pellet twice in buffer B, followed by homogenization by forcing through a 27-gauge needle three times. All membranes were stored at −70 °C until use.

NAD(P)H Oxidizing Activities. The oxidation of NAD(P)H was assayed at 37 °C in a Hitachi Model 100-60 spectrophotometer. The electron donors used were 300 μM NADH, 200 μM NADPH, or 1 mM succinate unless specified otherwise in the text. The acceptors were 750 μM ferricyanide, 40 μM cytochrome *c*/1 mM KCN, 3.5 mM dichlorophenolindophenol (DCPIP),[1] or 2.2 mM glyoxylate. The millimolar extinction coefficients and the corresponding wavelengths were as follows: cytochrome *c*, 19.0 (550 nm); ferricyanide, 0.96 (420 nm); DCPIP, 20.0 (600 nm); and NADH, 6.22 (340 nm). The buffer used in all experiments other than those in which pH was varied consisted of 50 mM Tris, pH 7.4, 0.5 mM EGTA, and 3 mM $MgCl_2$. All of the reagents were added prior to the membrane preparation so that the nonenzymatic reduction rate could be determined for each assay. This background rate was subtracted from the corresponding rate in the presence of enzyme. The data are presented as the specific activity: nanomoles of acceptor reduced per minute per milligram of membrane protein.

ATPase Assays. The Mg^{2+}-ATPase content was assayed by incubating the membrane preparation with 3 mM Tris–ATP in the same buffer used in the NADH dehydrogenase assays described above. In the analysis for the Na^+K^+-dependent ATPase, 30 mM KCl and 100 mM NaCl were added to the incubation medium. The mixture (1 mL of total volume) was incubated for 15 min at 37 °C, after which the reaction was stopped by the addition of 1 mL of ice-cold 10% trichloroacetic acid. Centrifugation resulted in a clear supernatant from which aliquots were taken for assay of free inorganic phosphate by the method of Fiske & Subbarow (1925). The Mg^{2+}-ATPase was assumed to be the activity in the absence of added Na^+ or K^+ ions, while the Na^+K^+-dependent ATPase was calculated by the difference between the activities in the presence and absence of the two ions together.

Marker Enzymes. The contamination of the plasma membrane preparation by endoplasmic reticulum was determined by the presence of RNA (Munro & Fleck, 1966), glucose 6-phosphatase (Swanson, 1955), and phosphorylcholine diglyceride transferase (Schneider, 1963; Schneider & Behki, 1963). Cytochrome *c* oxidase was estimated indirectly by the difference in the activity of NADH:cytochrome *c* reductase in the presence or absence of 1 mM KCN. In all other instances 1 mM KCN was included in assays involving cytochrome *c* reduction. The amount of outer mitochondrial membrane present was determined by assaying for the presence of monoamine oxidase (Wurtman & Axelrod, 1963). Protein determinations were performed by the Lowry method as modified by Markwell et al. (1978).

Results

Enzymatic Activities Demonstrating the Purity of the Plasma Membranes. Table I characterizes the homogenate and purified plasma membranes with respect to several commonly used subcellular membrane markers. In addition to the specific activity in both fractions, we have calculated a "relative specific activity" (RSA) by dividing the specific activity in the plasma membrane preparation by that found for the homogenate. We stress that the RSA is not the percentage of contamination, which would be a much smaller number.

In accordance with previous reports, the level of 5′-nucleotidase was too low to be used as a routine marker for plasma membrane (Rittenhouse et al., 1978). A more reliable plasma membrane marker, the Na^+K^+-ATPase, had an RSA of 27 and a specific activity of 26.2 (μmol/h)/mg of protein (Table I). These values demonstrate the high purity of the plasma membrane fraction. Colombini & Johnstone (1973) reported a specific activity of Na^+K^+-ATPase of 2.8 (μmol/h)/mg, and from their data one can calculate an RSA of 21.5. Forte et al. (1973) reported a specific activity of 19.7 (μmol/h)/mg, giving an RSA of 15.2. As shown in Table I, the Mg^{2+}-ATPase was not enriched in the isolated plasma membranes (RSA = 0.76). The low RSA may be due to loss of activity when the membranes are stored (Colombini & Johnstone, 1973; Im et al., 1976).

Contamination by outer mitochondrial membrane was tested for by the presence of monoamine oxidase (Sottocasa et al., 1967; Schnaitman et al., 1967; Schnaitman & Greenawalt, 1967). The value of 0.13 for the RSA (Table I) is slightly lower than that calculated from the data of Forte et al. (1973) (0.16) and suggests minimal contamination by the outer mitochondrial membrane. Succinate:cytochrome *c* reductase, a marker for inner mitochondrial membrane, yielded an RSA of 0.18. This demonstration of low contamination by mito-

[1] Abbreviations used: DCPIP, dichlorophenolindophenol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; RSA, relative specific activity; PCMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide; PMS, phenazine methosulfate.

Table II: Acceptor Specificity for the Plasma Membrane NADH Redox System[a]

system	act. [(nmol/min)/mg of protein]
NADH:ferricyanide	485 ± 30.0
NADH:cytochrome *c*	59.9 ± 1.5
NADH:cytochrome b_5	29.7 ± 1.4
NADH:glyoxylate	16.2 ± 3.5
NADH:DCPIP	12.5 ± 1.2

[a] The dichlorophenolindophenol (DCPIP) activity was measured at pH 6.0 to slow the nonenzymatic reduction that occurs at neutral pH. All other activities were assayed at pH 7.4 as described under Experimental Procedures. The nonenzymatic rate varied considerably for the different acceptors and was determined for each assay and subtracted from the rate in the presence of the enzyme. The values are averages (±SD) of at least three determinations.

chondrial membranes was substantiated when cytochrome *c* oxidase was estimated (Table I). Impurity due to endoplasmic reticulum was checked by assaying for glucose 6-phosphatase, RNA, and phosphorylcholine diglyceride transferase. The plasma membranes contained a significant amount of RNA as reported by others (Rittenhouse et al., 1978). The possibility that RNA may be a constituent of the plasma membrane cannot be ruled out (Colombini & Johnstone, 1973). Glucose 6-phosphatase, a popular marker for endoplasmic reticulum (Fleischer & Kervina, 1974), was found to have an RSA of 0.12 (Table I), indicating little if any microsomal contamination. Because the glucose 6-phosphatase activity has been questioned as a useful microsomal marker in the Ehrlich cell (Forte et al., 1973), phosphorylcholine diglyceride transferase was also determined. Measurement of this activity, which is thought to reside almost exclusively in the endoplasmic reticulum (Wilgram & Kennedy, 1963; Van Golde et al., 1971), resulted in an RSA of 0.25 (Table I), supporting the contention that the microsomal contamination of the plasma membranes was minimal.

Redox Activities of the Purified Plasma Membranes. As shown in Table I, the purified plasma membranes contained significant amounts of enzymes catalyzing the oxidation of reduced pyridine nucleotides. NADPH:cytochrome *c* reductase activity was consistently present in all plasma membranes although the activity was variable from preparation to preparation. A very high activity of NADPH:cytochrome *c* reductase in Ehrlich cell plasma membranes was reported by Awad & Spector (1976). This activity has also been studied in adipocyte plasma membranes where it appears to be regulated by insulin (Murkherjee & Lynn, 1977). Higher activity was observed when we assayed NADH:cytochrome *c* reductase (Table I). The RSA of about 2 suggests that this activity is inherent in the plasma membrane. An RSA of 35.6 was found for NADH:ferricyanide dehydrogenase (Table I). There have been previous reports of Ehrlich cell plasma membrane NADH:ferricyanide dehydrogenase with even greater specific activity than that found in the present study (Molnar et al., 1969; Wallach & Kamat, 1966). When compared to the RSA of 27 for the Na^+K^+-ATPase and the RSA's of less than 0.25 for enzymes of other subcellular membranes, this activity must represent a plasma membrane enzyme.

Acceptor Specificity. Interestingly, we find a high ratio of ferricyanide to cytochrome *c* acceptance in the plasma membranes (Table II). This property has also been noted in the red blood cell (Zamudio & Canessa, 1966) as well as other cell types (Löw & Crane, 1978). This ratio decreases slowly when the membranes are stored frozen for several weeks

Table III: Relative Change in the Activity of Plasma Membrane Redox Systems When Assayed in the Presence of Metabolic Inhibitors[a]

inhibitor	% of control: NADH:ferricyanide dehydrogenase	% of control: NADH:cytochrome *c* reductase
none	100 (543 ± 27)	100 (40.6 ± 0.4)
5 μM rotenone	96	102
5 μM antimycin A	97	103
50 mM azide	94	59
1 mM KCN	96	130
2 mM quinacrine		51
0.1 mM dicumarol	100	96

[a] The inhibitors were present during the determination of the nonenzymatic reduction, and the assay was started by the addition of plasma membrane. The percent of control was calculated from the average of at least three determinations. Typical specific activities [(nmol/min)/mg] for controls are shown in parentheses. Quinacrine inhibition of NADH:ferricyanide dehydrogenase was not determined because of quinacrine interference at 420 nm.

due to loss of NADH:ferricyanide dehydrogenase activity. The reduction of ferricyanide may reflect the turnover rate of electrons between NADH and flavoprotein (Hatefi et al., 1961). The plasma membranes also contained NADH:cytochrome b_5 reductase (Table II). This activity has been shown to occur in rabbit liver plasma membranes as well (Ichikawa & Yamano, 1970). Glyoxylate, which might be a natural acceptor, was one of the less active when assayed in vitro. The DCPIP activity was also quite small, and the high nonenzymatic reduction makes it unsuitable for routine analysis. The oxidase activity proved negligible when assayed as either oxidation of NADH or O_2 consumption with a Clark electrode.

Inhibitors of Electron Transport. To aid further in distinguishing the plasma membrane activity from that of the endoplasmic reticulum or mitochondria, we tested several metabolic inhibitors. Neither rotenone nor antimycin A significantly decreased the enzymatic activities measured (Table III). A mitochondrial preparation was used to show these agents were inhibitory. The failure to inhibit the plasma membrane activity demonstrates a definite difference between the two enzymes. Azide had no effect on the NADH:ferricyanide dehydrogenase, yet a 41% decrease in the cytochrome *c* reductase activity was observed (Table III). This result agrees with the report of Crane & Löw (1976) which showed azide inhibition of rat liver plasma membrane NADH:cytochrome *c* reductase but little or no inhibition of the corresponding activity in the microsomes or mitochondria. The NADH:ferricyanide dehydrogenase was not affected by 1 mM KCN, whereas reduction of cytochrome *c* was increased, suggesting cytochrome *c* oxidase contamination as discussed earlier. For this reason all cytochrome *c* reductase assays contained 1 mM KCN. To determine if any of the observed electron transfer was due to DT diaphorase, we tested inhibition by dicumarol. The DT diaphorase in rat liver reacts only weakly with either cytochrome *c* or ferricyanide and will oxidize NADPH as well as NADH (Ernster et al., 1962). As shown in Table I, these properties do not appear to hold for the plasma membrane activity. The absence of DT diaphorase is supported by the lack of inhibition by 0.1 mM dicumarol (Table III). Furthermore, dicumarol did not inhibit the small amount of NADPH:cytochrome *c* reductase activity present in the plasma membranes (data not shown). Quinacrine, a flavin antagonist, was shown to be a selective inhibitor of the

Table IV: Inhibition of Plasma Membrane Redox Activities by Sulfhydryl Modifying Reagents[a]

act. system	inhibitor	act. [(nmol/min)/mg]	% of control
NADH:ferricyanide dehydrogenase	none	1055	100
	0.1 mM PCMB	114	11
	0.1 mM PCMB + 3 mM NADH	833	79
	1 mM NEM	689	65
	1 mM NEM + 3 mM NADH	996	94
NADH:cytochrome *c* reductase	none	58.6	100
	0.1 mM PCMB	0.8	1
	0.1 mM PCMB + 3 mM NADH	52.6	90
	1 mM NEM	23.3	40
	1 mM NEM + 3 mM NADH	57.2	98
NADPH:cytochrome *c* reductase	none	10.2	100
	0.1 mM PCMB	2.3	23
	0.1 mM PCMB + 1.5 mM NADPH	8.7	85
	1 mM NEM	2.6	26
	1 mM NEM + 1.5 mM NADPH	8.9	87

[a] The inhibitors were added to the plasma membrane for 10 min prior to assaying for activity. When NADH protection was tested, this coenzyme was also present during the 10-min period. The results are the average of duplicate determinations. All assays were performed at 37 °C and pH 7.4 as described under Experimental Procedures.

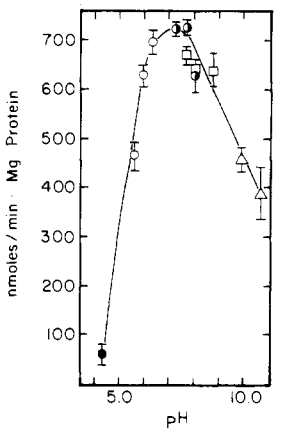

FIGURE 1: The change in NADH:ferricyanide dehydrogenase with respect to the pH of the assay medium. The enzyme activity was determined as described under Experimental Procedures except that the following buffers (10 mM) were substituted for the usual assay buffer: (●) ϵ-caproic acid, (○) maleate, (◑) phosphate, (□) Tris, (△) glycine. The pH values used to plot the data were determined immediately after each assay. The average (±SD) of at least three determinations is presented.

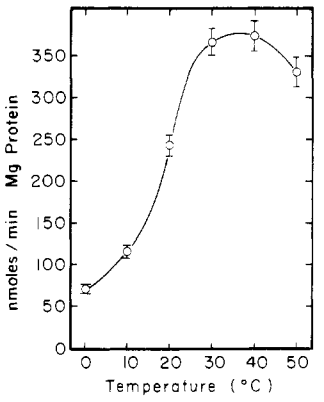

FIGURE 2: The effect of assay temperature on the NADH:ferricyanide dehydrogenase in plasma membrane vesicles. All components of the assay system, at the concentrations given under Experimental Procedures, were first incubated at the given temperature for 2 min prior to the assay. The results are presented as the average (±SD) of at least three determinations.

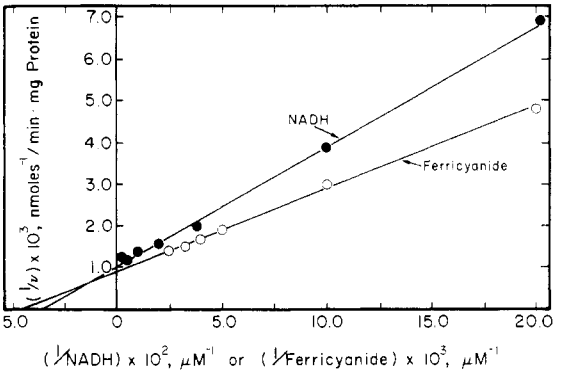

FIGURE 3: A double-reciprocal plot showing how the reaction rate increased with the concentration of either NADH or ferricyanide. All assays were performed at 37 °C and pH 7.4. Each point represents the average of at least three determinations. The lines indicated were derived from a computer program for linear regression analysis. When the concentration of ferricyanide was varied, NADH concentration was held constant at 300 μM, and when the concentration of NADH was varied, the ferricyanide concentration was maintained at 750 μM. Note the difference in magnitude for the variable concentrations of NADH or ferricyanide.

plasma membrane NADH:DCPIP dehydrogenase when compared to the same activity in mitochondria or microsomes (Crane & Löw, 1976). A 49% inhibition by quinacrine on the NADH:cytochrome *e* reductase was found in our studies (Table III).

Temperature and pH Sensitivity. Figure 1 shows the enzyme activity when the NADH:ferricyanide dehydrogenase was assayed at various pH values. In the pH range of 6–8, only a slight difference in activity occurred. The influence of assay temperature on the enzymatic rate can be seen in Figure 2. Although assay at 50 °C produced little inhibition, incubation at 50 °C for 15 min prior to assay led to a significant loss in activity. The enzyme was quite stable for several weeks at −70 °C and for several days at 4 °C.

Requirement for a Free SH Group. Several electron transfer enzymes have been shown to require a free sulfhydryl group for activity. When the plasma membranes were incubated with either PCMB or NEM for 10 min prior to assaying, all three redox activities were inhibited (Table IV). The NADH oxidizing reactions were more susceptible to inhibition by PCMB than NEM, whereas the NADPH:cytochrome *c* reductase was inhibited equally well by both. If an excess of either NADH or NADPH was present during the incubation with the sulfhydryl reagents, the respective enzyme activity was protected (Table IV). Similar results have been obtained by researchers using rat liver plasma membranes (Masuda et al., 1973a).

Table V: Solubilization of Plasma Membrane NADH:Ferricyanide Dehydrogenase by Detergents[a]

fraction	1% Triton X-100 protein (mg)	1% Triton X-100 enzyme (nmol/min)	2% cholate protein (mg)	2% cholate enzyme (nmol/min)
original in detergent	155	199 283	3.72	2164
pellet in detergent	49 (27%)	18 943 (11%)	0.16 (5%)	19 (1%)
supernatant	132 (73%)	152 618 (89%)	3.10 (95%)	1625 (99%)

[a] The membrane was incubated with the detergent indicated for 15 h at 4 °C. After centrifugation at 100000*g* for 1 h, the supernatants were passed through an 0.2-μm Millipore filter. The pellet was resuspended in assay buffer. The enzymatic activity represents the total activity present in the milligram of protein shown in the left-hand column. The numbers in parentheses are the percent of the total recovered. The respective detergent was added to the assays of the original preparation or pellet.

NADH and Ferricyanide Kinetics. Figure 3 shows by a double-reciprocal plot how the reaction rate increased with NADH concentration. The maximal velocity was about 1000 (nmol of ferricyanide reduced/min)/mg of protein, and the apparent K_m for NADH was calculated to be 30 μM. Molnar (1967) reported a similar K_m for both the microsomal and the mitochondrial NADH dehydrogenase from Ehrlich cells. As also shown in Figure 3, a linear double-reciprocal plot was obtained when the ferricyanide concentration was varied between 50 and 500 μM. A different precedent was set by Minakami et al. (1962) for a mitochondrial NADH oxidation, also by using ferricyanide as the acceptor. They concluded that their biphasic plot meant that reduction occurred at both cytochrome and flavin sites. The present linearity suggests that in the plasma membrane preparation ferricyanide reduction occurs only at the flavoprotein level and that therefore the rate can serve as an estimate of the turnover of the enzyme (Zamudio & Canessa, 1966). The maximal velocity was similar to that found in the studies on NADH kinetics, the apparent K_m for ferricyanide being about 220 μM. These results are considerably different from those found for rat liver microsomes by Strittmatter & Velick (1957); they do agree, however, with the findings for NADH:ferricyanide dehydrogenase in rat liver plasma membranes (Masuda et al., 1973a). At concentrations above 500 μM, inhibition by ferricyanide was observed.

Solubilization of the NADH:Ferricyanide Dehydrogenase. Membranes were incubated with either Triton X-100 or cholate to determine if the enzymatic activity could be removed from the membrane. Table V shows the results of the solubilization experiments. The enzyme was released by either of the two detergents; however, Triton X-100 resulted in a better ratio of enzyme/total protein solubilized. Storage at 4 °C for several days in 2% cholate caused some loss in activity of the solubilized enzyme; no loss of activity was observed with Triton X-100. After solubilization the NADH:cytochrome *c* reductase activity was reduced considerably, suggesting that components mediating electron transport between the dehydrogenase and the cytochrome *c* reductase either were not solubilized or were inhibited by the solubilization procedure. Although not tested in this report, deoxycholate has been used to solubilize a similar activity from rat liver plasma membranes (Masuda et al., 1973b).

Treatment of the membranes with organic solvents such as acetone, butanol, or pentane resulted in complete inactivation. Anhydrous butanol extraction has been used successfully on an NADH dehydrogenase from human red blood cells (Zamudio et al., 1969). Incubation with phospholipase A_2 for 15 h at 4 °C did not produce any significant solubilization (10–20%). Mitochondrial NADH dehydrogenase can be solubilized with phospholipase A (Minakami et al., 1962).

Discussion

The present report describes an NADH dehydrogenase activity in plasma membranes isolated from Ehrlich ascites tumor cells. Our work as well as that of several others (Löw & Crane, 1978) supports the conclusion that this redox system is intrinsic to the plasma membrane and not due to contamination by other subcellular membranes. Using intact Ehrlich cells, we could not detect any dehydrogenase activity. Therefore, the enzyme probably faces the cytoplasmic surface of the plasma membrane. The plasma membrane preparation described here is highly purified yet contains three redox activities not commonly associated with this structure. Contamination can be ruled out as the source of these enzymes by comparing the relative specific activities presented in Table I. Furthermore, several metabolic inhibitors have been used to distinguish the plasma membrane activity from that of the mitochondria or microsomes. It should be noted, however, that these differences may be due to the different membrane environments. There is some evidence that the redox components in plasma membrane, endoplasmic reticulum, and mitochondria are immunologically related (Raftell & Blomberg, 1973; Borgese & Medolesi, 1976; Löw & Crane, 1978).

We find that the NADH:cytochrome *c* reductase activity, which may require cytochrome b_5 and cytochrome b_5 reductase at intermediate steps (Jansson & Schenkman, 1977), is much less than that of the NADH dehydrogenase. This difference may be the result of low levels of plasma membrane cytochrome b_5 as is the case in rat liver plasma membranes. After treatment of rats with carbon tetrachloride, the NADH:cytochrome *c* reductase activity in the plasma membrane was increased threefold, the NADH dehydrogenase was increased twofold, and the NADH:cytochrome b_5 reductase was unchanged (Masuda et al., 1973a). The authors attribute these results to increases in plasma membrane bound NADH dehydrogenase and cytochrome b_5.

The actual number of electron transfer enzymes and their interactions remain to be established for the plasma membrane. Using both hydrophilic and hydrophobic metal chelators, we have preliminary evidence for the presence of non-heme iron centers in our plasma membrane preparation. Using similar conditions, Sun et al. (1975) have demonstrated an inhibition of *Escherichia coli* membrane bound ATPase by these chelators. Raftell & Blomberg (1974a, 1974b) find that the NADH dehydrogenase of rat liver plasma membranes may reside in a multienzyme complex with one or more ATPase activities. The Ca^{2+}-dependent ATPase of the red blood cell, presumably involved in Ca^{2+} transport, has been shown to be sensitive to flavin oxidizing agents such as DCPIP (Wins & Schoffeniels, 1968). The possibility that amino acid transport is coupled to an ATP-hydrolyzing activity remains to be firmly established (Im et al., 1976).

The observations that uphill transport occurs in the presence of low cellular ATP and unfavorable ion gradients impresses upon us the importance of investigating alternate forms of energization. Through the use of metabolic poisons, Schafer et al. (1977) showed that energy sources other than the two mentioned above must be considered as driving forces for amino acid transport. Lajtha and his associates came to a similar conclusion and postulated that the unknown energy

source, although mitochondrial in origin (Banay-Schwartz et al., 1976), was not ATP (Banay-Schwartz et al., 1974). Although the methionine gradient maintained across the plasma membrane of another ascites tumor cell correlates well with the apparent electrochemical gradient of Na^+, the former gradient is 1.8 times the value predicted by the latter (Philo & Eddy, 1978).

Our laboratory has proposed that NADH might serve as this undefined source of energy (Garcia-Sancho et al., 1977; Christensen, 1977; Ohsawa et al., unpublished experiments). The presence of a plasma membrane redox system for NADH supports this proposal and offers a mechanism for transfer of mitochondrial energy to membrane transport without the direct utilization of ATP. Furthermore, studies carried out with transport-competent plasma membrane vesicles show a relation between oxidation of NADH or PMS and transport of an amino acid (Ohsawa et al., unpublished experiments). Quinacrine, shown in the present report to inhibit the plasma membrane NADH:cytochrome *c* reductase by 50%, has been shown to depress amino acid transport to the same extent (Garcia-Sancho et al., 1977; Ohsawa et al., unpublished experiments). Although no inhibition of plasma membrane redox systems was found in our present work, rotenone has been shown to be inhibitory to amino acid transport in Ehrlich cell plasma membrane vesicles (Johnstone, 1976). We will continue to seek unambiguous evidence regarding the involvement of this redox system in energizing membrane transport.

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