

Characterization of the Membrane-Bound Succinic Dehydrogenase of *Micrococcus lysodeikticus*

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The occurrence of succinic dehydrogenase [succinic:(acceptor) oxidoreductase, EC 1.3.99.1] in membrane fractions of *Micrococcus lysodeikticus* was investigated. The enzyme could be purified 10-fold, by deoxycholate treatment. Butanol extraction of membranes yielded an active fraction, nonsedimentable at $130,000 \times g$ for 2 hr and altered in its phospholipid content relative to membranes. The activity of the enzyme in particulate preparations was decreased in the presence of competitive inhibitors and by compounds known to react with iron, sulfhydryl groups, and flavine. In this respect, the bacterial succinic dehydrogenase is similar to the enzyme derived from yeast and mammalian sources. In certain membrane fractions, Ca^{2+} and Mg^{2+} exhibited inhibitory effects whereas Triton X-100 caused activation. The enzyme could also be activated by substrate. In the phenazine reductase assay, incomplete reduction of electron acceptor was observed upon addition of divalent cations and iron binding agents.

In previous communications from this and several other laboratories, it has been suggested that succinic dehydrogenase (SDH) is localized in the membranes of *Micrococcus lysodeikticus* (20, 24, 31). However, apart from detecting this activity in membrane preparations, there have been very few attempts to purify the SDH or to investigate in detail the properties of the membrane-bound form. Such studies would be of special significance since a great deal is now known about the analogous enzyme from mammalian and yeast mitochondria and from anaerobic bacteria (9, 17, 18, 32, 33).

Mammalian SDH has been under intensive investigation for a considerable period of time but it has only been in fairly recent years that workers have obtained highly purified preparations relatively free from lipid (6). Owen and Freer (27) have recently studied some factors affecting the activity of SDH in washed membranes of *M. lysodeikticus* and observed inhibitory effects of Mg^{2+} and Ca^{2+} . We have concentrated our efforts on characterizing the properties of this enzyme from this aerobe in order to compare it with SDH enzymes from other sources, and to gain further insight into the functional organization of *M. lysodeikticus* membranes. It has been our hope that these studies will lead to the eventual purification and characterization of the enzyme in a soluble form.

MATERIALS AND METHODS

Chemicals. Hen's egg white lysozyme (three times recrystallized and salt free), pancreatic deoxyribonuclease, phenazine methosulfate (PMS), succinic acid (disodium salt), and fumaric acid (sodium salt) were obtained from Calbiochem (Los Angeles, Calif.). Triton X-100 was a gift from Rohm and Haas (Philadelphia, Pa.), 2,6-dichlorophenolindophenol (DCIP) and *p*-hydroxymercuribenzoate were products of Sigma Chemical Co. (St. Louis, Mo.), and sodium deoxycholate and malonic acid were supplied by Mann Fine Chemicals (Orangeburg, N.Y.). Sodium pyrophosphate and *o*-phenanthroline, were products of Fisher Scientific Co. (Fair Lawn, N.J.), and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate was purchased from Aldrich Chemical Co. (Milwaukee, Wis.). $Na_2H^{32}PO_4$ (specific activity, 500 μ Ci/mole) was obtained from New England Nuclear Corp. (Boston, Mass.), and dialysis tubing with a molecular weight exclusion of 12,000 was obtained from Arthur H. Thomas (Philadelphia, Pa.). All other materials used were of the highest purity available.

Growth of organisms and preparation of membranes.

M. lysodeikticus (NCTC 2665) was grown in peptone-water-yeast extract medium as described by Salton and Freer (30). When the cultures had reached an optical density of 4 as measured at 700 nm, the cells were harvested by centrifugation at 4 C, washed twice with distilled water, and then suspended in buffer for the preparation of membranes. When ^{32}P -labeled membranes were required, 100 μ Ci of $Na_2H^{32}PO_4$ was added to each culture flask (750 ml of medium) at the time of inoculation.

Bacteria were lysed with lysozyme and treated with deoxyribonuclease as previously described (29) except that 0.1 M ammonium acetate adjusted to pH 7.6 with concentrated NH_4OH was used as the buffer. The initial membrane preparation obtained (Fig. 1) was washed four times in this buffer, and after each wash the membrane suspensions were centrifuged at $30,000 \times g$ for 30 min at 4 C. Membranes were then suspended and washed an additional three times in low ionic strength buffer (0.01 M ammonium acetate buffer, pH 7.6) to yield the final seventh membrane pellet (Fig. 1). The washing of the membranes in the low ionic strength buffer will be referred to in the text as "shock" washing.

Deoxycholate extraction of membranes. The seventh membrane pellet at a protein concentration of 2 to 3 mg/ml, was suspended at 0 C in 0.01 M buffer containing 0.5% (w/v) sodium deoxycholate (DOC). The suspension was immediately centrifuged at $54,500 \times g$ for 1 hr at 0 C. The supernatant fluid was then removed and centrifuged at $105,000 \times g$ for 2 hr at 0 C. The resulting supernatant fluid was again centrifuged at 0 C for 2 hr, but at $130,000 \times g$ (Fig. 1). All fractions were extensively dialyzed against 0.01 M ammonium acetate buffer to reduce the concentration of DOC.

Butanol extraction of membranes. Butanol extraction was performed by a modification of the procedure described by Maddy (21). The seventh membrane pellet at a protein concentration of 4 to 6 mg/ml was suspended at 0 C in 0.005 M ammonium acetate buffer, pH 7.6. The suspension was made 20 mM with respect to sodium succinate, and two volumes of precooled butanol (-10 C) were added to three volumes of membrane suspension. The material was mixed periodically for 20 min while being maintained at -5 C and was then centrifuged at $12,000 \times g$ for 25 min at -10 C to yield a lower aqueous layer, an insoluble interfacial layer, and an upper nonaqueous phase. The aqueous material was immediately removed and transferred to dialysis tubing previously soaked in 1 mM ethylenediaminetetraacetate (EDTA). Dialysis was carried out at 4 C overnight against 0.005 M ammonium acetate buffer, pH 7.6. The dialyzed aqueous material, hereafter referred to as the "butanol-succinate extract" was centrifuged at $130,000 \times g$ for 2 hr at 0 C. The resulting supernatant and pellet fractions are designated as the "butanol 130,000 $\times g$ supernatant" and "butanol 130,000 $\times g$ pellet."

Analytical procedures. Fractions to be analyzed for phospholipids were extracted by the method of Bligh and Dyer (5). Lipids were dried under a stream of nitrogen, suspended in a known volume of chloroform, and then spotted on Whatman SG-81 silica gel loaded paper. Chromatograms were developed for 2.5 hr in an ascending solvent system (23) containing chloroform-methanol-diisobutylketone-acetic acid-water (45:15:20:30:4, v/v; CMDAW), and spots were demonstrated under ultraviolet light after staining in an aqueous solution of 0.0012% Rhodamine 6G. Radioactivity of ^{32}P -labeled phospholipids on chromatograms was determined by cutting out the ultraviolet-absorbing spots and counting them in a Nuclear-Chicago gas-flow counter. Autoradiography was carried out by exposing Dupont Cronex no. 4 X-ray film to chromatograms for

3 to 7 days before development. Samples (50 to 100 μliters) of membrane fractions were analyzed for ^{32}P content by counting in a gas-flow counter.

Protein concentrations were measured by the method of Lowry et al. (19), with bovine serum albumin as a standard.

Enzyme assay. Succinic dehydrogenase activity was assayed by a method similar to that used by Kimura et al. (16). The assay mixture contained 30 μliters of 0.2 M sodium succinate, pH 7.6, 10 μliters of 0.1 M EDTA, 20 μliters of 2.5×10^{-3} M DCIP, 10 μliters of 9 mg/ml PMS, enzyme, and 0.1 M ammonium acetate buffer, pH 7.6, to a final volume of 1.0 ml. Triton X-100 was also included where indicated. All components except DCIP and PMS were mixed in a quartz cuvette (light path, 1 cm), which was placed in the temperature-regulated compartment of a Cary model 15 recording spectrophotometer and was maintained at 38 C for 6 min except where otherwise indicated. The control cuvette contained all the components except for DCIP and PMS dyes. After this activation period, the DCIP was added and the absorbancy of the mixture at 600 nm was recorded for 10 sec. PMS was then rapidly added and mixed (4- to 5-sec period), and the drop in absorbancy or reduction of the DCIP dye was followed at 38 C. Enzyme activities were calculated on the basis of initial rates by use of the first 20% or less of the drop in absorbancy of the DCIP (millimolar extinction coefficient, 16.1). Corrections were made for any reduction of the DCIP (usually less than 5% of the phenazine reduction) by the enzyme in the absence of PMS. One unit of enzyme activity is the amount catalyzing the oxidation of 1 μmole of succinate per min.

RESULTS

Distribution of enzyme activity in membrane fractions. The preparation of membranes and deoxycholate-treated membranes is shown in Fig. 1. Quantitation of protein and SDH activity was carried out at each stage of the preparation, and specific activities, total protein, and total enzyme units were calculated. As indicated in Table 1, major protein losses from the membranes occurred during the first and fifth washes (25, 26). However, only a very small amount of SDH was released into the "shock" washes and, as a consequence, a fourfold increase in specific activity was obtained. Upon treatment of the seventh membranes with DOC and ultracentrifugation at $130,000 \times g$, the isolated pellet showed a further increase in specific activity (twofold as compared with seventh membranes).

Influence of activation and effect of inhibitors on SDH activity. The ability of substrate to activate the enzyme is illustrated in Fig. 2. As can be seen, the rate of activation increased most rapidly in the first 2 min and then leveled off to a maximum (about an eightfold increase in specific activity) in approximately 7 min. After 10 min of preincubation at 38 C, activity was found to decline (not shown in Fig. 2).

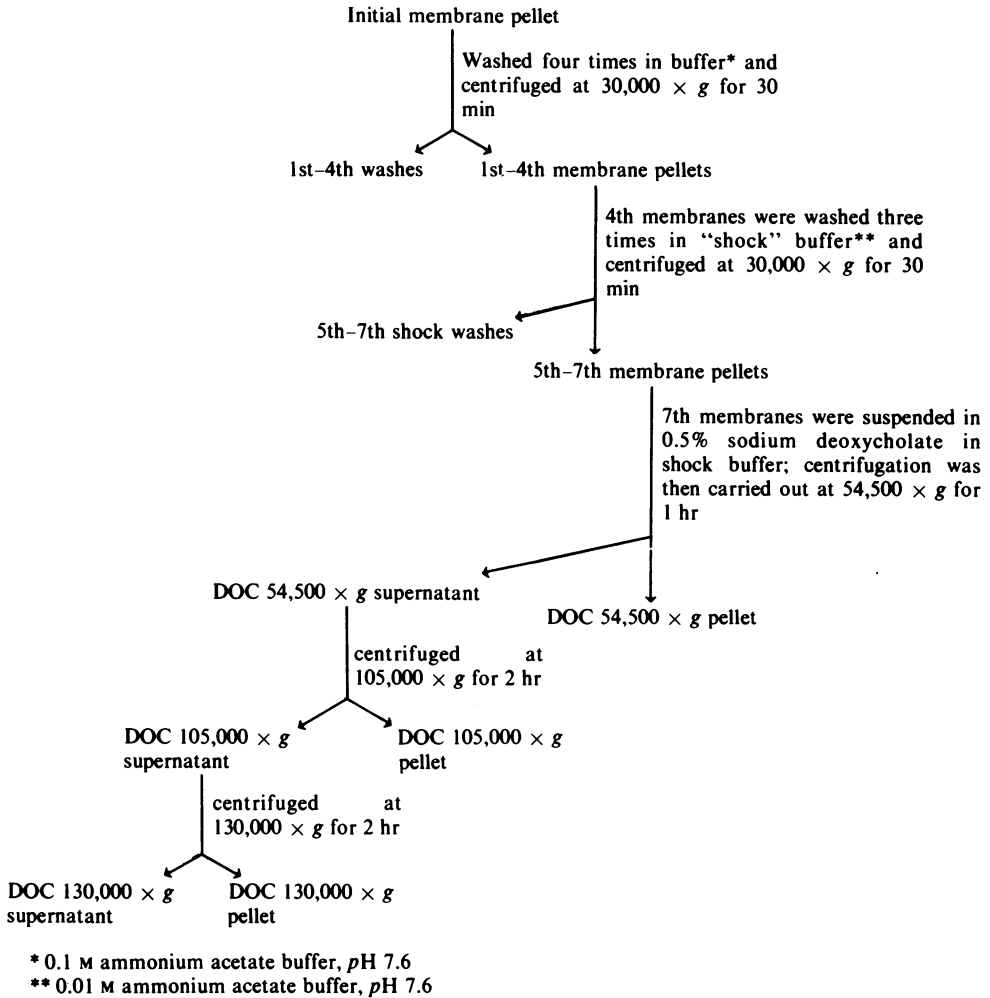


FIG. 1. Flow diagram of washing and deoxycholate extraction of *M. lysodeikticus* membranes. *M. lysodeikticus* cells were harvested and lysed as described in Materials and Methods, and the lysate was centrifuged to yield an initial membrane pellet. This pellet, at a protein concentration of approximately 1 mg/ml, was then treated as indicated.

To obtain complete reduction of the DCIP dye, the addition of Triton X-100 was required in assaying certain types of membrane preparations. The effect of Triton concentration on the assay of the fourth washed membranes is shown in Fig. 3. Curve *a*, representing a complete reduction of the DCIP dye, is typical of an acceptable enzyme assay, and could be used for the calculation of specific activity. Incomplete reduction of the dye was observed in curves *c* through *g* in which lower Triton concentrations were used. Curve *b* with a concentration of Triton higher than that used in *a* does exhibit a complete reduction, but the calculated specific activity for *b* was lower than that for *a*. The influence of EDTA in the assay mixture is shown in the comparison of curves *f* and *g*.

A plot of specific activity versus Triton X-100 concentration for three different types of preparations is shown in Fig. 4. When the Triton concentration used in the assay did not result in a complete reduction of the dye, the initial velocity was determined as usual by measuring the optical density change over the first 20% reduction. Certain types of preparations, for example, the DOC 130,000 \times g pellet (Fig. 4), were not as sensitive to high Triton concentrations and exhibited broad optima.

The effect of inhibitors or SDH activity is illustrated in Table 2. All of the compounds tested except sodium azide, sodium cyanide, and a water-soluble carbodiimide decreased the initial velocity, and the values for the per cent inhibition in Table 2 were calculated on this basis.

TABLE 1. Distribution of succinic dehydrogenase (SDH) activity during the washing and deoxycholate (DOC) extraction of *M. lysodeikticus* membranes^a

Prepn	Specific activity ^b	Total protein (mg)	Total SDH units
Initial membranes	0.250	1,020	255
1st membranes	0.309	720	222
1st wash	0.023	274	6
4th membranes	0.310	576	178
4th wash	0.041	18	1
5th membranes	0.905	252	226
5th wash	0.040	273	11
7th membranes	1.300	187	243
7th wash	0.710	12	9
DOC 54,500 × g pellet	0.058	18	1
supernatant	1.270	178	225
DOC 105,000 × g pellet	0.432	35	13
DOC 105,000 × g			
supernatant	2.280	80	182
DOC 130,000 × g pellet	2.600	15	39
DOC 130,000 × g			
supernatant	1.860	52	96

^a SDH activities and protein contents (see Materials and Methods) were measured at each stage of the washing and DOC extraction of membranes (Fig. 1). The assay mixture consisted of 30 μ liters of 0.2 M sodium succinate in 0.1 M ammonium acetate, pH 7.6, 10 μ liters of 0.1 M EDTA, 60 to 100 μ liters of 2.5% Triton X-100, enzyme (10 to 100 μ g of protein), and 0.1 M buffer to 980 μ liters. After 6-min activation at 38 C, 20 μ liters of 2.5 mM 2,6-dichlorophenol indophenol (millimolar extinction coefficient, 16.1) was added, and its optical density at 600 nm recorded for a period of 10 sec; 10 μ liters of PMS (9 mg/ml) was then quickly added to initiate the enzyme reaction, and the reduction of the 2,6-dichlorophenolindophenol was followed at 600 nm for a period of 3 to 4 min at 38 C.

^b Expressed as micromoles of succinate per minute per milligram of protein.

However, it should be noted that, although the inhibitors altered the initial velocities, complete dye reductions were observed with some (*p*-hydroxymercuribenzoate and Atabrine) and incomplete with others (Mg^{2+} , Ca^{2+} , *o*-phenanthroline, and sodium pyrophosphate). The latter response was unaffected by the inclusion of Triton X-100.

In the case of butanol-extracted preparations, *o*-phenanthroline and sodium pyrophosphate produced inhibition of activity as well as incomplete dye reductions, whereas neither Ca^{2+} nor Mg^{2+} did so. Moreover, in these preparations, specific activities were higher in the absence of Triton.

Kinetic studies. The K_m values for succinate and phenazine methosulfate and the K_i values for fumarate and malonate, found to be competitive inhibitors (Fig. 5), were determined on a DOC

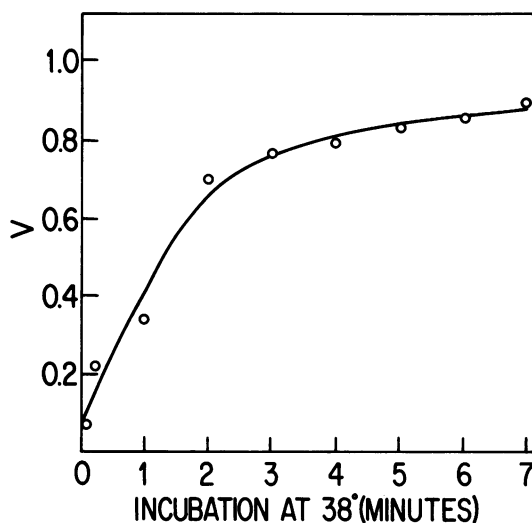


FIG. 2. Activation of succinic dehydrogenase activity by sodium succinate. Succinic dehydrogenase activity was measured by the usual procedure (Table 1) in a DOC 130,000 × g pellet (Fig. 1) (15 μ g of protein/assay) after preincubation of the enzyme with the substrate for periods of 0 to 7 min at 38 C. In the case of the zero-time sample, both DCIP and PMS were added simultaneously and the reduction of the DCIP dye was measured immediately at 600 nm. The initial velocity of the reaction (*v*) is expressed as the change in the optical density per minute.

130,000 × g pellet by the method of Dixon and Webb (10). The K_m for succinate was 0.26 mM, and the K_i values for fumarate and malonate were 2.24 mM and 1.30 mM, respectively.

The reaction velocity of the SDH of *M. lysodeikticus* is dependent upon the concentration of PMS with a DOC 130,000 × g pellet preparation (see Fig. 1) having a K_m for the dye of 0.25 mM at 38 C (2, 4). Final concentrations of PMS above 90 μ g/ml in the assay, however, were found to be inhibitory to SDH activity (28); as a result, a fixed PMS concentration of 90 μ g/ml has been routinely used in our assay system. At this concentration, the initial velocity of the SDH activity is maximum and is 80% of that obtained by extrapolation to V_{max} with respect to PMS. Furthermore, having used this concentration of PMS, the summation of total enzyme units in the various membrane fractions is in reasonably good agreement with the expected values (Table 1), suggesting no major change in K_m for the PMS.

The transfer of electrons from SDH to DCIP, which is independent of the DCIP concentration (5×10^{-5} M) used in the assay (36), proceeds at a very slow rate. The rate is approximately 5% of that of SDH to PMS in washed and DOC-

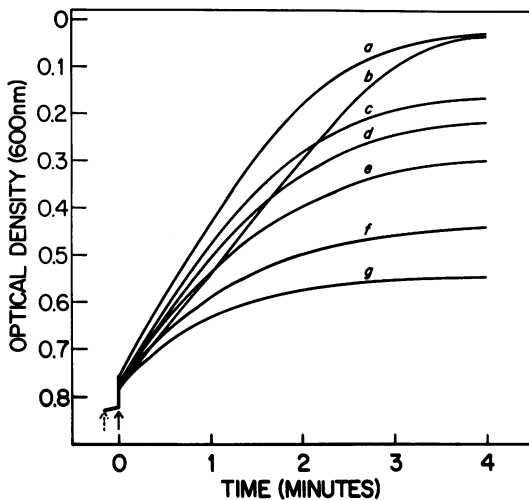


FIG. 3. Influence of Triton X-100 concentration upon the succinic dehydrogenase activity of *M. lysodeikticus* 4th membranes. Fourth-washed membranes (100 μ g of protein/assay), prepared as outlined in Fig. 1, were tested for succinic dehydrogenase activity (Table 1) in the presence of varying concentrations of Triton X-100. The absorbancy of the DCIP is represented by the broken arrow; that of the PMS by the solid arrow. Final concentrations of Triton in the assay mixture are as follows: a, 0.20%; b, 0.50%; c, 0.125%; d, 0.075%; e, 0.025%; f, no Triton; and g, no Triton, no EDTA.

extracted membrane preparations, and is even slower or negligible in butanol-extracted fractions. In the absence of enzyme, under our condition of assay where electron acceptors are added last to initiate the reaction, there is virtually no nonenzymatic photoreduction of the DCIP by PMS (27).

Properties of butanol-extracted fractions. The distribution of catalytic activity, phospholipids, carotenoid, and protein in 32 P-labeled membranes and in preparations obtained from butanol extraction of the membranes is shown in Table 3. Approximately 15% of the protein, 7.6% of the 32 P counts, and 5% of the carotenoids of the membrane were recovered in the aqueous phase. Significantly, the specific activity of the aqueous phase after butanol extraction was almost three times that of the membranes, and approximately 40% of the total SDH units were recovered. Upon high-speed centrifugation, most of the SDH activity was sedimented, although protein was distributed equally between the pellet and supernatant fractions. It is worthy of note that the ratio of phospholipid to protein in the supernatant fraction was twice that of the pellet, whereas the ratio of carotenoid to protein was half that of the pellet.

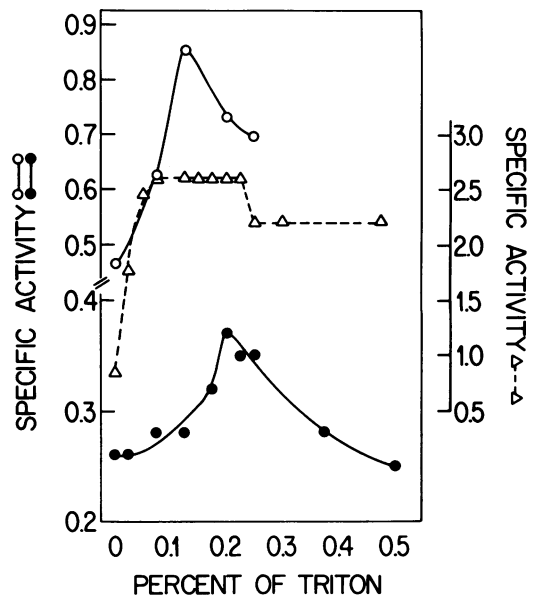


FIG. 4. Influence of Triton X-100 concentration upon the specific activities of membrane and deoxycholate-treated membrane preparations. Succinic dehydrogenase activity was measured after 3-min activation at 38 C in the presence of various concentrations of Triton X-100. Where the Triton concentration used in the assay did not result in complete reduction of the dye, the initial velocity was determined as usual by measuring the optical density change over the first 20% reduction of the dye. Specific activity values (μ moles of succinate oxidized per min per mg of protein) designated for the two preparations represented by open and closed circles refer to the axis on the left part of the figure and triangles refer to the axis on the right. (O) DOC 54,500 \times g supernatant, 50 μ g of protein/assay; (●) 4th membranes, 100 μ g of protein/assay; (Δ) DOC 130,000 \times g pellet, 25 μ g of protein/assay.

The ratios of the three major phospholipids present in *M. lysodeikticus* (A. De Siervo and M. R. J. Salton, *to be published*) were determined for the four types of preparations tested (Table 4). Upon extraction of seventh-washed membranes, there appeared to be an enrichment of cardiolipin relative to the other two phospholipids. The supernatant fraction was found to retain more lipid per milligram of protein than either the pellet or the whole extract; in fact, no loss of cardiolipin relative to protein was observed. These observations are qualitatively supported by the autoradiogram in Fig. 6.

DISCUSSION

For the membrane preparations shown in Fig. 1, the initial velocity of the reaction is linear with enzyme concentration provided that an optimal

TABLE 2. Effect of cations and inhibitors on the succinic dehydrogenase (SDH) activity of a deoxycholate (DOC) 130,000 \times g pellet^a

Compound	Concn (M)	Per cent inhibition
KCN	1×10^{-2}	0
NaN ₃	4×10^{-2}	0
1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho- <i>p</i> -toluene sulfonate	2×10^{-4}	0
CaCl ₂	1×10^{-3}	33
MgCl ₂	1×10^{-3}	34
<i>p</i> -Hydroxymercuribenzoate	4×10^{-7}	75
<i>o</i> -Phenanthroline	2×10^{-3}	31
Na ₂ P ₄ O ₇	3×10^{-2}	41
Atabrine	5×10^{-4}	35

^a Samples of DOC 130,000 \times g pellet (25 μ g of protein/assay) were tested for SDH activity in the presence of the compounds indicated after activation for 3 min. Triton X-100 (0.2%) was included in the assay of the DOC pellet. Per cent inhibitions are based on decreased initial velocities of the enzyme reaction.

amount of the nonionic detergent Triton X-100 is included in the incubation mixture. As indicated in Fig. 4, the optimal amount depends upon the type of membrane preparation under investigation. It does not depend upon protein concentration up to approximately 100 μ g of protein per assay, but when higher concentrations are used additional amounts of Triton do not produce optimal or "acceptable" assays (see below).

In Fig. 2, it can be seen that incubation of a DOC 130,000 \times g pellet with succinate for 6 to 7 min at 38 C results in an eightfold increase in specific activity. Although this approximates that found by Kimura et al. (16) for a mammalian SDH preparation, the increase in specific activity varied with the type of membrane fraction tested. This variation, however, may be affected to a certain extent by the purity of the preparation and the enzyme concentration used, as suggested by Kearny (14, 15). It is not surprising that the activation phenomenon occurs with *M. lysodeikticus* SDH, since this organism is a strict aerobe, and activation of SDH appears to be a general type of aerobic process occurring in a variety of mammalian tissues as well as in yeast mitochondria, but not in obligate anaerobes (4, 14, 32, 35). The specific activity of the seventh membrane pellet used in the study in Table 1 was determined to be 1.2 μ moles of succinate oxidized per min per mg of protein; however, this varied between 0.4 and 1.2 for different seventh membrane preparations and may well be a function of growth conditions.

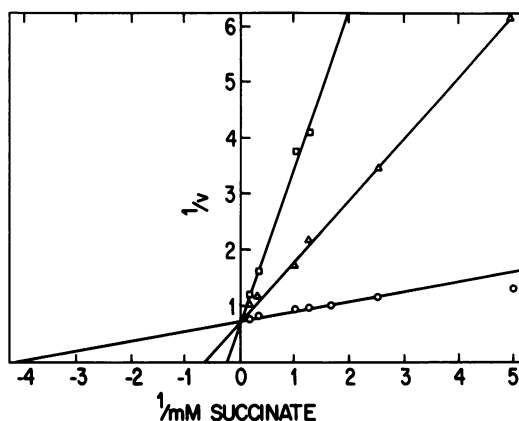


FIG. 5. Lineweaver-Burk plots of the variation of activity with succinate concentration in the absence and presence of competitive inhibitors. Succinic dehydrogenase activity of a DOC 130,000 \times g pellet (20 μ g of protein/assay) was measured in the presence of various concentrations of succinate with and without the addition of a constant concentration of either fumarate or malonate. The assays were conducted in the presence of 0.2% Triton, but the enzyme was not activated. The initial velocity of the reaction (v) is expressed as the change in optical density per minute. (O) Assay performed with concentrations of sodium succinate from 0.2 to 6.0 mM; (Δ) 3 mM sodium fumarate was added to the assay mixture for each concentration of succinate; (\square) 6 mM malonic acid, pH 7.6, was added to the assay mixture for each concentration of succinate.

Treatment of seventh membranes with DOC leads to a removal of both protein and lipid as well as most of the carotenoid pigment from the membranes. This removal of protein and lipid appears in part to involve the release of small vesicular particles (as determined by electron microscopy, unpublished data) sedimentable at 130,000 \times g. Such particles are highly active for SDH, having activity about 10 times that of the initial membrane pellet, and in addition contain cytochrome *b* activity (13) which can be reduced with dithionite but not with succinate.

Butanol extraction of seventh membranes yields an aqueous phase containing approximately 15% of the protein in the seventh membranes, and, although yields varied between 7 and 15%, the ratio of lipid to protein was constant and reproducible (see Table 3). When butanol-treated preparations were quantitatively analyzed for their component phospholipids, an enrichment of cardiolipin was noted in comparison with seventh membranes (see Table 4). This enrichment may be due to the relative insolubility of cardiolipin in organic solvents as compared with other phospholipids (11); it may also be influenced by the "multidentate" structure of

TABLE 3. *Distribution of catalytic activity, phospholipids, carotenoids, and protein in membranes and in preparations obtained from butanol extraction of the membranes^a*

Prepn	Total protein (mg)	Total ³² P (counts/min)	Counts per min per mg of protein	Ratio, ³² P to protein	Total OD ₄₇₅ units ^b	OD ₄₇₅ /mg of protein	Ratio, OD ₄₇₅ to protein	Specific activity SDH ^c	Total SDH units
7th membranes ..	37	2,398 × 10 ³	648 × 10 ²	1.0 ^d	119	3.2	1.0 ^d	0.48	17.8
Butanol-succinate extract	5.5	178 × 10 ³	324 × 10 ²	0.50	6	1.1	0.34	1.30	7.1
Butanol 130,000 × g pellet	2.3	54 × 10 ³	232 × 10 ²	0.36	3.7	1.6	0.50	2.40	5.5
Butanol 130,000 × g supernatant ..	2.0	96 × 10 ³	478 × 10 ²	0.71	1.4	0.7	0.22	0.45	0.9

^a ³²P-labeled 7th membranes were extracted with butanol in the presence of 20 mM succinate, and the resulting aqueous phase of the extract was dialyzed overnight and then subsequently centrifuged at 130,000 × g to give supernatant and pellet fractions. Protein, ³²P, and specific activities were determined in each preparation according to procedures outlined in Materials and Methods.

^b The optical density at 475 nm was used as a relative quantitation of the carotenoid present.

^c The specific activity is defined as the number of micromoles of succinate oxidized per minute per milligram of protein.

^d The 7th membrane ³²P to protein and carotenoid to protein ratios are arbitrarily set at 1.

TABLE 4. *Distribution of phospholipids and changes in the relative ratios of these phospholipids to protein in membranes and in preparations obtained from butanol extraction of the membranes^a*

Prepn	Percentage of ³² P in total phospholipid ^b	³² P counts per min per mg of protein	Individual phospholipids (³² P counts per min per mg of protein)	Comparative ratios of ³² P to protein
7th membranes		648 × 10 ^{2c}		
CL	60.4		391 × 10 ²	1.0 ^d
PG	32.0		207 × 10 ²	1.0 ^d
PI	7.6		49 × 10 ²	1.0 ^d
Butanol-succinate extract		324 × 10 ^{2c}		
CL	82.0		266 × 10 ²	0.68
PG	13.9		45 × 10 ²	0.22
PI	4.1		12 × 10 ²	0.25
Butanol 130,000 × g pellet		232 × 10 ^{2c}		
CL	80.0		185 × 10 ²	0.47
PG	17.0		39 × 10 ²	0.19
PI	3.0		7 × 10 ²	0.14
Butanol 130,000 × g supernatant ..		478 × 10 ^{2c}		
CL	84.3		404 × 10 ²	1.03
PG	11.2		54 × 10 ²	0.26
PI	4.5		20 × 10 ²	0.42

^a Lipid was extracted from each of the ³²P-labeled fractions according to the method of Bligh and Dyer (5), and samples from the extracts were subjected to paper chromatography as outlined under Materials and Methods. After Rhodamine 6G development, the ultraviolet-absorbing spots were cut out and counted for ³²P in the gas-flow counter. From the total counts obtained, the percentage of ³²P in each of the phospholipids was computed. CL, cardiolipin; PG, phosphatidyl glycerol; PI, phosphatidyl inositol.

^b Major phospholipids present in the lipid extract (A. DeSiervo and M. R. J. Salton, *to be published*). These areas represent 95% of the ³²P counts; 5% of the counts appeared in other areas of the chromatogram.

^c From Table 3.

^d Comparative ³²P to protein ratios of butanol-extracted preparations are expressed relative to 7th membranes which are arbitrarily set at 1.

cardiolipin (11). Although we have no direct evidence, it is conceivable that the selective recovery of SDH in the aqueous phase may then be due to its preferential association with cardiolipin (see Tables 3 and 4).

The K_m of SDH for succinate as determined for a DOC $130,000 \times g$ pellet is 0.26 mM. This value may be lower than the true value by approximately 20% since the assay was done at a fixed concentration of dye (see above). Nevertheless, the value obtained, allowing for correction, is within the range of values found for this enzyme isolated from other sources (22, 32). In the case of the competitive inhibitors fumarate and malonate, K_i values were found to be 2.24 mM and 1.30 mM, respectively. Again, because assays were not done at infinite dye concentration, these values should be somewhat lower. Although the K_i values for fumarate agree quite well with values obtained for other systems, our K_i value for malonate was very much higher (8, 32).

The effects of other inhibitors are shown in Table 2. Since succinic dehydrogenase isolated from mammalian and yeast mitochondria (3, 8, 17, 32, 34) have been shown to contain non-heme iron, free sulfhydryl, and flavine, all of which are involved in the mechanism of action of the enzyme, it is not surprising that compounds reacting with these groups are inhibitors of *M. lysodeikticus* SDH. Incomplete reductions of the DCIP dye occur in the presence of both *o*-phenanthroline and sodium pyrophosphate, perhaps suggesting that these compounds have similar effects on the enzyme. Since *o*-phenanthroline is a well-known iron chelator, it seems likely that sodium pyrophosphate also reacts with iron atoms (8), although both compounds may not necessarily bind those iron atoms involved in the electron-transfer process. The sulfhydryl group(s) of the enzyme is inhibited by *p*-hydroxymercuribenzoate. Inhibition by Atabrine may be a consequence of flavine interaction, although purified glucose dehydrogenase which does not possess flavine is also inhibited by this compound (12). The similarity in structure between Atabrine and PMS may suggest that the inhibition is due to competition of these compounds for the same region on the enzyme-active site. Cyanide is not an inhibitor of the yeast or brain succinic dehydrogenase (7, 33), nor does it inhibit the SDH of *M. lysodeikticus* (Table 2).

Acceptable assays. The explanation for the incomplete dye reduction is not clear. Triton X-100, which is capable of rupturing lipid-lipid bonds and lipid-protein bonds (the latter irreversibly; see reference 11) may alter the environment of the enzyme active site. Similarly, diva-

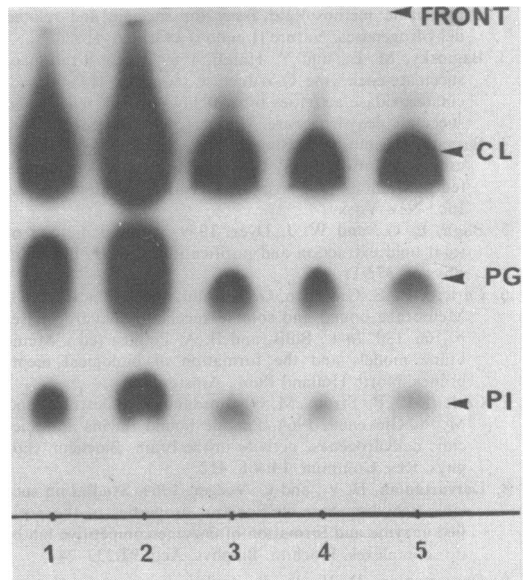


FIG. 6. Autoradiogram of ^{32}P -labeled phospholipids extracted from membranes and membrane fractions. Samples of total lipid extracts were spotted onto Whatman SG-81 silica gel-impregnated paper and developed in the CMDAW solvent. Phospholipid spots (Rhodamine 6G staining) were cut out from the paper, counted for radioactivity, and then reinserted back onto the chromatogram for autoradiography (see Materials and Methods). The phospholipids are indicated as follows: CL, cardiolipin; PG, phosphatidyl glycerol; PI, phosphatidyl inositol. The various lipid fractions are designated: 1, 7th membranes; 2, 7th membranes at twice the concentration of 1; 3, butanol-succinate extract; 4, butanol $130,000 \times g$ pellet and; 5, butanol $130,000 \times g$ supernatant.

lent cations can induce changes in micellar structure to produce an indirect influence on SDH activity (1). In *M. lysodeikticus*, these cations also have the capacity to aggregate membrane preparations (29). The incomplete reductions elicited by *o*-phenanthroline and sodium pyrophosphate may be due to a more direct effect at the active site of the enzyme.

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