RESPIRATION AND PROTEIN SYNTHESIS IN *ESCHERICHIA COLI* MEMBRANE-ENVELOPE FRAGMENTS

VI. Solubilization and Characterization of the Electron Transport Chain

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ABSTRACT

Membranes obtained from *Escherichia coli* have been solubilized with deoxycholate. The solubilized dehydrogenases and cytochromes are not sedimented at 105,000 *g*. These components readily penetrate the "included space" of Sepharose 4B (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) and polyacrylamide gels and have been fractionated on the basis of molecular size. Solubilization destroys nicotinamide adenine dinucleotide, reduced form (NADH) oxidase and D-lactate oxidase activities, but leaves an appreciable part of the original succinoxidase activity intact. Evidence for a succinate dehydrogenase-cytochrome b_1 complex is given. Menadione added to the solubilized preparation does not elicit NADH oxidase activity nor stimulate the existing succinoxidase activity, but does provoke an active D-lactate oxidase activity. This D-lactate oxidase activity, however, does not use cytochromes and is not sensitive to cyanide.

INTRODUCTION

Respiration can generate energy for active transport and biosynthesis. When energy-requiring processes occur at cell membrane sites where respiration-generated high-energy intermediates occur, the possibility exists that a direct utilization of these intermediates may take place. Examples of direct utilization of high-energy intermediates for a variety of work processes have been published (1–4). In order to continue our studies on the respiratory system of *Escherichia coli*, its structural arrangement in the membrane, and the possibility of its direct interaction with energy-requiring processes, we have tried to dissociate the active components from the membrane under controlled conditions. A straw-colored water-clear deoxycholate extract of the membranes, containing cytochromes and dehydrogenases for nicotinamide adenine dinucleotide, reduced form (NADH), D-lactate, and succinate, has been prepared. In this extract, however, only succinate dehydrogenase remained functionally linked to the cytochromes and oxygen as an oxidase unit. Evidence is presented for the existence of a succinate dehydrogenase-cytochrome complex.

METHODS

Preparation of Membrane-Envelope Fragments

E. coli W6 (ATCC No. 25377), a proline auxotroph, was grown and converted to spheroplasts as previously described (5). More than 90% of the sphero-

plasts were broken during harvesting and washing (6). This preparation was stored in liquid nitrogen. A combined particulate fraction T was obtained by treating the thawed suspension with a Branson sonifier (model S-75, Branson Instruments Co., Stamford, Conn.) for 15 sec at a power setting of 6 at 0°C and then centrifuging at 105,000 g for 60 min (7). About 35 ml of the suspension contained in a 50 ml beaker was sonicated, and the sonifier probe was moved around in the suspension during sonication. The supernatant fraction obtained from the centrifugation is called S_3. The fragments resulting from these procedures have been characterized with respect to respiratory activities, cytochromes, electron microscope appearance, involvement of nonheme iron in respiration, and general nature of the respiratory chain (5–9). The T fraction was stored in liquid nitrogen at a concentration of about 20 mg protein/ml in a solution of the following composition: 21.1 mM Na_2HPO_4, 11 mM KH_2PO_4, 25.6 mM NaCl, 0.39 mM Na_2SO_4, 0.7 mM $MgCl_2$, 25 mM Tris-HCl, and 4.5% (w/v) glycerol at pH 7.1. For pH studies, a GS_1 suspension (5) was prepared by removing particulates that sediment in 10 min at 3500 g from the broken spheroplast suspension. For the preparation of ^{55}Fe-labeled cells, 2 mCi of $^{55}FeCl_3$ (64 mCi/mg Fe, Amersham/Searle Corp., Arlington Heights, Ill.) was added to 70 ml of the growth medium (5).

Assay Methods

Most of the methods have been previously described (5, 9). NADH oxidase was determined both spectrophotometrically and polarographically, using the Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), succinate, malate, and isocitrate oxidase activities were determined polarographically. NADH dehydrogenase (NADH-ferricyanide reductase or NADH-dichlorophenol indophenol reductase), succinate dehydrogenase (succinate-dichlorophenol indophenol reductase), malate dehydrogenase (malate-dichlorophenol indophenol reductase and oxalacetate-NADH dehydrogenase), isocitrate dehydrogenase (isocitrate-nicotinamide adenine dinucleotide phosphate, oxidized form [NADP] reductase), and D-lactate dehydrogenase (D-lactate-dichlorophenol indophenol reductase) were determined spectrophotometrically with a Cary 16K automatic recording spectrophotometer (Cary Instruments, a Varian Subsidiary, Monrovia, Calif.). Cytochromes were assayed using an Aminco-Chance dual-wavelength split-beam recording spectrophotometer (American Instrument Co., Inc., Silver Spring, Md., reference 9).

^{55}Fe radioactivity was detected with a Beckman model LS-250 liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.) using 0.4% PPO (2,5-diphenyloxazole) in toluene. Nonheme iron was assayed using bathophenanthrolinedisulfonic acid, disodium salt (4,7-diphenyl-1,10-phenanthrolinedisulfonate; G. Frederick Smith Chemical Co., Columbus, Ohio, catalog item No. 286). The sample (0.3 ml) was mixed with 0.3 ml of 10% (w/v) trichloroacetic acid and 0.2 ml 10% $NH_2OH \cdot HCl$ and, after boiling for 20 min, was centrifuged in a clinical centrifuge. The supernatant fluid (0.6 ml) was mixed with 0.4 ml of 0.1% (w/v) disodium bathophenanthrolinedisulfonic acid and 0.15 ml of a 1:5 dilution of concentrated NH_4OH to bring the pH to about 7. Optical density at 535 nm was determined. For calibration, a standard curve was made using ferrous ammonium sulfate. The curve was linear to 5 μg Fe. Protein was determined by the method of Lowry et al. using bovine serum albumin as a standard (10). Small volumes of ethanolic solutions of quinones (10 μl/ml of incubation medium) were added to incubations where indicated. The concentrations of stock solutions were as follows: coenzyme-Q_6 (Sigma Chemical Co., St. Louis, Mo.) 10 mM, coenzyme-Q_{10} (Sigma Chemical Co.) 5 mM, menadione (from Dr. Roy Repaske) 50 mM, and benzoquinone (from Dr. Roy Repaske) 50 mM.

Dependence of Enzyme Activities on pH

The pH of the enzyme suspension (GS_1), cofactors as needed (5), and substrate, in 1 ml of 0.011 M histidine buffer containing 0.002 M magnesium sulfate was altered by small additions (less than 50 μl) of 0.2 N KOH, 1 N KOH, or 0.2 N H_2SO_4. For determination of pH, a Radiometer pH meter, type TTTIC (Radiometer, Copenhagen, Denmark) fitted with a microcombination glass electrode (Arthur H. Thomas Co., Philadelphia, Pa., No. 4858-L60) was used. For spectrophotometric analyses, the influence of pH on the color yield of chromophoric substance was detemined so that change of optical density could be expressed in terms of quantity of substrate consumed.

Preparation of Deoxycholate Extracts

A 0.1 vol of 1 M Tris-chloride pH 8.0 was added to T fraction followed by sufficient 10% sodium deoxycholate (DOC) to achieve the desired final DOC concentration. For all DOC extractions, the protein concentration was maintained at about 20 mg/ml. The preparation was used either directly or after centrifugation as indicated. Centrifugation at 105,000 g for 60 min yielded a yellow-colored but water-clear supernatant fraction and a pellet.

Chromatography on Sepharose 4B

An 80 × 5 cm column of Sepharose 4B (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) in 0.05 M

sodium phosphate buffer pH 7.1 containing 0.1% DOC was prepared. The sample (30 ml) was applied at the bottom of the column and the effluent was collected in 20 ml fractions from the top. The column was developed at 4°C with a 50 cm hydrostatic head, at a flow rate of 41 ml/hr. The sample was a supernatant fraction obtained by centrifuging a 0.3% DOC extract of *T* for 15 min at 20,000 *g*. Present in the 30 ml sample were: 330 mg protein, 2.64×10^6 cpm ^{55}Fe-labeled components, succinate dehydrogenase (capable of oxidizing 4.02 μmoles succinate/min), NADH dehydrogenase (capable of oxidizing 23.30 μmoles NADH/min with dichlorophenol indophenol as electron acceptor), D-lactate dehydrogenase (capable of oxidizing 14 μmoles D-lactate/min), and 600 μg nonheme iron. Also present were cytochromes, which, when assayed spectrophotometrically at 77°K by reduced vs. oxidized difference spectra, yielded the following number of optical density units: 21 for cytochrome $b_1(\gamma)$, 1.5 for cytochrome $b_1(\alpha)$, 0.75 for cytochrome $a_2(\gamma)$, 0.3 for cytochrome $a_1(\alpha)$, and 6.0 for the unidentified Soret II. The column was calibrated with blue dextran 2000 (Pharmacia Fine Chemicals Inc.) to determine void volume, radioactive glycine to determine salt elution volume, and standard molecular weight markers thyroglobulin (mol wt 330,000 and 660,000), fibrinogen (mol wt 340,000), and bovine serum albumin (mol wt 68,000). The elution volumes for these markers were found to lie along the curve published in the *Separation Bulletin* describing Sepharose 4B and Sepharose 6B (as supplied by Pharmacia Fine Chemicals Inc., Piscataway, N. J.) when the plot of K_{av} (which is equal to $V_e - V_o/V_t - V_o$; where V_e = elution volume, V_o = void volume, V_t = salt volume) vs. log of the molecular weight was used. For the range where K_{av} is a linear function of the log of the molecular weight (i.e., from mol wt of 10^4–10^6) the following equation was derived:

$$\text{mol wt} = 10^{p_1}$$

where

$$p_1 = \left[7.7 + \frac{V_o}{(V_t - V_o)\ 0.24}\right] - \left[\frac{V_e}{(V_t - V_o)\ 0.24}\right],$$

which for the column described here reduced to

$$\text{mol wt} = 10^{p_2}$$

where

$$p_2 = \left[9.87 - \frac{V_e}{274}\right].$$

The molecular weight can only be approximated by using Sepharose 4B because an error of 1 fraction (in this case 1% of total elution volume) leads to an error in estimated mol wt of ±9%.

Acrylamide Gel Electrophoresis

Gels were prepared with 4% cyanogum-41 (95% acrylamide and 5% bis-acrylamide; supplied by E-C Apparatus Corp., Philadelphia, Pa.) in 0.05 M phosphate buffer pH 7.1, 0.1 or 0.2% DOC, 0.1% ammonium persulfate (E-C Apparatus Corp.), and finally 0.1% (v/v) N,N,N^1,N^1-tetramethylethylenediamine (T MED, Eastman Organic Chemicals, Rochester, N. Y.). Gels were prepared about 18 hr before use and allowed to stand at room temperature with an overlay of H_2O. The gels were formed either in 6 × 72 mm columns in glass tubes or in a 3 or 6 mm thick 12 × 17 cm slab (model E-C 4% vertical gel cell supplied by E-C Apparatus Corp.). The electrophoresis buffer was 0.05 M phosphate, pH 7.1. For slabs, the upper compartment contained 0.2% DOC, the lower compartment no DOC, and the gel 0.2% DOC. During electrophoresis, the buffer circulated slowly between the two compartments so that the whole system approached a 0.1% DOC concentration during the electrophoresis period. For gel columns, the system contained 0.1% DOC in the gel and the buffer. Before applying the samples, gels were prerun for 30 min to establish equilibrium conditions and to remove any unpolymerized components. The prerun and the electrophoresis were performed at 10°C. Samples were mixed with a 50% vol of 60% glycerol containing 0.03% bromothymol blue for gel columns and 0.2% bromothymol blue for gel slabs. Gel columns were electrophoresed at a current of 12–15 mamp/gel and slabs at a voltage of 100 v. Electrophoresis was carried out for about 2 hr with the columns and about 3½ hr with slabs. The columns were electrophoresed in a model 3-1750 polyanalyst supplied by Buchler Instruments, Inc., Fort Lee, N. J., and the slabs were electrophoresed in the same container in which they were poured.

Location of Enzyme Activity in Gels and Estimation of Molecular Weights

NADH dehydrogenase was located by use of 0.8 mg/ml *p*-nitro-blue tetrazolium chloride (Nutritional Biochemicals Corporation, Cleveland, Ohio) in 0.1 M Tris, pH 7.0, plus 0.4 mM NADH. Succinate and D-lactate dehydrogenases were located with the same solution to which 0.14 mg/ml phenazine methosulfate (Calbiochem, Los Angeles, Calif.) was added and NADH was replaced by either 5 mM succinate or D-lactate. Gel columns were treated intact, but slabs were first split longitudinally with a metal wire held in a frame while the gel was held in place between two glass plates with abrased faces. Gels were incubated in the detection solution in the dark for about 45 min at 30°C. Excess reagent was removed by gentle agitation of the gels in the dark in 7% acetic acid (v/v). For molecular size calibration the follow-

ing standards were used: thyroglobulin (mol wt 330,000, 666,000, and 1,320,000), bovine serum albumin (mol wt 68,000 and 136,000), ovalbumin (dimer mol wt 86,000), pepsin (mol wt 35,000), and chymotrypsinogen (mol wt 26,000). The thyroglobulin was kindly supplied by Dr. H. Edelhoch and the other standards were high purity grades from various commercial sources. For molecular weight calibration (and determination), gel columns were formed in quartz tubes (Amersil, Inc., Hillside, N. J.) rather than glass, and the slabs were placed between two quartz plates for a 280 nm optical density scan in a Zeiss model PMQII scanning spectrophotometer (Carl Zeiss, Inc., New York). Staining with Coomassie blue is tedious because the DOC precipitates and the excess dye can not readily be removed to reveal stained bands.

RESULTS

Influence of pH on Respiratory Activities

The data in Fig. 1 show the pH response for several membrane-bound oxidases, two membrane-bound dehydrogenases (NADPH and succinate), and two soluble dehydrogenases (malate and isocitrate) (5). One of the soluble dehydrogenases (for malate) communicates with the electron transport chain via nicotinamide adenine dinucleotide (NAD) and the other (for isocitrate) uses NADP (5). There was a wide range of pH optima from a low of about 5.6 for NADPH oxidase to pH 9 for malate dehydrogenase measured by oxalacetate-NADH dehydrogenase activity. Malate-2,6-dichlorophenol indophenol (DCPIP) reductase activity had a pH optimum of 8.0. NADH-ferricyanide reductase was extremely active at all pH's from 6.0 to 9.0. Succinate dehydrogenase, isocitrate dehydrogenase, and NADH oxidase were optimally active at pH 7.0. Succinoxidase was optimally active from pH 7.5 to 8.5. Isocitrate oxidase activity was very low at all pH's, most likely because isocitrate dehydrogenase is minimally active at the pH where NADPH oxidase is maximal and vice versa. Malate oxidase showed its highest activity about pH 8.5.

D-Lactate has recently been implicated in a special role as a unique energy source in *E. coli* for amino acid and sugar transport (11, 12). The pH optimum for both D-lactate-DCPIP reductase and D-lactate oxidase activities was 7.0 (data not shown).

The influence of pH on the activities of succinate dehydrogenase and D-lactate dehydrogenase in a clear supernatant fraction obtained by centrifuging the DOC extract at 105,000 *g* was the same as shown in Fig. 1 for the membrane-bound activities. However, the released succinoxidase activity showed a pH optimum at 7 compared with about 8 for the membrane-bound system. As described below, greater activity of solubilized succinoxidase was obtained in a buffer containing 0.01 M Tris, 0.27 M $(NH_4)_2SO$, and 5 mM $MgSO_4$. In this more efficient system, the pH optimum was the same as found for the membrane-bound system (about pH 8).

Release of Dehydrogenases from the Membrane

When the membrane-envelope fragments were recentrifuged at 105,000 *g* to remove loosely held components (Fig. 2) and then resuspended with a Teflon ball, the succinate dehydrogenase remained tightly associated with the membranes as evidenced by the fact that a subsequent centrifugation at 105,000 *g* sedimented 95–100% of the original activity. However, this procedure released about 20% of the D-lactate dehydrogenase activity and about 15% of the NADH dehydrogenase activity. Moreover, there was a slight loss of total activity for D-lactate dehydrogenase and a great loss for NADH dehydrogenase. Deoxycholate released succinate dehydrogenase activity from the fragments with optimal recovery of the solubilized activity occurring at 0.3–0.4% concentration. A significant stimulation of the DOC-solubilized activity was obtained in the presence of 0.27 M $(NH_4)_2SO_4$ and 5 mM Mg^{++}. Similarly, DOC released D-lactate dehydrogenase activity from the fragments. This released activity was not inhibited by the higher range of DOC concentrations used, and an optimal concentration for DOC concentration is not indicated. Ammonium sulfate and Mg^{++} stimulated the solubilized activity leading to a greater than 100% recovery when compared with the intact fragments. Released NADH dehydrogenase activity was very sensitive to DOC so that the relation of release of activity to DOC concentration is not apparent. In order to see if more NADH dehydrogenase activity would survive the DOC treatment in the absence of the preliminary steps of recentrifugation at 105,000 *g* and resuspension with a Teflon ball, these steps were omitted. In the absence of DOC, a considerable background of soluble NADH dehydrogenase was present. The data indicate that 0.4% DOC may be the best concentration for releasing bound

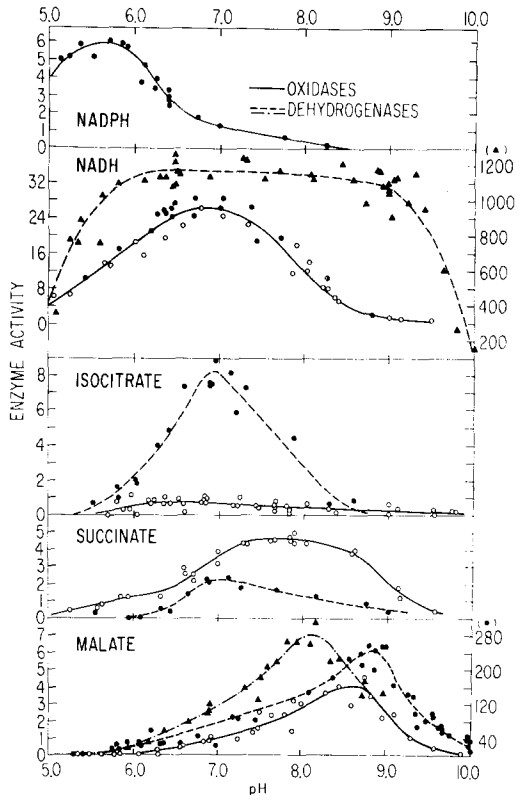

FIGURE 1 See table below.

Substrate	Conc	*GS*	Cofactor mixture	Key	Method
	M	*mg protein*			
NADPH	4×10^{-4}	0.2	−	●——● Oxidase	Spectrophotometric
NADH	4×10^{-4}	0.2	−	●——● Oxidase	Spectrophotometric
	1×10^{-3}	0.4	−	○——○ Oxidase	Oxygen electrode
	1.6×10^{-3}	0.01	−	▲- - - -▲ Ferricyanide reductase	Spectrophotometric
Isocitrate	5×10^{-3}	0.2	+	●- - - -● NADP reductase	Spectrophotometric
	5×10^{-3}		+	○——○ Oxidase	Oxygen electrode
Succinate	5×10^{-3}	0.2	−	●- - - -● 2,6-Dichlorophenol inophenol reductase	Spectrophotometric
	5×10^{-3}	1.0	−	○——○ Oxidase	Oxygen electrode
Malate	5×10^{-3}	0.2	+	▲—·—·—▲ 2,6-Dichlorophenol indophenol reductase	Spectrophotometric
	5×10^{-3}	0.5	+	○——○ Oxidase	Oxygen electrode
Oxalacetate	5×10^{-3}	0.2	+	●- - - -● NADH dehydrogenase	Spectrophotometric

Volume of reaction mixture was 1 ml. Except in the case of oxidase 12 mM HCN was present. Cofactor mixture ("+" if present) provided 10 mM adenosine 5′-triphosphate, 9 mM NAD, 4 mM NADP, and 20 mM nicotinamide. Units for enzyme activity are μmoles of substrate changed/10 min per 10 mg protein.

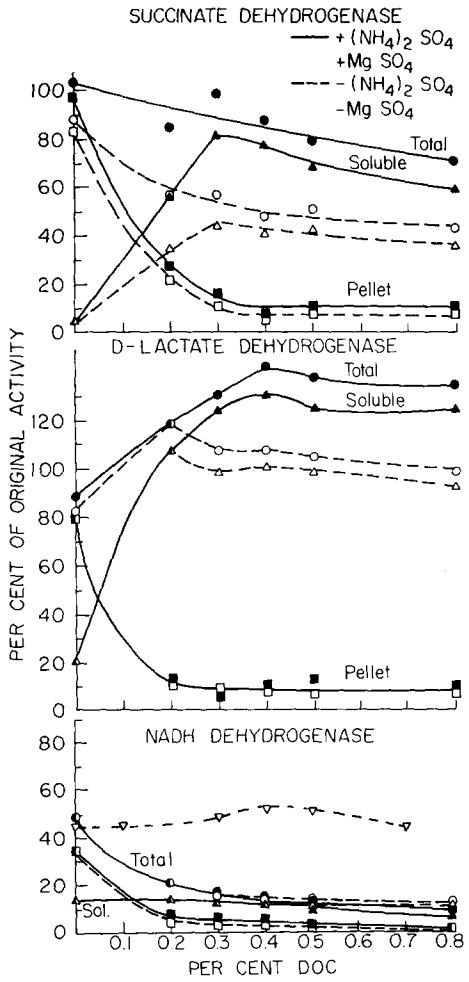

FIGURE 2 Release of dehydrogenases by DOC. *T* fraction (all particulate components of the cell) was centrifuged at 105,000 *g* for 60 min and the pellet was resuspended to its original volume with 0.01 M Tris buffer pH 8. The protein concentration was 15.2 mg/ml. 0.05 ml of 1 M Tris, pH 8, was added to 0.5 ml portions of particle suspension followed by appropriate volumes of 10% DOC to attain desired final DOC concentrations. After sitting 15 min at room temperature, the suspensions were centrifuged 1 hr at 105,000 *g*. The supernatant solutions (5- or 10-μl samples) were analyzed directly in 0.01 M Tris buffer, pH 7.3, $\pm$0.27 M $(NH_4)_2SO_4$, and $\pm$5 mM $MgSO_4$. The pellets were resuspended in 0.01 M Tris buffer, pH 7.3, and then assayed in the same buffer (10- or 20-μl samples) $\pm$0.27 M $(NH_4)_2SO_4$ and $\pm$5 mM $MgSO_4$. Solid lines and filled symbols show results with the added salts and dashed lines with open symbols, without. For the experiments with NADH dehydrogenase, the top dashed line shows the results for nonsedimentable activity when the first centrifugation of *T* fraction was omitted. In this case, no ammonium sulfate or magnesium sulfate was present during the assay. Original or 100% starting activities for the resuspended fragments were: 38 nmoles/min per mg protein for succinate dehydrogenase, 510 nmoles/min per mg protein for NADH dehydrogenase, and 53 nmoles/min per mg protein for D-lactate dehydrogenase. The following symbol designations were used: particulate activity (□), soluble activity (△), and total activity (○).

NADH dehydrogenase from the fragments. Ammonium sulfate and Mg^{++} stimulated the NADH dehydrogenase activity of both the membrane fragments and the DOC extracts by about 50%.

Release of Cytochromes and Protein from the Membrane

Fig. 3 shows the release of cytochromes and protein as a function of DOC concentration. The 100% or starting levels for cytochromes in terms of optical density units were 0.215 for cytochrome $b_1(\gamma)$, 0.021 for cytochrome $b_1(\alpha)$, 0.010 for cytochrome $a_2(\alpha)$, and 0.0032 for cytochrome $a_1(\alpha)$. The protein concentration was 20 mg/ml. Because of the extremely low optical absorbance of cytochrome $a_1(\alpha)$, its quantitation is only approximate. Per cent recoveries for pellets plus DOC extracts, averaged over the whole DOC concentration range ($\pm$error of the mean) were 85 $\pm$ 3 for cytochrome $b_1(\gamma)$, 88 $\pm$ 2 for cytochrome $b_1(\alpha)$, 99 $\pm$ 2 for cytochrome $a_2(\alpha)$, 84 $\pm$ 7 for cytochrome $a_1(\alpha)$, and 99 $\pm$ 3 for protein. The release of cytochrome b_1, determined by both its α and γ absorptions was almost a linear function of DOC concentration. At the lower concentrations of DOC, cytochrome a_2 was more resistant to release than cytochrome b_1. Cytochrome a_1 seems to be more easily released than cytochrome a_2.

Release of Oxidase Activities

E. coli membranes have very high NADH oxidase activity, and although several-fold lower activities are evident for D-lactate and succinate oxidases, these substrates are also readily consumed. In the extracts solubilized with DOC, both NADH dehydrogenase activity and D-lactate dehydrogenase activity are about two to three times that found for succinate dehydrogenase. However, only succinoxidase activity survives in the DOC extracts; NADH oxidase activity is absent and only a few per cent of the original D-lactate oxidase activity is apparent. Fig. 4 shows the release of

succinoxidase activity. When fresh fragments (not resedimented and rehomogenized) were used, (having an initial oxidase activity of 90 nmoles/min per mg protein), 90–100% of the original oxidase activity was recovered in the 0.5% DOC extract. Succinate within 8 min of its addition reduced 60% of the cytochrome b_1 and 80% of the cytochrome a_2 in the extract. No reduction of the cytochromes by NADH or D-lactate was evident even 35 min after additions of either substrate. The succinoxidase activity of the DOC extract was inhibited 80–90% by 4 mM HCN. The low level of residual D-lactate oxidase activity was not inhibited by 12 mM HCN (Table I). Attempts

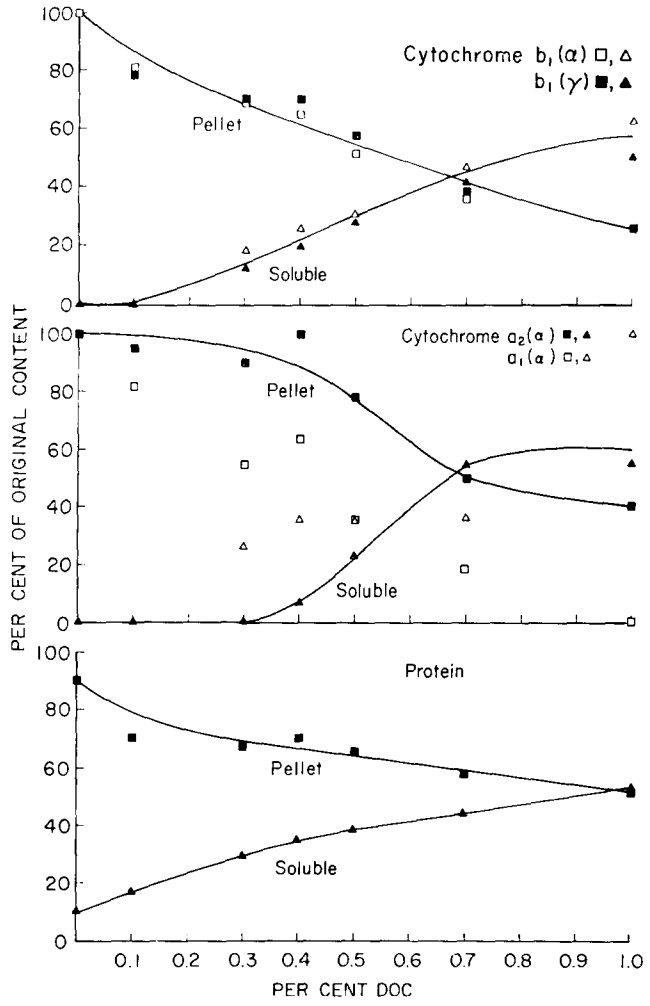

FIGURE 3 Release of cytochromes and protein by DOC. In this experiment the preliminary centrifugation of *T* fraction was omitted. Protein concentration of the original *T* fraction was 20 mg/ml. Cytochrome assays were performed as described in the methods. See legend to Fig. 2 for other experimental details. The total optical density for each of the cytochrome absorption bands was 0.215 for cytochrome $b_1(\gamma)$, 0.021 for cytochrome $b_1(\alpha)$, 0.010 for cytochrome $a_2(\alpha)$, and 0.0032 for cytochrome $a_1(\alpha)$. Triangle symbols were used to denote contents for the 105,000 *g* supernatant fractions and square symbols for the contents of the centrifugal pellets. The symbols were filled or unfilled as designated in the figure legends. The points shown for cytochrome $a_1(\alpha)$ represent approximations, because of its extremely low optical absorbance. These points were not connected by lines. Averaged over the whole DOC concentration range, per cent recoveries ± standard error were 85 ± 3 for cytochrome $b_1(\gamma)$, 88 ± 2 for cytochrome $b_1(\alpha)$, 99 ± 2 for cytochrome $a_2(\alpha)$, 84 ± 7 for cytochrome $a_1(\alpha)$, and 99 ± 3 for protein.

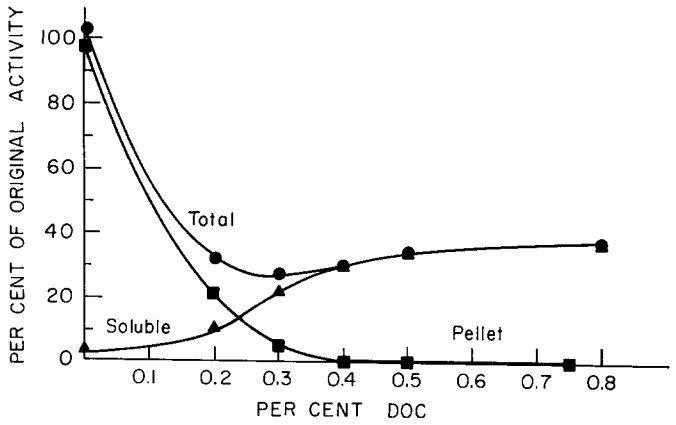

FIGURE 4 Release of succinoxidase by DOC. See legend to Fig. 2 for experimental details. Oxidase activity was measured with a Clark oxygen electrode in a 1 ml vol of 0.01 M Tris buffer pH 7.3 plus 0.27 M $(NH_4)_2SO_4$ and 5 mM $MgSO_4$. The oxidase activity of the resuspended fragments was 59 nmoles/min per mg protein. Sample portions were 50 μl. Protein present per assay varied from 0.23 mg using the 0.2% DOC extract to 0.33 mg using the 0.8% DOC extract and from 0.76 mg using the resuspended particles from the 0% DOC treatment to 0.18 mg using the resuspended particles from the 0.8% DOC treatment.

TABLE I

Effects of Vitamin K_3 and HCN on Membrane-Bound and DOC-Solubilized D-Lactate Oxidase Activity

Enzyme source	Additions	Oxidase activity (nmoles/min per 50 μl)
T fraction	None	68
	First +12 mM HCN	9.8
	Then +200 μM K_3	89
0.5% DOC extract	None	8
	First +12 mM HCN	8
	Then +200 μM K_3	71
0.5% DOC extract	None	9
	First 200 μM K_3	84
	Then 12 mM HCN	68

The DOC extract was first centrifuged at 105,000 *g* for 60 min and the clear supernatant fraction was used.

Oxidase activity was measured at 23°C in a 1 ml vol using 0.01 M Tris, 5 mM $MgSO_4$, and 0.27 M $(NH_4)_2SO_4$ at pH 7.8. Protein concentration was 0.9 mg/ml for *T* fraction and 0.24 mg/ml for the DOC extract.

were made to stimulate the solubilized activities by addition of quinones. None of the three oxidases were noticeably stimulated by ubiquinones Q_6 or Q_{10} or benzoquinone up to 30 μM concentration (data not shown). The same was true for higher concentrations of Q_6 (100 μM) and menadione (100 μM) for succinate and NADH oxidases and for Q_6 (100 μM) for D-lactate oxidase. However, menadione (vitamin K_3) at 50–200 μM concentrations was able to dramatically stimulate D-lactate oxidase activity. As a matter of fact, the oxidase activity observed in the presence of 200 μM K_3 was higher than the activity of the original unsupplemented membrane-envelope fragments (Table I). This stimulated D-lactate oxidase activity was only slightly sensitive to 12 mM HCN (about 19% inhibition) whereas the original fragments were inhibited by about 90% by 12 mM HCN. In the presence of 12 mM HCN, vitamin K_3 could also invoke strong stimulation of D-lactate oxidase activity with the original fragments. The rapid uptake of oxygen observed in the presence of D-lactate and vitamin K_3 in the DOC extracts was not accompanied by cytochrome reduction.

Effects of Ammonium Sulfate (AMS) and Magnesium Sulfate on Dehydrogenase Activities in DOC Extracts

Dehydrogenase activities for the substrates, succinate, D-lactate, and NADH could be precipitated out of a 0.4% DOC extract by the addition of AMS. There was no evidence of enrichment of any of the dehydrogenases relative to protein in these precipitates. However, solutions of precipitates obtained from greater than 90% saturation with

AMS, yielded more than 100% recovery for succinate and D-lactate dehydrogenases. The simple addition of low levels of AMS to the DOC extract stimulated all dehydrogenase activities. For example the succinate dehydrogenase activity of a 0.4% DOC extract was stimulated 60% by 0.27 M AMS (5% of saturation at 0°C), 47% by 0.54 M AMS, and 7% by 1.08 M AMS. $MgSO_4$ also caused a stimulation of activity to the extent of 36% at 0.005 M, 43% at 0.01 M, and 61% at 0.02 M. The presence of AMS at 0.27 M and $MgSO_4$ at 0.005 M caused a stimulation of 63%.

Fractionation of DOC Extracts

Fig. 5 shows the chromatography of a DOC extract obtained from ^{55}Fe-labeled cells on Sepharose 4B. The extract was first centrifuged at 20,000 *g* for 15 min and was opalescent in appearance. The column void fractions were also opalescent, but all other fractions were transparent. Radioactive iron-labeled material occurred in the void volume and in a broad peak centered at an elution volume corresponding to a molecular weight of about 350,000. Succinate dehydrogenase appeared heterogeneous and the presence of four species with molecular weights from about 60,000 to about 400,000 was indicated. D-Lactate dehydrogenase was extremely active and heterogeneity was indicated in the molecular weight range of 50,000–100,000. NADH dehydrogenase was present in one or two components at about mol wt 60,000. Nonheme iron analyses of the column effluent were somewhat erratic, but a small amount

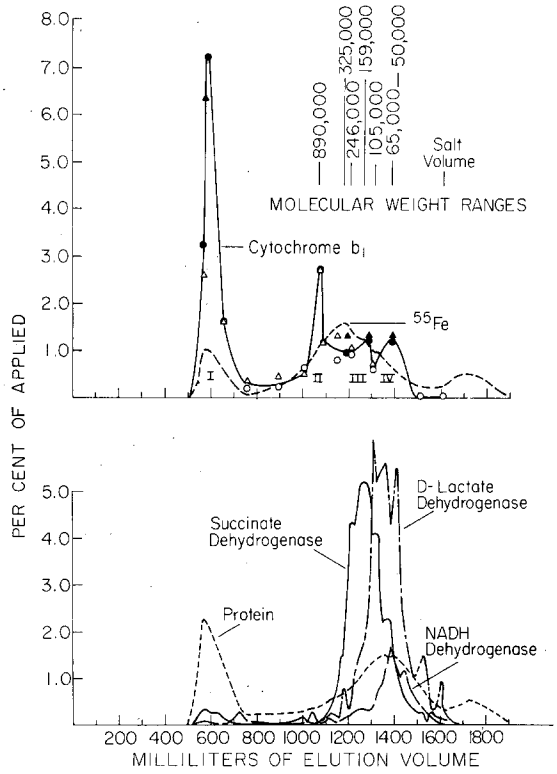

FIGURE 5 Sepharose 4B chromatography of a 20,000 *g* (15 min) supernatant fraction from a 0.3% DOC extract of *T* fraction. The ordinate is per cent of each applied component depicted in the figure. A full description of the experimental details is given in Chromatography on Sepharose 4B in Methods. The Roman numerals in the top figure represent pooled fractions for cytochrome analyses shown in Figs. 6 and 7. Symbol designations shown in the top figure are as follows: ●, cytochrome $b_1(\gamma)$ (77°K spectrum); ○, cytochrome $b_1(\gamma)$ (room temperature spectrum); ▲, cytochrome $b_1(\alpha)$ (77°K spectrum); △, cytochrome $b_1(\alpha)$ (room temperature spectrum). Fraction sizes were 10 ml and the curves were drawn through points taken for every other fraction. The points are not shown in order to simplify the figure.

was present in all fractions with the major amounts being found in both the void and salt elution volumes. The salt elution volume of the column occurred between 1600 and 1900 ml. Of the applied material, the eluted components accounted for 100% of the protein, cytochrome b_1, succinate dehydrogenase, and D-lactate dehydrogenase, 115% of the nonheme iron, 80% of the ^{55}Fe, and 29% of the NADH dehydrogenase.

At least four separate chromatographic entities containing cytochrome b_1 are indicated by the effluent pattern shown in Fig. 5. Each of these materials was examined by difference spectroscopy both at room temperature and at 77°K. The original DOC extract showed characteristic absorption peaks for cytochrome $b_1(\gamma)$ at 428 nm, Soret II (not yet identified) at about 438 nm, cytochrome $b_1(\alpha)$ at 557 nm, cytochrome $a_1(\alpha)$ at 591 nm, and cytochrome $a_2(\alpha)$ at 628 nm (Fig. 6, reference 9). The absorption for cytochrome $b_1(\beta)$ at 528 nm is partially obscured by an optical artifact of the gratings. The chromatographed fractions showed spectra altered with respect to the relative amounts of cytochromes present. The void volume fraction (I) showed an enrichment for the component responsible for the Soret II absorption relative to the cytochrome b_1 and the absorption bands for cytochromes a_1 and a_2 stand out more clearly. The most striking aspect of the absorption spectra of the pooled fractions II, III, and IV is that they all present absorption bands characteristic of only cytochrome b_1 (Fig. 7). The absence of absorptions for Soret II and cytochromes a_1 and a_2 is quite evident. It should also be noticed that, although cytochromes occurred in the same fractions with radioactive iron (Fig. 5), the distribution

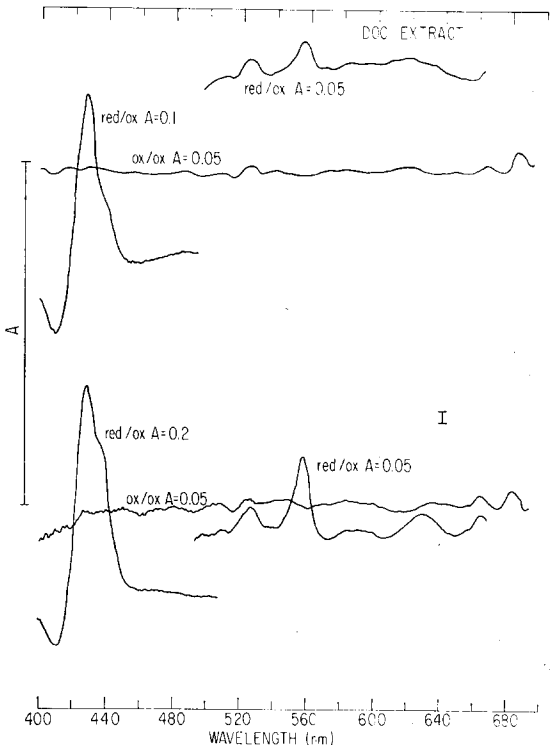

FIGURE 6 Difference spectra at 77°K in 50% glycerol for original DOC extract applied to Sepharose 4B column in experiment shown in Fig. 5 (top) for pooled fraction I from that experiment (bottom). Sensitivity is indicated for each trace by the value assigned to A in optical density units. The redox state of the material in the sample and reference cuvettes is shown by the expression (*red* or *ox*)/(*red* or *ox*) where the numerator (*red* = reduced with $Na_2S_2O_4$, *ox* = oxidized with air) represents the sample cuvette and the denominator, the reference cuvette. Absorption peaks are identified as follows: cytochrome $b_1(\gamma)$ at 427 nm, Soret II (unidentified) at about 437 nm, cytochrome $b_1(\beta)$ at 526 nm, cytochrome $b_1(\alpha)$ at 558 nm, cytochrome $a_1(\alpha)$ at 588 nm, and cytochrome $a_2(\alpha)$ with a peak at 626 nm and a trough at 647 nm (9).

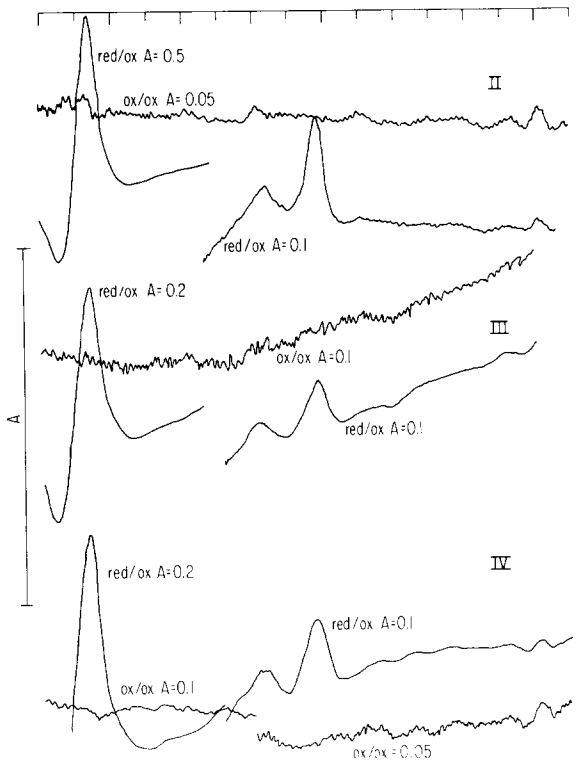

FIGURE 7 Difference spectra at 77°K for pooled column fractions II (top), III (middle), and IV (bottom) from the Sepharose 4B chromatography experiment shown in Fig. 6. See Fig. 6 for other details.

of cytochromes did not coincide with the distribution of iron in the chromatographed species. Cytochrome b_1 showed peaks of concentration in eluted volumes of 1070, 1290, and 1400 ml with the first being the most prominent. Radioactive iron, on the other hand, showed a diffuse distribution with highest concentration at an elution volume of about 1200 ml.

Fig. 8 shows the results of disc gel electrophoresis on 4% cyanogum impregnated with 0.1% DOC. The location of dehydrogenase activity was established with nitro-blue tetrazolium and specific substrate. Succinate dehydrogenase activity of the DOC-treated membranes is found at the top of the gel and in several bands that move through the gel. NADH dehydrogenase activity of the DOC-treated membranes is found in a single band, and D-lactate dehydrogenase activity appears as a fast-moving major band and two minor bands that are slightly faster in migration. The pellets obtained at 20,000 and 105,000 *g* from DOC-treated *T* fraction show no enzyme bands. No enzyme bands are found for D-lactate or succinate dehydrogenases when the whole membranes are electrophoresed in the absence of DOC. Enzyme activity is found in both the 20,000 and 105,000 *g* supernatant fractions of the DOC extracts. For succinate dehydrogenase and D-lactate dehydrogenase, little or no activity was seen in the soluble fraction from the original broken spheroplast preparation (S_3). NADH dehydrogenase activity is found in two forms in the soluble fraction. The slower moving NADH dehydrogenase is characteristic of the DOC extract of membranes, whereas an additional faster moving band is found predominantly in the soluble (S_3) fraction. A gel slab was used for electrophoresis of a DOC extract made from ^{55}Fe-labeled cell fragments. The gel was split and one-half was exposed to X-ray film while the other half was stained to locate succinate dehydrogenase activity. Fig. 9 shows at least three distinct bands containing ^{55}Fe. One of these was in the same location as one of the succinate dehydrogenase bands. Only two of the usual two or four dehydrogenase bands are apparent in this experiment. The electrophoretic mobilities on DOC-acrylamide gels of the proteins pepsin, bovine serum albumin (monomer), ovalbumin

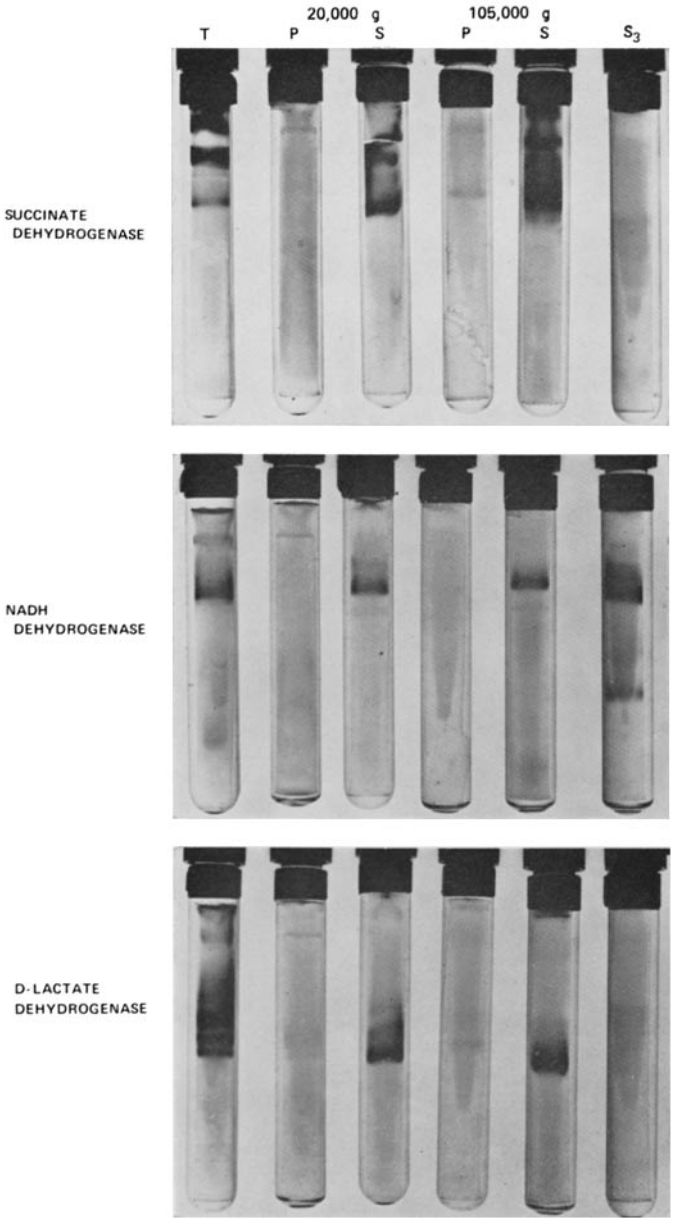

FIGURE 8 Electrophoresis of *E. coli* dehydrogenases in 0.1% DOC in polyacrylamide gels. The gels shown contained the following samples from left to right: *T*, *T* fraction in 0.3% DOC; 20,000 *g P*, the pellet obtained by 15 min centrifugation of DOC-treated *T*; 20,000 *g S*, the supernatant fraction obtained from DOC-treated *T*; 105,000 *g P*, the pellet obtained from 20,000 *g S* by centrifuging 60 min at 105,000 *g*; 105,000 *g S*, the supernatant fraction obtained above; S_3, the supernatant fraction obtained from the original broken spheroplast preparation (not treated with DOC) by centrifuging 60 min at 105,000 *g*. Enzyme location was revealed by incubation with the indicated substrate and nitro-blue tetrazolium.

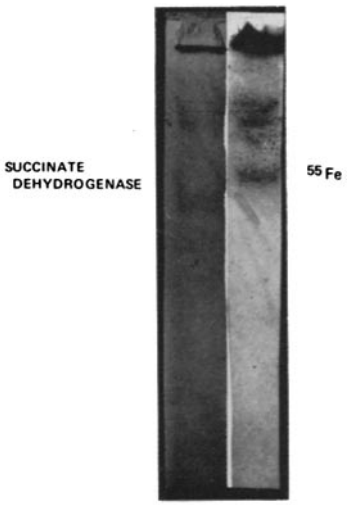

FIGURE 9 A 0.3% DOC extract of ^{55}Fe-labeled *T* fraction was electrophoresed and the gel split longitudinally. One-half was stained to localize succinate dehydrogenase activity and the other half wrapped in Saran Wrap and exposed to X-ray film.

(dimer), bovine serum albumin (dimer), and thyroglobulin (monomer) are linearly related to the logarithms of their molecular weights. The location of the ^{55}Fe-succinate dehydrogenase band in relation to these standards corresponds to a molecular weight of about 500,000.

DISCUSSION

The DOC-extracted succinoxidase described in the present work appears to be unique in that the activity did not sediment at 105,000 *g* for 60 min either in the presence of DOC or after extensive dialysis. Present in the same optically clear solution were active dehydrogenases for NADH and D-lactate, but these no longer were functionally connected to the respiratory chain. There are several possible ways for succinoxidase activity to occur in these extracts: (*a*) separate molecules of each of the respiratory carriers and succinate dehydrogenase interact upon random fortuitous collisions. (*b*) Succinate dehydrogenase but not NADH or D-lactate dehydrogenases is structurally associated with cytochrome b_1. This complex reacts with a cytochrome oxidase complex either by random collision or by reforming a membrane upon dilution with the assay buffer solution. (*c*) A structural unit consisting of succinate dehydrogenase connected to the entire respiratory chain is present. The first explanation seems most unlikely because both NADH dehydrogenase and D-lactate dehydrogenase are also present and more active than succinate dehydrogenase, but oxidase activities for the first two substrates are largely or entirely absent. The second and third explanations are suggested by the relatively high efficiency observed for succinoxidase in the extracts compared with the activity in the original membranes, by the polyacrylamide gel electrophoresis showing the migration of succinate dehydrogenase and ^{55}Fe (identified as cytochrome b_1 in the experiment describing chromatography on Sepharose 4B) and by the high apparent molecular weight for the succinate dehydrogenase activity indicated by its behavior on Sepharose 4B and gel electrophoresis. The finding that upon chromatography on Sepharose 4B, activity occurs in a region containing cytochrome b_1 but no observable cytochrome a_1 or a_2 indicates that explanation *b* is the most likely.

The electron transport chain of mammalian mitochondria has been dissected into individual dehydrogenases, cytochromes, and complexes of respiratory carriers (see references 13 and 14 for reviews). Sedimentable, reconstituted succinoxidase systems have been described by several groups (14–16).

The dissection and reconstruction of bacterial respiratory chains have not been as systematically pursued or reviewed as that of mammalian mitochrondria but many isolated findings have been reported. In order to place our findings in perspective a brief review of some of the work follows.

Salton et al. found that membranes of *Micrococcus lysodeikticus* retained their cytochromes and succinate dehydrogenase even after treatment with 1% DOC (17). The same was found for *Sarcina lutea* and *Bacillus subtilis* membranes. In later work (18), however, part of the succinate dehydrogenase was solubilized by treatment with DOC or by butanol extraction. NADH dehydrogenase could be removed from the membrane by simply washing with a dilute Tris-ethylenediaminetetraacetate (EDTA) buffer (19). Gutman et al. reported that lyophilization of *E. coli* membranes liberated a soluble NADH dehydrogenase (20). Both that group and we (5) have observed that there is an extremely active soluble NADH dehydrogenase in *E. coli*. Kashket and Brodie reported the solubilization of NADH dehydrogenase from *E. coli* membranes by use of DOC (21). The serious possibility that a significant portion of the "liberated" NADH dehydrogenase was due to adsorbed or occluded

soluble enzyme has not been ruled out in either case. The release of a soluble, masked NADH oxidase (that could be unmasked by divalent cations) by treatment of *B. megaterium* with DOC has been reported (22, 23). The participation of cytochromes in this activity was not demonstrated. We have found that divalent cations do not unmask NADH oxidase activity in DOC extracts from *E. coli* (unpublished observations). DOC plus ammonium sulfate released two NADH dehydrogenases from *E. coli* membranes while destroying over 90% of the NADH oxidase activity (24, 25). Some soluble NADH oxidase activity was observed but it was not inhibited by cyanide and the involvement of cytochromes was not shown.

Cytochrome b_1 released from *E. coli* membranes by ultrasonic irradiation has been crystallized and extensively studied (26). Its molecular weight was about 65,000. A soluble functional formate dehydrogenase-cytochrome b_1 complex has been released from *E. coli* by use of DOC and ammonium sulfate (27). No NADH oxidase or succinoxidase activity survived the treatment. Another formate dehydrogenase-cytochrome b_1 complex was released from *E. coli* by crude snake venom, but in this case the cytochrome could not be reduced by formate (28, 29). Treatment of membrane fragments of *Azotobacter vinelandii* with DOC and KCl led to the isolation of red and green membrane fragments (30) similar to those previously described for a mitochondrial system (31). The red particles possessed succinoxidase activity whereas the green particles, although containing cytochromes, were inactive. Pappenheimer et al. found a nonsedimentable succinoxidase activity after prolonged (30 min) sonication of *Corynebacterium diptheriae*, particularly from a strain with a deficient terminal oxidase system and lacking cytochrome *c* (32). The oxidase activity was not sensitive to cyanide and carbon monoxide (although the normal chain is), and the authors suggest that the oxidative activity was dependent on the autooxidation of cytochrome *b*-558.

Baillie et al. have recently described a soluble respiratory complex obtained from *E. coli* by use of sodium cholate and ammonium sulfate (33). Although our work parallels theirs in some respects, there are numerous significant differences. Analytical data and enzymatic data in their work were obtained on two different kinds of preparations and therefore the analyses given may not truly represent the composition of their respiratory complex. They reported that no cytochrome b_1 was released by cholate alone whereas in our work deoxycholate alone readily released cytochrome b_1. Their membrane preparation was reported to have cytochrome *o* but no cytochromes a_1 and a_2. We have observed (9) that optical absorptions attributed to cytochrome *o* could be largely due to an optical artifact and that cytochromes a_1 and a_2 are present and functional. They report a molecular weight of 2×10^6 for their soluble respiratory complex; we find a molecular weight of about 0.5×10^6 for our indicated soluble succinate dehydrogenase–cytochrome unit. Their soluble preparation possessed no NADH oxidase or succinoxidase activities unless quinones were added. Our soluble preparation possessed succinoxidase but no NADH oxidase activity without added quinones and the addition of quinones did not help either activity. They did not analyze for D-lactate oxidase activity in their preparations. We found that menadione gave a very great stimulation to D-lactate oxidase, but by a route not involving the cytochromes. In our work we recovered from 30 to 100% of the succinoxidase activity originally present in the membranes. (The higher recoveries were obtained when unwashed envelope fragments were used.) In the work of Baillie et al. the quinone-stimulated succinoxidase represents 425% recovery compared with the activity originally present in the membranes.[1] The cytochrome b_1 of their soluble preparation became reduced even when there was no oxidase activity, whereas in our preparation cytochrome b_1 was reduced only by the naturally occurring (unsupplemented) succinoxidase system. The conclusion, therefore, that the quinone-stimulated succinoxidase of Baillie et al. represents the same phenomenon as the succinoxidase activity of the particles is not definitely established at this time. In our studies, NADH did not reduce the cytochromes nor did D-lactate even in the presence of menadione which induced a very active D-lactate oxidase activity.

[1] That is, 1 mg of their original small respiratory particles containing 0.03 units of succinate dehydrogenase activity, 0.161 nmoles cytochrome b_1 (reference 33, Table I), and 0.009 units of succinoxidase activity (reference 24, Table I) yielded 0.44 mg of soluble respiratory complex fraction which contained 0.067 nmoles cytochrome b_1 (reference 33, Table I) and 0.553 units of succinoxidase activity/nmole of cytochrome b_1 or a total of 34.2 units (reference 33, Table VI).

The solubilization studies reported here reveal some striking differences in the mode of coupling of dehydrogenases to the cytochrome chain and to other electron acceptors. In the solubilized preparation, succinate dehydrogenase remained functionally and probably physically associated with cytochrome b_1 whereas NADH dehydrogenase and D-lactate dehydrogenase were removed. Upon addition of menadione, electrons from D-lactate were readily transferred to the quinone but electrons from NADH or succinate were not. This apparently unique aspect of D-lactate dehydrogenase may have some bearing on the findings of Kaback et al. that although electrons from NADH, succinate, and D-lactate can readily be inserted into the electron transport chain, only D-lactate can markedly stimulate active transport (11, 12, 34). Kaback and Barnes have interpreted this to mean that all of the various active transport carriers are also electron transport components of individual respiratory chains operating between D-lactate dehydrogenase and cytochrome b_1 (35).

In order to achieve active transport in the systems studied by Kaback and Barnes, at least two requirements must be met: (*a*) energy must be available and (*b*) the active transport carrier must be reduced. Succinate and NADH can very efficiently satisfy requirement *a* but perhaps not *b*. If D-lactate was acting in the same manner as we find here by being able to reduce efficiently a particular electron acceptor outside of the electron transport chain, (whereas NADH and succinate cannot) it could explain the observations of Kaback et al. This would also explain why the external electron pump of ascorbate + phenazine methosulfate is able to support active transport efficiently by supplying electrons for both requirements (36). The observation that D-lactate oxidation is much more sensitive to sulfhydryl reactants (34, 35) than is NADH oxidation has been interpreted by Kaback and Barnes to mean that it is the sulfhydryl group of the active transport protein that is involved. The inhibition of D-lactate oxidation by sulfhydryl reactants, however, could reflect some other unique aspect of coupling of D-lactate to the electron transport chain perhaps involving a nonheme iron protein (7).

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