Biochimica et Biophysica Acta, 357 (1974) 215–230
© Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

BBA 46768

FRACTIONATION OF THE ELECTRON-TRANSPORT CHAIN OF *ESCHERICHIA COLI*

RICHARD W. HENDLER and AMELIA H. BURGESS

Section on Cellular Physiology, Laboratory of Biochemistry, National Heart and Lung Institute, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)

(Received January 21st, 1974)

SUMMARY

This paper describes the resolution of the electron-transport chain of *Escherichia coli* into fractions containing dehydrogenases and/or cytochromes. The part of the chain including dehydrogenases and cytochrome b_1 can be solubilized with 0.4 % deoxycholate. This extract was fractionated by chromatography on hydroxyapatite followed by chromatography on DEAE-cellulose. D-Lactate dehydrogenase and NADH dehydrogenase were isolated virtually free of cytochromes whereas succinate dehydrogenase was isolated in a complex with cytochrome b_1. The complex exhibited succinoxidase activity. NADH dehydrogenase was found in two separable molecular species both of which were present in membranes and cytoplasm of *E. coli*. Cytochrome b_1 was present in a fraction free of dehydrogenases and other cytochromes. A 1 % deoxycholate extract, after chromatography on DEAE-cellulose, yielded fractions containing cytochromes a_1 and d in addition to cytochrome b_1.

INTRODUCTION

Remarkable success has been obtained in the fractionation of the respiratory chain of the mitochondrial inner membrane and in its reconstitution from individual components and complexes of components (for references see refs 1 and 2). Although individual dehydrogenases and cytochromes have been isolated from a variety of bacteria, a systematic effort to fractionate the entire respiratory chain of a single bacterial species has not been reported (for references, see refs 1, 3 and 4). In a continuation of our earlier studies on the characterization and solubilization of the electron-transport chain of *Escherichia coli* W-6 [1, 5–8], we report here procedures for separating the chain into fractions containing NADH dehydrogenase, D-lactate dehydrogenase, succinate dehydrogenase associated with nonheme iron protein and cytochrome b_1, cytochrome b_1, and cytochrome b_1 plus cytochromes a and d*. All of these components are membrane-bound in *E. coli* except for NADH dehydrogenase

* Bacterial cytochrome d is a newer designation replacing the earlier designation of cytochrome a_2 (see ref. 4).

which occurs mostly in soluble form [1]. We have found that the soluble and bound forms of NADH dehydrogenase are the same.

METHODS

General

The total particulate fraction, "T", was prepared from penicillin spheroplasts of *E. coli* W6 (ATCCNo. 25377), a proline auxotroph, and the deoxycholate extract made as previously described [1]. In brief, T is the centrifugal pellet obtained from broken spheroplasts by centrifugation at 105 000 × *g* for 60 min. It consists of about 16 % ribosomes and 84 % cell envelopes on the basis of protein content. The 0.4 % deoxycholate extract was prepared by diluting a suspension of T with a 1/10th vol. of 1 M Tris (pH 8.0), adding 10 % (w/v) deoxycholate in the amount of 1/25th the combined volume of T plus Tris and centrifuging at 105 000 × *g* for 60 min. The supernatant fluid is the deoxycholate extract, and the pellet is the deoxycholate pellet. Another supernatant fraction referred to, "S_3", is the aqueous soluble fraction obtained in the preparation of T.

Column chromatography

All columns were prepared and used at 4 °C. Hydroxyapatite was made according to the procedure of Levin [9] or when specified, purchased from Bio-Rad Laboratories. The adsorbent was slowly added to 0.01 M potassium phosphate buffer, pH 7.0, containing 0.05 % (w/v) Triton X-100 (Rohm and Haas, Philadelphia, Pa.) and a slurry made by gentle stirring. A column bed 2.2 cm × 23.5 cm was poured, allowed to settle by gravity, and washed with about 10 column volumes of the dilute phosphate–Triton solution. The sample was allowed to sink into the top of the column and the first 20 fractions (approx. 5.1 ml each) were eluted with the same phosphate-Triton solution. Five chambers of a Varigrad (Buchler Instruments Inc., Fort Lee, N.J.) were then connected to the column in order to accomplish a phosphate-gradient elution. The Varigrad chambers were loaded with a series of potassium phosphate (pH 7.0) solutions containing 0.05 % (w/v) Triton X-100 as follows: 1) 0.01 M (100 ml); 2) 0.02 M (99.5 ml); 3) 0.07 M (99 ml); 4) 0.12 M (98.5 ml); 5) 0.35 M (95.5 ml). A pressure head of about 127 cm of the eluting buffers induced a flow rate of about 0.4 ml/min.

Whatman brand, DEAE-cellulose adsorbent (DE52) was freed of fine particles by decantation and washed successively with 0.5 M NaOH, water, 0.5 M HCl, water, 0.5 M NaOH, and finally water. The washed adsorbent was suspended in 0.01 M sodium phosphate, pH 6.4, containing 0.05 % (w/v) Triton X-100 and titrated to pH 6.4 with 4 M H_3PO_4. A column was prepared and washed with at least 10 column volumes of 0.01 M sodium phosphate, pH 6.4, containing 0.05 % Triton X-100. A flow rate of 0.5–1.0 ml/min was provided by a peristaltic pump (LKB Instruments, Bromma, Sweden; type 4912A). The sample was pumped into the top of the column and the first 20 fractions (about 5 ml each) were eluted with the 0.01 M phosphate, Triton X-100 solution. Elution was continued with 500 ml of a linear gradient of NaCl from 0 to 0.6 or 0.7 M in the 0.01 M phosphate–Triton solution.

Before chromatography, the hydroxyapatite-column fraction in dilute (0.01 M) buffer was titrated to pH 6.4 with 0.125 M H_3PO_4. The hydroxyapatite-column frac-

tion in strong (0.3 M) buffer was titrated to pH 6.4 with 1.25 M H_3PO_4 and dialyzed overnight at 4 °C against the starting buffer prior to application to the DEAE-cellulose column. Fraction S_3 was dialyzed overnight against starting buffer before use.

Analysis of column fractions

The protein content of the DEAE-cellulose fractions was assayed by the procedure of Lowry et al. [10], using bovine serum albumin as a standard. This method could not be used with hydroxyapatite fractions because of extensive turbidity developed during the assay procedure. The hydroxyapatite effluent was assayed as follows. A mixture of 0.2 ml sample, 0.3 ml water, and 0.2 ml of 5.85 M NaOH in a 1.1 cm × 10 cm test tube was covered with aluminum foil and held at 90 °C for 4 h. The solution was allowed to cool, and 0.2 ml 39.8 % glacial acetic acid and 1 drop of chlorophenol red were added. The indicator insured that the solution was at pH 5.6±0.4 before color was developed with ninhydrin. Three sealed standard solutions containing the indicator at pH 5.2, 5.6 and 6.0 were used for comparison with the color of the neutralized solution. If needed, a drop of NaOH or acetic acid solution was added to adjust the pH. 0.4 ml ninhydrin reagent (12 ml methyl cellosolve, 0.8 g ninhydrin, 4 ml water, 8 mg $SnCl_2$) was added and the covered solution heated at 90 °C for 15 min. The solution, diluted with *n*-propanol–water (1:1, v/v) to bring the adsorbance into range, was read at 560 nm in a spectrophotometer (Model 16K, Cary Instruments, Monrovia, Calif.). A standard curve using bovine serum albumin was simultaneously generated by including the standards in the group of column fractions being assayed. Curves for bovine serum albumin were either linear or showed a slightly increasing slope. When 2 ml of *n*-propanol–water (1:1, v/v) was used for dilution, 100 μg of albumin yielded an $A_{560\ nm}$ of 2.9.

Succinate dehydrogenase and D-lactate dehydrogenase were assayed at 23 °C by diluting a sample of the column fraction (200 μl for succinate dehydrogenase and 50 μl for D-lactate dehydrogenase), to 0.9 ml with 0.01 M Tris–HCl buffer, pH 7, containing 0.43 M $(NH_4)_2SO_4$, and then adding 0.15 M of a solution consisting of 5 ml dichlorophenolindophenol (9 mg/32 ml), 0.8 ml of 0.8 M KCN, 0.8 ml 0.8 M HCl and 0.5 ml of 0.5 M sodium succinate or D-lactate. The initial rate of change of absorbance at 600 nm was taken as a measure of enzyme activity. For NADH dehydrogenase, 200 μl of sample was taken and the Tris–$(NH_4)_2SO_4$ buffer used as described above. However, the dichlorophenolindophenol–HCN reaction mixture was made without substrate and the reaction was started by adding 10 μl of a solution of NADH (10 mg/0.35 ml). A blank representing the non-enzymatic reduction of dichlorophenolindophenol by NADH was substracted in order to determine the rate of enzymatic reduction.

O_2 uptake was measured with an O_2 electrode [5], and cytochrome b_1 was measured by dual-wavelength spectroscopy at 428 and 411 nm before and after reduction with $Na_2S_2O_4$ [7]. ^{55}Fe was determined in a liquid scintillation system, using a narrow ^3H channel (Beckman Instruments Inc., Fullerton, Calif., Model LS-250). The sample (50 μl) was assayed in 15 ml of Bray's [11] scintillation solution. Nonheme iron was determined chemically [1] and nonheme iron protein by its electron paramagnetic resonance signal [8].

Polyacrylamide gel electrophoresis

The discontinuous sodium dodecylsulfate system of Neville [12] was used. For

the deoxycholate gel electrophoresis, 0.1 % deoxycholate was present in the upper reservoir buffer (pH 8.3) instead of 0.1 % sodium dodecylsulfate. The upper or stacking gel (pH 6.1) was 2.5 times the volume of the sample applied and contained 3 % (w/v) acrylamide and 0.2 % (w/v) bisacrylamide. The lower or running gel (pH 9.18) was 6 cm long and contained 7.88 % (w/v) acrylamide and 0.12 % (w/v) bisacrylamide. Protein was detected with Coomassie Brilliant Blue according to Fairbanks et al. [13]. Succinate, D-lactate, and NADH dehydrogenase were detected with nitrobluetetrazolium as described [1].

RESULTS

General characteristics of the membrane-bound and solubilized dehydrogenases described in this work

1. Succinate dehydrogenase (dichlorophenolindophenol electron acceptor). In 0.01 M Tris–HCl containing 0.35 M $(NH_4)_2SO_4$ at pH 7.0, the apparent K_m of both the membrane-bound and deoxycholate-solubilized enzyme (cytochrome b_1 complex) was about 1.7 mM.

Phenazine methosulfate (10–150 μg/ml) did not enhance the activity of the membrane-bound enzyme but at a level of 50–100 μg/ml it did stimulate the unfractionated solubilized extract by 60 %. Preincubation of the bound enzyme (0.45 mg protein/ml) in buffer containing 5 mM succinate and 12 mM HCN at 23 °C, increased activity of the enzyme to a maximum of 300 % of its initial activity in 20 min. A similar preincubation of the solubilized extract (0.13 mg protein/ml) increased activity to a maximum of 160 % of initial activity in less than 1 min. (See ref. 14 for comparative properties of succinate dehydrogenase from mammalian and some microbial sources.)

2. D-Lactate dehydrogenase (dichlorophenolindophenol electron acceptor). In the Tris–$(NH_4)_2SO_4$ buffer used above, the apparent K_m of the membrane-bound enzyme was about 1.2 mM and that of the crude solubilized extract 2.3 mM. Phenazine methosulfate did not stimulate the soluble or membrane-bound enzyme. Preincubation with D-lactate under the conditions described above for succinate, stimulated the membrane bound enzyme to a maximum level of 300 % initial activity in 2 min. The activity of the crude extract was instantly stimulated by 50 % if the substrate was added before dichlorophenolindophenol.

3. NADH dehydrogenase (1.5 mM $K_3Fe(CN)_6$ electron acceptor). In the Tris–$(NH_4)_2SO_4$ buffer, the apparent K_m for the bound activity was $1.7 \cdot 10^{-3}$ mM and for the deoxycholate-solubilized activity it was 0.6 mM.

Description of the fractionation procedure and nomenclature of the fractions

Fig. 1 represents a flow chart for the fractionation of the 0.4 % deoxycholate extract. The 0.4 % deoxycholate extract of the T fraction contains cytochrome (principally cytochrome b_1); dehydrogenases for succinate, D-lactate, and NADH; succinoxidase, and nonheme iron proteins ([1] as well as this report). A cytochrome oxidase-enriched fraction was obtained from a 1 % deoxycholate extract of a residue of T fraction that was previously extracted with 0.5 % deoxycholate. This extract was then chromatographed on a DEAE-cellulose column. The fraction names are prefixed with either HA or DE to designate the column (hydroxyapatite or DEAE-cellulose, respectively) used. The substrate named indicates the principal dehydroge-

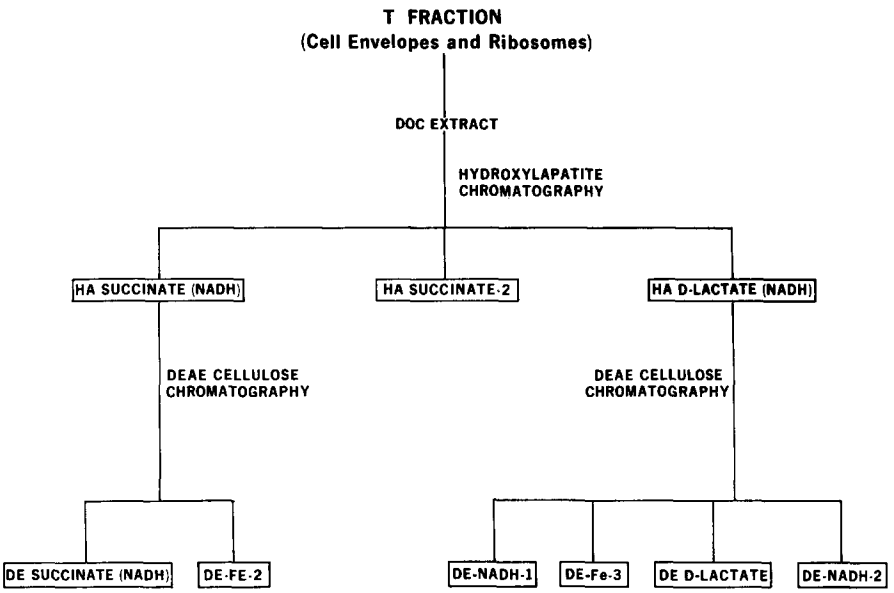
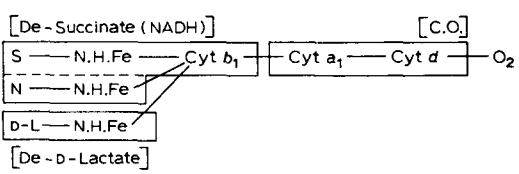


Fig. 1. Flow sheet for fractionation of electron-transport chain components of *E. coli*. The particulate components were obtained by centrifuging a suspension of broken spheroplasts for 60 min at 105 000 × *g*. The pellet (T fraction consisting of cell envelopes and ribosomes) was extracted with 0.4 % (w/v) deoxycholate and recentrifuged for 60 min at 105 000 × *g*. Chromatography of the clear extract on a column of hydroxyapatite (HA) yields two major fractions, HA-succinate (NADH) and HA-D-lactate (NADH), and one minor fraction, HA-succinate-2. The two major fractions are each chromatographed on DEAE-cellulose (DE). HA-succinate (NADH) then yields DE-succinate (NADH) and DE-Fe-2. HA-D-lactate (NADH) yields DE-NADH-1, DE-Fe-3, DE-D-lactate, and DE-NADH-2.

nase activity present. The designation "(NADH)" following "succinate" or "D-lactate" means that NADH dehydrogenase was also present in the fractions.

Fractions containing iron but having no appreciable dehydrogenase activity are designated as "Fe". Cytochrome oxidase-enriched fractions are designated C.O.

The reader will be aided in following the fractionation described in this paper by referring to the basic protein skeleton of the electron-transport chain of *E. coli*, arguments for which have been summarized previously [8].



S, succinate dehydrogenase; N, NADH dehydrogenase; D-L, D-lactate dehydrogenase; N.H.Fe, nonheme iron protein. The blocks drawn indicate the kind of fragmentation

that gives rise to the fractions that we have isolated and designated as DE-succinate (NADH), DE-D-lactate, and C.O. DE-Fe-2 appears to be essentially free cytochrome b_1 and the fractions designated DE-NADH-1 and DE-NADH-2 are most likely two forms of the NADH dehydrogenase–nonheme iron protein segment. HA-succinate-2, a minor component, may be the DE-succinate (NADH) fragment minus the NADH dehydrogenase–nonheme iron protein component.

Fractionation of the deoxycholate extract on hydroxyapatite

Fig. 2 shows that hydroxyapatite completely separates D-lactate dehydrogenase from succinate dehydrogenase. Both the major succinate dehydrogenase peak (HA-succinate dehydrogenase (NADH)) and the D-lactate dehydrogenase peak (HA-D-lactate (NADH)) contain NADH dehydrogenase activity. The two peaks containing succinate dehydrogenase are seen over a wide range of sample loads and therefore do not represent a column overload phenomenon. The heterogeneity most likely results from a difference in the extent of depolymerization and dissociation from other membrane components. Deoxycholate extract prepared from cells grown in the pres-

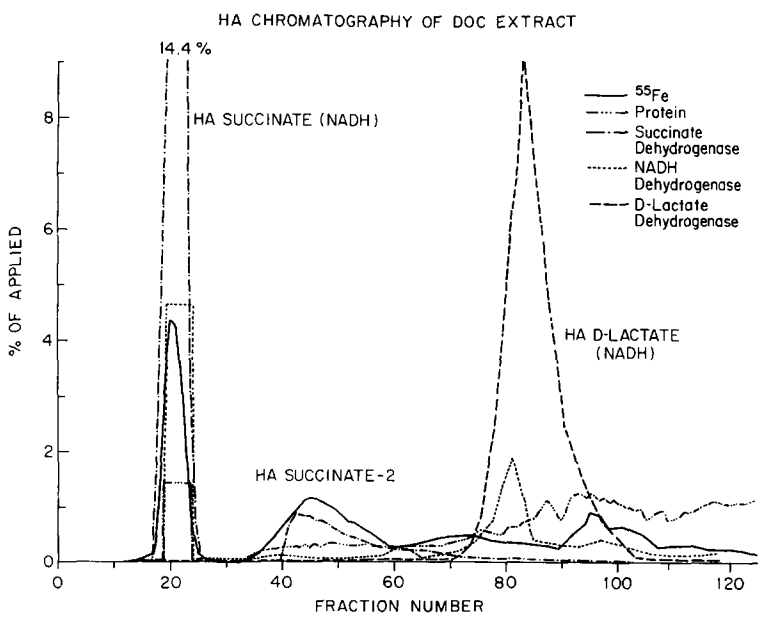


Fig. 2. 38 ml of 0.4 % deoxycholate (DOC) extract containing Triton X-100 (0.5 %, w/v) was applied to a 2.2 cm × 23 cm column of hydroxyapatite and 20 fractions (5.0 ml each) were eluted with 0.01 M potassium phosphate, 0.05 % Triton X-100, pH 7.0. A gradient of increasing concentration of potassium phosphate was then generated with 5 chambers of a Varigrad loaded in sequence with increasing strengths of potassium phosphate buffer, pH 7.0, in 0.05 % Triton X-100: 100 ml at 0.01 M, 100 ml at 0.02 M, 99.8 ml at 0.07 M, 99 ml at 0.12 M and 95 ml at 0.35 M. Flow rate was 0.4 ml/min. Applied to the column were 228 mg protein, $5.89 \cdot 10^6$ cpm of ^{55}Fe-labeled protein, 12.4 units of succinate dehydrogenase, 44.2 units of D-lactate dehydrogenase and 53.5 units of NADH dehydrogenase. (1 unit = 1 μmole substrate oxidized at 23 °C per min). Recovered from the column were 60 % of the protein, 46 % of the ^{55}Fe, 64 % of succinate dehydrogenase, 82 % of D-lactate dehydrogenase, and 49 % of NADH dehydrogenase. The protein and NADH dehydrogenase are shown as bars in the first peak because they were determined on pooled fractions.

ence of ^{55}Fe was previously shown to contain ^{55}Fe and cytochrome b_1 in material which co-chromatographed on Sepharose 4B [1]. Cytochrome a_1, d and b_1 were shown to be present in large-molecular weight components which were excluded from the Sepharose. Cytochrome b_1 was the only detectable cytochrome in components of molecular weight under 10^6. Fig. 2 shows that peaks for ^{55}Fe were coincident with HA-succinate (NADH) and HA-succinate-2 peaks, but not with the HA-D-lactate (NADH) peak. The material in the ^{55}Fe-containing peaks exhibited Soret absorptions for cytochrome b_1 (not shown). Both succinate dehydrogenase peaks had succinoxidase activity. The peak for this activity occurred in Fraction 20 and accounted for 9.5 % of the applied activity (data not shown). HA-succinate (NADH) from Fraction 17 to Fraction 25 contained 42 % of the applied succinoxidase compared to 51 % of the applied succinate dehydrogenase activity. The succinoxidase activity was not inhibited by 12 mM HCN and therefore cytochrome d probably was not present. Reduced vs oxidized difference spectra obtained with this fraction also indicate the absence of cytochrome d (e.g. Fig. 6). A total of about 2–3 % of applied succinoxidase was present in Fractions 42 and 43 with no detectable activity in the rest of the fractions of HA-succinate-2. This may be compared to the 9 % of applied succinate dehydrogenase in Fractions 41–60.

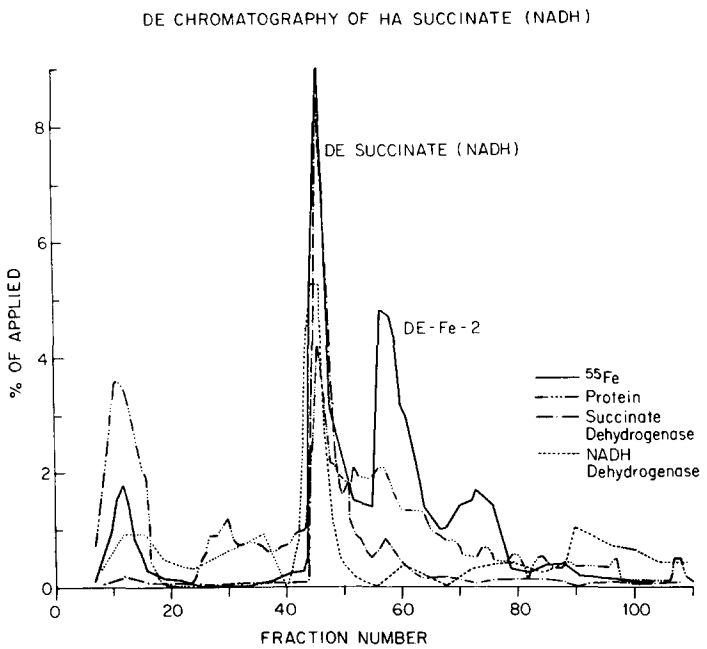


Fig. 3. 29 ml of the pooled Fractions 19–24 (HA-succinate (NADH)) from the hydroxyapatite column described in Fig. 2 was titrated to pH 6.4 with 0.125 M H_3PO_4 and applied to a 1.6 cm × 24.5 cm column of Whatman DE52. 20 fractions (5.4 ml each) were then eluted with 0.01 M sodium phosphate, 0.05 % Triton X-100, pH 6.4. A linear gradient to 0.6 M NaCl (500 ml) was then used to complete the chromatography. The flow rate was 1 ml/min. Applied to the column were 19 mg protein, $8.8 \cdot 10^5$ cpm of ^{55}Fe-labeled protein, 4.65 units of succinate dehydrogenase and 11 units of NADH dehydrogenase (1 unit = 1 μmole substrate oxidized per min at 23 °C). Recovered from the column were 100 % of the protein, 74 % of the ^{55}Fe, 38 % of the succinate dehydrogenase and 55 % of the NADH dehydrogenase.

Sequential fractionation of deoxycholate extract on hydroxyapatite and DEAE-cellulose columns

Chromatography of HA-succinate (NADH) directly (without storage) on DEAE-cellulose gave the results shown in Fig. 3. Succinate dehydrogenase occurred in a single major peak (DE-succinate (NADH)) which was also defined by the profiles for ^{55}Fe and protein. The peak for NADH dehydrogenase preceded the succinate dehydrogenase peak by one fraction. Fractions 45 to 49 of the DE-succinate (NADH) peak contained 35 % of the applied succinoxidase activity (data not shown). This may be compared to 25 % of the applied succinate dehydrogenase activity contained in these fractions. In addition to the major peak for ^{55}Fe present in DE-succinate (NADH), another major peak was seen in Fractions 55–65. These two peaks were also outlined on the basis of their Soret-band absorption for cytochrome b_1 (not shown). Two minor ^{55}Fe-containing peaks were also seen – one at the beginning and one near the end of the chromatogram.

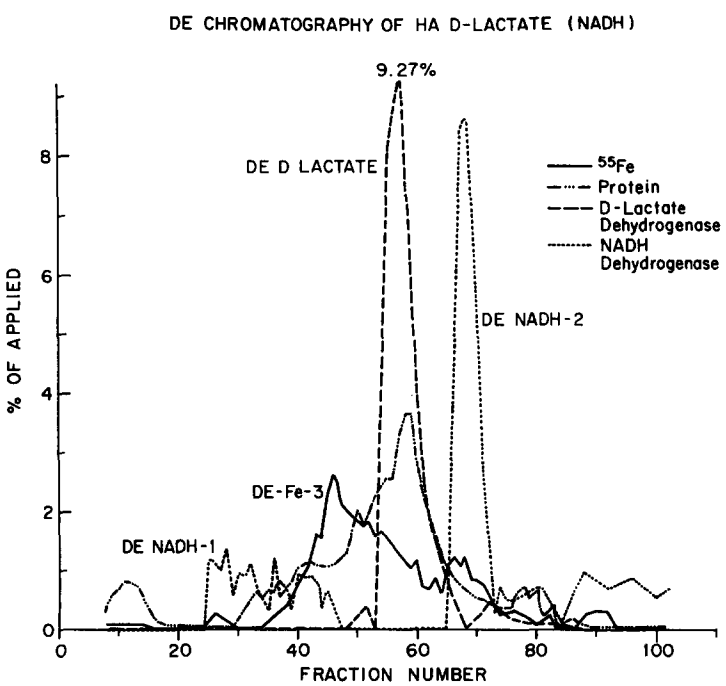


Fig. 4. The pooled fractions 78–88, HA-D-lactate (NADH), from the hydroxyapatite column described in Fig. 2 was thawed after being stored in liquid N_2. After titrating to pH 6.4 with a 1.25 M H_3PO_4, the solution was dialyzed overnight at 4 °C against 0.01 M sodium phosphate–0.05 % Triton X-100, pH 6.4. 38 ml was applied to a 1.6 cm × 26 cm column of Whatman DE52 and 20 fractions (5 ml each) were eluted with 0.01 M sodium phosphate–0.05 % Triton X-100, pH 6.4. A linear gradient to 0.6 M NaCl (500 ml) was then used to complete the chromatography. The flow rate was 1.1 ml/min. Applied to the column were 12.4 mg protein, $1.3 \cdot 10^5$ cpm of ^{55}Fe-labeled, protein, 3.5 units of D-lactate dehydrogenase and 6.5 units of NADH dehydrogenase (1 unit = 1 μmole of substrate oxidized per min at 23 °C). Recovered from the column were 70 % of the protein, 50 % of the ^{55}Fe, 59 % of the D-lactate dehydrogenase and 68 % of the NADH dehydrogenase.

HA-D-lactate (NADH) applied to DEAE-cellulose gave the elution pattern shown in Fig. 4. D-Lactate dehydrogenase was found in a peak (DE-D-lactate) virtually free of NADH dehydrogenase activity, and the major NADH dehydrogenase activity occurred in a peak (DE-NADH-2) almost free of D-lactate dehydrogenase activity. A protein peak corresponding to the D-lactate dehydrogenase peak is evident. The broad low erratic distribution of NADH dehydrogenase activity (DE-NADH-1) seen in Fractions 25–46 is repeatedly observed. The low amount of ^{55}Fe present*, peaked around Fraction 46 (DE-Fe-3), was not studied further.

Fractionation of cytochromes b_1, a_1 and d (cytochrome oxidase)

In anticipation of subsequent reconstitution experiments, extraction of T fraction with 0.5 % deoxycholate was performed to remove virtually all of the succi-

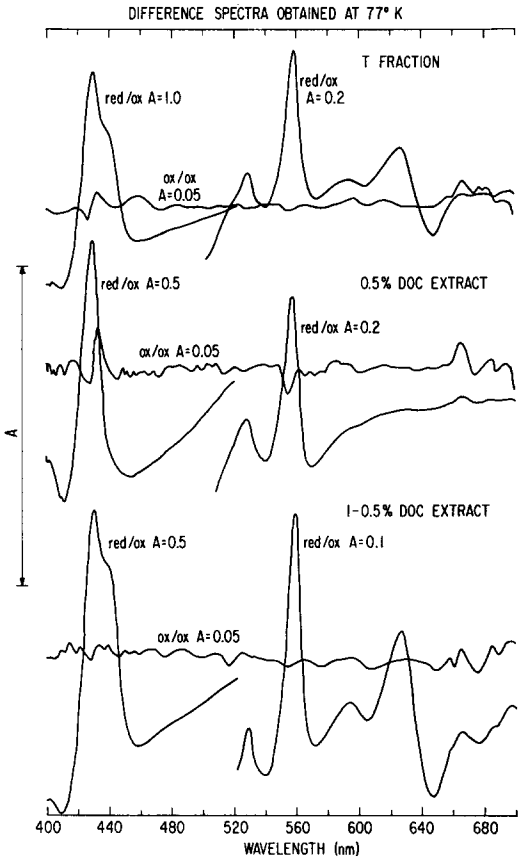


Fig. 5. Sensitivity is indicated for each curve by the value assigned (in absorbance units) to the dimension "A". The redox state of contents in the sample and reference cuvettes is indicated by the expression (red or ox)/ox where the numerator (red = reduced, ox = oxidized) represents the sample and the denominator represents the reference. Protein concentrations were 4.5 mg/ml for T fraction, 3.8 mg/ml for the 0.5 % deoxycholate extract and 1 mg/ml for the 1–0.5 % deoxycholate (DOC) extract. HA, hydroxyapatite.

* 3 % of ^{55}Fe applied to hydroxyapatite was present in HA-D-lactate (NADH) and only 50 % of this is recovered in all of the fractions eluted from the DEAE-cellulose column.

nate dehydrogenase and very little of the cytochrome oxidase [1]. This was followed by extraction of the 105 000 × *g* pellet with 1 % deoxycholate to remove a large amount of cytochrome oxidase. Fig. 5 (top) shows the presence of cytochromes b_1, a_1 and *d* in *E. coli* cell envelopes (T fraction). The Soret region shows a strong absorption for cytochrome b_1 (γ) (peak 430 nm, trough 410 nm) and a shoulder for cytochrome a_1 or *d* (peak approx. 439 nm, trough 456 nm). The other absorption peaks are: cytochrome b_1 (β) (529 nm), cytochrome b_1 (α) (558 nm), cytochrome a_1 (α) (594 nm) and cytochrome *d* (626 nm with a deep trough at 648 nm). Fig. 5 (middle) shows that a 0.5 % deoxycholate extract contains mainly cytochrome b_1. The Soret absorption shoulder at approx. 439 nm is virtually absent as are the α-absorptions for cytochromes a_1 and *d*. A 1 % deoxycholate extract of the insoluble pellet obtained after extraction with 0.5 % deoxycholate (Fig. 5) bottom, shows a relatively high content of cytochrome oxidase (cytochromes *d* and a_1). The Soret band of fraction HA-succinate (NADH) (Fig. 6, top) had even less absorption (by actual measurement) in the Soret shoulder region (characteristic of cytochrome a_1 or *d*) than did the 0.5 % deoxycholate

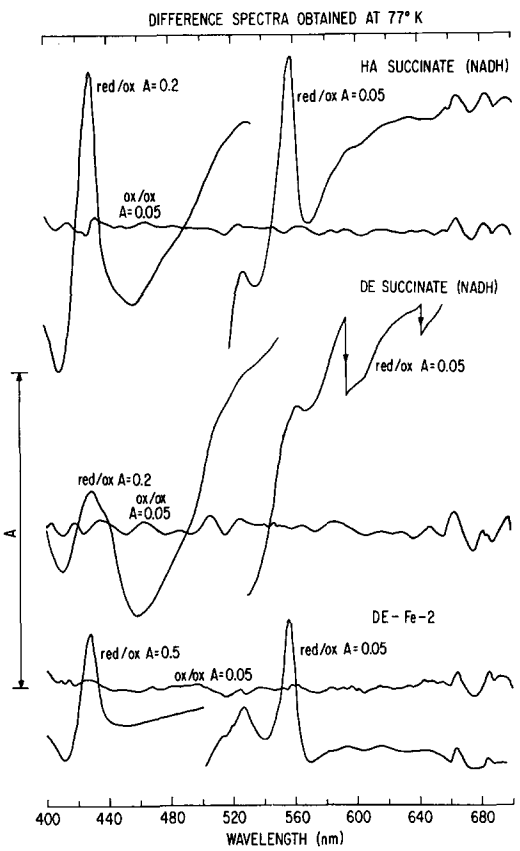


Fig. 6. Designations are the same as for Fig. 5. Protein concentrations were 1.4 mg/ml for HA-succinate (NADH), 0.39 mg/ml for DE-succinate (NADH) and 0.34 mg/ml for DE-Fe-2. The arrows pointing downwards show where the base line was lowered in order to keep the tracing on scale.

extract. The spectrum obtained with DE-Fe-2 (Fig. 6, bottom) is characteristic of crystalline cytochrome b_1 [26]. DE-succinate (NADH) (Fig. 6, middle) shows the Soret and α-absorption for cytochrome b_1. In addition there is a deep trough with a minimum at about 452 nm and a steep rise breaking at about 515 nm. This is characteristic of the absorption properties of flavoprotein [15] and nonheme iron protein [16, 17], both of which are present in the DE-succinate (NADH) complex. A shoulder is also evident in the Soret region at about 440 nm. Whether this is due to cytochromes a_1 or d or something else, cannot be decided at this time.

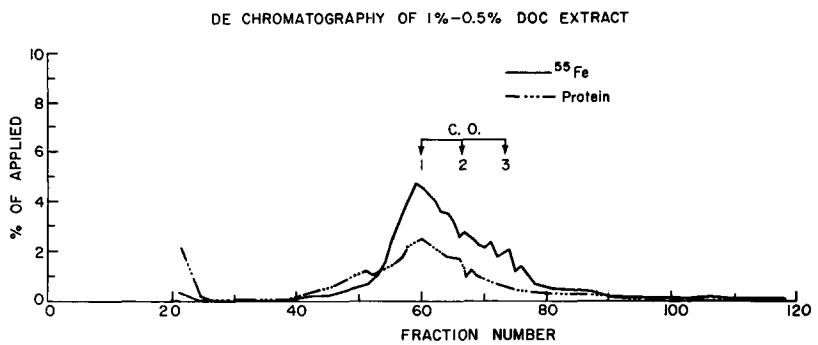


Fig. 7. A 1 % deoxycholate extract of a 0.5 % deoxycholate-extracted pellet of cell envelopes was prepared as follows. The pellet (575 mg protein), obtained after extracting 48 ml (960 mg protein) of ^{55}Fe-labeled T fraction with 0.5 % deoxycholate, was suspended in 20 ml of original medium (i.e. 21.1 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) plus 2 ml 1 M Tris–HCl (pH 8.1) and 2.2 ml 10 % (w/v) deoxycholate. The suspension after standing 15 min at room temperature was centrifuged at 105 000 × g for 60 min at 4 °C. 20 ml of this 1 % (–0.5 %) deoxycholate extract (4 mg protein/ml) was diluted with 20 ml of starting DEAE-cellulose column buffer (0.01 M sodium phosphate, 0.05 % Triton X-100 (pH 6.4), titrated to pH 6.4 with 1.25 M H_3PO_4, adjusted to 0.5 % Triton X-100 with a 10 % (w/v) solution and applied to a 17 cm × 1.25 cm column of Whatman DE52 adsorbent. After washing the column with 10 ml of DEAE-cellulose buffer, the column was eluted with 250 ml of a linear gradient of NaCl from 0 to 0.6 M in the DEAE-cellulose column buffer, at a flow rate of 1 ml/min. The first 50 ml (sample volume plus 10 ml of DEAE-cellulose buffer) was collected in bulk followed by 2.5-ml fractions of the gradient numbered from 21 to 120. Of the applied protein (80 mg) and ^{55}Fe ($7 \cdot 10^5$ cpm), 92 % and 70 %, respectively, were recovered. The first bulk fraction contained 39 % and 0.4 %, respectively, of the applied protein and ^{55}Fe. The indicated pooled fractions C.O.1, C.O.2, and C.O.3 were used for low-temperature difference spectra as shown in Fig. 8.

The difference spectra for fractions obtained by chromatography of the 1 % deoxycholate extract on DEAE-cellulose (Fig. 7) are shown in Fig. 8. The three spectra show a rich content of cytochrome oxidase (cytochromes a_1 and d). The amount of oxidase relative to cytochrome b_1 decreases in the later-eluted fractions. Table I presents a summary of the characteristics of the major fractions described in this work.

Polyacrylamide gel electrophoresis of major fractions

All major fractions were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate or deoxycholate. The results in terms of number of major bands present are summarized in Table I.

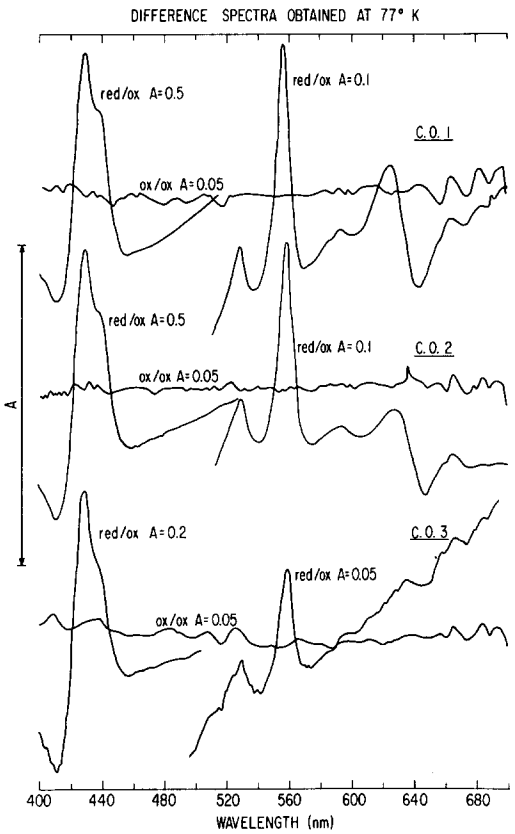


Fig. 8. Designations are the same as for Fig. 5. Protein concentrations were 0.85 mg/ml for cytochrome oxidase-enriched fraction 1, 0.77 mg/ml for cytochrome oxidase-enriched fraction 2 and 0.29 mg/ml for cytochrome oxidase-enriched fraction 3.

On the relation of NADH dehydrogenase in the soluble and membrane-containing fractions

The NADH dehydrogenase activity of the soluble fraction (S_3) is about 20 times that of the deoxycholate extract of the particulate fractions [5, 19]. In order to see if the activity in both of these fractions resides in the same or different molecular species, the following experiments were done. First, S_3 was chromatographed on DEAE-cellulose under the same conditions used for the deoxycholate extract of the particulate fractions. Peaks were obtained for NADH dehydrogenase activity in the same locations as that obtained from the membrane-containing fraction (cf. Fig. 4). Polyacrylamide gel electrophoresis was performed for NADH-1 and NADH-2 obtained from both particulate and soluble sources. The fractions were analyzed both in sodium dodecylsulfate- and deoxycholate-containing gels and both for the separate fractions and for mixtures of the same NADH dehydrogenase species from the two separate sources. All of the data showed that the same molecular species of DE-NADH-1 and -2 exist in both the membrane and cytosol fractions of the cell (gels are not shown).

TABLE I

SUMMARY OF CHARACTERISTICS OF MAJOR FRACTIONS

A cross means the component is present in the fraction. The major polypeptide bands were revealed by Coomassie Brilliant Blue.

Fraction	Dehydrogenases present			Cytochromes present		Number of major polypeptide bands in polyacrylamide gel electrophoresis	
	Succinate	D-Lactate	NADH	b_1	a_1-d	Sodium dodecyl-sulfate	Deoxycho-late
DE-Succinate (NADH)	×		×	×	?	3	1(0.37)*
DE-Fe-2				×		5	3
DE-NADH-1			×			6	1(0.35)*
DE-NADH-2			×			5	0
DE-D-Lactate		×				8	3(0.55)*
C.O. 1, 2, 3				×	×	16	—

* The number in parenthesis is the R_F for the major-stained band as well as the location of specific enzyme activity. For DE-D-lactate the most intensely stained band was coincident with the enzyme activity at R_F 0.55. The enzyme activity and major-stained band of DE-NADH-2 did not enter the stacking or running gels of the deoxycholate system. Radioactive iron was found coincident with the protein and enzyme activity in the deoxycholate gel of DE-succinate (NADH).

TABLE II

DISTRIBUTION OF PROTEIN, NONHEME IRON AND NONHEME IRON PROTEIN AMONG MAJOR FRACTIONS OF AN *E. COLI* HOMOGENATE

The protein concentration of the spheroplast suspension was 26.8 mg/ml. All of the numbers shown are the averages from duplicate determinations. The duplicate values for both the protein and nonheme iron determinations agreed to with ±2% of the mean values. There was a wider variation, however, in the electron paramagnetic resonance signals used in the determination of nonheme iron protein content. This variation is shown in the table.

	Protein; percent of		Nonheme iron			Nonheme iron protein; percent of	
			μg Fe/ mg protein	percent of			
	Sonicate	"T"		Sonicate	"T"	Sonicate	"T"
Spheroplast suspension	100		0.60	100		100	
T Fraction	42	100	0.69	56	100	75±20	100
S_3 Fraction	58		0.40	44		10±3	
0.4% deoxycholate extract		30	0.51		22		35±0.5
0.4% deoxycholate pellet		65	0.58		54		28±16
HA-Succinate (NADH)		3	3.2		6		24±5

Distribution of nonheme iron proteins

Nonheme iron proteins can be assayed by the electron paramagnetic resonance (EPR) signal of their reduced form and total nonheme iron can be determined chemically. Table II shows the distribution of nonheme iron measured both ways. The EPR measurements are not strictly comparable because the shape of the EPR signal is somewhat different in the different fractions. Nevertheless, the table shows that about 75 (or 90 % calculated by subtracting the 10 % found in S_3 from the starting 100 %) of the nonheme iron protein is in the particulate fraction, compared to about 56 % of the total nonheme iron. The deoxycholate extract contained about 1/3 of the nonheme iron protein and about 1/5 of the total nonheme iron originally present in the particulate fraction. HA-succinate (NADH) contained about 2/3 of the nonheme iron protein and 1/3 of the total nonheme iron of the deoxycholate extract or 24 % and 6 %, respectively, of the amounts present in the T fraction. The concentration of protein-bound and free nonheme iron in all of the other fractions was too low for accurate quantification.

DISCUSSION

We report here methods for separating the electron-transport chain of *E. coli* into fractions containing various parts of the overall activity. The fractions obtained (except for the cytochrome oxidase-enriched fractions) are soluble by the following criteria: 1) they are not sedimented at $105\,000 \times g$ for 60 min; 2) the fractions containing dehydrogenases for NADH, succinate, and D-lactate penetrate the included space of Sepharose 4B [1] and 8 % acylamide gels; 3) cytochrome b_1 co-chromatographs with succinate dehydrogenase on Sepharose 4B [1] and in vivo-incorporated ^{55}Fe can be co-electrophoresed with succinate dehydrogenase in the deoxycholate–acrylamide electrophoresis system.

One of the fractions obtained (DE-succinate (NADH)) contains succinate dehydrogenase functionally linked to cytochrome b_1 presumably through a nonheme iron protein [8]. Although NADH dehydrogenase activity is also present in the fraction it is not coupled to cytochrome b_1. A more convincing proof of the actual structural association of succinate dehydrogenase to cytochrome b_1 and the absence of such an association with the NADH dehydrogenase could be obtained by using a mutant lacking the succinate dehydrogenase protein and determining which of the components are no longer present in the DE-succinate (NADH) fraction. Thus far, we have found no reports of deletion mutants for succinate dehydrogenase.

The cleanest preparation of the functional complex (DE-succinate (NADH)) exhibits succinoxidase activity that is insensitive to HCN and presumably is linked to oxygen through the autooxidizability of cytochrome b_1. However, a shoulder in the Soret region in the difference spectrum suggests the possible presence of a small amount of cytochrome oxidase.

In addition to the succinate dehydrogenase–cytochrome complex, the following major fractions were obtained: 1) DE-Fe-2 showing absorption characteristics for only cytochrome b_1 and possessing none of the three dehydrogenases; 2) DE-NADH-1 possessing only NADH dehydrogenase activity and isolated both from the membrane and soluble fractions of the cell; 3) DE-NADH-2 different than DE-NADH-1 and possessing only NADH dehydrogenase activity and isolated both from

the membrane and soluble fractions of the cell; 4) DE-D-lactate possessing only D-lactate dehydrogenase activity and just traces of cytochrome; 5) cytochrome oxidase-enriched preparations 1, 2 and 3 containing cytochrome b_1, a_1 and *d*.

We have discussed previously ([1] and in this Introduction) other work on the solubilization and fractionation of electron-transport chains (see also refs 2–4 and 20). A recent related work has described the solubilization of succinate dehydrogenase from *E. coli*, achieved by extensive sonication followed by acetone treatment [21]. This succinate dehydrogenase, although also capable of activation by preincubation with substrate as described here, was isolated separate from cytochrome b_1 and required phenazine methosulfate for full reactivity with dichlorophenolindophenol. Cowell et al. [18] reported the isolation of a preparation of succinate dehydrogenase from *E. coli* that contained cytochrome b_1 and may be similar to the complex described here. D-Lactate dehydrogenase from *E. coli* was recently solubilized and purified [22, 23].

It has been proposed that in mammalian electron-transport chains several forms of cytochrome *b* exist and that one is capable of existing in two different states having different half-reduction potentials and capable of forming one of the high-energy intermediates of the respiratory chain [24, 25]. The separation of several fractions described in this paper, each containing cytochrome b_1, may represent the actual physical separation of the suspected different redox-potential forms of this cytochrome. We are proceeding to determine the midpoint oxidation–reduction potentials of cytochrome b_1 in the various fractions obtained. It will also be of interest to try to reconstruct a completely integrated and CN^--sensitive electron-transport chain from the various fractions that have been isolated. Experiments to accomplish this goal are currently underway.

ACKNOWLEDGEMENTS

The authors are indebted to Dr E. A. Peterson for his advice and guidance during the development of the column-chromatographic procedures. Mr David Rogerson, Jr prepared the hydroxyapatite. The presentation of the material in this manuscript was greatly aided through discussions with Drs Raymond Scharff and Michael Pereira.

REFERENCES

1 Hendler, R. W. and Burgess, A. H. (1972) J. Cell Biol. 55, 266–281
2 Racker, E. (1970) in Membranes of Mitochondria and Chloroplasts (Racker, E., ed.), pp. 127–171 Van Nostrand Reinhold, New York
3 Gelman, N. S., Lukoyanova, M. A. and Ostrovskii, D. N. (1967) in Respiration and Phosphorylation of Bacteria, pp. 71–159, Plenum Press, New York
4 Kamen, M. D. and Horio, T. (1970) Annu. Rev. Biochem. 39, 673–700
5 Hendler, R. W., Burgess, A. H. and Scharff, R. (1969) J. Cell Biol. 42, 715–732
6 Hendler, R. W., Burgess, A. H. and Scharff, R. (1970) J. Cell Biol. 44, 376–384
7 Hendler, R. W. and Nanninga, N. (1970) J. Cell Biol. 46, 114–129
8 Hendler, R. W. (1971) J. Cell Biol. 51, 664–673
9 Levin, O. (1962) Methods Enzymol. 5, 27–32
10 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275

11 Bray, G. A. (1960) Anal. Biochem. 1, 279–285
12 Neville, Jr, D. M. (1971) J. Biol. Chem. 246, 6328–6334
13 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617
14 Singer, T. P. (1974) in Methods of Biochemical Analysis (Glick, D., ed.), Vol. 22, Interscience Publishers Inc., New York
15 Chance, B. and Williams, G. R. (1955) J. Biol. Chem. 217, 395–407
16 Ringler, R. L., Minakami, S. and Singer, T. P. (1963) J. Biol. Chem. 238, 801–810
17 Singer, T. P., Gutman, M. and Massey, V. (1973) in Iron–Sulfur Proteins (Lovenberg, W., ed.), Vol. 1, pp. 225–300, Academic Press, New York
18 Cowell, J. L., Raffeld, M. and Friedberg, I. (1973) Abstr. 73rd Annu. Meet. Am. Soc. Microbiol. 157, 100
19 Scharff, R., Hendler, R. W., Nanninga, N. and Burgess, A. H. (1972) J. Cell Biol. 53, 1–23
20 Razin, S. (1972) Biochim. Biophys. Acta 265, 241–296
21 Kim, I. C. and Bragg, P. D. (1971) Can. J. Biochem. 49, 1098–1104
22 Futai, M. (1973) Biochemistry 13, 2468–2474
23 Kohn, L. D. and Kaback, H. R. (1973) J. Biol. Chem. 248, 7012–7017
24 Chance, B., Wilson, D. F., Dutton, P. L. and Erecinska, M. (1970) Proc. Natl. Acad. Sci. U.S. 66, 1175–1182
25 Slater, E. C., Lee, C. P., Berden, J. A. and Wegdam, H. J. (1970) Biochim. Biophys. Acta 223, 354–364
26 Deeb, S. S. and Hager, L. P. (1964) J. Biol. Chem. 239, 1024–1031