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

V. On the Reduction of Nonheme Iron and the Cytochromes by Nicotinamide Adenine Dinucleotide and Succinate

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ABSTRACT

The sensitivity of nicotinamide adenine dinucleotide (NADH) oxidase and succinoxidase to metal chelators, the generation of an electron paramagnetic resonance (EPR) signal upon addition of these substrates, and the rate of formation of the EPR signal relative to the rate of the cytochrome reduction suggest the participation of nonheme iron proteins in the respiratory process of *Escherichia coli*. The most inhibitory metal chelator, thenoyltrifluoro acetone, inhibited the reduction of nonheme iron and cytochromes but did not prevent the reoxidation of the reduced forms. The EPR signal, dehydrogenase, and oxidase activities evoked by NADH are considerably greater than the corresponding activities evoked by succinate. Because both substrates can reduce almost all of the cytochromes, a model in which fewer succinate dehydrogenase–nonheme iron protein complexes are linked to a common cytochrome chain than NADH dehydrogenase–nonheme iron protein complexes is considered likely.

INTRODUCTION

Succinoxidase activity of *Escherichia coli* W6 can be completely inhibited by bovine serum albumin (BSA)[1] and an unidentified cell factor under conditions where succinate dehydrogenase is only 10% inhibited and nicotinamide adenine dinucleotide (NADH) oxidase is completely uninhibited (1). Since we have presented evidence previously (2) and in the current paper, that both NADH and succinate share a common cytochrome chain, the location of the sensitive component in succinate oxidation must be between succinate dehydrogenase and the cytochrome chain. Current concepts of the respiratory chain in mitochondria place a nonheme iron protein in this location (3). We present evidence for the involvement of nonheme iron proteins in the respiration of *E. coli*, A quantitation of the maximum extent of reduction of nonheme iron and cytochromes by NADH and succinate plus a measure of the NADH and succinate dehydrogenase activities leads to a picture of a single cytochrome chain joined to a small number of succinate dehydrogenase–nonheme iron protein

[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; EPR, electron paramagnetic resonance; NADH, nicotinamide adenine dinucleotide; PAB, Penn Assay Broth or Difco Antibiotic Medium No. 3; S, soluble fraction; TFTB, 4,4,4-trifluoro-1,2(2-thienyl)-1,3-butane dione.

units compared to the number of NADH dehydrogenase–nonheme iron protein units.

METHODS

Preparation of Particle Suspension

E. coli W6 (ATCC No. 25377), a proline auxotroph, was converted to spheroplasts by treatment with penicillin as previously described (4). More than 90% of the spheroplasts were broken during the procedures of harvesting by centrifugation and washing. The acellular suspension in "storage medium" (9% glycerol w/v, 0.05 M Tris, and 10^{-3} M $MgCl_2$ at pH 7.4) was stored in liquid nitrogen. After thawing in tepid water, the suspension was treated with a Branson sonifier (Branson Instruments, Inc., Stamford, Conn.), model S-75, at power setting 6–15 sec at 0°C. Small volumes were used at this stage, usually about 30–35 ml in a 50 ml beaker. The suspension was then centrifuged at 105,000 *g* for 60 min in a Beckman Spinco Model L preparative centrifuge. The pellet was resuspended in "suspending medium" with a Teflon pestle. Suspending medium was made by mixing equal volumes of storage medium and "C" medium (C medium contains 6 g Na_2HPO_4, 3 g KH_2PO_4, g 3 NaCl, 110.9 mg Na_2SO_4, and 83.5 mg $MgCl_2 \cdot 6 H_2O$/liter). The usual preparation contained 5–6 mg protein/ml and is sometimes referred to as the unit or 1 × concentration. A 3.5 × concentration (about 19 mg protein/ml) was also prepared. The suspensions were stored in liquid nitrogen.

Electron Paramagnetic Resonance (EPR) Measurements

The thawed suspension was vigorously shaken in air for 60 sec before each sampling. Kinetic studies were performed in the following manner. A 0.5 ml tuberculin syringe was fitted with a small piece of Tygon tubing so that it would easily be attached to and detached from an 9 × 0.5 cm length of glass tubing joined to a 26 cm long capillary tube (inner diameter = 1 mm). The suspension (0.3 ml) was drawn into the capillary tube by use of the syringe at the top. The suspension was drawn far enough into the capillary tube to leave an empty space of about 8 cm at the open end of the tube. 7 μl of substrate (either 0.2 M NADH or 0.5 M sodium succinate) was inserted into this free space with a 10 μl Hamilton microsyringe (Hamilton Co., Whittier, Calif.). Incubations were started by expelling the substrate and particle suspension into a 3 × 250 mm quartz Varian EPR tube and simultaneously mixing with the capillary tube. Experiments using dye in place of substrate showed that complete mixing occurred rapidly. Incubations were stopped by quick freezing. The rate of cooling was measured by a thermocouple of 0.004 inch Teflon insulated copper wire and 0.003 inch constantan wire inserted into the quartz tube and attached to an oscilloscope. Whereas plunging the tube directly into liquid nitrogen cooled the suspension at a rate of about 7°C/sec, the suspension could be frozen within 0.5 sec by immersing it in Freon-22 contained in a copper tube partially immersed in liquid nitrogen. For all kinetic measurements, the latter technique was used.

After freezing, the tubes were placed in liquid nitrogen in preparation for the EPR measurements which were performed at liquid nitrogen temperature. All tubes used for EPR measurements were calibrated in advance by measuring the strength of the EPR signal produced from 10^{-4} M diphenyl picryl hydrazyl in toluene. EPR measurements were made with a Varian EPR Spectrometer E-4. A small Dewar flask containing liquid nitrogen was placed in the sample compartment of the instrument. The instrument settings were as follows: field set, 3320 G; scan range, 1000 G; time constant, 3 sec; scan time, 16 min; receiver gain, 2×10^3; modulation amplitude, 2×10^1; microwave power, 4 db; microwave frequency, 9.177 GHz. All samples were scanned at least three times and the average distance from the trough of the spectrum at $g = 1.92$ to the maximum shoulder downfield (see Fig. 4) was determined.

Spectrophotometric Assay of Cytochrome Reduction

An Aminco-Chance Dual wavelength/splitbeam recording spectrophotometer was used. A portion of the suspension at room temperature was vigorously shaken in air for 60 sec. 3 ml was placed in an Aminco (American Instrument Company, Silver Spring, Md.) anaerobic cell and a stopcock-plunger mixer assembly containing 45 μl of 0.25 M NADH or 50 μl of 0.5 M sodium succinate was fitted to the top. The gas space was flushed with nitrogen gas for 30 sec before closing the stopcock. A kinetic scan was taken in the dual wavelength mode to measure the endogenous rate of cytochrome reduction. Reduction by substrate was started by rapidly plunging the substrate-containing mixer into the solution two times. The wavelength pairs (in nanometers) for peaks and isosbestic points were as follows: Cytochrome $b_1(\gamma)$, 430-420; cytochrome $b_1(\alpha)$, 561-550; cytochrome $a_1(\alpha)$, 596-588; cytochrome $a_2(\alpha)$, 629-619. At the 1 × concentration level of particle suspension, dithionite or 100% reduction gave the following optical absorbancies for a 1 cm light path: Cytochrome $b_1(\gamma)$, 0.24; cytochrome $b_1(\alpha)$, 0.04; cytochrome $a_1(\alpha)$, 0.002; cytochrome $a_2(\alpha)$, 0.0065.

Rates of oxygen uptake were measured as pre-

viously described using a Clark-type oxygen electrode (4).

RESULTS

Sensitivity of Succinoxidase Activity to Bovine Serum Albumin (BSA)

Fig. 1 shows that 0.1 mM BSA can completely inhibit the succinoxidase activity of a suspension of *E. coli* membrane-envelope fragments. The NADH oxidase activity, however, is still completely active. At 0.01 mM BSA concentration, succinoxidase activity was still completely inhibited, whereas succinate dehydrogenase was 90% active (1). The sensitivity of succinoxidase to BSA, however, is not typical of the average acellular *E. coli* preparation. Out of 20 different cell preparations, four possessed the behavior illustrated in Fig. 1. Three of these were from cells grown in glucose-PAB (Penn Assay Broth or Difco Antibiotic Medium No. 3) (4) and one from cells grown in a malate medium (4). Because the phenomenon is reproducible and the preparations are stable over long periods of storage in liquid nitrogen, it has been possible to learn something about the phenomenon.

When the particulate components of a sensitive preparation were separated from the soluble fraction by centrifugation at 105,000 *g* for 60 min it was found that these fragments, which accounted for virtually all of the succinoxidase activity, were no longer sensitive to BSA. The addition of the soluble fraction S_3 (105,000 *g* supernatant fraction [4]) to the resuspended pellet fully restored the sensitivity. The addition of S_3 from a sensitive preparation to any insensitive preparation invoked BSA sensitivity for the succinoxidase activity present (for example, see Table I). Boiling the active S_3 fraction for 5 min in pH 6.5 histidine buffer (4) totally destroyed activity (for invoking BSA sensitivity). The active principle passed through both Sephadex G25 and G100 with blue dextran, indicating a mol wt above 150,000.

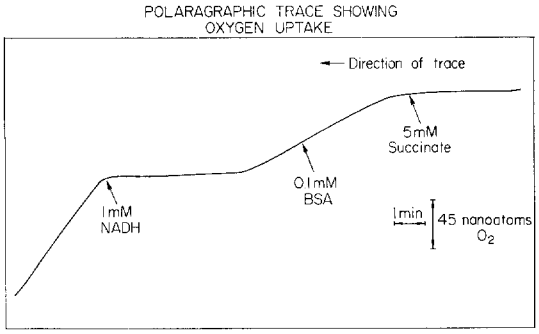


FIGURE 1 Polaragraphic trace showing oxygen uptake. Spheroplasts from malate-grown cells were sonicated and centrifuged at 3500 *g* for 10 min (4). The supernatant fraction (0.6 mg protein) plus 0.2 mg protein of S_3 (105,000 *g* supernatant fraction [4]) from BSA-sensitive glucose-grown cells were present in 1 ml of 0.01 M histidine buffer containing 0.002 M $MgSO_4$ at pH 6.8.

TABLE I
Succinoxidase Activity

	Before BSA	After BSA*	% of change
0.1 ml MP_2‡	5.5§	8.1	+45
0.1 ml MP_2	6.2	0	−100
0.1 ml S_3‖			
0.1 ml MP_2	6.2	7.1	+15
0.1 ml Boiled S_3			

* Bovine serum albumin present at 0.1 mM concentration.

‡ P_2 fraction (pellet obtained from 3500 *g* supernatant fraction by centrifugation 15 min at 20,000 *g* [4]), from malate-grown cells (0.21 mg protein) in 1 ml 0.01 M histidine buffer with 0.002 M $MgSO_4$ at pH 6.8.

§ Net rate of oxygen uptake in nanoatoms per minute. Endogenous rate of 2 was subtracted.

‖ S_3 fraction (supernatant fraction obtained by centrifugation at 105,000 *g* for 60 min [4]) from a BSA-sensitive preparation (0.4 mg protein).

Inhibition of NADH and Succinate Oxidases by Metal Chelators

The sensitivity of respiration to metal chelators has been taken as partial evidence in support of a direct role for nonheme iron-containing proteins in electron transport (5–10). We have tested four chelators which have been shown to be inhibitory to respiration in mammals, parasites, and bacteria. Figs. 2 and 3 show that both NADH and succinate oxidases from *E. coli* were markedly inhibited by all four chelators. By far the most potent inhibitor was 4,4,4-trifluoro-1,2(2-thienyl)-1,3-butane dione (TFTB). It was, therefore, decided to study directly the effects of TFTB on nonheme iron reduction and oxidation using electron paramagnetic resonance (EPR).

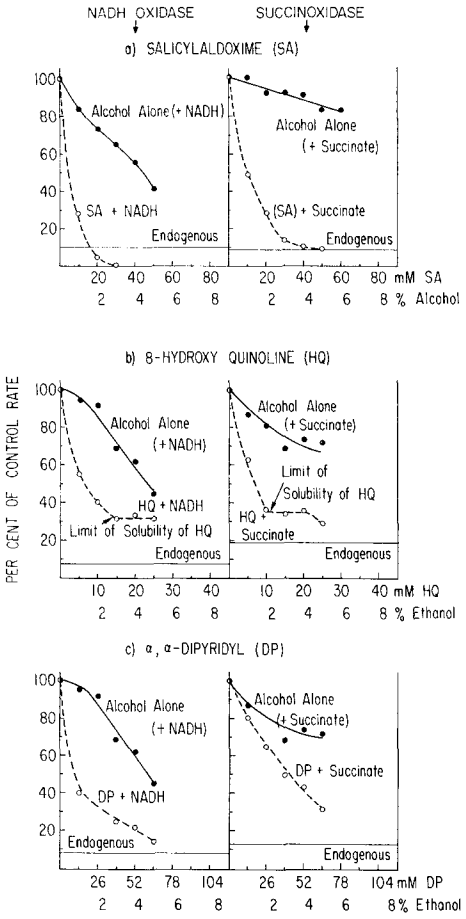


FIGURE 2 Metal chelator studies. Inhibition of NADH oxidase and succinoxidase activities by metal chelators. The 3500 *g* supernatant fraction (GS 1 [4]) from glucose-grown cells was used in 0.01 M histidine buffer containing 0.002 M $MgSO_4$ at pH 6.8. Oxidase activities were measured with a Clark oxygen electrode. For NADH oxidase assays, 0.44 mg protein/ml was present and the initial rate of uptake was 19 μatoms oxygen/10 min per 10 mg protein. For succinoxidase, 1.1 mg protein/ml was present and the initial rate of uptake was 2.2 μatoms oxygen/10 min per 10 mg protein. The ordinate shows per cent of initial rates. The chelators were introduced in ethanol. The concentration of both ethanol and chelator is shown along the abscissa. Inhibition due to ethanol alone is shown in each case. The inhibitors were reagent-grade chemicals purchased from Eastman Kodak (salicylaldoxime) and Fisher Scientific Co., Pittsburgh, Pa. (8-hydroxyquinoline and dipyridyl).

EPR Studies

Reduced nonheme iron, because of its unpaired electron, can be detected by electron paramagnetic resonance methods (11). This means of detection, however, is relatively much less sensitive than either polaragraphic measurements of oxygen uptake or spectrophotometric measurements of cytochrome reduction, and therefore higher cell fragment concentrations are required for analysis. As in the case of the cytochromes (2), we have found that the nonheme iron-containing proteins are concentrated in the membrane-envelope fractions. The usual preparation for EPR analysis was about 20 mg protein/ml. For routine polarographic measurements the suspension was diluted to about 0.3 mg protein/ml. The inhibition of oxidase activity shown in Fig. 3 *b* was at the higher concentration of particulate components which was about 70-fold greater than used for the experiments shown in Figs. 2 and 3 *a*. The inhibitor concentration for 50% inhibition of oxidase activity at the higher protein concentration was 10 times greater than at the lower protein concentration when NADH was the substrate, but only two times higher when

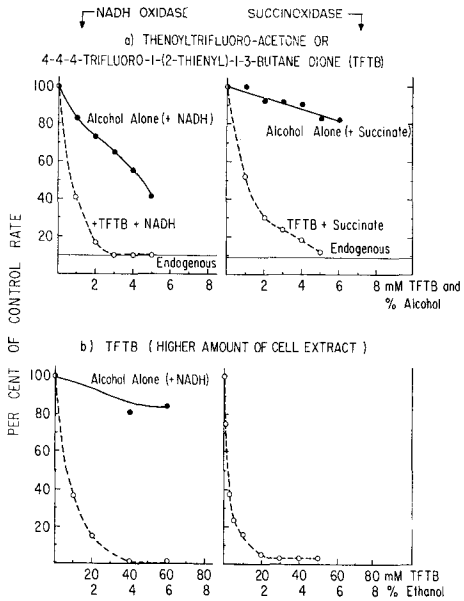


FIGURE 3 Metal chelator studies. The details described for Fig. 2 also apply to Fig. 3 *a*. For the experiments shown in Fig. 3 *b*, 18 mg protein/ml of the membrane-envelope fragments (described in Methods) in suspending medium were used. This is typical of the preparations used for the EPR studies and (at a concentration of about 5 mg protein/ml) for the cytochrome studies. NADH oxidase is shown on the left and succinoxidase on the right. The TFTB was Eastman Kodak Co. reagent grade.

succinate was the substrate. A concentration of 30 mM TFTB was capable of inhibiting more than 95% of the oxidase activity with either substrate at the higher protein concentration.

Beinert et al. have shown that an EPR signal at g = 1.94 is usually generated upon reduction of respiratory particles from a variety of mammalian and bacterial cells (11). The point of maximum deflection (the minimum of the spectrum) occurred at a g value greater than 1.94. This signal has been identified as arising from reduced nonheme iron. In the case of suspended membrane-envelope fragments from *E. coli*, we find the signal to occur at a slightly lower g value (see Fig. 4). This has been confirmed by examining the EPR spectra from both *E. coli* particles and rat liver mitochondria. Using calibrated tubes and known concentrations of *E. coli* envelope fragments, reduced with dithionite, it was established that the magnitude of the EPR signal with its trough at g = 1.92 was proportional to the amount of membranes. The time course of generation of signal from NADH or succinate

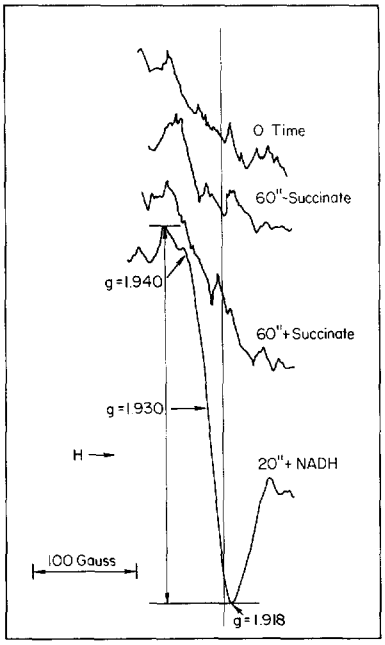


FIGURE 4 EPR spectra. *E. coli* membrane-envelope fragments in suspending medium at a concentration of 19 mg protein/ml examined for generation of an EPR signal during an incubation with either NADH or succinate. Experimental details described in Methods section.

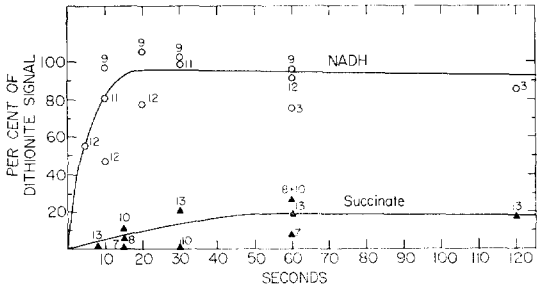


FIGURE 5 EPR signal generated by NADH or succinate. Rate of generation of EPR signal during incubation with either NADH or succinate. *E. coli* membrane-envelope fragments in suspending medium were used. The data shown were obtained from eight different experiments using four different preparations of *E. coli* fragments with the protein concentration ranging from 11 mg/ml to 19 mg/ml. The numbers shown refer to individual experiments.

was subject to appreciable variation as shown in Fig. 5. In spite of this variation from experiment to experiment, it became quite evident that NADH could evoke an EPR signal comparable in strength to that generated by dithionite, whereas succinate could produce only a fraction of this signal. The actual magnitude of the signal evoked with succinate is difficult to quantitate since the irregularities of the oxidized and slightly reduced curves make the precise locations for peak and trough uncertain (see Fig. 4). This situation is quite different than what we observed with rat liver mitochondria where succinate could evoke as strong a signal as NADH or dithionite and the build-up of signal was as fast as with NADH (data not shown).

Effects of TFTB on Reduction and Oxidation of Nonheme Iron

Fig. 6 (phase 1) shows that 30 mM TFTB prevented the generation of EPR signal by NADH. After the full EPR signal had been generated by NADH, the suspension was vigorously shaken in air for 60 sec to reoxidize the electron-transport chain (2). A subsequent 20 sec incubation allowed the regeneration of about 80% of the full signal (Fig. 6 [phase 3]). However, when TFTB was added before shaking in air and then 20 sec of incubation performed, a signal no larger than endogenous was developed. This shows that TFTB prevents reduction of oxidized nonheme iron, but not the oxidation of reduced nonheme

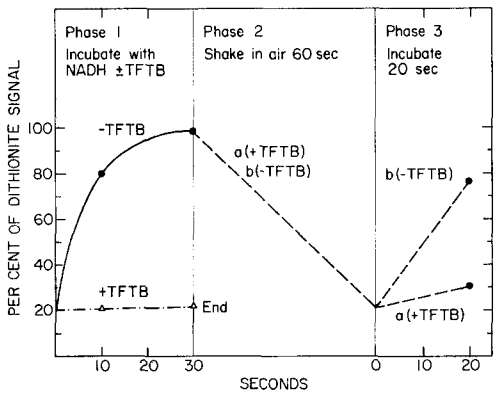


FIGURE 6 Effect of TFTB on reduction and oxidation of nonheme iron. 16 mg protein/ml of *E. coli* membrane-envelope fragments in suspending medium were used. TFTB when present was at 30 mM. In phase 1, an incubation of the fragments in the presence of 4.8 mM NADH ± TFTB was performed. For phase 2, two samples were preincubated for 30 sec in the absence of TFTB. One of these, *a*, was supplemented with TFTB and shaken in air for 60 sec. The other, *b*, was simply shaken in air for 60 sec. The procedure reoxidizes the nonheme iron. A subsequent incubation of 20 sec comprises phase 3. Case *a* shows that the reduced nonheme iron was reoxidized by air, even in the presence of TFTB. Residual NADH could re-reduce the nonheme iron in the absence of TFTB (case *b*) but not in its presence (case *a*).

iron. In the case of succinate, the same kind of experiment was inconclusive because of the small differences between endogenous and succinate-induced signals.

Reduction of Cytochromes by NADH and Succinate

The rates of cytochrome reduction were followed at the usual concentration of *E. coli* particles and also at the higher concentration used for the EPR studies. Data obtained at the lower (6 mg protein/ml) concentration are shown in Fig. 7 using NADH as substrate and in Fig. 8 using succinate as substrate. Both figures show that upon addition of substrate, the cytochromes are quickly reduced to a steady state level which is abruptly terminated when the oxygen tension is too low to remove electrons at the rate at which they are introduced by the substrate. Since NADH is oxidized much more rapidly than succinate, the end of the steady state occurs at a higher oxygen tension with NADH as substrate than with succinate. Although cytochrome reduction is slower with succinate as substrate the level of maximum reduction is comparable for both substrates. At the higher particle suspension concentration which was used for the EPR studies it was found that 63% of

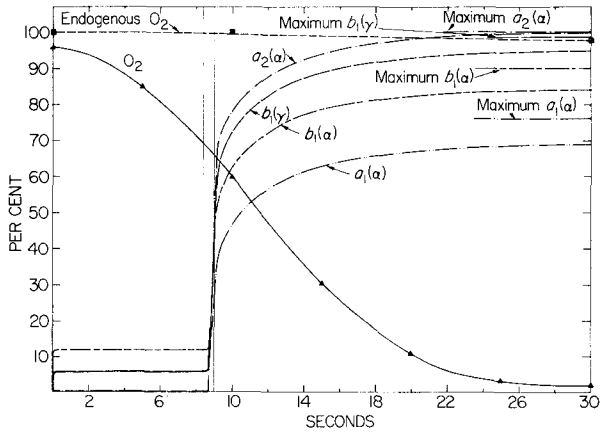


FIGURE 7 Cytochrome reduction with NADH (I× level). In Figs. 7–10, the kinetics of reduction of cytochromes a_1 (α), a_2 (α), b_1 (α and γ) are shown. The *E. coli* membrane-envelope fragments in suspending medium were present at a concentration of 6.1 mg protein/ml for the experiments shown in Figs. 9 and 10. The NADH concentration was 4 mM (Figs. 7 and 9) and the succinate concentration was 10 mM (Figs. 8 and 10). The figures show the steady-state levels of cytochrome reduction, the time at which the steady state ends, and the maximum levels of reduction attained for each cytochrome. The 100% level was attained by reduction with dithionite. Also shown are the rates of endogenous oxygen consumption and the rates in the presence of the substrate. These were determined in separate experiments with each of the cell-fragment suspensions by use of the Clark oxygen electrode. The actual oxygen tension in the suspension is probably lower than indicated at times shown because of the reponse delay of the electrode.

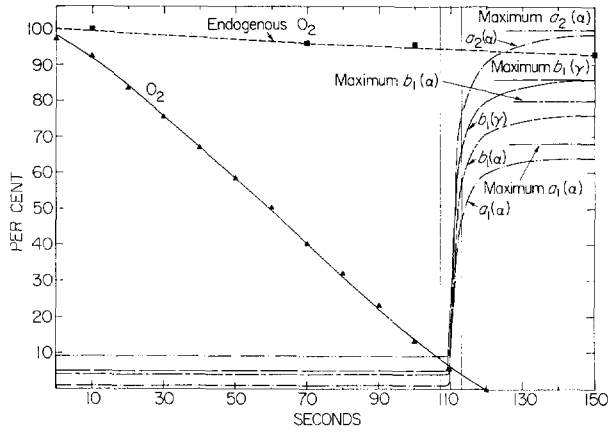


FIGURE 8 Cytochrome reduction with succinate (I× level). See explanation under Fig. 7.

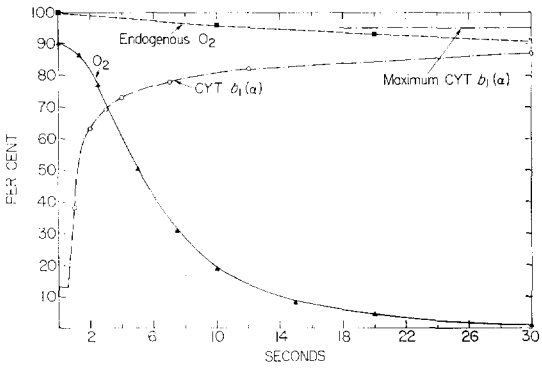


FIGURE 9 Cytochrome reduction with NADH (3.5× level). See explanation under Fig. 7.

cytochrome b_1 could be reduced in 2 sec by NADH (Fig. 9). The variability encountered in the EPR measurements makes a direct comparison between the extent of cytochrome and nonheme iron reduction at this early time interval undependable, but it does appear that a far smaller percentage of nonheme iron was reduced in 2 sec of incubation. At the higher concentration of *E. coli* particles, succinate reduced about 68% of cytochrome b_1 in 30 sec (Fig. 10). The EPR signal at this time was between 0 and 20% (Fig. 5).

Effects of TFTB and "BSA-Sensitive Soluble Factor" Plus BSA on Reduction and Oxidation of Cytochromes

At a concentration of 6 mg protein/ml of *E. coli* particles, 15 mM TFTB did not prevent the reoxidation of cytochromes reduced by NADH (Exp. 1, Table II) or succinate (Exp. 3, Table II). However, in the presence of 15 mM TFTB, reduction of cytochromes by NADH (Exp. 2, Table II) or succinate (Exp. 4, Table II) was markedly inhibited. In the absence of chelator, NADH caused cytochrome reduction in about 15 sec and succinate in about 4 min. BSA-sensitive S_3 fraction at a concentration of 0.8 mg/ml in the presence of 0.25 mM BSA did not prevent the reoxidation of cytochromes reduced by succinate (Exp. 5, Table II). In the presence of soluble factor and 0.25 mM BSA, the reduction of cytochromes by succinate was inhibited (Exp. 6, Table II). At any time when the reduction of the cytochromes by succinate was inhibited by the S_3 factor plus BSA, the addition of NADH caused complete reduction within 20 sec.

DISCUSSION

NADH and succinate oxidase activities are associated with the membranes of *E. coli*. Both activities appear to involve a single cytochrome chain (2). The inhibition of succinoxidase activity by bovine serum albumin and a required cell factor under conditions where succinate dehydrogenase was only slightly inhibited and NADH oxidase was uninhibited, indicated the participation of a sensitive component between succinate dehydrogenase and the cytochrome chain. A nonheme iron protein is believed to function between succinate dehydrogenase and cytochrome *b* in mammalian mitochondria (3). Metal chelators have been used by many investigators studying mammalian, bacterial, and parasitic preparations to show the dependency of electron transport on non-

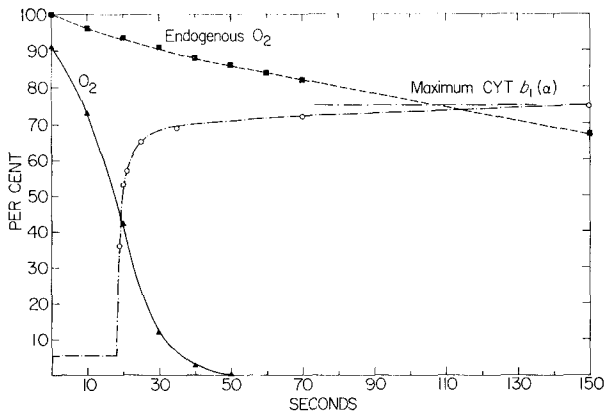


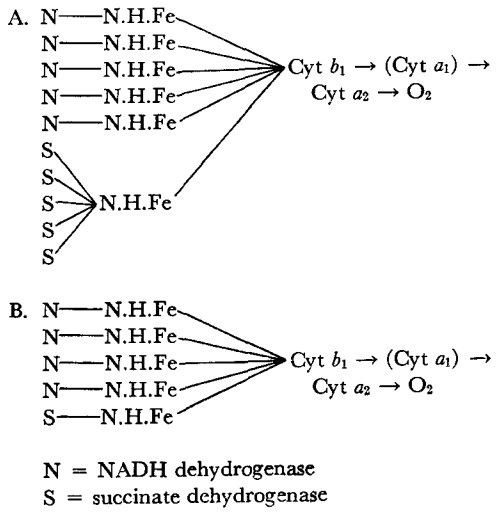
FIGURE 10 Cytochrome reduction with succinate (3.5× level). See explanation under Fig. 7.

heme-linked metals (5–10). An NADH oxidase preparation from *E. coli* was partially inhibited by the chelators *o*-phenanthroline and 2-2′ dipyridyl (8). Gutman et al. have presented evidence that nonheme iron is bound to NADH dehydrogenase and that it can be reduced by NADH (9).

We have shown that respiration with either succinate or NADH as substrate is markedly inhibited by each of four metal chelators. The most potent inhibitor, thenoyl trifluoroacetone (TFTB), inhibited the reduction of nonheme iron but not its oxidation.

TFTB also inhibited the reduction of all cytochromes by NADH or succinate. Once the cytochromes were reduced, however, TFTB could not inhibit their reoxidation. The effects of the BSA-sensitive soluble factor plus BSA on the reduction of the cytochromes by succinate and their subsequent reoxidation were analogous to those of the metal chelator (Table II). The soluble factor plus BSA could not inhibit the reduction of the cytochromes by NADH. These observations are consistent with but do not establish the hypothesis that the BSA-sensitive soluble factor plus BSA acts at the site of a nonheme iron complex involved in linking succinate dehydrogenase to the cytochrome chain.

We have also found that although NADH could produce as strong an EPR signal for reduced nonheme iron as dithionite, succinate could produce only a fraction of this signal (<20%). Since the total amount of cytochrome reduction is virtually the same with either substrate, we are considering the following two alternatives: (*a*) Electrons from many succinate dehydrogenases are funnelled through a limiting number of nonheme iron proteins. (*b*) More NADH dehydrogenase–nonheme iron units are linked to the cytochrome chain than succinate dehydrogenase–nonheme iron units. Two models compatible with these conclusions are shown below. At this time, we favor model B because, using the same electron acceptor, dichlorphenol indophenol, there is much more NADH dehydrogenase activity than succinate dehydrogenase activity (4).



N = NADH dehydrogenase
S = succinate dehydrogenase

The participation of cytochromes b_1, a_1, and a_2 in a single electron-transport chain available to both NADH and succinate is shown by (*a*) the ability of either substrate to reduce almost all of each cytochrome; (*b*) the fact that the duration of the steady state is the same for all three cytochromes when either substrate is present; (*c*) the fact that when the cytochromes have been reduced by succinate, an oxygen pulse can oxidize 50–60%

TABLE II

Effects of TFTB and BSA-Sensitive S_3 and BSA on Reduction and Oxidation of Cytochromes

Exp. No.	Substrate	Conditions	Per cent of reduction* Cyt b_1 (α)	Cyt b_1 (γ)	Cyt a_1 (α)	Cyt a_2 (α)
1	NADH	*a*) 60 sec incubation	100‡	100	100	100
		b) Bubble air 3 min	9	8	0	0
		c) 60 sec incubation	98	100	115	95
		d) 15 mM TFTB + bubbling air 2 min	7	8	0	0
2	NADH	*a*) 15 mM TFTB–incubation	0	0	0	0
		b) 15 mM TFTB–incubation 12 min (total time)	84	89	87	95
		c) Shake in air 60 sec	1	1	0	0
3	Succinate	*a*) 4 min incubation	100§	100	100	100
		b) Shake in air 60 sec	1	1	0	0
		c) 4 min incubation	96	96	100	96
		d) 15 mM TFTB–shake in air 60 sec	1	0	0	0
4	Succinate	*a*) 15 mM TFTB–incubation 40 min	0	0	0	0
		b) 15 mM TFTB–incubation 47 min (total time)	85	96	100	68
		c) Shake in air 60 sec	1.6	1	0	2
5	Succinate	*a*) 4 min incubation	100	100	100	100
		b) Shake in air 60 sec	1	1	0	0
		c) 4 min incubation	99	100	95	95
		d) +0.8 mg/ml BSA-sensitive S_3 + 0.25 mM BSA–shake in air 60 sec	0	0	0	0
6	Succinate	*a*) 0.8 mg/ml BSA-sensitive S_3 + 0.25 mM BSA–incubation 20 min	0	0	0	0
		b) 27 min incubation (total time)	98	100	94	110
		c) Shake in air 60 sec	0	0	0	0

* The cytochromes were measured by reduced vs. oxidized difference spectra and the wavelength pairs used were 430 and 412 nm for $b_1(\gamma)$, 560 and 540 nm for $b_1(\alpha)$, 593 and 578 nm for $a_1(\alpha)$, and 628 and 650 nm for $a_2(\alpha)$.

‡ The 100% level of reduction caused by NADH when related to the extent of reduction caused by dithionite was 99% for $b_1(\gamma)$, 90% for $b_1(\alpha)$, 76% for $a_1(\alpha)$, and 100% for $a_2(\alpha)$.

§ The 100% level of reduction caused by succinate when related to the extent of reduction caused by dithionite was 85% for $b_1(\gamma)$, 80% for $b_1(\alpha)$, 68% for $a_1(\alpha)$, and 100% for $a_2(\alpha)$.

Concentrations present were 6 mg protein/ml of *E. coli* fragments, 2 mM NADH, 5 mM succinate, 0.8 mg protein/ml BSA-sensitive S_3, 0.25 mM BSA. The buffer was suspending medium (2) at pH 7.0. The volume of each reaction mixture was 3.5 ml.

of each of the cytochromes in 6 msec.[2] The parenthesis around cytochrome a_1 indicates that, because of its small absorbance, we are less certain of its role in the electron-transport chain than of cytochrome b_1 or cytochrome a_2.

[2] Unpublished observations of R. W. Hendler, B. Chance, and M. Erecinska.

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