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MUTATIONS AFFECTING THE REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE DEHYDROGENASE COMPLEX OF *ESCHERICHIA COLI*

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SUMMARY

A strain carrying a point mutation affecting the NADH dehydrogenase complex of *Escherichia coli* has been isolated and its properties examined. The gene carrying the mutation (designated *ndh*) was located on the *E. coli* chromosome at about minute 23 and was shown to be cotransducible with the *pyrC* gene. Strains carrying the *ndh*$^-$ allele were found to be unable to grow on mannitol and to grow very poorly on glucose unless the medium was supplemented with succinate, acetate or casamino acids.

The following properties of strains carrying the *ndh*$^-$ allele were established which suggest that the mutation affects the NADH dehydrogenase complex but apparently not the primary dehydrogenase. Membrane preparations possess normal to elevated levels of D-lactate oxidase and succinate oxidase activities but NADH oxidase is absent. NADH is unable to reduce ubiquinone in the aerobic steady state and reduces cytochrome *b* very slowly when the membranes become anaerobic. NADH dehydrogenase, measured as NADH-dichlorophenolindophenol reductase is reduced but not absent. NADH oxidase is stimulated by menadione although not by Q-3 or MK-1 and in the presence of menadione, cytochrome *b* is reduced normally by NADH.

Further mutants affected in NADH oxidase were isolated using a screening procedure based on the growth characteristics of the original *ndh*$^-$ strain. The mutations carried by these strains were all cotransducible with the *pyrC* gene and the biochemical properties of the additional mutants were similar to those of the original mutant.

The properties of the group of *ndh*$^-$ mutants established so far suggest that they are affected in the transfer of reducing equivalents from the NADH dehydrogenase complex to ubiquinone.

INTRODUCTION

The NADH dehydrogenase complex** in mammalian mitochondria, certain yeast mitochondria and in *Escherichia coli* carries the first site of energy conservation

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** The NADH dehydrogenase itself together with the other respiratory carriers involved in electron transport and energy conservation between NADH and ubiquinone are referred to throughout this paper as the NADH dehydrogenase complex.

of the respiratory chain. A number of different approaches have been used to study the complex from different organisms. The NADH dehydrogenase of mammalian mitochondria has been solubilized and purified by a number of procedures [1]. EPR spectroscopy has been used to demonstrate the presence of multiple iron-sulphur centres in the NADH dehydrogenase of mitochondria from bovine heart and *Candida utilis* [2, 3]. The possible function of the iron-sulphur components in electron transport and in energy coupling has been studied using iron-limited or sulphate-limited cultures of *C. utilis* and *E. coli* [3, 4].

To date, no mutants have been described which are affected in the NADH dehydrogenase complex. In the present paper we describe the isolation and initial genetic and biochemical characterization of strains carrying mutations affecting the NADH dehydrogenase complex of *E. coli* K12.

MATERIALS AND METHODS

Bacterial strains. The strains used are described in Table I.

Chemicals. Q-3 and MK-1 were kindly donated by Dr O. Isler of F. Hoffmann-La Roche and Co., Basle, Switzerland.

Media. The mineral salts medium employed has been described elsewhere [5] and was used at normal strength except when growing cells for the preparation of membranes where double strength medium was used. Glucose and other supplements were sterilized separately and were added where appropriate, at the following concentrations: 30 mM glucose, 30 mM mannitol, 30 mM succinate as sole source of carbon or 4 mM when used with glucose, 4 mM acetate, 0.1 % Casamino acids, 0.2 mM L-methionine, 0.2 mM uracil, 0.15 mM adenine-HCl, 1 mM L-proline, 0.15 mM L-histidine HCl, 0.3 mM L-isoleucine, 0.3 mM L-valine, 0.2 mM L-tryptophan and

TABLE I

STRAINS OF *E. COLI* K12 USED

Strain	Sex	Relevant genetic loci[a]	Source or other information[b]
AN589	Hfr	*metB*$^-$, *ndh-401*	Derived from AB3311 using NTG[c]
AN591	F$^-$	*ilv*$^-$, *str*^{R}, *ndh-401*	Recombinant from AN589 × AN595
AN592	F$^-$	*ilv*$^-$, *str*^{R}, *pyrC*$^-$	Derived from AN591 by transduction
AN594	F$^-$	*ilv*$^-$, *str*^{R}	
AN595	F$^-$	*his*$^-$, *ilv*$^-$, *trp*$^-$, *str*^{R}	
AB3311	Hfr	*metB*$^-$	
RE110	F$^-$	*pyrD*$^-$	
30SO-U6	Hfr	*pyrC*$^-$	CGSC 5153
AT1325	F$^-$	*proA*$^-$, *his*$^-$, *purB*$^-$	A. Taylor
KLF23/181	F′	F123/*pyrD*$^-$, *his*$^-$, *trp*$^-$, *recA1*	CGSC 4256
KLF26/181	F′	F126/*pyrD*$^-$, *his*$^-$, *trp*$^-$, *recA1*	CGSC 4253
AB259	Hfr		Hfr Hayes
KL16	Hfr		

[a]Genetic nomenclature is that used by Bachmann et al. [15].
[b]CGSC indicates that the strain was obtained from the Coli Genetic Stock Center, New Haven, Connecticut.
[c]NTG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.

1 μM thiamine. Mannitol-bromothymol blue plates consisted of Luria broth [6] pH 7.5, containing mannitol (30 mM), agar (2 %) and bromothymol blue (0.001 %).

Growth tests. Growth tests were carried out under aerobic conditions using 125 ml flasks which contained 10 ml of growth medium and which were shaken at 37 °C in a water bath. The flasks were fitted with a sidearm which allowed the turbidity of the culture to be followed using a Klett-Summerson colorimeter fitted with a blue filter.

Isolation of mutants lacking NADH oxidase. Strain AN595 was mutagenized using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [7] and the cells grown overnight in minimal medium containing glucose (6 mM) and Casamino acids (0.1 %) to allow phenotypic expression. The cells were then harvested by centrifugation, washed once with basal medium and resuspended to a Klett of 40 (approx. 10^8 cells/ml) in mannitol-minimal medium. This culture was shaken at 37 °C until one division had occurred then diluted 1/100 in mannitol-minimal medium containing 300 U/ml of benzylpenicillin and shaken at 37 °C for a further 16 h. Samples of the penicillin treated culture were plated in agar onto plates of mannitol-minimal medium and after incubation at 37 °C for 3 days all colonies were marked and the plates overlaid with glucose plus succinate. Any colonies which appeared during a further three days incubation were examined further and strains isolated which were able to grow on glucose plus succinate and glucose plus acetate plates but not on mannitol-minimal plates and which fermented mannitol slowly on mannitol-bromothymol blue plates. The screening was initially carried out by replica plating and the growth characteristics of promising strains confirmed by plating for single colonies. Mutant strains of the desired phenotype were then lysed using the freeze-thaw-lysozyme method (described below) and the lysates examined for NADH oxidase.

Genetic techniques. Methods described elsewhere were followed for bacterial matings [8], transduction experiments using the generalized transducing phage P1Kc [9], and the isolation of strains resistant to nalidixic acid [5]. For matings using *recA*$^-$ F-prime strains as donors the following method was employed.

The female recipient strain was grown overnight in glucose-minimal medium, the cells harvested by centrifugation and resuspended in one-tenth the original volume of basal medium. A sample (0.1 ml) of this suspension was spread onto the surface of the appropriate selective medium. Single colonies of the F-prime strains were streaked onto complete medium as patches about 1 cm square, incubated until a light growth had been achieved and printed first onto the selective medium alone and then onto the plate with the female strain. After 1–2 days near confluent patches of recombinants indicated that the gene transferred was carried on the episome.

Preparation of membranes and assay of membrane components. Methods which have been described elsewhere were followed for the growth of cells and preparation of membranes, the estimation of ubiquinone and menaquinone, cytochromes, oxidase rates and the degree of reduction of ubiquinone (Wallace, B. J. and Young, I. G., in preparation), FAD and FMN [10] and protein concentrations [11]. NADH-dichlorophenolindophenol reductase activity was measured at 30 °C by following the rate of reduction of the absorbance at 600 nm (extinction coefficient, $\varepsilon_{600} = 16\,000$). The reaction mixture consisted of 2.5 ml STM buffer*, 50 μl freshly

* STM buffer: sucrose, 0.25 M/*N*-Tris-(hydroxymethyl)methyl-2-aminoethanesulphonic acid, 0.1 M/magnesium acetate, 0.02 M, adjusted to pH 7.5 with 10 M NaOH.

prepared dichlorophenolindophenol (10 mM) and 5 μl membranes (about 0.3 mg protein) and the reaction was started by the addition of 20 μl NADH (0.1 M). For each membrane preparation the rate obtained was corrected for the endogenous rate which occurred in the absence of NADH and for the rate of nonenzymic reduction of dichlorophenolindophenol by NADH which occurred in the absence of membranes. The rates obtained were approximately linear with time and were proportional to membrane concentration.

Freeze-thaw-lysozyme method for lysing cells. The method used was a modification of the method of Ron et al. [12] and was as follows. Strains were grown at 37 °C in 25 ml of glucose-minimal medium supplemented with Casamino acids (see Methods) to cell densities corresponding to Klett readings of 150–200. Cells were collected by centrifugation and resuspended in 0.3 ml of STM buffer containing lysozyme (Sigma) at 1 mg/ml. The cell suspensions were twice frozen and thawed after which 2.5 μl of deoxycholate solution (10 %) and 2.5 μl of deoxyribonuclease (Sigma, 5 mg/ml) was added. 100 μl samples of lysates were used to test for NADH oxidase.

Reduction of cytochrome b by NADH. Experiments were performed at 25 °C with an Aminco Chance dual wavelength spectrophotometer using 5 % transmission, a spectral bandpass of 1.1 nm and settings of 558 nm and 573 nm for the sample and reference beams respectively. In each case the cuvette contained 2.8 ml STM buffer, 0.2 ml AN591 (*ndh*$^-$) membranes and, where indicated, 3 μl of 125 mM ethanolic menadione. The membrane suspension was aerated and the beams balanced. The substrate, 20 μl of 0.1 M NADH, was then added and the reduction of cytochrome *b* followed.

RESULTS

The first mutant isolated (AN589), which was deficient in NADH oxidase, was found amongst mutant strains growing poorly on succinate as sole carbon source. This strain also grew poorly on plates of glucose-minimal medium. It was important to establish a phenotype for mutants lacking NADH oxidase to facilitate the isolation of further mutants and to allow genetic analysis. Inspection of the pathway for the catabolism of mannitol [13] suggested that mutants lacking NADH oxidase might be unable to grow on mannitol. This is because the conversion of mannitol-1-phosphate to fructose-6-phosphate generates one molecule of NADH which is not reoxidized in the subsequent metabolism of fructose to lactate and which is presumably oxidized via NADH oxidase in a wild type cell. The mutant lacking NADH oxidase was indeed found to be unable to grow on mannitol as sole carbon source. A number of transductants and spontaneous revertants were isolated which had regained the ability to grow on mannitol and in each case they had regained normal levels of NADH oxidase. This suggested that strain AN589 probably carried a single point mutation affecting NADH oxidase. The gene carrying this mutation was designated *ndh* as further work indicated that the mutation affected the NADH dehydrogenase complex.

Genetic analysis of the NADH oxidase mutant

The phenotype used for scoring strains lacking NADH oxidase in genetic experiments was growth on glucose plus succinate but not on mannitol as sole carbon source. In order to get a preliminary location of the *ndh* gene a streptomycin-resistant

derivative of strain AN589 was converted to a female phenocopy and used as a recipient in "time of entry" experiments using *nalA*^{R} derivatives of the Hfr strains AB259 and KL16. In both cases the times of entry obtained for the *nalA*^{R} allele were very much slower than expected but in each case the *ndh* gene behaved as though it was anticlockwise from the *nalA* gene (minute 48) in about the 10–30 minute segment of the chromosome.

To facilitate further mapping of the *ndh* gene, the *ndh*$^-$ allele carried by strain AN589 was transferred to strain AN595 (F$^-$, *his*$^-$, *trp*$^-$, *ilv*$^-$, *str*^{R}) by uninterrupted mating. Recombinants (*his*$^+$, *trp*$^+$ or *ilv*$^+$) were isolated and one of the *his*$^+$ recombinants (AN591) was found to be *ndh*$^-$. It was verified that strain AN591 lacked NADH oxidase and this strain was used as a recipient for matings with F-prime strains. It was found that the *ndh* gene was carried on the F126 episome (strain KLF26/181) but not on the F123 episome (strain KLF23/181) indicating that the *ndh* gene was in the segment of the chromosome between minute 17 and minute 27 [15].

The generalised transducing phage P1Kc was used to test cotransduction between *ndh* and a variety of markers present in this region. The *ndh* gene was found to be cotransducible with *pyrC* at a frequency of 3–9 % depending on the marker selected (Table II) which is consistent with the *ndh* gene being located about 1 minute away from *pyrC*. No cotransduction was detected between *ndh* and the *purB* or *pyrD* genes which are located clockwise and anticlockwise respectively from *pyrC*. Since *pyrC* and *purB* have been reported to be cotransducible at a frequency of 2–4 % [14] then the above data suggests that the *ndh* gene lies between *pyrD* and *pyrC* and that these genes may be separated by slightly more than the 2 minutes shown on the map of Bachmann et al. [15].

Physiology of the NADH oxidase mutant

Studies of the effect of the loss of NADH oxidase on growth in liquid medium were made using the recombinant strain AN591. As well as being unable to grow on mannitol-minimal medium, strain AN591 grew very poorly on glucose-minimal medium but could grow at rates close to those of the *ndh*$^+$ strain (AN592) if the glucose-minimal medium was supplemented with either Casamino acids (0.1 %), succinate (4 mM) or acetate (4 mM). These growth requirements presumably reflect defective operation of the tricarboxylic acid cycle in the absence of NADH oxidase.

The effect of menadione, which was found to partially restore NADH oxidase in membrane preparations of the mutant (see below), was also examined using whole cells. Menadione (20 μM) noticeably stimulated the growth of AN591 on both

TABLE II

TRANSDUCTION DATA FOR THE *ndh* GENE

Donor strain	Recipient strain	Marker selected	Unselected marker frequencies
30SO-U6 (*pyrC*$^-$)	AN591 (*ndh*$^-$)	*ndh*$^+$	2/80 *pyrC*$^-$
AN591 (*ndh*$^-$)	AN592 (*pyrC*$^-$)	*pyrC*$^+$	15/160 *ndh*$^-$
RE110 (*pyrD*$^-$)	AN591 (*ndh*$^-$)	*ndh*$^+$	0/80 *pyrD*$^-$
AT1325 (*purB*$^-$)	AN591 (*ndh*$^-$)	*ndh*$^+$	0/46 *purB*$^-$

glucose- and mannitol-minimal medium but the growth rate (generation time about 2.5 h) was still much less than that of AN592 (*ndh*$^+$) (generation time about 1 h). The growth yield of AN591 on glucose or mannitol in the presence of menadione (20 μM) was 85–90 % of that of AN592.

Biochemical properties of the NADH oxidase mutant

Respiratory membranes were prepared from cells of strain AN591 (*ndh*$^-$) grown into late logarithmic phase on glucose-minimal medium supplemented with succinate (4 mM) and Casamino acids (0.1 %). NADH oxidase was absent, but good levels of succinate and D-lactate oxidase were present (Table III). Membranes prepared from cells of AN591 grown into early logarithmic phase using glycerol as carbon source also lacked NADH oxidase.

TABLE III

OXIDASE RATES IN MEMBRANES FROM *ndh*$^+$ AND *ndh*$^-$ STRAINS

Substrate	Addition*	Oxidase rates (ng atom O · min^{-1} · mg protein^{-1})	
		AN591 (*ndh*$^-$)	AN592 (*ndh*$^+$)
NADH		<10	670
	Q-3	<10	
	MK-1	<10	
	menadione	290	
D-lactate		250	260
Succinate		250	360

* Q-3, MK-1 and menadione were added in 3 μl of ethanol to give final concentrations of 48 μM, 48 μM, and 150 μM, respectively.

Comparison of the quinone levels in glucose-grown cells of AN591 (*ndh*$^-$) and AN592 (*ndh*$^+$) indicated that the *ndh*$^-$ strain was not affected in the synthesis of ubiquinone or menaquinone. Similarly the levels of FAD, FMN and cytochromes *b* and *o* in membranes from AN591 were shown to be comparable to the levels present in membranes of AN592 (data not shown).

Since electron transport to oxygen from succinate and D-lactate was not impaired in AN591 membranes (Table III) this indicated that the lesion in the oxidation of NADH was in the NADH dehydrogenase complex and not in the common portion of the respiratory chain of *E. coli* (ref. 4 and Wallace, B. J. and Young, I. G., in preparation). In order to confirm the site of the lesion, the ability of membrane preparations of the *ndh*$^-$ strain to carry out electron transport from NADH to cytochrome *b* and from NADH to ubiquinone was measured as described below.

The kinetics of reduction of cytochrome *b* with NADH as substrate were compared using membranes from AN591 (*ndh*$^-$) and AN592 (*ndh*$^+$). In the case of strain AN592, the addition of NADH to an aerobic membrane suspension resulted in the reduction of a small proportion of the cytochrome *b* during the aerobic steady state (Fig. 1). The membrane suspension soon became anaerobic and the majority of the cytochrome *b* was then reduced. With membranes from AN591 (*ndh*$^-$) the aerobic

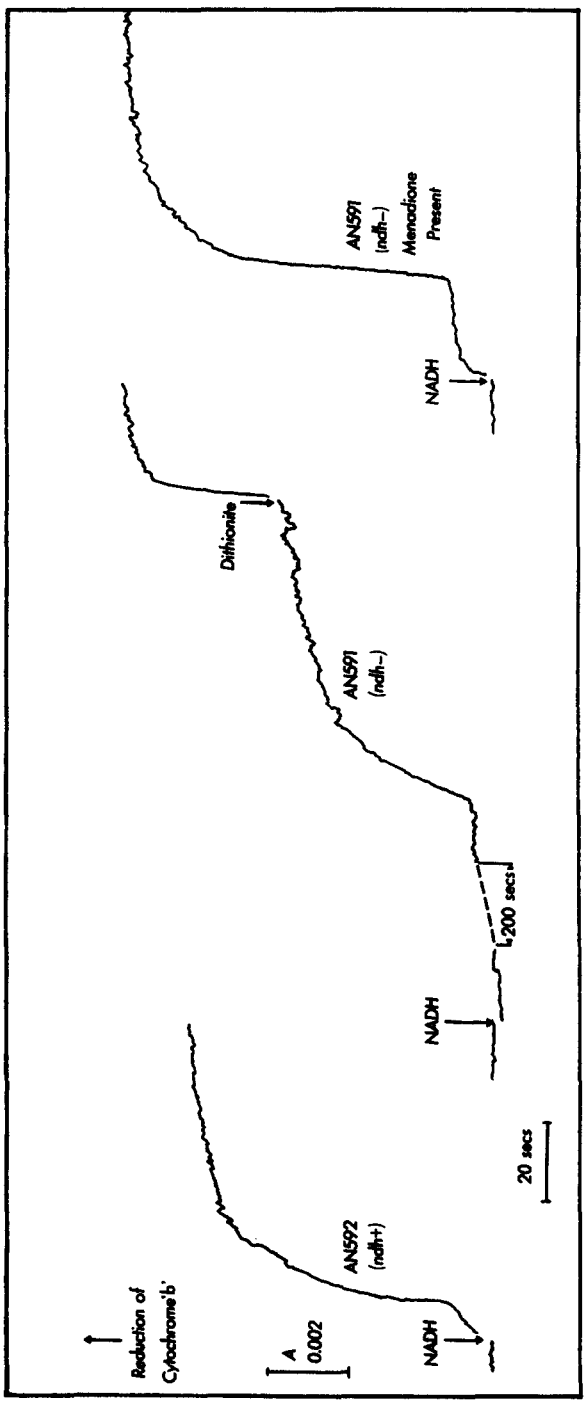

Fig. 1. Reduction of cytochrome *b* by NADH in membranes of AN591 (*ndh*$^-$) and AN592 (*ndh*$^+$). Experiments were performed as described in Methods and the arrows indicate when the addition of substrates were made to the aerobic membrane suspensions.

TABLE IV

REDUCTION OF UBIQUINONE BY NADH IN AEROBIC MEMBRANE SUSPENSIONS OF AN591 (*ndh*$^-$) AND AN592 (*ndh*$^+$)

The method used for determination of the % ubiquinone reduced in the aerobic steady state with NADH as substrate has been described elsewhere (Wallace, B. J. and Young, I. G. (1976), in preparation).

Substrate	% ubiquinone reduced	
	AN591 (*ndh*$^-$)	AN592 (*ndh*$^+$)
Endogenous	3	2
NADH	2	55

steady state was greatly prolonged because of the very low NADH oxidase activity of these membranes. During this period a slow reduction of a small proportion of the cytochrome *b* occurred and when the suspension eventually became anaerobic the reduction of the rest of the cytochrome *b* was considerably slower than that observed with AN592 (*ndh*$^+$) and was noticeably biphasic. This behaviour is consistent with the lesion in electron transport in the *ndh*$^-$ strain being prior to cytochrome *b*. It was also shown that aerobic membrane suspensions from the *ndh*$^-$ strain, in contrast to similar preparations from the *ndh*$^+$ strain, were unable to reduce ubiquinone using NADH as substrate (Table IV). This finding provides additional confirmation that the block in electron transfer in the *ndh*$^-$ strain is in the NADH dehydrogenase complex.

Several short chain quinone analogues were tested for their ability to stimulate NADH oxidation in the *ndh*$^-$ strain. Neither Q-3 nor MK-1, the short chain homologues of the naturally occurring quinones formed by *E. coli*, showed any activity but menadione was found to be active (Table III). In the presence of menadione, cytochrome *b* was reduced normally (Fig. 1).

The restoration of NADH oxidation by menadione suggested that the primary dehydrogenase of the NADH dehydrogenase complex might not be completely inactivated in strain AN591 but that the lesion could be later in the sequence of electron transfer and possibly at the normal site of interaction with ubiquinone. Accordingly, NADH dehydrogenase activity was measured using dichlorophenolindophenol as acceptor. The *ndh*$^-$ strain showed 28 % of the dichlorophenolindophenol reductase activity with NADH as substrate of the *ndh*$^+$ strain AN592 which gave 680 nmol dichlorophenolindophenol reduced/min/mg protein.

Isolation of further mutants affected in NADH oxidase

A procedure was developed (see Methods), based on the growth characteristics of the original mutant to enable further mutants affected in the NADH dehydrogenase complex to be isolated. Briefly, AN595 was mutagenized using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and then the cells incubated in mannitol-minimal medium in the presence of penicillin to select against cells capable of growth on mannitol as sole carbon source. Some of the remaining cells were then screened and mutant strains isolated which were unable to grow on minimal medium with mannitol as carbon source but which could grow with glucose plus succinate or glucose plus acetate and in

addition were capable of slow fermentation of mannitol on mannitol-bromothymol blue plates. The use of the latter plates enabled strains affected directly in the utilization of mannitol to be eliminated and the criterion of growth on both glucose plus succinate and glucose plus acetate, allowed the elimination of mutants affected in various reactions of the tricarboxylic acid cycle which respond to either acetate or succinate but not to both [16]. Those mutants having the appropriate phenotype were then examined for NADH oxidase using the freeze-thaw-lysozyme method. Of 54 strains examined, 20 were found to be affected in NADH oxidase and the cell smashes of these strains were retested for NADH oxidase in the presence of Q-3 in order to test whether any of these strains were ubiquinone-deficient. Eight of the original 20 strains showed reconstitution of NADH oxidase in the presence of Q-3 and these were confirmed to be "leaky" ubiquinone mutants by quinone estimation of whole cells. The remaining 12 mutants were shown to be unaffected in ubiquinone synthesis.

Genetic and biochemical properties of the additional ndh⁻ mutants

Phage preparations were grown on 11 of the new isolates and used to transduce *pyrC*$^+$ into strain AN592. The mutations carried by these strains were all found to be cotransducible with *pyrC* at a frequency of about 8 % suggesting that these mutations and that carried by the original *ndh*$^-$ mutant are closely linked. The twelfth mutant was not examined as this strain proved to be phage resistant.

Respiratory membranes were also prepared from each of the new isolates and it was found that their properties were generally similar to those of the original mutant. In each strain, D-lactate and succinate oxidase were present and NADH oxidase was absent but was stimulated by menadione. In no strain was NADH-dichlorophenol-indophenol reductase activity completely absent. Further studies of these mutants are currently in progress.

DISCUSSION

The isolation of mutants affected in the NADH dehydrogenase complex of *E. coli* opens up another approach to the study of the site one region of the respiratory chain of *E. coli* and possibly other microorganisms. Based on the properties of the class of mutants obtained it may be possible to devise different selection procedures so that mutants affected in other components of the NADH dehydrogenase complex can be isolated. In the present study it is puzzling that no mutants were obtained which were lacking the primary dehydrogenase. This could be explained on the basis of the low number of mutants isolated thus far or in terms of the suggestion that there may be two NADH dehydrogenases in *E. coli* [4].

The *ndh*$^-$ mutants obtained all behave like ubiquinone-deficient mutants (Wallace, B. J. and Young, I. G., in preparation) with respect to NADH oxidase but like wild type strains with respect to D-lactate and succinate oxidation. They appear to be affected in the terminal stages of electron transfer within the NADH dehydrogenase complex possibly in the component which normally transfers electrons to ubiquinone. Menadione appears to overcome the block in electron transfer in these strains and may transfer reducing equivalents to ubiquinone or directly to the *b* cytochromes. A detailed understanding of these mutants, however, awaits further work.

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REFERENCES

1 Huennekens, F. M. and Mackler, B. (1971) in Electron and Coupled Energy Transfer in Biological Systems (King, T. E. and Klingenberg, M., eds.), Vol. 1, pp. 135–157, M. Dekker Inc., New York
2 Orme-Johnson, N. R., Hansen, R. E. and Beinert, H. (1974) J. Biol. Chem. 249, 1922–1927
3 Ohnishi, T. (1973) Biochim. Biophys. Acta 301, 105–128
4 Poole, R. K. and Haddock, B. A. (1975) Biochem. J. 152, 537–546
5 Stroobant, P., Young, I. G. and Gibson, F. (1972) J. Bacteriol. 109, 134–139
6 Luria, S. E. and Burrous, J. W. (1957) J. Bacteriol. 74, 461–476
7 Adelberg, E. A., Mandel, M. and Chen, G. C. (1965) Biochem. Biophys. Res. Commun. 18, 788–795
8 Taylor, A. L. and Thoman, M. S. (1964) Genetics 50, 659–677
9 Pittard, J. (1965) J. Bacteriol. 89, 680–686
10 Udenfriend, S. (1962) Fluorescence Assay in Biology and Medicine, p. 241, Academic Press, New York
11 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
12 Ron, E. Z., Kohler, R. E. and Davis, B. D. (1966) Science 153, 1119–1120
13 Solomon, E. and Lin, E. C. C. (1972) J. Bacteriol. 111, 566–574
14 Signer, E. R., Beckwith, J. R. and Brenner, S. (1965) J. Mol. Biol. 14, 153–166
15 Bachmann, B. J., Brooks Low, K. and Taylor, A. L. (1976) Bact. Rev. 40, 116–167
16 Herbert, A. A. and Guest, J. R. (1968) J. Gen. Microbiol. 53, 363–381