The Purification and Properties of the Respiratory-Chain Reduced Nicotinamide–Adenine Dinucleotide Dehydrogenase of *Torulopsis utilis*

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1. An NADH–ferricyanide reductase activity has been isolated from the respiratory chain of *Torulopsis utilis* by using detergents. The isolated enzyme contains non-haem iron, acid-labile sulphide and FMN in the molar proportions 27.5:28.4:1. The preparation is free of FAD and largely free of cytochrome. 2. The enzyme catalyses ferricyanide reduction by NADPH at about 1% of the rate with NADH, and reacts poorly with acceptors other than ferricyanide. The rates of reduction of some acceptors are, as percentages of the rate with ferricyanide: menadione, 0.35%; lipate, 0.01%; cytochrome *c*, 0.065%; dichlorophenolindophenol, 0.35%; ubiquinone-1, 0.08%. 3. Several properties of submitochondrial particles of *T. utilis* (non-haem iron, acid-labile sulphide, FMN and an NADH-reducible electron-paramagnetic-resonance signal) were found to co-purify with the NADH–ferricyanide reductase activity. Thus about 70% of the FMN and, within the limits of accuracy of the experiments, 100% of the non-haem iron and acid-labile sulphide of submitochondrial particles derived from *T. utilis* cells grown under conditions of glycerol limitation (but relatively low iron availability) can be attributed to the NADH–ferricyanide reductase. 4. It was also shown that the component of submitochondrial particles specifically bleached at 460 nm by NADH [species 1 of Ragan & Garland (1971)] co-purifies with the NADH–ferricyanide reductase. 5. This successful purification of an NADH dehydrogenase from *T. utilis* forms a starting point for investigating the molecular properties of phenotypically modified mitochondrial NADH oxidation pathways that lack energy conservation between NADH and the cytochromes.

Several preparations of mitochondrial NADH dehydrogenase from mammalian sources have been reported (Mahler, Sarkar, Vernon & Alberty, 1952; De Bernard, 1957; Mackler, 1961; King & Howard, 1962; Ringler, Minakami & Singer, 1963; A. G. Chapman & V. Jagannathan, personal communication; Pharo, Sordahl, Edelhoef & Sanadi, 1968; Hatefi & Stempel, 1967). Despite the extensive literature on these enzymes, neither the sequence of redox carriers acting between NADH and cytochrome *b*, nor the factors involved in energy conservation at site I (Lehninger, 1964), have been determined. This lack of success can be ascribed to the lability of this region of the respiratory chain, the ease with which artifactual activities may be created during solubilization (e.g. Singer & Gutman, 1970) and the absence of analytical methods by which redox changes of non-haem iron proteins and

flavoproteins can be unambiguously distinguished (Garland, Clegg, Light & Ragan, 1969). This latter difficulty has prevented adequate comparison of the isolated dehydrogenases with the intact respiratory chain. Iron or sulphate limited growth of the yeast *Torulopsis utilis* leads to loss of site I energy conservation and loss of sensitivity to the respiratory inhibitors rotenone or piericidin A (Light, Ragan, Clegg & Garland, 1968; Light & Garland, 1971) as well as to loss of mitochondrial non-haem iron and acid-labile sulphide (Clegg *et al.* 1969; Clegg & Garland, 1971; Haddock & Garland, 1971). Spectroscopic modification of the electron transport particles accompanying these phenotypic changes enabled us to characterize more closely the nature of the respiratory carriers involved in the oxidation of NADH, in the site I energy conservation and in the inhibition by piericidin A (Ragan, Clegg, Haddock, Light & Garland, 1970; Ragan & Garland, 1971). The obvious extension of this work was to attempt an isolation of these carriers from the different phenotypes. In this paper, we report the

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isolation and properties of a mitochondrial NADH dehydrogenase from *T. utilis* grown under glycerol-limited conditions, when the mitochondria conserve energy at site I and are sensitive to rotenone and piericidin A. A particular advantage of using continuous culture of *T. utilis* in these studies arises from the fact that although one growth nutrient alone was limiting for growth (in this case, glycerol), the concentration of another nutrient in the culture could be decreased until it was nearly limiting. Use of low but not quite growth-limiting concentrations of iron in the culture decreased the concentration of mitochondrial non-haem iron to relatively low values, which are thought to exclude functionally redundant or superfluous non-haem iron which may make up the bulk of the mitochondrial non-haem iron under the usual conditions of extensive iron supplementation (Clegg & Garland, 1971).

MATERIALS AND METHODS

Continuous culture conditions for the production of glycerol-limited cells were as described by Light & Garland (1971). The entering iron and glycerol concentrations were 2 μM and 70 mM respectively. Electron-transport particles (ETP) were prepared either from whole cells (when they are referred to as ETP_c) or from isolated mitochondria (ETP_m) as described by Clegg & Garland (1971). Acid-labile sulphide was measured as described by King & Morris (1967). Iron in the purified enzyme was assayed by the total-iron method of Doeg & Ziegler (1962), except that 10% (w/v) hydroxylamine hydrochloride replaced the 5% (v/v) thioglycolic acid. Non-haem iron in ETP preparations was extracted with trichloroacetic acid and dithionite as described by Kurup & Brodie (1967) and then assayed as described above. Flavin was extracted with 10% (w/v) trichloroacetic acid, with or without prior digestion with trypsin, and assayed in the neutralized extracts either by the extinction change at 450 nm–530 nm on reduction with dithionite, or fluorimetrically by the method of Burch (1957). An ϵ value of $9.8001 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for the oxidized-minus-reduced difference at 450 nm–530 nm was used (V. Massey & B. E. P. Swoboda, personal communication). Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), except with samples that contained high concentrations of Triton X-100, which causes precipitation with the Folin-Ciocalteu reagent, and spurious colour development. Under these circumstances, the microbiuret assay of Itzhaki & Gill (1964) was used. The precipitate formed by Triton X-100 in the assay did not interfere with colour development, and could be removed by filtration through a 2.5 cm diameter filter of 0.40 μm pore size.

Spectrophotometric measurements were performed with a dual wavelength or a wavelength scanning spectrophotometer constructed in general as described by Chance & Legallais (1951) and Yang & Legallais (1951) respectively. The optical light-path of the cuvettes was 1 cm. Measurements of flavoprotein fluorescence were made with a modified Eppendorf fluorimeter (Garland, Shepherd & Yates, 1965) by using a 10 nm bandwidth interference filter

centred at 456 nm to obtain the exciting light from a 100 W tungsten lamp. The secondary filter cut-off was at 520 nm and below. Electron-paramagnetic-resonance spectroscopy was performed with a Varian 4500-10A instrument, operating at a microwave power and a frequency of 7.5 mW and 9.3 GHz respectively, and a temperature of 77°K. The modulation amplitude was 5 G.

NADH–ferricyanide reductase was assayed at 22°C by the decrease in extinction at 420 nm–500 nm. To 2.0 ml of 10 mM-tris chloride, pH 7.4, were added 5 μl of 0.25 M-potassium ferricyanide and a suitable concentration of enzyme. The reaction was started by the addition of 10 μl of 25 mM-NADH. The presence of 10 μg of antimycin A did not affect the reaction. The rate was linear for a change in extinction of up to 0.2. The concentration of the enzyme was usually chosen to give a change in extinction of approximately 0.1/min. Enzyme specific activity is expressed as μmol of ferricyanide reduced/min per mg of protein. NADH–lipoate reductase was assayed by the decrease in extinction of NADH at 340 nm in a cuvette containing 10 mM-tris chloride, pH 7.4, 0.7 mM-lipoate, 0.1 mM-NAD⁺, 0.125 mM-NADH and the enzyme. NADH–menadione reductase and NADH–ubiquinone-1 reductase were measured similarly, except that 0.25 mM-menadione or 0.04 mM-ubiquinone-1 respectively replaced the lipoate and NAD⁺. NADH–cytochrome *c* reductase was assayed at 550 nm–540 nm ($\epsilon = 190001 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$; Ohnishi, Kröger, Heldt, Pfaff & Klingenberg, 1967) in a cuvette containing 10 mM-tris chloride, pH 7.4, 0.125 mM-NADH, 0.09 mM-cytochrome *c* and the enzyme. NADH–dichlorophenolindophenol reductase was measured at 600 nm ($\epsilon = 210001 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$; King & Howard, 1967) in 10 mM-tris chloride buffer, pH 7.4, containing enzyme, 0.125 mM-NADH and 0.04 mM-dichlorophenolindophenol. Where necessary, corrections were made for non-enzymic reduction of the acceptor.

NADH, NADPH and cytochrome *c* were obtained from the Boehringer Corp. (London) Ltd., London W.5, U.K. Ubiquinone-1 and piericidin A were gifts from Professor K. Folkers. Reduced acetylpyridine adenine dinucleotide was prepared by reduction of the oxidized form as described by Rafter & Colowick (1957) for the preparation of NADH.

RESULTS AND DISCUSSION

Enzyme assay. The reduction of ferricyanide by NADH is the most commonly used assay for preparations of respiratory chain NADH dehydrogenase (see, e.g., King & Howard, 1967). Minakami, Ringler & Singer (1962) have shown that the assay can give a true measure of the activity of the enzyme from ox heart, whether membrane-bound or in solution. The reduction of ferricyanide by NADH, catalysed by ETP_c from glycerol-limited *T. utilis*, resembles the activity of ox heart ETP_m in that the rate greatly exceeds the rate of NADH oxidase activity (Ragan & Garland, 1971), the reaction is insensitive to piericidin A, rotenone or antimycin A, and lastly, that the rate of ferricyanide reduction greatly exceeds the rates of reduction of other acceptors, e.g. cytochrome *c*,

menadione and dichlorophenolindophenol. However, yeast ETP_c preparations are much less pure than ETP_m, and contain other enzymes that could catalyse ferricyanide reduction, e.g. lipoamide dehydrogenase (EC 1.6.4.3) (Ragan & Garland, 1971). Two lines of evidence suggest that the NADH-ferricyanide reductase of ETP_c has a function within the respiratory chain. Firstly, ETP_m contain threefold higher concentrations of cytochrome than ETP_c (Clegg & Garland, 1971), and the ferricyanide reductase activity of the former is higher by the same factor (Tables 1 and 5). Secondly, ETP_c prepared from iron-limited cells, which have a modified NADH dehydrogenase (Ragan & Garland, 1971), catalyse ferricyanide reduction at tenfold lower rates than ETP_c from glycerol-limited cells, although the two varieties of ETP_c contain similar concentrations of lipoamide dehydrogenase (Ragan & Garland, 1971). Therefore it is probable that at least 90% of the NADH-ferricyanide reductase activity of ETP_c from glycerol-limited cells is catalysed by the respiratory chain NADH dehydrogenase.

Solubilization of the enzyme. Many solubilization treatments which are effective in removing the enzyme from ox heart ETP were totally ineffective with the *T. utilis* ETP_c. Thus ethanol at low pH and high temperature (Mahler *et al.* 1952; De Bernard, 1957; Mackler, 1961), urea (Davis & Hatefi, 1969), or sodium perchlorate (Davis & Hatefi, 1969) caused no solubilization and eventual loss of all activity. This confirms the results of Biggs, Nakamura, Kearney, Rocca & Singer (1970), who also found that phospholipase digestion (King & Howard, 1962; Ringler *et al.* 1963) was ineffective. However, certain detergents caused a solubilization of the ETP_c preparations without destroying their ferricyanide reductase activity. These were the bile salts sodium deoxycholate (1%, w/v), sodium cholate (1%, w/v) and the non-ionic detergent Triton X-100 (1%, v/v). The anionic detergent sodium dodecyl sulphate (0.5%, w/v) and the cationic detergent cetyltrimethylammonium bromide (0.5%, w/v) caused loss of enzyme activity.

Purification of the enzyme. A summary of the preparation procedure is shown in Scheme 1. All operations were performed at 0–4°C. ETP_c were suspended in buffer containing 0.25 M-sucrose–10 mM-tris chloride, pH 7.8, at a protein concentration of 16.5 mg/ml. A 10% (w/v) aqueous solution of sodium deoxycholate was then added to give a final concentration of 0.6 mg of sodium deoxycholate/mg of protein (the final protein concentration was therefore 15 mg/ml). Saturated, neutralized ammonium sulphate solution was then added to give 20% saturation and the solution was stirred for 10 min. After centrifugation at 34 000g for 10 min in the 8 × 50 ml head of the MSE 18 centrifuge, the

supernatant was decanted off, brought to 25% saturation with ammonium sulphate, and again centrifuged after 10 min. The pellet was discarded and the ammonium sulphate concentration of the supernatant was increased to 35% saturation. After centrifugation, the pellet (P₃) was dissolved in sucrose-tris buffer and centrifuged to remove insoluble protein. Occasionally the enzyme was precipitated between 20% and 30% saturation.

The protein concentration was adjusted to 6 mg/ml by adding sucrose-tris buffer, and sodium deoxycholate solution was added to give a final concentration of 0.45 mg/mg of protein. The solution was then fractionated with ammonium sulphate as described above. The protein that precipitated at 15% and 20% saturation was discarded, and the enzyme was obtained in the precipitate formed at 35% saturation (P₆). This pellet was dissolved in sucrose-tris buffer.

After adjusting the protein concentration to 4 mg/ml, a 10% (v/v) aqueous solution of Triton X-100 was added to give a final detergent concentration of 1% (v/v). Ammonium sulphate solution was then added to 27% saturation and the suspension centrifuged at 34 000g for 15 min. A small red pellet (P₇) and a green-brown layer (F₇) floating on the supernatant (S₇) were obtained. The supernatant was removed from beneath the floating layer with a fine Pasteur pipette and adjusted to 29% saturation with ammonium sulphate, and centrifuged at 34 000g for 15 min. This step removed a little more red material in the pellet (P₈) and the last traces of the floating layer (F₈). The enzyme was precipitated from the supernatant (S₈) at 35% saturation with ammonium sulphate. The pellet (P₉) was dissolved in 20 mM-tris chloride, pH 7.4, containing 1% (v/v) Triton X-100, and adjusted to a protein concentration of approximately 4 mg/ml. Approximately 1500 units of activity were then applied to a 20 cm × 1.5 cm column of DEAE-cellulose equilibrated with the same buffer, and eluted, also with the same buffer. The enzyme passed through the column without being bound, whilst a red band (cytochrome *b*) remained bound at the top of the column. A small proportion of the cytochrome *b* was eluted just after the enzyme. The behaviour of the cytochrome *b* is very much dependent on the residual salt concentration of P₉, and occasionally this chromatography method does not give a good separation of enzyme and cytochrome *b*. If this was the case, P₉ was dissolved in a small volume of sucrose-tris buffer (protein concentration 10–15 mg/ml) and applied to a 30 cm × 1.5 cm column of Sephadex G-25 (coarse) equilibrated with 20 mM-tris chloride, pH 7.4. The enzyme was eluted with this buffer and was obtained in the void volume free from ammonium sulphate and substantially free from deoxy-

Scheme 1. Flow diagram of the purification procedure. Abbreviations: S, supernatant; P, pellet; F, floating layer; AS, ammonium sulphate.

cholate. Triton X-100 was then added to a final concentration of 1% (v/v) and the enzyme applied to the DEAE-cellulose column as described above. The column was eluted with 20mM-tris chloride (pH 7.4)–1% (v/v) Triton X-100, and a linear gradient of sodium chloride between 0 and 0.8M in a total volume of 200ml. The enzyme eluted at about 0.2M-sodium chloride and the cytochrome *b* between 0.3M- and 0.5M-sodium chloride. The two methods of DEAE-cellulose chromatography were equally effective in separating the enzyme from the total protein.

For some purposes the enzyme was used at this stage, since the solution was optically clear. However, the presence of Triton X-100 was undesirable in other cases, since it interfered with acid extraction of flavin and estimation of protein by the Lowry method. Triton X-100 was removed by applying the DEAE eluent to a 30cm × 1.5cm column of Sephadex G-200 equilibrated with 20mM-tris chloride, pH 7.4. The enzyme was obtained in the excluded fraction as a turbid, yellow solution. Triton X-100 was eluted immediately after the enzyme and often some small sacrifice in yield was necessary to ensure that the preparation was completely free from Triton X-100. Some low-molecular-weight impurities were also removed by this gel-filtration step, but the purification with respect to total protein was negligible.

Data from a number of preparations are summarized in Table 1.

Purification of cytochrome oxidase. The greenish floating layer F₇ contained a high concentration of cytochrome *a* and some cytochrome *b*. Most of the Triton X-100 was removed by gel filtration on Sephadex G-25 as described above, and the turbid solution obtained in the excluded fraction was applied to a 20cm × 1.5cm column of DEAE-cellulose equilibrated with 20mM-tris chloride, pH 7.4, containing 0.1% (v/v) Triton X-100 and 0.10M-sodium chloride. After washing the column through with this buffer, cytochrome oxidase was eluted with 20mM-tris chloride (pH 7.4)–0.1% (v/v) Triton X-100–0.15M-sodium chloride. Most of the cytochrome *b* remained bound under these conditions. Repetition of the Sephadex G-25 and DEAE-cellulose steps resulted in further removal of cytochrome *b*.

Purification of cytochrome b. The enzyme fraction P₉ contained a large concentration of cytochrome *b* as the sole cytochrome contaminant. The cytochrome *b* fraction obtained from the DEAE-cellulose chromatography was further purified by desalting on Sephadex G-25 as described above and then applying the excluded fraction to a 20cm × 1.5cm column of DEAE-cellulose equilibrated with 20mM-tris chloride, pH 7.4, containing 0.1% (v/v) Triton X-100. The column was then washed with this buffer containing 0.10M-sodium chloride and the cytochrome *b* eluted with the same buffer containing 0.30M-sodium chloride.

Composition of the enzyme: content of flavin, iron

Table 1. *Purification of NADH dehydrogenase*

The values in parentheses are averages.

Fraction	No. of preparations	Specific activity of NADH dehydrogenase (μmol of ferricyanide reduced/min per mg of protein)	Yield (%)
ETP _c	12	2.12–3.38 (2.68)	100
P ₃	11	7.2–16.6 (12.2)	63–81 (71)
P ₆	11	18.8–48 (29.4)	35–68 (48)
P ₉	6	62–94.6 (83.4)	26–62 (40)
Fraction from Sephadex G-200	6	84–134 (108.8)	15–28 (20)

Table 2. *Non-haem iron, acid-labile sulphide and flavin analyses*

The specific activity of the enzyme is expressed as μmol of ferricyanide reduced/min per mg of protein.

Preparation	Specific activity of NADH dehydrogenase	Fe content (ng-atom/mg of protein)	Acid-labile sulphide (ng-atom/mg of protein)	Total flavin (nmol/mg of protein)	FMN (by fluorescence) (nmol/mg of protein)
1	100	15.7	—	—	—
2	110	13.3	15.7	—	—
3	111	17.2	15.4	0.56	0.54
4	107	15.5	16.6	—	0.60
5	134	—	—	0.54	0.60
6	95	—	—	0.57	—

and acid-labile sulphide. Results of a number of estimations are given in Table 2. All flavin was found to be acid extractable, and shown to be FMN by fluorimetric analysis. The haem-iron content, estimated by the extinction changes in the Soret region of the spectrum elicited by dithionite, was at most 0.1 nmol/mg of protein, and as low as 0.01 nmol/mg of protein in the best preparations obtained. The near equality of the iron and acid-labile sulphide concentrations is a feature of most non-haem iron proteins. The proportions of non-haem iron to acid-labile sulphide to FMN were, on average, 27.5:28.4:1.

Visible spectroscopy. Fig. 1 shows oxidized and NADH-reduced spectra of the enzyme. The peak at 420 nm does not appear to be due to haem contamination. The extinction at wavelengths greater than 500 nm is characteristic of proteins containing non-haem iron (Rajagopalan & Handler, 1963). Reduction with NADH caused bleaching between 400 nm and 560 nm. The reduced-minus-oxidized difference spectrum (Fig. 1 inset) shows a broad minimum between 420 nm and 450 nm. Dithionite did not cause any further bleaching. The bleaching at 450 nm ($\Delta E = 0.0070$) cannot all be accounted for by reduction of the flavin component of the dehydrogenase. The FMN content of 0.51 nmol/mg of protein can account for an extinction change at 450 nm of 0.0040 assuming an ϵ value of $9.81 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (the protein concentration was 0.8 mg/ml).

Thus the non-haem iron moiety must contribute to the spectroscopic change. Fig. 2 is a recording of the extinction changes at 450 nm–560 nm. In trace (a), NADH caused rapid bleaching at 450 nm. After oxidation of the NADH, the extinction at 450 nm increased, but not to the original value. NADPH caused no significant extinction change. After reduction by a second addition of NADH, dithionite had no further effect. Trace (b) shows the very small bleaching obtained with NADPH, and the effect of the mercurial, mersalyl, which inhibits the enzyme activity. After bleaching with mersalyl, NADH had no further effect.

Fluorescence. The fluorescence of the flavin is highly quenched. Excitation at 456 nm produced no detectable emission at 520 nm and above, i.e. the emission was less than 1% of that of the flavin after liberation by trichloroacetic acid.

Electron-paramagnetic-resonance spectroscopy. The enzyme, reduced with NADH, gave rise to the $g = 1.94$ signal characteristic of many non-haem containing proteins (see, e.g., Hall & Evans, 1969). Fig. 3(a) shows the first derivative spectrum of the reduced enzyme, and (b) the absence of the signal from the oxidized enzyme. Dithionite failed to intensify the signal obtained with NADH.

Kinetic properties. The enzyme was inhibited by high concentrations of both substrates, as shown in Fig. 4. NADH was a competitive inhibitor with respect to ferricyanide. The V_{max} obtained by

Fig. 1. Absorption spectra of the purified NADH dehydrogenase. Enzyme (specific activity 100) from the DEAE-cellulose step was used. The protein concentration was 0.8 mg/ml. Trace (a), oxidized enzyme; trace (b), after addition of NADH (0.1 mM). Inset: NADH reduced-minus-oxidized difference spectrum. This enzyme preparation was apparently free from cytochrome.

Fig. 2. Dual-wavelength spectrophotometric measurements of extinction changes at 450 nm–560 nm. The same enzyme preparation as that in Fig. 1 was used. The protein concentration was 0.8 mg/ml. Final concentrations of reagents were: NADH, 0.1 mM; NADPH, 0.1 mM. Dithionite and mersalyl were added in the solid form.

extrapolation was 560 μ mol of ferricyanide reduced/min per mg of protein at 22°C. The ferricyanide concentration used for the assay during purification is indicated by the arrow in Fig. 4. At this concentration the enzyme has its maximum activity and the rate is relatively insensitive to changes in the ferricyanide concentration. The K_m for ferricyanide was found to be 1.0 mM and the K_i for NADH was about 0.2 mM. Reaction rates at low concentrations of NADH were determined at a number of ferricyanide concentrations and an apparent V_{max} was determined for each NADH concentration by extrapolation. These values were then used to determine the K_m for NADH as shown in Fig. 5. The value obtained was 0.083 mM.

Other electron acceptors were very slowly reduced compared with ferricyanide. Relative rates for a number of commonly used acceptors are given in Table 3. With ferricyanide as acceptor, NADPH and reduced acetylpyridine adenine

Fig. 3. Electron-spin-resonance spectra of NADH dehydrogenase. Trace (a), 0.3 ml of enzyme (fraction P₉, specific activity 96, protein concentration 14 mg/ml) was mixed with 10 μ l of 75 mM-NADH and frozen in a 3 mm (inside diam.) quartz tube after 30 s. Trace (b), as (a) but without NADH.

dinucleotide (both at 0.1 mM) were oxidized at 1.1–1.3% and 10% respectively of the rate of oxidation of NADH (0.1 mM).

Fate of NADPH–ferricyanide reductase during purification of the enzyme. Ragan & Garland (1971) found that ETP_m from *T. utilis* could oxidize NADPH at appreciable rates, and it was suggested that two distinct dehydrogenases, one specific for NADH and the other specific for NADPH, were in operation. Table 4 gives the relative rates of oxidation of NADH, NADPH and L-glycerol 3-phosphate by ferricyanide catalysed by the enzyme at various stages of purification. It can be seen that NADPH–ferricyanide reductase did not co-purify with NADH–ferricyanide reductase. Triton X-100 appeared to stimulate NADPH oxidation (cf. rates with P₆ and P₉ in Table 4), but subsequent steps in the purification did not cause any change in the relative rate. This residual activity is therefore likely to be a property of the NADH dehydrogenase rather than being caused by an impurity.

Fate of flavin, non-haem iron and acid-labile sulphide during purification of the enzyme. As the ETP_c preparation contains considerable concentrations of non-respiratory chain enzymes, in Table 5 the NADH dehydrogenase is compared with ETP_m. The purification of NADH–ferricyanide reductase activity is very closely paralleled by the purification of non-haem iron, acid-labile sulphide and, to a lesser extent, by FMN. Thus approximately 100% of the non-haem iron and 70% of the FMN of ETP_m can be accounted for in the NADH dehydrogenase.

Fig. 4. Kinetic properties of NADH dehydrogenase. All assays were performed at 22°C in 10 mM-tris chloride, pH 7.4. The concentration of enzyme (specific activity 134) was 0.94 μ g of protein/ml. NADH concentrations were: $\circ$, 0.126 mM; Δ , 0.253 mM; $\square$, 0.632 mM. The arrow indicates the ferriyanide concentration used for the assay during purification.

Fig. 5. Kinetic properties of NADH dehydrogenase. All assays were performed as described in the legend to Fig. 4. Plots of $1/v$ against $[\text{ferriyanide}]^{-1}$ at various NADH concentrations were used to determine the V_{max}^{-1} values.

Table 3. Activity of NADH dehydrogenase with other acceptors

Activities were assayed at 22°C as described in the Materials and Methods section. Rates were calculated as μ mol of NADH oxidized/min per mg of protein and then expressed as a percentage of the rate with ferriyanide as the acceptor. (The specific activity of the enzyme was 100.)

Acceptor	Relative rate of NADH oxidation
Ferriyanide (0.625 mM)	100
Menadione (0.25 mM)	0.35
Lipoate (0.7 mM)	<0.01
Cytochrome <i>c</i> (0.09 mM)	0.065
Dichlorophenolindophenol (0.04 mM)	0.35
Ubiquinone-1 (0.04 mM)	0.08

Recovery of the extinction change at 460 nm on reduction by NADH. Studies on ETP from a number of phenotypic modifications of *T. utilis* led us to conclude that there were two species of non-haem iron involved in the oxidation of NADH (Ragan *et al.* 1970; Ragan & Garland, 1971). One of these, denoted species 1, was supposedly a non-haem-iron-containing flavoprotein, reducible only by NADH, and located on the substrate side of the site of action of piericidin A. The second, species 2, was thought to be a pure non-haem iron protein, reducible by NADH, NADPH or DL-glycerol 3-phosphate, and possibly the site of action of piericidin A. These species were identified by the bleaching at around 460 nm after reduction. Table 6 compares the concentration of these two species in ETP_m in extinction units with the bleaching of the purified enzyme at 460 nm–530 nm caused by addition of NADH. It is clear that purification of species 1 rather than 1 and 2 has occurred. As further evidence for this, in Fig. 6, the reduced-minus-oxidized difference spectrum of species 1 in ETP_m is compared with the difference spectra of a partially purified enzyme and of a highly purified enzyme. After allowance for the Soret bands of cytochrome *b* in the spectrum of species 1 (Fig. 6a) and cytochrome *c* in the spectrum of the impure enzyme (Fig. 6b) it can be seen that the spectra are quantitatively similar. A species 1', similar in properties to species 1, but reducible only by NADPH, was also identified in ETP_m (Ragan & Garland, 1971). The inability of NADPH to cause significant reduction of the pure enzyme (Fig. 2) strengthens the conclusion that these were originally two separate pyridine nucleotide dehydrogenases, and is in agreement with the results given in Table 4.

The characterization of species 1 and 2 in ETP_m

Table 4. Fate of ADPH- and L-glycerol 3-phosphate-ferricyanide reductases during purification of NADH dehydrogenase

NADPH- and L-glycerol 3-phosphate-ferricyanide reductases were assayed exactly as described for the assay of NADH-ferricyanide reductase except that 0.1 mM-NADPH and 2.5 mM-DL-glycerol 3-phosphate respectively replaced the NADH. All rates were calculated as μmol of ferricyanide reduced/min per mg of protein, and then expressed as percentages of the rates with NADH as donor.

Fraction	Relative extent of purification of NADH-ferricyanide reductase	Rates (percentage of rate with NADH as donor)	
		with NADPH	with DL-glycerol 3-phosphate
ETP _c	1	12.2	5.4
P ₃	2.9	1.35	0.0
P ₆	10.3	0.34	0.0
P ₉	27.6	1.3	0.0
Eluate from DEAE-cellulose step	41.0	1.3	0.0

Table 5. Comparison of ETP_m with NADH dehydrogenase

Property	Specific activity ($\mu\text{mol}/\text{min}$ per mg of protein) or concentration (nmol/mg of protein)		Ratio of parameter in NADH dehydrogenase to that in ETP _m
	NADH dehydrogenase	ETP _m	
NADH-ferricyanide reductase	100	8.4	11.9
Non-haem Fe	15.4	1.46	10.6
Acid-labile sulphide	15.9	1.34	11.9
FMN content	0.51	0.06	8.5
FAD content	0	0.36	0

Table 6. Comparison of ETP_m with NADH dehydrogenase

Species 1 and 2 contents of ETP_m were assayed at 460 nm–530 nm as described by Ragan & Garland (1971). The units are $10^3 \times \Delta E$ for a suspension of ETP_m of 1 mg of protein/ml. The value for NADH dehydrogenase is the extinction change at 460 nm–530 nm obtained on adding NADH to the enzyme, expressed in the same units.

Property	Activity or concentration		Ratio of parameter in NADH dehydrogenase to that in ETP _m
	NADH dehydrogenase	ETP _m	
NADH-ferricyanide reductase ($\mu\text{mol}/\text{min}$ per mg)	100	8.4	11.9
Species 1	7.0	0.60	11.7
Species 1 + species 2	7.0	3.10	2.26

was based on the spectroscopic changes accompanying variations in the non-haem iron content of ETP. The conclusion that species 1 and 2 were respectively a non-haem-iron-containing flavo-protein and a non-haem iron protein agreed with conclusions of Hatefi & Stempel (1967) and Hatefi (1968) concerning the structure of the particulate NADH-ubiquinone reductase from ox heart (Hatefi, Haavik & Griffiths, 1962). Therefore the recovery of all the original non-haem iron of the ETP_m in the NADH dehydrogenase would suggest, on the basis

of this interpretation, that both species 1 and 2 should be present in the enzyme. The absence of species 2 therefore implies either that it is not a non-haem iron protein or that it is present but inactive. It was found that treatment of ETP_c with deoxycholate did, in fact, cause inactivation of species 2, i.e. there was no component reducible by NADH, NADPH and DL-glycerol 3-phosphate. The concentrations of species 1, species 1' and species 3 [L-glycerol 3-phosphate dehydrogenase (EC 1.1.2.1); Ragan & Garland (1971)] were, however, quite

Fig. 6. Purification of NADH dehydrogenase. Trace (a) is the difference spectrum of species 1 of ETP_m obtained as described by Ragan & Garland (1971); the specific activity of NADH-ferri-cyanide reductase was 8.0, and the protein concentration was 1.76 mg/ml; trace (b), NADH reduced-minus-oxidized difference spectrum of a partially purified NADH dehydrogenase preparation (fraction P₆) of specific activity 30.4 and protein concentration 0.8 mg/ml; trace (c), NADH reduced-minus-oxidized difference spectrum of purified NADH dehydrogenase re-drawn from Fig. 1. The ΔE scales are drawn in inverse proportion to the number of enzyme units/ml to facilitate comparison of the three spectra.

unaffected by deoxycholate. The inactivation of species 2 was accompanied by loss of NADH-ubiquinone-1 reductase activity, which is piericidin A-sensitive in ETP_m under the conditions of assay described in the Materials and Methods section.

The co-purification of ferricyanide reductase activity, non-haem iron, acid-labile sulphide, FMN, species 1 and, at least qualitatively, the NADH-specific $g = 1.94$ electron-paramagnetic-resonance signal, suggests very strongly that the isolated enzyme is a fragment of the respiratory chain which still retains many of the properties present in the intact state. The component responsible for NADH-ferri-cyanide reductase activity is located on the substrate side of the site of action of piericidin A, and of the region responsible for site I energy conservation. The same is true of species 1 (Ragan & Garland, 1971) and the $g = 1.94$ signal (Lee, 1970). The absence of rotenone or piericidin A-sensitive NADH-ubiquinone-1 reductase, which can be

coupled to energy conservation at site I in ox heart ETP_m (Schatz & Racker, 1966a), and the inactivation of species 2 suggests that the solubilization procedure damages the respiratory chain in the region of site I energy conservation, but leaves the other properties intact. The recovery of all the non-haem iron in the enzyme, however, suggests that all the respiratory chain components between NADH and ubiquinone are present in a damaged or undamaged form. The presence of species 2 is suggested also by the presence of the $g = 1.94$ electron-paramagnetic-resonance signal specifically reduced by NADH, which, although not a direct property of species 2, is only found in ETP_m which contain both species 1 and species 2 (C. I. Ragan, J. C. Swann & R. C. Bray, unpublished work).

Evidence that the NADH dehydrogenase is a multi-enzyme complex. The ratio of non-haem iron to FMN in ETP_m from glycerol-limited cells varies widely depending on the iron concentration available to the culture (Clegg & Garland, 1971). Although high ratios of non-haem iron to FMN are thought to be caused by non-functional iron, ETP_m from glycerol-limited cells (i.e. cells with mitochondria still conserving energy at site I and sensitive to piericidin A) can be prepared with non-haem iron:FMN ratios of about 10:1 (Clegg & Garland, 1971), and ETP_m from iron-recovered cells, which are functionally similar, have a non-haem iron:FMN ratio of about 7:1 (Clegg & Garland, 1971). Unless only a small proportion of the FMN of these ETP_m is accounted for by the NADH dehydrogenase, the iron:flavin ratio of NADH dehydrogenases isolated from glycerol-limited cells with low iron content, and from iron-recovered cells will be respectively about one-half and one-third of the ratio of the enzyme described here. One explanation would be that the NADH dehydrogenase is a multi-enzyme complex and moreover that the relative proportions of the constituent proteins is variable. It is well known that high-molecular-weight dehydrogenases from ox heart may be fragmented into smaller units (Singer & Gutman, 1970; Hatefi & Stempel, 1967). Chromatography of the NADH dehydrogenase on Sepharose 4B in the presence of 1% Triton X-100 and 0.1 M-sodium chloride produced a single peak of enzyme activity. Accurate determination of the molecular weight was not possible since it was found that Triton X-100 altered the gel-filtration characteristics of Sepharose 4B, and no marker proteins were found which eluted as sharp peaks under these conditions. However, the extent to which the enzyme was excluded from the gel suggested that it had a mol.wt. of approx. 5×10^5 . Polyacrylamide-gel electrophoresis with Triton X-100 revealed one major band accounting for approximately 60% of the protein, and three minor bands (R. A. Clegg, personal com-

munication). The major band was shown to be NADH dehydrogenase by staining with NADH and Nitro Blue Tetrazolium. However, electrophoresis of the enzyme in sodium dodecyl sulphate as described by Weber & Osborn (1969) revealed about nine bands, the two major bands having molecular weights of 63 000 and 57 000 respectively (R. A. Clegg, personal communication). Thus it is apparent that the enzyme contains at least two different types of polypeptide chain and may well contain about ten polypeptide chains altogether.

Purification of cytochrome oxidase and cytochrome *b*. Fig. 7 shows reduced-minus-oxidized difference spectra of cytochrome *b* and cytochrome oxidase prepared as described earlier. The cytochrome oxidase difference spectrum has peaks at 603 nm, 444 nm and a minor peak at about 517 nm. The peak at about 562 nm is due to residual cytochrome *b*. The oxidized enzyme has peaks at 425 and 598 nm, the reduced enzyme at 603, 517 (approx.) and 444 nm. The cytochrome *a* content, based on the results for the ox heart enzyme was, for two preparations, 4.5 and 4.6 nmol/mg of protein ($\epsilon = 165001 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for the reduced enzyme at 603 nm–630 nm; Griffiths & Wharton, 1961) and the purity was 50%. This is about a 40-fold purification from ETP_C.

The cytochrome *b* difference spectrum has peaks at 561, 532 and 430 nm, and appears to be free from contamination by other cytochromes. The oxidized enzyme has a peak at 418 nm, and the reduced enzyme at 430, 532 and 561 nm. Based on the ox

heart protein, the cytochrome *b* preparation had a haem content of 7.4 nmol/mg of protein ($\epsilon = 132001 \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for the reduced enzyme at 561 nm–600 nm; Goldberger, Smith, Tisdale & Bornstein, 1961) and an estimated purity of 21%. This is about a 50-fold purification from ETP_C. Both of these preparations would serve as useful starting points for complete purification of cytochrome oxidase and cytochrome *b*.

During the purification of NADH dehydrogenase, cytochromes *c* and *c*₁ are largely absent from P₃ (Scheme 1) and almost completely absent from P₆. P₆, however, still contains most of the cytochrome *b* of the starting material, and a considerable concentration of cytochrome oxidase, both of which have co-purified with the enzyme. Cytochrome *b* co-purifies with the enzyme to the P₃ stage, whilst cytochrome oxidase is removed in F₇ and F₈. The ease with which the *b* and *c* cytochromes are separable and the co-purification of cytochrome oxidase with cytochrome *b* and the enzyme was unexpected. There is therefore no evidence that the respiratory chain of *T. utilis* is organized into multienzyme complexes as is proposed for the respiratory chain of ox heart (Hatefi, 1966) and *Saccharomyces cerevisiae* (Tzagoloff, 1969). The association of one respiratory carrier with another appears to be entirely unrelated to the actual sequence in which the carriers operate in the mitochondrial membrane.

Comparison with other NADH dehydrogenases. The NADH dehydrogenase described here has a number of features in common with the high-molecular-weight NADH dehydrogenase described by Ringler *et al.* (1963), isolated from ox heart ETP by phospholipase digestion, and also with the particulate NADH-ubiquinone reductase described by Hatefi *et al.* (1962), isolated from ox heart ETP with the use of bile salts and ammonium sulphate. Table 7 gives a comparison of the three preparations. The only other NADH dehydrogenase prepared from yeast is that isolated by Duncan & Mackler (1966) from *Saccharomyces cerevisiae*. This was solubilized by using ethanol at low pH and high temperature and is therefore not directly comparable with the enzyme described here. However, the absence of non-haem iron and labile sulphide, and the presence of FAD rather than FMN in the *Saccharomyces* enzyme are differences which could hardly be caused by the isolation procedure, and undoubtedly reflect the differences in respiratory chain function between the two organisms, namely, the absence of site I energy conservation in *S. cerevisiae*, and the insensitivity of the mitochondria to rotenone and piericidin A (Schatz & Racker, 1966b). However, our reason for isolating the NADH dehydrogenase from *T. utilis* was not to add further confusion with yet another preparation

Fig. 7. Reduced-minus-oxidized difference spectra of partially purified cytochrome oxidase and cytochrome *b*. Trace (a), dithionite reduced-minus-oxidized difference spectrum of cytochrome oxidase. The protein concentration was 0.136 mg/ml; trace (b), dithionite reduced-minus-oxidized difference spectrum of cytochrome *b*. The protein concentration was 0.10 mg/ml.

Table 7. *Comparison of T. utilis NADH dehydrogenase with other preparations*

Values for the ox heart preparations are taken from Singer, Rocca & Kearney (1966). Turnover numbers are mol of NADH oxidized by the acceptor/min per mol of FMN at 30°C. N.T., not tested; e.p.r., electron paramagnetic resonance.

Property	Ox heart NADH dehydrogenase (Ringler <i>et al.</i> 1963)	Ox heart NADH-ubiquinone reductase (Hatefi <i>et al.</i> 1962)	<i>T. utilis</i> NADH dehydrogenase
Non-haem iron/FMN ratio	16–18	17–18	27.5
Labile sulphide/Fe ratio	1.5–1.6	1.0	1.0
Substrate-reducible e.p.r. signal at $g = 1.94$	Yes	Yes	Yes
Reactivity with cytochrome <i>c</i>	Very low	Very low	Very low
Fragmentation and conversion into cytochrome reductase by heat or acid-ethanol	Yes	Yes	No*
Turnover no. (ferricyanide)	1.3×10^6 – 1.5×10^6	0.65×10^6	0.64×10^6
Turnover no. (ubiquinone-1)	0.5×10^3 – 0.7×10^3	11.7×10^3	approx. 0.5×10^3
Ubiquinone content (nmol/mg of protein)	0	4.5	N.T.
Lipid content (mg/mg of protein)	0	0.22	N.T.

* All enzyme activities are lost on heating (S.O.C. Tottmar, unpublished work).

but to serve as a starting point for the isolation of NADH dehydrogenases from phenotypic modifications of the respiratory chain of *T. utilis*, and ultimately to a characterization of the pathways of NADH oxidation in these phenotypes and their relationship to energy conservation.

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