Spectroscopic Studies of Flavoproteins and Non-Haem Iron Proteins of Submitochondrial Particles of *Torulopsis utilis* Modified by Iron- and Sulphate-Limited Growth in Continuous Culture

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1. A spectroscopic resolution has been made of the components contributing to the 'iron-flavoprotein' trough extending from 450 to 520 nm in the reduced-minus-oxidized difference spectrum of submitochondrial particles of *Torulopsis utilis*. 2. Seven components were identified other than cytochrome *b*, ubiquinone and succinate dehydrogenase. On the basis of the effects of iron- and sulphate-limited growth of cells on their subsequently derived electron-transport particles, and also by consideration of analytical measurements of the concentration of FMN, FAD, non-haem iron and acid-labile sulphide in the electron-transport particles in relation to the magnitude of the spectroscopic changes, it was possible to identify five of these components as follows: species 1a, the flavin of NADH dehydrogenase ferroflavoprotein; species 1b, the iron-sulphur component of NADH dehydrogenase ferroflavoprotein; species 1', the flavin of an NADPH dehydrogenase; species 2, an iron-sulphur or ferroflavoprotein component; species 3, the flavin of L-3-glycerophosphate dehydrogenase. Two additional components were a fluorescent flavoprotein, probably lipoamide dehydrogenase, and a *b*-type cytochrome reducible by NADH or NADPH but not reoxidizable by the respiratory chain. 3. Species 1b and 2 were undetectable in electron-transport particles from iron- or sulphate-limited cells, but could be recovered *in vivo* under non-growing conditions. 4. The recovery *in vivo* of species 2 but not species 1b was inhibited by cycloheximide. 5. The recovery of species 1b correlates with the recovery of site 1 conservation. 6. The recovery of species 1b with species 2 correlates with the recovery of piericidin A sensitivity. 7. Evidence is presented for an NADPH dehydrogenase distinct from NADH dehydrogenase. The oxidation of NADH and NADPH by the respiratory chain is sensitive to piericidin A, and an iron-sulphur protein common to both pathways (species 2) is suggested as the piericidin A-sensitive component. 8. The approximate E'_0 (pH 7.0) values of species 1 (a and b, low potential) and species 2 (high potential) indicate that site 1 energy conservation occurs between the levels of species 1 (a and b) and species 2.

The various contributions made by flavoproteins and non-haem iron proteins to electron transport, energy conservation and the phenomenon of piericidin A sensitivity in that segment of the respiratory chain leading from intramitochondrial NADH to the cytochromes is poorly understood (Slater, 1966; Garland, Clegg, Light & Ragan, 1969). A major difficulty arises, not only from the overlapping spectral characteristics of flavoproteins and non-haem iron proteins, but also from the high concentration of non-haem iron (e.g. 5 nmol/mg of

mitochondrial protein: Clegg & Garland, 1971) relative to flavoprotein (e.g. 0.25 nmol/mg of mitochondrial protein; Clegg & Garland, 1971). Since the extinction coefficients in the most approachable region (e.g. 450–520 nm) are not grossly dissimilar (see the Discussion section), it is not possible to allocate unequivocally to either flavoprotein or non-haem iron protein spectral changes observed in this region in complex systems such as mitochondria or submitochondrial particles. A new way of overcoming this limitation of spectroscopic studies is to decrease drastically the concentration of mitochondrial non-haem iron by iron-limited non-fermentative growth of the eukaryotic

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organism, *Torulopsis utilis* (Light, Ragan, Clegg & Garland, 1968; Clegg & Garland, 1971). Spectroscopic changes in such iron-deficient submitochondrial particles can then, on the basis of their magnitude and growth-condition dependence, be interpreted in some cases quite unequivocally in terms of either flavoproteins or non-haem iron proteins. A similar approach is offered by sulphate-limited growth of *T. utilis* (Haddock & Garland, 1971) which, although not affecting greatly the concentration of non-haem iron of mitochondria, so lowers the concentration of acid-labile sulphide that the amount of non-haem iron able to exist as a functional iron-sulphur protein is similarly lowered. The outcome is functionally the same as that obtained with iron-limited growth.

Table 1 summarizes the phenotypic variations of *T. utilis* used in this study. An obvious objective of making a detailed spectroscopic study of the submitochondrial particles derived from these cells was to ascertain whether the changes in mitochondrial function were paralleled by changes in the concentration of spectroscopically identifiable non-haem iron proteins. A preliminary account of this work has been published (Ragan, Clegg, Haddock, Light & Garland, 1970).

MATERIALS AND METHODS

Culture conditions for the production of G- and F-cells were as described by Light & Garland (1971). FR-(anaerobic)- and FR(cycloheximide)-cells were prepared as described by Clegg & Garland (1971), and S-, SR₀-, SR₀(cycloheximide)- and SR₁-cells as described by Haddock & Garland (1971). Submitochondrial or electron-transporting particles (ETP) were prepared either from isolated mitochondria (when they are referred to as ETP_m) or from whole cells (ETP_c) as described by Clegg & Garland (1971). After preparation the ETP were suspended in 10 mM-tris-chloride or -sulphate buffer,

pH 7.4, and stored at liquid-N₂ temperature if not used immediately. ETP_c preparations differ from those of ETP_m in having lower specific activities for NADH or L-glycerol 3-phosphate oxidation, and also in having lower cytochrome concentrations. Spectroscopic measurements were made with a dual-wavelength spectrophotometer or wavelength-scanning spectrophotometer constructed in general as described by Chance & Legallais (1951) and Yang & Legallais (1951) respectively. Measurements of flavoprotein fluorescence were made with a modified Eppendorf fluorimeter (Garland, Shepherd & Yates, 1965) with a 10 nm-band-width 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. Oxygen uptake was measured polarographically (Chappell, 1964) in the medium described by Light & Garland (1971). Lipoamide dehydrogenase (EC 1.6.4.3) was assayed as described by Veeger & Massey (1962), in the direction from NADH to lipoamide. The assay medium also included 1 μ M-antimycin A. Transhydrogenase activity from NADH to NADP⁺ was assayed as described by Danielson & Ernster (1963), except that the assay-medium buffer was 10 mM-tris-sulphate, pH 7.4. Transhydrogenase activity from NADPH to NAD⁺ was assayed at 25°C by measuring the decrease in extinction at 340 nm in a cuvette containing 10 mM-tris-sulphate, pH 7.4, 0.5 mM-KCN, 20 μ M-NAD⁺, 5 mM-sodium pyruvate, 50 units of L-lactate dehydrogenase (EC 1.1.1.27), 0.1 mM-NADPH, and 0.5 mg of ETP_m preparation protein. Protein was assayed as described by Lowry *et al.* (1951).

RESULTS

Properties of submitochondrial particles from G-cells

Oxidase activities. Submitochondrial particles from the G-cells catalyse the oxidation of NADH, NADPH and DL-glycerol 3-phosphate, and the rates of oxygen uptake with these substrates and the effects of added cytochrome *c*, piericidin A and antimycin A are shown in Table 2. It is assumed that the oxygen uptake occurring on addition of DL-glycerol 3-phosphate to submitochondrial par-

Table 1. *Phenotypic modifications of T. utilis*

(+) and (−) indicate presence and absence of the property listed. References are: *, Light & Garland (1971); †, Haddock & Garland (1971); ‡, Clegg & Garland (1971).

Abbreviation	Growth or other conditions	Properties of derived mitochondria	
		Site 1 energy conservation	Piericidin A sensitivity
G*	Glycerol-limited	+	+
F*	Iron-limited	−	−
S†	Sulphate-limited	−	−
FR*	F-cells recovered with iron	+	+
FR(cycloheximide)‡	F-cells recovered with iron and cycloheximide	+	−
FR(anaerobic)‡	F-cells recovered with iron anaerobically	−	−
SR ₀ †	S-cells recovered without sulphate	+	+
SR ₀ (cycloheximide)†	S-cells recovered without sulphate in the presence of cycloheximide	+	−
SR ₁ †	S-cells recovered with 1 mM-sulphate	+	+

ticles is in fact due to the oxidation of the L-isomer. Table 2 shows that the addition of cytochrome *c* stimulated all oxidase rates. However, with NADPH oxidation, cytochrome *c* caused a decrease in the percentage inhibition observed with piericidin A or antimycin A. The rate of NADPH oxidation that was sensitive to these inhibitors was largely unchanged by cytochrome *c*, whereas the inhibitor-insensitive rate was stimulated. With NADH oxidation, both the sensitive and insensitive rates were stimulated by cytochrome *c*. It appears that both NADH and NADPH oxidase activities involve not only a conventional route via the inhibitor-sensitive respiratory chain but also an uncharacterized alternative route. The oxidation of NADPH was not stimulated by the presence of NAD⁺ (100 μ M), nor did the particles catalyse transhydrogenation between NADPH and NAD⁺, or NADP⁺ and NADH, at rates greater than 2 nmol/min per mg of protein. This rate is not greater than 2% of the observed oxidase rates (Table 2), and it therefore seems unlikely that NADPH was oxidized by transhydrogenation to endogenous NAD⁺. There must therefore be either a common dehydrogenase that will accept both NADH and NADPH, but that does not act as a transhydrogenase, or two distinct dehydrogenases, one specific for NADH and the other specific for NADPH. Half-maximal inhibition of either NADH or NADPH oxidase activity was observed with 15 pmol of piericidin A/mg of protein.

Spectroscopic studies. Fig. 1 shows the dual-wavelength spectroscopic responses at 470–510 nm of ETP_m preparations from G-cells. In trace (a) of Fig. 1, addition of a low concentration of NADH resulted in a cyclic decrease and increase in extinction as the NADH was oxidized. There was a small residual extinction change remaining even after complete oxidation of the NADH. A difference spectrum of particles before and after a single cycle of NADH oxidation showed that a *b*-type of

cytochrome remained reduced (Fig. 2). The residual decrease at 470–510 nm after a single cycle of NADH oxidation is similar to that described for electron-transport particles from ox heart by Minakami, Schindler & Estabrook (1966) and Bois & Estabrook (1969). Subsequent addition (Fig. 1a) of DL-glycerol 3-phosphate and antimycin A led to a considerable decrease in extinction at 470–510 nm and the further addition of NADH caused yet another decrease, presumably by the reduction of low-potential material, since the decrease was elicited with NADH but not with DL-glycerol 3-phosphate. [We are using 'low potential' and 'high potential' to indicate likely E_0' values at pH 7.0 of very approx. -0.32 V and 0 V respectively (Ragan & Garland, 1969)].

Trace (b) of Fig. 1 shows that a cycle of NADH oxidation in the presence of piericidin A differs from the uninhibited cycle (trace a) in that it exhibits (i) a longer duration for the spectroscopic change, (ii) a diminished fall in extinction at 470–510 nm at the onset of the cycle, and (iii) a greater residual extinction decrease remaining at the end of the cycle. The further addition of antimycin A and glycerol 3-phosphate caused a decrease in extinction identical with that observed in the absence of piericidin A (trace a), whereas a final addition of NADH had no effect. We conclude from these observations that the material reducible by NADH but not by glycerol 3-phosphate remains reduced after a cycle of NADH oxidation in the presence of piericidin A, and that this material is located functionally on the NADH side of the site of respiratory inhibition caused by piericidin A. This situation is similar to that in electron-transport particles from ox heart (Bois & Estabrook, 1969). If the experiment of trace (b) was extended for a further 5 min, then the extinction at 470–510 nm slowly increased until a final position similar to that of trace (a) was observed.

Traces (c) and (d) of Fig. 1 show that addition

Table 2. *Oxidase rates of ETP_m preparations from G-cells*

ETP_m preparations from G-cells were suspended at 25°C in an air-saturated medium as described by Light & Garland (1971). Concentrations of reagents were NADH (0.2 mM), NADPH (0.11 mM), cytochrome *c* (1 μ M), DL-3-glycerophosphate (5 mM), piericidin A (0.2 μ M), antimycin A (0.5 μ M). The final volume was 3.0 ml and the concentration of ETP protein was about 0.3 mg/ml.

Substrate	Rate of oxygen uptake (μ g-atoms/min per mg of protein)	Inhibition by piericidin A (%)	Inhibition by antimycin A (%)
NADH	0.29	84	100
NADH + cytochrome <i>c</i>	0.38	83	100
NADPH	0.100	80	85
NADPH + cytochrome <i>c</i>	0.190	61	63
DL-3-Glycerophosphate	0.25	<5	100
DL-3-Glycerophosphate + cytochrome <i>c</i>	0.33	<5	100

Fig. 1. Dual-wavelength spectrophotometer measurements of extinction changes at 470–510 nm for ETP_m preparations from G-cells. In each experiment ETP (2.0 mg of protein) was suspended in a final volume of 2.0 ml of 10 mM-tris-sulphate buffer, pH 7.4, at 25°C. An upward deflexion of the recorder tracings indicates an increase of extinction at 470 nm with respect to 510 nm. The light-path was 1.0 cm. Additions, abbreviations and final concentrations were: NADH (0.1 mM), antimycin A (AA, 1 μ M); DL-glycerol 3-phosphate (G-3-P, 5 mM), and piericidin A (PA, 0.3 μ M).

of glycerol 3-phosphate in the presence of antimycin A reduces two distinct components, one of which is also reducible by NADH. Comparison of the two traces shows that reduction of antimycin A-treated particles by NADH decreased the extinction change observed with glycerol 3-phosphate (trace d) compared with that observed in the absence of NADH (trace c). The experiments in Fig. 1 therefore indicate four functionally distinct species characterized spectroscopically at 470–510 nm, and their properties are summarized in Table 3. For convenience, these four species are referred to as species 0, 1, 2 and 3 to avoid introducing non-operational terminology, which may complicate discussion of their chemical nature or functional role.

A succinate-reducible flavoprotein (or 460–520 nm-absorbing species) was not detectable in ETP_m preparations from G-cells. Addition of succinate (5 mM) to antimycin A-treated particles failed to cause a detectable extinction change at 470–510 nm under conditions where glycerol 3-phosphate did (Fig. 1c). Nor was it possible in similar experiments to detect reduction of cytochrome *b* (at 562–575 nm) on addition of succinate to mitochondria treated with antimycin A.

The experiments in Fig. 3 explored the spectroscopic effects of NADPH on ETP_m preparations from G-cells. In trace (a), addition of a low concentration of NADPH resulted in a decrease in extinc-

tion at 460–520 nm, which did not altogether reverse when the oxidation of NADPH was completed. After reduction of species 2 and 3 by the addition of antimycin A and glycerol 3-phosphate, the final addition of NADPH indicated that there was a low-potential pool analogous to species 1. Traces (b) and (c) show that species 2 but not species 3 is reducible by NADPH. Thus NADPH behaved analogously to NADH in the reduction of three distinct 460–520 nm-absorbing species, and one of these (species 2) was commonly reduced by NADPH and NADH.

The experiments in Table 4 were undertaken to determine whether species 0 and 1 characterized with NADH were identical or not with the analogous species characterized with NADPH. The assumption underlying the experiments and their interpretation is that additive behaviour of the extinction changes with NADH and NADPH would indicate separate species, whereas non-additive behaviour would indicate a commonly reducible species. The first two experiments of Table 4 show that, with additions of NADH and NADPH in either order after those of antimycin A and glycerol 3-phosphate, the total extinction change at 460–520 nm attributable to the addition of NADH and NADPH was 3.8×10^{-3} . This value includes species 0, and repetition of the experiments after a preliminary cycle of either NADH (Expts. 3 and 4) or NADPH (Expts. 5 and 6) decreased the value to 1.65×10^{-3} or very closely so in each case. Since the total of 1.65×10^{-3} was independent of whether NADH or NADPH was used for the preliminary cycle, the species remaining after a cycle must be the same in each case. Therefore species 0 is reducible by both NADH and NADPH, and its contribution to the extinction change was 2.2×10^{-3} . This conclusion was confirmed by repeating the experiment of Fig. 1(a) with cycles of oxidation of NADH, NADH followed by NADPH, NADPH followed by NADH, and NADPH. In all cases the residual decrease in extinction at 460–520 nm at the completion of the experiment was the same.

From the results in Table 4 it is possible to calculate, for each of the three pretreatments ('none', 'NADH', 'NADPH'), the subsequent extinction changes attributable to NADH specifically, NADPH specifically, or to either. These values are given in Table 5, and their derivation from the four right-hand columns of Table 4 is quite apparent. The values based on Expts. 1 and 2 of Table 4 show that, in the absence of a preliminary cycle to reduce species 0, the extinction change commonly elicited with NADH or NADPH was 2.4×10^{-3} . Since this value includes a contribution of 2.2×10^{-3} from species 0, the contribution from other species was 0.2×10^{-3} , which is close to the limit of spectroscopic sensitivity available (0.1×10^{-3}). Therefore,

Table 3. *Properties of spectroscopic species of ETP_m preparations from G-cells*

Species	Substrate specificity	Presumed redox potential
0	Reduced by NADH, but not reoxidized by oxygen	—
1	Reduced by NADH, located on the substrate side of the piericidin A sensitive site	Low, i.e. approx. that of NADH/NAD ⁺
2	Reduced by NADH or L-3-glycerophosphate. Located between the sites of action of piericidin A and antimycin A	High, i.e. approx. that of ubiquinone+cytochrome <i>b</i>
3	Reduced by L-3-glycerophosphate only	Intermediate between that of the ubiquinone, cytochrome <i>b</i> region and L-3-glycerophosphate

Fig. 2. Reduced-minus-oxidized difference spectrum of species 0. In the upper trace (a) 30 mg of ETP_m protein from F-cells was suspended in 5.0 ml of air-saturated potassium phosphate buffer (5 mM, pH 7.4) at 25°C and divided between the test and reference cuvettes of a wavelength-scanning spectrophotometer. The light-path was 1 cm. A base line was recorded. NADH (0.4 μmol) was then added to the test cuvette and, after a delay of 3 min (sufficient for the oxidation of the NADH), the spectrum shown was recorded. The peak at 550 nm indicates that some c-type cytochrome was also present. In the lower trace (b), 15 mg of ETP_c protein from G-cells was used in an otherwise similar experiment. The broken line is the base line. An upward deflexion of the trace indicates an increase of extinction of the reduced-minus-oxidized difference.

in the absence of any previous cycle of NADH or NADPH oxidation, the low-potential species reduced by NADH (species 1) is not reduced by NADPH. Similarly, the low-potential species reduced by NADPH (Fig. 3a), and now called species 1', is not reduced by NADH. However, in the

experiments preceded by a cycle of NADH or NADPH oxidation (Tables 4 and 5, Expts 3, 4, 5 and 6) the results were different. First, after a cycle of NADH oxidation, there is no species specifically reduced by NADH, whereas the species specifically reduced by NADPH is virtually unchanged (Tables 4 and 5, Expts. 3 and 4). Thus it appears that, in the presence of NAD⁺ (remaining from the previous cycle of NADH oxidation), NADPH could reduce species 1', presumably via a transhydrogenation to NAD⁺. The low transhydrogenase activity is not an obstacle to this interpretation, since the respiratory chain was inhibited with antimycin A. A second difference was observed after an initial cycle of NADPH oxidation (Tables 4 and 5, Expts. 5 and 6), when the two species specifically reduced by NADH and NADPH respectively were apparently decreased, i.e. in the presence of NADP⁺, NADH can partially reduce species 1'. This could occur via a transhydrogenase. More complete reduction of species 1' by NADH in the presence of NADPH is perhaps prevented by the relatively active, antimycin A-insensitive, NADPH oxidase (Table 2). The partial reduction of species 1 by NADPH in the presence of NADP⁺ cannot be explained unless one postulates a conversion of NADP⁺ into NAD⁺ and P_i by the sub-mitochondrial particles or their contaminating enzymes. This apart, these results lead to the conclusion that there are two separate low-potential species, species 1 reduced by NADH and species 1' reduced by NADPH. Both species are functionally linked to the respiratory chain. Species 1' was found to remain reduced after a cycle of NADPH oxidation in the presence of piericidin A when experiments analogous to those of Fig. 3 were performed with NADPH rather than NADH. Thus species 1', like species 1, can be located on the substrate side of the site of respiratory inhibition by piericidin A.

Fluorimetric studies of flavoprotein fluorescence. Fig. 4(a) shows that the addition of NADH to ETP_m preparations caused a decrease in flavoprotein fluorescence (i.e. flavoprotein reduction),

Fig. 3. Dual-wavelength spectrophotometer measurements of extinction changes at 460–520 nm for ETP_m preparations from G-cells: the effects of NADPH. In each experiment ETP preparation (6.1 mg of protein) was suspended in a final volume of 2.0 ml of 10 mM-tris-sulphate buffer, pH 7.4, at 25°C. An upward deflexion of the recorder tracings indicates an increase in extinction at 460 nm with respect to 520 nm. The light-path was 1 cm. Additions, abbreviations and final concentrations were: NADPH (0.1 mM); antimycin A (AA, 1 μ M); DL-glycerol 3-phosphate (G-3-P, 5 mM). The recording of trace (a) was interrupted for 2 min as shown. The wavelength pair 460–520 nm was used to obtain greater sensitivity than that offered by 470–510 nm (Fig. 1).

Table 4. *Substrate specificity of species 0 and 1 in ETP_c preparations from G-cells*

Experimental conditions were as described in the legend to Fig. 3. In each case, the protein concentration was 2.1 mg/ml. In each experiment DL-3-glycerophosphate (5 mM) and antimycin A (1 μ M) were added, preceded, in Expts. 3–6 inclusive, by an addition of NADH (0.1 mM) or NADPH (0.1 mM) as in Fig. 1(a) and Fig. 2(a) respectively (NADH or NADPH 'cycle'). Further additions were, in Expts. 1, 3 and 5, NADH (0.1 mM) followed by NADPH (0.1 mM) and, in Expts. 2, 4 and 6, NADPH (0.1 mM) followed by NADH (0.1 mM). The total extinction changes for the last two additions in each experiment are given in the last column.

Expt. no.	Pretreatment	$10^3 \times \Delta E$ at 460–520 nm after addition of:				Total change with NADH and NADPH
		DL-3-Glycero-phosphate	NADH before NADPH	NADPH	NADH after NADPH	
1	None	5.0	3.0	0.85	—	3.85
2	None	4.6	—	3.2	0.6	3.8
3	NADH 'cycle'	5.0	0.9	0.75	—	1.65
4	NADH 'cycle'	5.0	—	1.65	0	1.65
5	NADPH 'cycle'	4.9	1.3	0.3	—	1.6
6	NADPH 'cycle'	4.8	—	1.4	0.3	1.7

which returned to its original value after oxidation of the NADH. Further addition of antimycin A and glycerol 3-phosphate caused a small increase in flavoprotein fluorescence, which is attributable to an increase in the intensity of exciting light (456 nm) received by the sample arising from the decrease in extinction of the sample at that wavelength (Ragan & Garland, 1969). A final addition of NADH reduced the fluorescent flavoprotein again, establishing it as of low potential (i.e. not reducible by glycerol 3-phosphate in these non-phosphorylating

submitochondrial particles). NADPH can also reduce this fluorescent flavoprotein, as shown in Fig. 4(b). Therefore this fluorescent flavoprotein cannot be identified as species 1 or 1'. Nor is it species 0, since it is capable of cyclic reduction and oxidation in an uninhibited system. Its properties allocate it to the barely detectable pool of low-potential material that can be reduced either by NADH or NADPH. The decrease in fluorescence after the addition of NADH occurred independently of whether or not reduction of the cytochromes

Table 5. *Substrate specificities of species 0 and 1 in ETP_c preparations from G-cells*

Results taken from Table 4. For details see the Legend to Table 4 and the text.

Pretreatment (and Expt. no. from Table 4)	$10^3 \times \Delta E$ at 460 – 520 nm			
	NADH specific	NADPH specific	Reduced by either substrate	Total reduced by both substrates
None (1, 2)	0.6	0.85	2.4, 2.35	3.85
NADH 'cycle' (3, 4)	0	0.75	0.9, 0.9	1.65
NADPH 'cycle' (5, 6)	0.3	0.3	1.0, 1.1	1.65

could occur (see Fig. 4*a*, and the effects of NADH both before and after the addition of glycerol 3-phosphate and antimycin A). It is therefore unlikely that these changes of fluorescence arose from increases of extinction of the suspension either at the exciting wavelength at 450 nm or at the measuring wavelengths above the cut-off (520 nm) of the secondary filter.

Fig. 4(*b*) also shows that the reduction of the low-potential fluorescent flavoprotein by NADPH was prevented after a previous cycle of NADH oxidation. It is reasonable to suppose that the rate of oxidation of the fluorescent flavoprotein by NAD⁺ and the respiratory chain was in excess of the rate of reduction by NADPH, with the result that the steady-state reduction of the fluorescent flavoprotein was low and undetectable. In keeping with this interpretation, the final addition of antimycin A (Fig. 4*b*) resulted in reduction of the fluorescent flavoprotein. Since NADPH oxidation was insensitive to NAD⁺ (100 μ M), whereas the steady-state reduction of the fluorescent flavoprotein was lowered by NAD⁺, it seems unlikely that this flavoprotein was part of the respiratory chain between NADH or NADPH and oxygen. The most likely candidate for the fluorescent flavoprotein is lipoamide dehydrogenase. The lipoamide dehydrogenase activity of ETP_m preparations from G- or F-cells is about 0.24 unit/mg of protein and, like the low-potential fluorescent flavoprotein, is not removed from the particles by extensive treatment with ultrasonic vibration and washing (O. Tottmar & C. I. Ragan, unpublished work). The lipoamide dehydrogenase activity of the particle may be responsible for the transhydrogenase activity noted above.

In summary, the species discussed so far in ETP_m preparations from G-cells are shown in Scheme 1. It has not been established that species 1, 1', and 2 are obligatory respiratory-chain carriers, although species 0 and the fluorescent flavoprotein are unlikely to be so and species 3 is not implicated in the oxidation of NADH or NADPH.

Properties of submitochondrial particles from F-cells

Oxidase activities. ETP_m preparations from F-cells catalyse a piericidin A-insensitive but

Fig. 4. Flavoprotein fluorescence measurements of ETP_m preparations from G-cells. For optical details, see the Materials and Methods section. In each experiment an upward deflexion of the recorder tracing indicates an increase in flavoprotein fluorescence which, if artifacts were absent (Ragan & Garland, 1969), would correspond to oxidation of flavoprotein. 100% fluorescence is defined as the total signal received by the photomultiplier. The experiments of traces (a) and (b) were performed on different ETP_m preparations. In trace (a), 8.0 mg of ETP protein was suspended in 2.0 ml of 10 mM-tris-sulphate buffer, pH 7.4, at 25°C. In trace (b), 2.0 mg of protein was used and the recording was also interrupted for 3 min as shown. Additions, abbreviations and final concentrations were: NADH (0.3 mM); antimycin A (AA, 1 μ M); DL-glycerol 3-phosphate (G-3-P, 5 mM); NADPH (0.1 mM).

antimycin A-sensitive oxidation of NADH and NADPH. The oxidase activities and inhibitor sensitivities are given in Table 6, and for comparison the rates observed with ETP_m preparations from G-cells (Table 2) are included. The oxidase rates for ETP_m preparations from F-cells were 54%, 64% and 30% of the comparable rates for those of G-cells oxidizing NADH, NADPH and glycerol 3-phosphate respectively.

Scheme 1. Reactions of species 0, 1, 1', 2 and 3 in ETP preparations from G-cells. It is not established that species 2 is the first oxidant of species 1, 1' and 3, and cytochrome *b* and ubiquinone are included in parentheses to indicate the uncertainties of sequence in this region.

Table 6. *Oxidase rates of ETP_m preparations from F-cells*

Assay conditions, and reagent concentrations as described in Table 2. Comparable values for ETP_m preparations from G-cells from Table 2 are given in parentheses.

Substrate	Rate of oxygen uptake ($\mu\text{g-atoms of O}_2/\text{min}$ per mg of protein)	Inhibition by piericidin A	Inhibition by antimycin A (%)
NADH + cytochrome <i>c</i>	0.20 (0.38)	0 (83)	100 (100)
NADPH + cytochrome <i>c</i>	0.12 (0.190)	0 (61)	71 (63)
DL-3-Glycerophosphate + cytochrome <i>c</i>	0.10 (0.33)	0 (<5)	100 (100)

Spectroscopic studies. Experiments similar to those described above for ETP_m preparations from G-cells were repeated with those from F-cells with the object of investigating the effects of iron deficiency on the various species (0, 1, 1', 2 and 3) characterized by their spectroscopic behaviour at 460–520 nm in ETP_m preparations from G-cells. Dual-wavelength spectrophotometer recordings for ETP_m preparations from F-cells are shown in Fig. 5, and the wavelength pair of 476–510 nm was chosen as being preferably closer without diminishing greatly the magnitude of the changes observed. The conclusions to be drawn from Fig. 5 are (i) that a species 0 was present [note the residual change after a single cycle of NADH oxidation in recordings (a) and (b)], (ii) that a species 1 was present [note the change elicited by a final addition of NADH in recordings (a), (b) and (d)], and (iii) that species 2, defined as reducible by either NADH or glycerol 3-phosphate, was greatly decreased. Piericidin A

was without effect on these spectroscopic properties as was anticipated from its inability to inhibit respiration. A full investigation of the spectroscopic effects of NADPH was not attempted, although it should be noted that a species 1' was identified in ETP_m preparations from F-cells.

Fluorimetric studies of flavoprotein fluorescence. The experiments of Fig. 6 showed the presence in ETP_m preparations from F-cells of a fluorescent flavoprotein reduced by NADH or NADPH but not by glycerol 3-phosphate. A fluorescent flavoprotein reducible by glycerol 3-phosphate was not demonstrable. In these respects ETP_m preparations from F-cells resemble those from G-cells.

Difference spectra in species 1, 2 and 3 in submitochondrial particles from G- and F-cells

The experiments of Figs. 1 and 5 were repeated at different wavelength pairs to obtain difference

Fig. 5. Dual-wavelength spectrophotometer measurements of extinction changes at 470–510 nm for ETP_m preparations from F-cells. In each experiment 3.3 mg of protein was used. Otherwise the conditions were as in Fig. 1.

spectra (oxidized minus reduced) from 420 to 530 nm. This procedure, though expensive both of time and material, is more sensitive than the conventional wavelength-scanning technique. Fig. 7 presents the results for ETP_m preparations from G- and F-cells. The spectrum of species 1 exhibited a peak at 420 nm and a minimum at 460 nm in both G- and F-cells. However, in G-cells the trough extended to higher wavelengths than in F-cells. Thus, a considerable extinction decrease for species 1 was obtained at 500–530 nm for the former but none for the latter. It can be concluded, therefore, that species 1 consists of two components, 1a, maximally bleached at 460 nm and unaffected by iron limitation, and 1b, maximally bleached at around 500 nm and deleted by iron limitation.

The difference spectrum of species 2, reducible by NADH, NADPH or glycerol 3-phosphate had, in ETP_m preparations from G-cells, a minimum at 460 nm and an inflexion at about 510 nm. In ETP_m preparations from F-cells, species 2 was undetectable after allowance had been made for the contribution of cytochrome *b* towards the extinction changes accompanying the reduction of species 2. This correction for cytochrome *b* was made by measuring

Fig. 6. Flavoprotein fluorescence measurements of ETP_m preparations from F-cells. The conditions were as in Fig. 4 except that 6.0 mg of protein was used. Traces (a) and (b) are from separate experiments.

the α -band extinction change at 562–575 nm occurring on the addition of substrate to antimycin A-treated particles, and then calculating the β -band extinction change at 500–530 nm as being 0.33 of the α -band change (Goldberger, Smith, Tisdale & Bomstein, 1961). Studies with a partially purified preparation of cytochrome *b* from *T. utilis* confirmed the validity of this correction and also showed that no further correction was needed between 500 and 460 nm (O. Tottmar & C. I. Ragan, unpublished work). Application of the same correction technique for species 2 in ETP_m preparations from G-cells showed that the contribution from cytochrome *b* was not greater than 10–20% of the uncorrected trough depth of species 2.

The extent to which ubiquinone reduction contributed towards species 2 was also investigated. Ubiquinone was directly measured at 280–289 nm (Kröger & Klingenberg, 1966) during the transition after the addition of glycerol 3-phosphate to antimycin A-treated particles in the absence of NADH or NADPH. By using ϵ_{mM} values of $8.8 \text{ mmol}^{-1} \cdot \text{cm}^{-1}$ at 280–289 nm (Kröger & Klingenberg, 1966) and $0.176 \text{ mmol}^{-1} \cdot \text{cm}^{-1}$ at 465–510 nm (Albracht & Slater, 1969), the contribution of ubiquinone extinction changes to the species 2 spectrum of ETP_m preparations from G-cells was calculated to be less than 4% of the uncorrected trough depth for species 2.

Species 3, reduced specifically by glycerol 3-phosphate, had a difference spectrum similar in ETP_m preparations from both G- and F-cells, except that the trough depth was greater in the latter. The spectrum exhibited a peak at 430 nm and a minimum at 460 nm. The Soret band of cytochrome *b* probably does not cause the 430 nm

Fig. 7. Reduced-minus-oxidized difference spectra of species 1, 2 and 3 in ETP_m preparations from G- and F-cells. Species 1, 2 and 3 were identified as shown in Fig. 1, except that the protein concentrations were 2.0 mg/ml for G- ETP_m and 1.7 mg/ml for F- ETP_m . Spectra were plotted at 10 nm intervals with a dual-wavelength spectrophotometer using 520 nm as the reference wavelength. Changes in extinction were plotted with the higher of any chosen pair as the reference. In such plots, no oxidized-minus-oxidized base line is shown as in conventional wavelength-scanning difference spectroscopy. However, use of the wavelength-scanning technique demonstrated the presence of an isosbestic point at about 520 nm. The left-hand traces in the figure are for G- ETP_m , the right-hand traces for F- ETP_m . Traces (a) and (a') are for species 1, (b) and (b') for species 2 and (c) and (c') for species 3.

maximum in the spectrum of species 2, since independent measurements at 562–575 nm showed that the addition of glycerol 3-phosphate to NADH- and antimycin-treated particles did not cause further reduction of cytochrome *b*. The shape of the species 3 spectrum resembles that observed by Garland, Chance, Ernster, Lee & Wong (1967) for electron-transfer flavoprotein in mitochondria from rat liver. The concentrations (as extinction changes per mg of protein) for species 1a, 1b, 2 and 3 in ETP_m preparations from G- and F-cells are summarized in Table 7. The concentration of species 1a was unchanged by iron-limitation, species 1b and 2 were removed, and species 3 was increased.

Difference spectra of species 1, 2 and 3 in submitochondrial particles from other cell types

All varieties of ETP_c preparations examined (Fig. 8) contained species 0, 1a, and 3. The difference

spectra of species 3 were similar in all cases. Fig. 8 shows that sulphate limitation, like iron limitation, led to the loss of species 1b and 2. Fig. 8 also shows that in all ETP_c preparations from recovered cells [FR, FR(cycloheximide), SR_0 , SR_1 and SR_0 (cycloheximide)], species 1b reappeared. Species 2 also reappeared during the recovery procedure provided that cycloheximide was absent. Neither species 1b nor species 2 reappeared during the anaerobic recovery of F-cells (Clegg & Garland, 1971). The problem of correcting for the contribution of cytochrome *b* to the difference spectra of species 2 was simplified in all the cases shown in Fig. 7, as the cytochrome *b* contents of ETP_c preparations from G-, S-, SR_0 -, SR_1 - and SR_0 (cycloheximide)-cells were identical (Haddock & Garland, 1971), as is also the case for ETP_c preparations from FR- and FR(cycloheximide)-cells (Clegg & Garland, 1971). In these cases, therefore, the differences between the species 2 difference spectra are independent of

Table 7. Concentration of species 1a, 1b, 2 and 3 in ETP_m preparations from G- and F-cells

For species 1a, 2 and 3, the concentrations are expressed as $-10^3 \times$ extinction difference at 460–530 nm after reduction, for a 1 mg of protein/ml suspension of ETP_m. For species 1b, the wavelength pair is 500–530 nm. The light-path was 1 cm.

Species	10 ³ × Concn. of species ETP _m preparations from:	
	G-cells ($-\Delta E/\text{mg}$ of protein per ml)	F-cells
1a	0.59	0.6
1b	0.36	<0.04
2	2.8	<0.1
3	1.25	3.0

the cytochrome *b* contribution when comparison is made within groups of identical cytochrome *b* concentration. The concentrations of cytochrome *b* were assayed with the same transition as that used to reduce species 2, namely the addition of glycerol 3-phosphate to antimycin A-treated particles in the presence and absence of NADH.

E'_0 (pH 7.0) values for species 1a, 1b, 2 and 3

After reduction of species 1, 2 and 3 of ETP_m preparations from G-cells by NADH and glycerol 3-phosphate in the presence of antimycin A, as shown in Fig. 1(a), the addition of 2.5 mM-pyruvate and 5 units of lactate dehydrogenase caused a rapid reoxidation corresponding in magnitude to species 1. Further additions of 2.5 mM-lithium lactate caused only slight reduction. The identification of this reoxidizable pool as species 1 is complicated by the presence of species 2 which, however, is absent from ETP_m preparations from F-cells. After a cycle of NADH oxidation, treatment of ETP_m preparations from F-cells with NADH in the presence of antimycin A leads to reduction of species 1 only. This may be readily reoxidized by addition of pyruvate and lactate dehydrogenase. This strengthens the conclusions already drawn from Fig. 1, namely that the E'_0 (pH 7.0) value for species 1 is low (<-250 mV), whereas the values for species 2 and 3 are higher. Treatment of ETP_m preparations from G-cells with dihydroxyacetone phosphate did not have any significant effect on the extent of reduction of species 2 and 3 obtained by addition of DL-glycerol 3-phosphate and antimycin A, even at a molar ratio of dihydroxyacetone phosphate to L-glycerol 3-phosphate of 5:1. This indicates that these species have E'_0 (pH 7.0) values of -150 mV or higher. These results suggest that site 1 energy conservation occurs at a level between species 1

and the group composed of ubiquinone, cytochrome *b* and species 2.

DISCUSSION

Oxidation of NADPH and NADH. Electron-transport particles from *Saccharomyces cerevisiae* do not catalyse the oxidation of NADPH (Mackler, Collip, Duncan, Rao & Huennekens, 1962), and the relatively rapid rate of NADPH oxidation described in Table 1 for electron-transport particles from *T. utilis* is unusual. In the presence of cytochrome *c*, the rate of oxidation of NADPH by ETP_m preparations from G-cells sensitive to piericidin A and antimycin A was 118 ng-atoms of O₂/min per mg of protein. This is about one-third of the comparable rate with NADH. The transhydrogenase activity of ETP_m preparations from G-cells was less than 2 nmol/min per mg of protein and therefore incapable of supporting the observed NADPH oxidase rates. As both NADH and NADPH can reduce both species 2 and cytochrome *b* in antimycin A-treated electron-transport particles, it appears that the paths of oxidation of NADH and NADPH meet on the substrate side of the site of respiratory inhibition by antimycin A. A single NAD(P)H dehydrogenase utilizing both NADH and NADPH would be difficult to reconcile with the lack of transhydrogenase activity, since most isolated NADH dehydrogenases catalyse transhydrogenation between NADH and NAD⁺ analogues (see, e.g., Minakami, Cremona, Ringler & Singer, 1963). If species 1 and 1' are identified as NADH dehydrogenase and NADPH dehydrogenase respectively, then there are two possibilities for the piericidin A inhibition: first, that each dehydrogenase is separately inhibited by piericidin A and secondly, that inhibition occurs at or after the point at which the two pathways meet. This second possibility provides the simpler explanation for the observation that the same concentration of piericidin A (15 pmol/mg of protein) is required for half-maximal inhibition of either NADH or NADPH oxidation. If the first component commonly reducible by each pathway were of high potential (E'_0 at pH 7.0 of about 0 V), then the lack of transhydrogenase activity would be anticipated on thermodynamic grounds. On the other hand, if the first commonly reducible component were of low potential (which we could not demonstrate) then the lack of transhydrogenase activity would require a kinetic rather than thermodynamic explanation. It has not proved possible to prepare phosphorylating electron-transport particles from *T. utilis*, and the question as to whether NADPH oxidation is coupled to energy conservation at site 1 must remain unanswered at present.

Chemical nature of species 1, 2 and 3. There are

Fig. 8. Reduced-minus-oxidized difference spectra of species 1 and 2 in ETP_c preparations from other phenotypic modifications of *T. utilis*. Spectra were obtained as described in the legend to Fig. 7. The protein concentrations expressed as mg/ml were: S, 3.0; FR, 3.0; FR(cycloheximide), 2.4; SR₀, 4.1; SR₁, 3.9; and SR₀(cycloheximide), 3.3.

four types of protein that on reduction may exhibit bleaching in the region mainly from 450 to 530 nm. They are (i) iron-less flavoproteins, (ii) iron-containing flavoproteins, or ferroflavoproteins, (iii) non-haem iron proteins containing both iron and acid-labile sulphide in equimolar amounts, or iron-sulphur proteins, and (iv) non-haem iron proteins not containing acid-labile sulphide, or iron proteins (Hall & Evans, 1969). Members of the last category are rare, and are unlikely to be involved in those phenomena that arose from both iron- and sulphate-limited cell growth. There is no single criterion applicable to submitochondrial particles or mito-

chondria that can unequivocally distinguish the spectroscopic properties of flavoproteins from non-haem-iron proteins. The limitations of flavoprotein fluorescence have been described (Ragan & Garland, 1969), and there are two major restraints imposed on the interpretation of the effects on the respiratory chain of growth limitation by iron or sulphate. These are (i) that the effects may be exerted on the iron-sulphur component of a ferroflavoprotein, thereby affecting the behaviour of a flavin prosthetic group rather than an iron-sulphur protein *per se*, and (ii) that iron- and possibly sulphate-limited growth could act, as in *Clostridium pasteurianum*,

to cause substitution of a new flavoprotein (flavodoxin) for an iron-sulphur protein (ferredoxin) (Knight & Hardy, 1966).

A combination of growth-limitation by iron or sulphate, direct spectroscopic measurements made on electron-transport particles (Table 8), measurements of the concentrations of FAD, FMN, non-haem iron and acid-labile sulphide (Table 8), and a knowledge of the likely extinction coefficients for flavoproteins and non-haem iron proteins (Table 9) can, under favourable circumstances, lead to an unequivocal statement that a certain spectral change in the 450–530nm region is not due to non-haem iron protein. Provided then that cytochrome interference can be corrected for, the spectral change can be attributed to the flavin of a flavoprotein.

Consider first the results of Table 8, which presents both direct spectroscopic data and analytical data for the F to FR and FR(cycloheximide) transitions. These transitions were chosen since they involve minimal but significant changes in the acid-labile sulphide concentration of ETP_m preparations. The non-haem iron/acid-labile sulphide ratio in electron-transport particles can vary widely according to the conditions of cell growth and treatment (Clegg & Garland, 1971; Haddock & Garland, 1971), and the preferred method of estimating the concentration of iron and acid-labile sulphide occurring in the one-to-one ratio (which appears to be the normal state for function; Table 9) is to take the lower of the two measurements (one for non-haem iron, one for acid-labile sulphide). If the spectroscopic species listed in Table 8 arise

from the oxidoreduction behaviour of the carriers (FMN, FAD, iron-sulphur protein), then the spectroscopic and analytical values are related by the appropriate extinction coefficients. By using millimolar extinction coefficients of $-10.6 \text{ mmol}^{-1} \cdot \text{cm}^{-1}$ at 460–530nm for flavoprotein reduction (Mahler, 1954), and $-2.4 \text{ mmol}^{-1} \cdot \text{cm}^{-1}$ for a representative iron-sulphur protein (Table 9), the following conclusions may be drawn from the results in Table 8. First, the iron-sulphur content of ETP_m preparations from F-cells would permit a maximal extinction change at 460nm of 0.072 in the units of Table 8. The actual changes for species 1a and species 3 were 0.54 and 2.7 respectively (Table 8). From this it follows that neither species 1a nor species 3 can be accounted for by reduction of iron-sulphur protein and, since the spectral changes leading to the identification of species 1a and 3 were free from interference by cytochrome it can be concluded that these two species are flavoproteins and that the extinction changes were due to the reduction of the flavin prosthetic group. NADH dehydrogenase from mammalian sources contains FMN, and the FMN content of ETP_m preparations from F-cells could support an extinction change of 0.6–0.7 (units as in Table 8). Thus, it is quantitatively possible that species 1a is the FMN of NADH dehydrogenase. There is no precedent for an NADPH dehydrogenase in mitochondria and this was not investigated in all of the conditions listed in Table 8.

A second conclusion that may be drawn from Table 8 requires that a further assumption must be made, namely that the reappearance of species 1b

Table 8. Concentrations of FMN, FAD, non-haem iron, acid-labile sulphide and species 1a, 1b, 2 and 3 in ETP_m preparations from various cell types

The values for FMN and FAD are taken from Light *et al.* (1968), Clegg & Garland (1971), and Light (1969). The measurements of non-haem iron, acid-labile sulphide and spectroscopic species were made on single preparations of ETP_m, and confirmed by repetition on at least two other preparations. The methods for recovery with $1 \mu\text{M}$ -FeSO₄ labelled with $^{59}\text{FeCl}_3$, the assay of acid-labile sulphide and non-haem iron were as described by Clegg & Garland (1971). G-cells were grown with a low ($1 \mu\text{M}$) concentration of iron and with 55mM-glycerol as the growth-limiting nutrient. Concentrations of species 1a, 1b, 2 and 3 are given as $10^3 \times -\Delta E/\text{mg}$ of protein per ml for a 1 cm light-path at 460–530nm for species 1a, 2 and 3 and at 500–530nm for species 1b. The maximal possible concentration of iron-sulphur protein with an iron/sulphur ratio of unity is represented as Fe-S and corresponds either to the non-haem iron concentration or acid-labile sulphide concentration, whichever is the lower. N.T., not tested.

	Analytical concentrations (nmol or ng-atom/mg of ETP _m protein)					Spectroscopic concentrations (see the legend for units)			
	FMN	FAD	Non-haem iron	Acid-labile sulphide	Fe-S	Species 1a	Species 1b	Species 2	Species 3
F	0.07	0.24	0.11	0.03	0.03	0.54	0.04	0.1	2.7
G	0.06	0.27	0.85	0.58	0.58	0.54	0.33	2.5	1.75
FR	0.06	0.26	0.44	0.38	0.38	0.62	0.44	0.52	1.0
FR(cycloheximide)	0.07	N.T.	0.27	0.31	0.27	0.80	0.40	0.03	1.45
FR(anaerobic)	N.T.	N.T.	0.24	0.07	0.07	N.T.	N.T.	N.T.	N.T.

Table 9. *Properties of isolated non-haem iron proteins*

'e.p.r. detectable Fe' refers to the number of iron atoms that can be accounted for by the electron paramagnetic resonance signal in the $g = 1.9$ region after reduction by substrate. Millimolar extinction coefficients are for the oxidized-minus-reduced difference at 460 nm, expressed per mmol of total iron (or labile sulphide) or per mmol of 'e.p.r. detectable Fe'. In many cases, it had to be assumed that spectra were obtained by using a 1 cm light-path. References: ¹Lovenberg, Buchanan & Rabinowitz (1963), ²Mortenson (1964), ³Malkin & Rabinowitz (1965), ⁴Palmer, Sands & Mortenson (1966), ⁵Tagawa & Arnon (1962), ⁶Bachofen & Arnon (1966), ⁷Fry & San Pietro (1962), ⁸Palmer & Sands (1966), ⁹Kimura (1968), ¹⁰Lovenberg & Sobel (1965), ¹¹Dus, De Klerk, Sletton & Bartsch (1967), ¹²Der Vartanian (1968), ¹³Shethna, Wilson, Hansen & Beinert (1964), ¹⁴Rieske, MacLennan & Coleman (1964), ¹⁵Hatefi & Stempel (1967). Rubredoxin lacks acid-labile sulphide, whereas the others have approximately equimolar amounts of acid-labile sulphide and iron.

Non-haem iron protein	Iron or labile sulphide (atoms/molecule)	e.p.r. detectable Fe (atoms/molecule)	$-\epsilon_{\text{mM}}$ at 460 nm for total iron ($\text{mmol}^{-1} \cdot \text{cm}^{-1}$)	ϵ_{mM} at 460 nm for e.p.r. detectable Fe ($\text{mmol}^{-1} \cdot \text{cm}^{-1}$)
Clostridial ferredoxin	6-8 ^{1,2,3,4}	1 ⁴	0.95→0.71 ⁵	5.7 ⁵
<i>Chromatium</i> ferredoxin	4 ⁶	None*	0.93 ⁶	—
Spinach ferredoxin	2 ⁷	1 ⁸	2.3 ⁵	4.6 ⁵
Andrenodoxin	2 ⁹	1 ⁹	1.9 ⁹	3.8 ⁹
Rubredoxin	1 ¹⁰	None†	6.3 ¹⁰	—
<i>Chromatium</i> high-potential protein	4 ¹¹	None*	2.4 ¹¹	—
<i>Azotobacter</i> protein 1	2 ¹²	1 ¹²	3.5 ¹³	7.0 ¹³
Complex III protein	2 ¹⁴	None‡	1.2 ¹⁴	—
Complex I fraction B	—	None‡	1.5 ¹⁵	—

* No signals in the reduced state.

† No signals in oxidized or reduced state.

‡ $g = 1.9$ signals in reduced state, but no quantitative data available.

and 2 as measured spectroscopically is due to the increases in iron-sulphur content of the electron-transport particles as measured analytically. This assumption is compatible with a role for the increased iron-sulphur content either in a ferroflavoprotein or iron-sulphur protein. The recovery of species 1b in the F to FR(cycloheximide) transition was associated with an increase in iron-sulphur concentration of 0.28 ng-atom/mg of protein, and an increase of species 1b from <0.04 to 0.40 in the units of Table 8. The corresponding millimolar extinction coefficient for species 1b at 500–530 nm would therefore be 0.40/0.28, or $-1.43 \text{ mmol}^{-1} \cdot \text{cm}^{-1}$. This value is consistent with the properties of ferroflavoproteins or iron-sulphur proteins, and can be used for ETP_m preparations from FR- or G-cells to calculate the amount of iron and sulphur that could be attributed to species 1b. Subtraction of this amount from the total iron and sulphur of these ETP_m preparations leaves a difference that can be regarded as the iron and sulphur of species 2. From the results in Table 8, this calculation leads to values for iron and sulphur (ng-atom/mg of protein) for species 2 of 0.07 for ETP_m preparations from FR-cells and 0.35 for those from G-cells. This fivefold difference is reflected in the corresponding extinction changes for species 2 (0.52 for FR-cells; 2.5 for G-cells: Table 8) and the corresponding millimolar extinction coefficients for species 2 come

to -7.4 and $-7.1 \text{ mmol}^{-1} \cdot \text{cm}^{-1}$ at 460–530 nm. Whether species 2 represents ferroflavoprotein or iron-sulphur protein cannot be stated with certainty: the intermediate value for the extinction coefficient and the errors inherent in its calculation leave room for adjustment either to greater values (ferroflavoprotein) or lesser values (iron-sulphur proteins).

A noteworthy corollary to these conclusions is that the iron and sulphur content of ETP_m preparations from both G- and FR-cells is as active in the sense of being capable of reduction as is the iron-sulphur content of a number of purified ferroflavoproteins or iron-sulphur proteins. This is not the case with ETP_m preparations as they are normally prepared from ox heart or *T. utilis* grown on iron-supplemented media. In these latter instances the iron-sulphur concentration is as high as 5 ng-atoms/mg of protein, and not more than a small percentage can be identified by either visible or electron-paramagnetic-resonance spectroscopy (Beinert, 1965; Beinert & Lee, 1961).

Identification of species 1, 2 and 3 with previously known respiratory-chain components. The reduced-minus-oxidized difference spectrum of species 1 in ETP_m preparations from G-cells closely resembles the difference spectrum in ox heart electron-transport particles for reduction by succinate plus NADH minus reduction by succinate (Estabrook, Tyler,

Gonze & Peterson, 1968). The spectrum also resembles the NADH reduced minus oxidized difference spectrum of crude NADH dehydrogenase in the phospholipase A digest of ox heart electron-transport particles (Singer, Minakami & Ringler, 1963). Species 1 therefore has the spectral properties expected for NADH dehydrogenase. The ratio of iron-sulphur protein to FMN in ETP_m preparations from F-cells is 0.43. It therefore follows that, if either the FMN or species 1a content of ETP_m preparations from F-cells is equated with the flavin of NADH dehydrogenase, then neither a low-molecular-weight NADH dehydrogenase (iron-sulphur/FMN ratio about 2:1 or 4:1; Hatefi & Stempel, 1967) nor a high-molecular-weight NADH dehydrogenase (iron-sulphur/FMN ratio 10:1 or greater) would be present to account for the FMN or species 1a. The iron-sulphur/FMN ratio in ETP_m preparations from FR(cycloheximide) cells is 3.8:1, and in those from FR-cells 6.3:1. It is therefore reasonable to suggest that species 1b corresponds to the iron-sulphur component of a low-molecular-weight type of NADH dehydrogenase ferroflavoprotein, whereas species 2 corresponds to the additional iron-sulphur of the high-molecular-weight type NADH dehydrogenase. The iron-sulphur/FMN ratio in ETP_m preparations from G-cells was 9.8:1 (Table 8).

A spectroscopically characterized component with properties reminiscent of species 2 is also found in electron-transport particles from mammalian sources. Chance, Azzi, Lee, Lee & Mela (1969) believe that it is a flavoprotein (Fp_{D2} of Chance *et al.* 1967), but the results presented above are also consistent with the suggestion that it is a non-haem iron protein (Ragan & Garland, 1969; Hatefi, 1966, 1968). Albracht & Slater (1969) considered that the spectroscopic basis for inferring the presence of this species in submitochondrial particles was invalidated by interference from ubiquinone in the 460nm region. This objection has now been withdrawn (E. C. Slater, personal communication), and is also shown to be untenable for *T. utilis* (see the Results section). Nor can interference by cytochrome (Gutman *et al.* 1970) account for the spectroscopic behaviour of species 2 in electron-transport particles from G-, FR- and SR-cells.

Species 3 appears to be the flavin of L-glycerol 3-phosphate dehydrogenase (EC 1.1.99.5). The enzyme isolated from pig brain contains flavin, non-haem iron and acid-labile sulphide (Ringler, 1961), but little is known of the detailed functioning of these components. The extinction changes observed on adding substrate to the isolated enzyme were small (Ringler, 1961), but more extensive bleaching was caused by dithionite. The dithionite-reduced-minus-oxidized difference spectrum had a trough at 460nm and a peak at 430nm, very similar to that of species 3.

Function of species 1a, 1b, 2 and 3. Species 1a and 3 are flavoproteins which can be identified with NADH dehydrogenase and glycerol 3-phosphate dehydrogenase respectively. Species 1b is not detectable in electron-transport particles when site 1 energy conservation is missing from the mitochondria (e.g. F- and S-mitochondria). It is therefore possible that species 1b is essential for energy conservation but not for electron flow between NADH and the cytochromes. This does not exclude a role for species 1b as a carrier member of the respiratory chain in G-cell mitochondria, but does imply that the route or mechanism of reduction of the cytochromes is different in the absence of species 1b. The functional correlation of species 2 is with piericidin A sensitivity. Since we have not yet been able to produce piericidin A-sensitive mitochondria lacking site 1, we cannot decide whether species 1b is also a requirement for piericidin A sensitivity.

If species 2 is the piericidin-sensitive component, then there is agreement with the conclusions drawn above about distinct NADH and NADPH dehydrogenases and only one site of piericidin A action at or below the point where the two pathways join each other. Further, if the two pathways met by commonly reducing species 2, then its high potential would exclude a transhydrogenase activity between NAD and NADP in non-phosphorylating electron-transport particles. To postulate that species 2 is the piericidin A-sensitive component is preferable to the common custom of putting the site of action of this inhibitor on a reaction arrow between two adjacent respiratory carriers, a region which by definition contains nothing. It must be admitted that piericidin A sensitivity is scarcely a biological role for species 2, and why *T. utilis* should make this species when it is not apparently essential for electron flow or energy conservation is not known.

A further correlation of species 1b and 2 in combination is with the presence of an NADH-reducible electron paramagnetic resonance spectroscopy signal at $g = 1.94$ (Clegg *et al.* 1969). Thus, this signal is present in electron-transport particles from G-, FR-, and SR-cells but absent from those from F, FR, FR(anaerobic)-, FR(cycloheximide)-, S- and SR(cycloheximide)-cells (Clegg *et al.* 1969; C. I. Ragan, J. Swann & R. C. Bray, unpublished work). This particular $g = 1.94$ signal is considered to arise from a low-potential component of NADH dehydrogenase, and the most likely candidate for this would be species 1b. Nevertheless the additional non-haem iron of species 2 appears to be required for the signal to appear. In this respect, it is noteworthy that analogous behaviour is shown by isolated NADH dehydrogenases from ox heart. The low-molecular-weight enzyme (iron-sulphur/FMN of about 2:1 or 4:1) does not yield a

$g = 1.94$ electron-paramagnetic-resonance signal on reduction with NADH, whereas the high-molecular-weight enzyme does (Singer, 1966; King *et al.* 1966).

It is not possible to provide an absolute proof for the 'absence' of species 1b and 2 from electron-transport particles F- and S-cells, for failure to detect these species could be due to lack of instrumental sensitivity. Nevertheless, the parallel changes of other respiratory-chain properties (energy conservation at site 1, piericidin A sensitivity) do indicate that the chain is indeed different and not merely proceeding as before through smaller concentrations of the same carriers.

The role of iron-sulphur proteins in site 1 energy conservation and in piericidin A sensitivity could be merely structural as, for example, is the role of cytochrome *b* in conferring 4,4,4-trifluoro-1-thien-2-ylbutane-1,3-dione sensitivity on succinate-ubiquinone reductase (Racker & Bruni, 1968). We consider that this is unlikely to be entirely so for the magnitude of the spectroscopic changes occurring on reduction of species 1b and 2 in electron-transport particles from FR-cells (Table 8) and even G-cells (provided that the entering iron concentration was nearly but not quite growth-limiting) indicates that most, if not all, of the non-haem iron of the electron-transport particles can undergo oxidoreductions. This, of course, does not exclude a structural role, but indicates the likelihood of a catalytic role as well.

Although the evidence for the deletion of species 1b and 2 from electron-transport particles from F- and S-cells was strong, no evidence was forthcoming to suggest that an alternative species had been synthesized as a replacement. If species 1b and 2 are indeed respiratory carriers, it is difficult to understand how respiration could be so little affected by their deletion. However, this difficulty may arise from our inadequate and perhaps too inflexible concepts of the mechanisms of the respiratory chain, rather than from any molecular cause.

Mechanisms of oxidoreductions in ETP_m preparations from F- and FR(cycloheximide)-cells. The spectral and analytical characteristics associated with species 1a in ETP_m preparations from F-cells indicate that the reduced form corresponds to fully reduced flavin (FMNH₂). Since species 2 is absent, the likely acceptor for the NADH dehydrogenase is ubiquinone. This sequence of two hydrogen carriers would not, according to Mitchell's (1966) chemiosmotic hypothesis, result in energy conservation. In ETP_m preparations from FR(cycloheximide)-cells, it is not possible to draw such a definite conclusion about the reduced forms of species 1a and 1b. If the sequence in electron-transport particles FR(cycloheximide)-cells is from species 1a

to ubiquinone via the iron-sulphur protein of species 1b, then the alternating sequence of hydrogen and electron carriers required by the Mitchell (1966) hypothesis would be possible and also in keeping with the correlation between the presence of site 1 and species 1b. The demonstration of a functional NADH dehydrogenase coupled to energy conservation at site 1 but insensitive to piericidin A must lead to a reappraisal of the acceptor specificities of solubilized NADH dehydrogenases. Although some of these preparations act as an NADH-coenzyme Q reductase, the significance of this activity has been doubted on the grounds of its lacking rotenone sensitivity which, in this context, is equivalent to piericidin A sensitivity (Singer, 1966; Pharo, Sordahl, Vyas & Sanadi, 1966). However, it is apparent from our results that an NADH-coenzyme Q reductase need not be regarded as an artifactual activity on the grounds that it is piericidin insensitive. The kinetic interrelationships of species 1a, 1b, ubiquinone and cytochrome *b* in FR(cycloheximide)-mitochondria and ETP_m preparations should permit a relatively unequivocal identification of the carrier sequence and an end to the controversy about the physiological acceptor for the NADH dehydrogenase (Slater, 1966).

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