The Effects of Iron-limited Growth on the Reduced Nicotinamide–Adenine Dinucleotide Dehydrogenase Activity and the Membrane Proteins of *Candida utilis* Mitochondria

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(Received 20 June 1973)

1. The mitochondrial NADH dehydrogenase (EC 1.6.99.3) of *Candida utilis* exhibited altered properties when the organism was grown under iron-limited conditions. No suitable acceptor was found for assay of this enzyme from iron-limited cells. 2. Mitochondrial membrane proteins from *C. utilis* were analysed by polyacrylamide-gel electrophoresis. Compared with glycerol-limited cells, iron limitation resulted in the loss of at least two polypeptides from the mitochondrial membrane. 3. Neither of the polypeptides affected by iron limitation was part of a cytochrome, although one of them was part of the mitochondrial NADH dehydrogenase. 4. Non-haem iron of mitochondrial membranes was released in the presence of sodium dodecyl sulphate, and electrophoresis in solutions of this detergent cannot be used directly to identify iron–sulphur proteins. Non-ionic detergents do not release non-haem iron but nor do they provide a satisfactory system for electrophoretic separation.

Continuous culture of *Candida utilis* under conditions in which iron is the growth-limiting nutrient, has been shown to result in several modifications in both the composition and activity of the mitochondrial respiratory chain between NADH and cytochromes *b* (Light *et al.*, 1968; Clegg *et al.*, 1969; Light & Garland, 1971; Clegg & Garland, 1971; Ragan & Garland, 1971; Clegg & Light, 1971). Similar phenotypic changes of *C. utilis* may also be induced by growth of this organism in batch cultures in which the initial iron concentration is below $1.0\mu\text{M}$ (Ohnishi *et al.*, 1969; Ohnishi & Schleyer, 1969; Ohnishi & Chance, 1971; Demaille *et al.*, 1970). Several independent lines of evidence suggest that the altered behaviour of the respiratory chain in mitochondria from iron-limited *C. utilis* is due to the loss or modification of iron–sulphur proteins which, in the mitochondria of glycerol-limited *C. utilis* (or of *C. utilis* whose growth is limited by any one of several other nutrients in the presence of a non-limiting concentration of iron), participate in the functions of electron transport and energy conservation (Ragan & Garland, 1971; Ohnishi & Chance, 1971). In the present study we describe the effects of iron-limited growth of *C. utilis* on the protein composition of mitochondrial membranes as measured by electrophoresis in the presence of detergents. We also describe the effects of iron limitation on the oxidation of NADH by a variety of artificial acceptors catalysed by mitochondrial membrane fragments.

Materials and Methods

The apparatus and growth conditions for the continuous culture of *C. utilis* (N.C.Y.C. 193) were as described by Light & Garland (1971). The dilution rate was 0.2h^{-1} ; pH was controlled at 5 ± 0.2 , and the temperature at $30\pm 1^\circ\text{C}$. For certain experiments, batch cultures (in 100 ml of the glycerol- or iron-limited media described by Light & Garland, 1971) were used. They were inoculated with 0.1 ml of a liquid culture grown in iron-free medium, incubated at 30°C on a rotary shaker and harvested in late exponential phase. Growth medium for glycerol-limited continuous culture contained $2\mu\text{M}$ -iron; no iron was added to media supplied to iron-limited continuous cultures (Clegg & Garland, 1971).

Preparations of mitochondria (Light & Garland, 1971) and submitochondrial particles (Clegg & Garland, 1971) were by methods previously described.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis was performed in gels, 5 mm in diameter, whose acrylamide composition was normally $T = 10\%$ (w/v), $C = 2.5\%$ (w/w) (this nomenclature is that suggested by Hjerten, 1962), in approximate accordance with the method of Weber & Osborn (1969). Disaggregation of samples was performed at 100°C for 10 min in the buffer described by these authors with the addition of 1 mM-phenylmethane-sulphonyl fluoride. Samples underwent some proteolytic degradation in the absence of this inhibitor.

Densitometric traces of gels stained in Coomassie Blue were obtained by using an Eppendorf photometer (Netheler und Hinz G.m.b.H., Hamburg, Germany) modified for densitometry.

Polyacrylamide-gel electrophoresis in the presence of other detergents was usually performed in gels of the above composition, with the detergent at a concentration of 0.1% in the gel and electrode compartment buffers.

To measure the distribution of radioactivity in gels, they were sliced into discs 1 mm thick. These slices were then dissolved as described by Tishler & Epstein (1968) and their radioactivity was counted after the addition of 1 ml of NCS (Amersham-Searle Corporation, High Wycombe, Bucks., U.K.) and 10 ml of scintillation fluid containing 3 g of 2,5-diphenyloxazole and 0.1 g 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of toluene. Other aqueous samples were also counted in this NCS-toluene scintillation mixture. A Nuclear Chicago Unilux II or Isocap 3000 liquid-scintillation spectrometer was used. Quenching was determined by either external standard ratio or channels ratio methods. Results are expressed as c.p.m. above background, corrected to zero quenching. At least 2000 counts in excess of background were collected from each sample.

Spectrophotometric measurements were made with a dual wavelength spectrophotometer constructed in general as described by Chance & Legallais (1951). The optical path-length of the cuvettes was 1 cm.

NADH-ferricyanide reductase was prepared from glycerol-limited *C. utilis* and assayed by the methods of Tottmar & Ragan (1971). An initial ferricyanide concentration of 0.625 mM was used in the enzyme assay. O₂ uptake was measured polarographically (Light & Garland, 1971). The reduction by NADH of ubiquinones, other benzoquinones and naphthaquinones (added to reactions as methanolic solutions) was assayed as described by Ruzicka & Crane (1971). FMN and FAD were measured fluorimetrically by the method of Burch (1957). Protein was measured by the method of Lowry *et al.* (1951), after precipitation by 10% (w/v) trichloroacetic acid from solutions containing detergent, with bovine serum albumin as standard.

Horse heart cytochrome *c* and NADH were obtained from the Boehringer Corp. (London) Ltd., London W.5, U.K. Ubiquinone-1 and piericidin A were gifts from Professor K. Folkers. Duroquinone (2,3,4,6-tetramethyl-1,4-benzoquinone) was obtained from Ralph N. Emanuel Ltd., Wembley, London, U.K. Juglone (5-hydroxyl-1,4-naphthaquinone) and 1,2-naphthaquinone were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Lubrol-WX was a gift from I.C.I. Ltd. (Organics Division), Manchester, U.K. Tween 80, Tween 40, Brij 35, sodium deoxycholate, phenylmethanesulphonyl fluoride, antimycin A and Coomassie

Brilliant Blue R were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. L-[U-¹⁴C]Leucine (0.34 Ci/mmol), L-[4,5-³H]leucine (3.2 Ci/mmol) and ⁵⁹Fe (as FeCl₃ in 0.1 M-HCl, 0.0027 Ci/mg of iron) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Cytochrome *b* and cytochrome oxidase (EC 1.9.3.1) were prepared from *C. utilis* by Dr C. I. Ragan and Dr S. O. C. Tottmar by the methods they have described (Tottmar & Ragan, 1971). Scintillants were supplied by Thorn Electronics Ltd., Surbiton, Surrey, U.K.

All other chemicals and reagents were purchased from BDH Ltd., Poole, Dorset, U.K., and were of the highest purity available. Water was distilled twice from an all-glass apparatus.

Results and Discussion

NADH dehydrogenase activity of submitochondrial particles

The respiratory-chain NADH dehydrogenase defined as the enzyme responsible for NADH oxidation by the next carrier, presumably ubiquinone (Singer & Gutman, 1971) can be assayed after release from the mitochondrial membrane only as an NADH-ferricyanide reductase (Minakami *et al.*, 1962) or as a reductase for other artificial acceptors such as naphthoquinones or benzoquinones. These NADH-acceptor reductase activities are also characteristic of many other flavoproteins (Singer, 1961), and there is therefore no guarantee that such an activity, measured in native or detergent-solubilized mitochondrial membranes, represents the respiratory chain NADH dehydrogenase. Tottmar & Ragan (1971) have shown that the NADH-ferricyanide reductase activity of submitochondrial particles from glycerol-limited *C. utilis* can be attributed to a respiratory chain NADH dehydrogenase with properties similar to the enzyme of mammalian mitochondria (Singer & Gutman, 1971). This conclusion can not validly be extrapolated to iron-limited submitochondrial particles, and we have therefore attempted a partial characterization of the NADH-ferricyanide reductase from this source.

Oxidation of NADH by submitochondrial particles with various acceptors

Table 1 lists the rates of NADH oxidation by submitochondrial particles from glycerol-limited and iron-limited *C. utilis*, with several acceptors. These results show that iron-limitation caused (1) an eight-fold fall in NADH-ferricyanide reductase activity, (2) complete loss of piericidin A-sensitive reduction of ubiquinone-1 and duroquinone by NADH, (3) a fall in the rates of reduction of substituted naphthaquinones which is twofold or smaller, and (4) a two-

Table 1. Oxidation of NADH by submitochondrial particles from iron-limited and glycerol-limited *C. utilis* in the presence of various acceptors, with and without piericidin A

For all assays except those in which O₂ was the acceptor, antimycin A (0.1 µg/ml) and KCN (3 mM) were present in the reaction mixture. The final concentration of protein ranged between 0.05 mg/ml in the assay of ferricyanide reduction and 2 mg/ml in the assay of O₂ uptake. The concentration of piericidin A during the determinations 'with piericidin A' was 1 nmol/mg of protein. Other methods were as described in the Materials and Methods section.

Acceptor	NADH oxidation rate (nmol of NADH · min ⁻¹ · mg of protein ⁻¹)		
	Glycerol-limited submitochondrial particles		Iron-limited submitochondrial particles
	Without piericidin A	With piericidin A	With or without piericidin A
Ferricyanide	4300	4300	500
Ubiquinone-1	62	10	14.5
Duroquinone	94	12	20
Juglone	66	66	31
1,2-Naphthaquinone	180	180	132
O ₂	390	59	200

fold fall in the rate of NADH oxidation by O₂. Despite the large decrease of NADH–ferricyanide reductase activity resulting from iron-limitation, this activity was still in excess of the NADH oxidase rate.

Fractionation of NADH–ferricyanide reductase from iron-limited submitochondrial particles

We have investigated the recovery of NADH–ferricyanide reductase activity in fractions prepared either by extraction of iron-limited submitochondrial particles at pH 10 (Table 2) or with ether followed by 1% (w/v) Lubrol-WX (Table 3). It was not significantly released from glycerol-limited particles at pH 10, whereas it was from the iron-limited particles. The FMN was not significantly solubilized from either type of submitochondrial particle although Tottmar & Ragan (1971) showed that the FMN of glycerol-limited submitochondrial particles is quantitatively recovered on purification of the NADH–ferricyanide reductase activity. Together, these results indicate that the FMN of iron-limited submitochondrial particles is not associated with an NADH–ferricyanide reductase of more than 2% of the activity, per mol of FMN, of that in glycerol-limited particles.

A similar conclusion can be drawn from the experiments of Table 3, where the extraction procedure released most of the NADH–ferricyanide reductase activity and FMN from glycerol-limited particles, but failed to release NADH–ferricyanide reductase from iron-limited particles despite the solubilization of FMN.

Analyses of the protein composition of submitochondrial particles by using polyacrylamide-gel electrophoresis

Although the above results show that the NADH–ferricyanide reductase activity of the respiratory-chain NADH dehydrogenase is diminished by up to 98% by iron-limited growth, they do not provide direct information on the possible absence of proteins in response to iron limitation. There does not appear to be any general rule relating the absence of the prosthetic group of an enzyme during cell growth to the synthesis of the apoprotein. For instance, the apoproteins of catalase (EC 1.11.1.6, Beljanski & Beljanski, 1957) and the *b*-type respiratory-chain cytochromes (Haddock & Schairer, 1973) of *Escherichia coli* are synthesized in the absence of haem synthesis, as are the apoproteins of nitrate reductase (EC 1.9.6.1) in *Staphylococcus aureus* (Chang & Lascelles, 1963) and the cytochrome *c* of *Spirillum itersonii* (Garrard, 1972). By contrast, the apoproteins associated with bacteriochlorophyll are not synthesized in *Rhodospseudomonas spheroides* in the absence of bacteriochlorophyll synthesis (Takemoto & Lascelles, 1973), and a similar relationship holds for haemoglobin synthesis in rabbit reticulocytes (Gross & Rabinowitz, 1972).

We have therefore attempted to identify the NADH dehydrogenase by means of polyacrylamide-gel electrophoresis of disaggregated mitochondrial membranes in detergent solutions. Electrophoretic profiles of the NADH dehydrogenase, of other iron-

Table 2. *Distribution of protein, FMN and NADH-ferricyanide reductase activity after treatment, at pH 10, of submitochondrial particles from iron-limited and glycerol-limited C. utilis*

Submitochondrial particles were suspended in 0.1 M-glycine-KOH buffer, pH 10, at a protein concentration of 4 mg/ml, and stirred for 30 min at 4°C. A part of this extract was removed for the assays represented in the columns headed 'submitochondrial particles'. The remainder was centrifuged, at 4°C, for 50 min at 105000g. The supernatants were poured off and the tubes and pellets rinsed carefully with 1 ml of the glycine-KOH buffer. The pellets were re-suspended by gentle homogenization in this buffer. The volumes of all fractions were recorded. Compared with a control sample incubated at pH 7.4 for 30 min at 4°C, the pH 10 treatment caused activation of between 5 and 12% of the NADH-ferricyanide reductase activity. N.A., Not applicable.

	Glycerol-limited				Iron-limited			
	Submitochondrial particles	Supernatant	Pellet	Recovery (%)	Submitochondrial particles	Supernatant	Pellet	Recovery (%)
Protein (mg)	114	59	51	97	104	59	51	106
FMN (nmol)	3.3	0.33	3.1	104	3.4	0.28	2.8	91
FMN (nmol/mg of protein)	0.029	0.006	0.061	N.A.	0.033	0.005	0.055	N.A.
NADH-ferricyanide reductase activity (μ mol of NADH oxidized/min)								
(1) Total	546	10.4	588	110	46.8	36.5	1.2	79
(2) Per mg of protein	4.8	0.176	11.5	N.A.	0.45	0.62	0.024	N.A.
(3) Per nmol of FMN	165	31.5	189	N.A.	13.8	130	0.43	N.A.

Table 3. *Protein FMN and NADH-ferricyanide reductase distribution after treatment with Lubrol WX of ether-extracted submitochondrial particles from iron-limited and glycerol-limited C. utilis*

Submitochondrial particles were suspended in 10mm-Tris-HCl, pH 7.4, at an approximate protein concentration of 50 mg/ml, and extracted with 5 vol. of ether. This, and all subsequent steps were carried out at 0-4°C. The ether and aqueous phases were separated by centrifugation in a clinical centrifuge, and the aqueous residue was re-extracted with a further 5 vol. of ether. The aqueous residue from this extraction (P₀) was suspended in 10mm-Tris-HCl, pH 7.4, plus 1 % (w/v) Lubrol-WX at a protein concentration of 10 mg/ml. The suspension was stirred for 30 min, then centrifuged at 105 000g for 50 min. The supernatant (S₁) was poured off, and the residual pellet (P₁) was re-suspended in the same buffer to the same final volume as during the detergent-extraction step. Portions of each fraction were set aside for the assay of protein, FMN and NADH-ferricyanide reductase as described in the Materials and Methods section. This fractionation procedure is a modified form of that used by Kaniuga & Gardas (1967) as an initial step in the preparation of NADH dehydrogenase from ox heart submitochondrial particles. One unit of NADH-ferricyanide reductase is defined as the catalysis of the oxidation of 1 μmol of NADH/min. N.T., Not tested.

		Glycerol-limited				Iron-limited			
		Submitochondrial particles				Submitochondrial particles			
		P ₀	P ₁	S ₁		P ₀	P ₁	S ₁	
Protein	{ mg % }	16.5 100	13.7 83	2.3 14		13 100	10.3 79	2.2 17	
NADH-ferricyanide reductase	{ units/mg of protein % }	97 5.9	35 2.6	57 24.8		5.4 0.42	4.3 0.42	0.54 0.25	
FMN	{ nmol/mg of protein % }	100 0.483 0.029 100	85 N.T. N.T. N.T.	59 0.361 0.157 75		98 N.T. N.T. N.T.	80 0.041 0.004 9	10 0.37 0.168 84

Fig. 1. Densitometer tracings of sodium dodecyl sulphate-polyacrylamide-gel electrophoretic separations of mitochondrial membrane proteins prepared from glycerol-limited *C. utilis*

Sample disaggregation and electrophoresis in 10% (w/v) gels was performed as described in the Materials and Methods section. From above downwards, tracing A (—) is of submitochondrial particles; tracing B (---), cytochrome oxidase; tracing C (·····), cytochrome *b*; tracing D (-.-.-), NADH dehydrogenase. The zero extinction value on the vertical axis is different for each of these tracings to allow their display without an unacceptable degree of overlap. Therefore the axis has no scale markings. Since the densitometer output is always adjusted to read zero extinction at the most transmitting region of the gel, the absolute extinction values depend only upon the degree of destaining of the gel, and are arbitrary. The cytochrome *b* preparation contained 7.4 nmol of haem/mg of protein; cytochrome oxidase contained 4.8 nmol of haem/mg of protein. The specific activity of the NADH dehydrogenase was 52 μmol of NADH oxidized $\text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$.

containing proteins (cytochrome oxidase and cytochrome *b*) and of submitochondrial particles from glycerol-limited *C. utilis* are shown in Fig. 1. Each of the purified preparations was free from the characteristic chromophore or activity of the others; consequently, the polypeptides which are not unique to each preparation are probably impurities. Additionally, the most slowly migrating polypeptide of NADH dehydrogenase preparations purified together with the enzyme activity, whereas a number of other bands present did not.

Fig. 2. Densitometer tracings of sodium dodecyl sulphate-polyacrylamide-gel electrophoretic analysis of submitochondrial particles from iron-limited and glycerol-limited *C. utilis*

Sample disaggregation and electrophoresis in 10% gels was performed as described in the Materials and Methods section. The upper tracing A is of glycerol-limited submitochondrial particles; the lower tracing B, iron-limited submitochondrial particles. The arrow indicates the area of these gels in which the greatest difference is reproducibly obtained between these two preparations.

The membrane proteins of mitochondria from iron-limited and glycerol-limited phenotypes are compared in Fig. 2. Of the several quantitative differences between these two qualitatively similar profiles, only two (indicated by an arrow in Fig. 2) remained when the comparison was made by a method which eliminates artifactual differences attributable to different conditions of preparation and electrophoresis. Fig. 3 shows the results of this type of comparison. The two major peaks locate the positions of two or more polypeptides present in twofold greater concentration in glycerol-limited than in iron-limited mitochondrial membranes. The relative mobility of the more slowly migrating of these two peaks is the same as that of one of the major polypeptides of NADH dehydrogenase. Neither of these two peaks has the same relative mobility as any of the component polypeptides of cyto-

Fig. 3. Co-electrophoresis of ^3H -labelled iron-supplemented and ^{14}C -labelled low-iron submitochondrial particles in sodium dodecyl sulphate-polyacrylamide gels

Batch-grown cells were used in this experiment. Low-iron cells were grown in the presence of [^{14}C]leucine ($0.5\mu\text{Ci/ml}$) and in the absence of added iron. Iron-supplemented cells were grown in the presence of [^3H]leucine ($5\mu\text{Ci/ml}$) and $2\mu\text{M}$ -iron. Inoculation times of these cultures were staggered such that both could be harvested together at the same cell density in late exponential phase, 1h after the addition of 10mM -L-leucine to each. Submitochondrial particles were prepared from the pooled cells; all buffers contained 10mM -L-leucine. Disaggregation and electrophoresis in sodium dodecyl sulphate-10% polyacrylamide gels was performed as described in the Materials and Methods section. Gels were first stained, then sliced; the ^3H and ^{14}C radioactivity of each slice was determined. In the experiment shown, recovery of ^3H was 103% and of ^{14}C was 101%. The staining pattern and radioactivity profile for each isotope was similar to that found in submitochondrial particles from the corresponding chemostat-grown cell type.

chrome oxidase or cytochrome *b* (see Fig. 1) or of cytochrome *c* (see the following section).

Behaviour of iron-sulphur proteins in sodium dodecyl sulphate

Results in the previous section have shown that iron-limited continuous culture (or low-iron batch culture) of *C. utilis* causes specific alterations in the polypeptide chain composition of mitochondrial membranes. Further, they suggest, but do not prove, that one of the affected polypeptides is part of the NADH dehydrogenase multienzyme complex. From e.p.r. (electron paramagnetic resonance) studies of this enzyme in submitochondrial particles, it is known that it contains, in iron-supplemented *C. utilis*, several paramagnetic iron-sulphur centres (Ohnishi *et al.*, 1972), each of which may be a separate iron-sulphur protein. Our earlier analytical and e.p.r. results (Clegg & Garland, 1971; Clegg *et al.*, 1969) imply that at least some of the mitochondrial membrane proteins whose concentrations are diminished by iron-limited growth may be iron-sulphur proteins. We therefore attempted to identify, as iron-sulphur proteins, the mitochondrial polypeptides affected by iron-limited growth.

Fig. 4 shows the distribution of protein and ^{59}Fe in a sodium dodecyl sulphate-polyacrylamide gel after electrophoresis of iron-supplemented mitochondrial membranes whose iron proteins had been labelled *in vivo* by growth in the presence of ^{59}Fe . Some 87% of the iron in the preparation was no longer in specific association with protein after disaggregation and electrophoresis.

Fig. 5 shows the time-course of this labilization, by sodium dodecyl sulphate, of membrane-bound iron at room temperature. These membrane preparations contain approximately equimolar amounts of haem and non-haem iron (Clegg & Garland, 1971). Therefore the extent of iron loss in the presence of sodium dodecyl sulphate demonstrates that both types of prosthetic group are dislocated from their specific associations with their respective apoproteins. The residual 13% of sodium dodecyl sulphate-stable iron is probably in the haem of cytochromes *c*, these being the only respiratory-chain cytochromes in which the haem groups are covalently linked to their proteins. This identification is supported both by its quantity and by its mobility in sodium dodecyl sulphate-polyacrylamide gels, which is the same as that determined for horse heart cytochrome *c*.

The denaturation of haem and non-haem iron proteins effected by sodium dodecyl sulphate is, therefore, such that the proteins dissociate from their iron-containing chromophores. Consequently we were unable to identify directly any mitochondrial protein as an iron-sulphur protein after separating it from other membrane proteins by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis.

Behaviour of mitochondrial iron-sulphur proteins in other detergents

We investigated the possibility of using other detergents to disaggregate membrane proteins before their quantitative separation, on an analytical scale, by electrophoretic and other methods.

Fig. 4. Distribution of ^{59}Fe in sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of submitochondrial particles labelled *in vivo* from iron-supplemented *C. utilis*

Batch-grown cells were used in this experiment. The culture medium contained ^{59}Fe at a concentration of $5\mu\text{Ci/ml}$. Submitochondrial particles were prepared from late exponential-phase cells and disaggregated and electrophoresed in 10% gels as described in the Materials and Methods section. Slicing and counting of gels immediately after electrophoresis gave an almost uniformly high ^{59}Fe concentration in each slice; isotope recovery under these circumstances was between 90 and 100% (not shown). When the gels were soaked in four changes of electrophoresis buffer for a total of 6–8 h, the above ^{59}Fe (----) distribution was obtained, and the isotope recovery was 13%. Slicing and counting of a stained gel (densitometer tracing shown above, —) gave the same distribution and low recovery of ^{59}Fe as obtained from the buffer-soaked gels.

Deoxycholate and several non-ionic detergents (Triton X-100, Lubrol-WX, Brij 35, Tween 80, Tween 40) were found to be more or less equally effective in solubilizing the proteins of *C. utilis* mitochondrial membranes (i.e. converting into a non-sedimenting state on centrifugation at 100000g for 1 h). Iron-sulphur proteins remained intact in the presence of these detergents (i.e. the iron remained non-diffusible, as in the control curve of Fig. 5). However, their electrophoretic fractionation in gels was not possible since 90% of the loaded protein failed to enter the gels to a significant extent over the normal range of protein-separating polyacrylamide concentrations.

Fig. 5. Loss of ^{59}Fe from submitochondrial particles labelled *in vivo* suspended in sodium dodecyl sulphate-containing buffer

Submitochondrial particles were prepared from glycerol-limited cells to whose media had been added $2\mu\text{Ci}$ of ^{59}Fe /litre. These particles were suspended, at a protein concentration of 4 mg/ml in 10mM-sodium phosphate buffer, pH 7.1, containing 1% sodium dodecyl sulphate, and dispensed in 0.5ml portions into dialysis sacs. These were placed in 2 litres of the same buffer, which was changed every 2 h, and stirred at room temperature. A sac was removed at zero time and each 1 h thereafter. The sac contents and the washings from the emptied sac were combined and made up to 2ml with more buffer. Portions (0.1 ml) of each sample thus collected were counted in duplicate. The experimental points are the means of four separate experiments ($\circ$). Control experiments (Δ) were performed in the same way except that sodium dodecyl sulphate was omitted from all buffers. The presence of 1mM-sodium citrate in the dialysis buffer, or of 50mM-ferric citrate in the initial solubilized membrane mixture had no influence on the result.

Polyacrylamide-agarose gels (Peacock & Dingman, 1968) of sufficiently large pore-size to allow sample entry achieved only poor resolution of the sample proteins.

We were equally unsuccessful in our attempts to fractionate these detergent-solubilized preparations by Sephadex chromatography, DEAE-cellulose chromatography or by isoelectric focusing in detergent-containing gels (polyacrylamide or polyacrylamide-agarose) or in detergent-containing sucrose gradients.

Conclusions

The difficulties of solubilizing and characterizing membrane proteins are well documented, and are aggravated in the case of non-haem iron proteins of *C. utilis* mitochondria by the loss of enzymic activity and the prosthetic group(s) under the conditions required for electrophoretic analysis. Nevertheless, the results presented provide circumstantial evidence that one of the polypeptides of the NADH dehydrogenase is diminished in concentration in mitochondrial membranes from iron-limited *C. utilis*. Although iron limitation causes a 5–10-fold decrease in the mitochondrial content of cytochromes (Light & Garland, 1971), no comparable changes were detected in the electrophoretic analyses of mitochondrial polypeptides. It seems likely therefore that in *C. utilis* as in *E. coli* (Haddock & Schairer, 1973), the synthesis of cytochrome apoproteins and their insertion into the energy-transducing membrane is not controlled by or co-ordinated with synthesis of the haem prosthetic groups. This conclusion is conditional upon the resolution of the electrophoretic technique, and requires confirmation by reconstitution of cytochromes *in vitro* in the membrane (Haddock, 1973).

This work was generously supported by the Medical Research Council. R. A. C. was in receipt of an M.R.C. Junior Postdoctoral Fellowship and J. E. S. of an M.R.C. Postgraduate Studentship. We are grateful to Professor P. B. Garland for guidance and encouragement, to Mr. A. Tucker and Mr. D. Coutie for skilled technical assistance, and Dr. C. I. Ragan and Dr. S. O. C. Tottmar for samples of cytochrome oxidase and cytochrome *b* from *Candida utilis* mitochondria.

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