Non-Haem Iron and the Dissociation of Piericidin A Sensitivity from Site 1 Energy Conservation in Mitochondria from *Torulopsis utilis*

By R. A. CLEGG AND P. B. GARLAND*

Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

(Received 18 January 1971)

1. The aerobic incubation of iron-deficient *Torulopsis utilis* cells for 12h under non-growing conditions results in the recovery by mitochondria of the previously absent site 1 energy conservation and sensitivity to piericidin A. 2. The recovery of piericidin A sensitivity but not site 1 is prevented by the presence of cycloheximide (100 $\mu\text{g/ml}$) in the medium used for aerobic incubation of the cells. Rotenone sensitivity behaved similarly. 3. Chloramphenicol, erythromycin and tetracycline were without effect on the recovery of site 1 and piericidin A sensitivity. 4. Inclusion of ^{59}Fe in the growth medium can be used as the basis for a highly sensitive assay for non-haem iron. 5. Iron-limited growth of *T. utilis* lowers the concentration of both non-haem iron and acid-labile sulphide of submitochondrial particles by over 20-fold compared with the 'normal' situation with iron-supplemented glycerol-limited growth. 6. Increases in the non-haem iron and acid-labile sulphide concentrations of submitochondrial particles occur when site 1 and piericidin A sensitivity are recovered. The increase is approximately halved by the presence of cycloheximide. 7. The non-haem iron of *T. utilis* submitochondrial particles does not exchange with added iron. 8. Continuous culture of *T. utilis* at the transition between glycerol- and iron-limitation results in cells where mitochondria possess site 1 energy conservation but lack piericidin A sensitivity. 8. It is concluded, in contrast with widely held views to the opposite, that energy conservation at site 1 does not require electron flow to proceed through a piericidin A- or rotenone-sensitive route. 9. Restriction of the iron supplied to growing *T. utilis* to a concentration just above that required for growth limitation demonstrates that a 10- to 20-fold decrease of the 'normal' non-haem iron concentration of both cells and mitochondria is without effect on the growth yield per unit of carbon source. Submitochondrial particles prepared from such iron-restricted but otherwise functionally normal cells have a non-haem iron concentration of about 0.5–0.8 ng-atom/mg of protein. It is concluded that the concentration of iron-sulphur protein required for normal function by the respiratory chain is close to the concentrations of cytochromes and flavoproteins.

In mammalian mitochondria reduction of the respiratory-chain cytochromes by intramitochondrial NADH is associated with the synthesis of one terminal pyrophosphate bond of ATP for each pair of electrons that pass from NADH to the level of cytochrome *b*. The mechanism of the energy-conservation process occurring in this region (site 1) or elsewhere (i.e. site 2 between cytochromes *b* and *c*₁, and site 3 between cytochrome *c* and oxygen) is not known with any certainty. An additional feature of electron flow from intramitochondrial NADH to cytochrome *b* is that the flow is inhibited by several compounds that are thought to have a common site

of action: Amytal (Quastel & Wheatley, 1933; Ernster, Jalling, Löw & Lindberg, 1955), rotenone (Ernster, Dallner & Azzone, 1963) and piericidin A (Hall *et al.* 1966; Jeng *et al.* 1968). The behaviour of yeast mitochondria is different. Vitols & Linnane (1961) reported that site 1 energy conservation was absent from the mitochondria of *Saccharomyces cerevisiae*, and Mackler, Collipp, Duncan, Rao & Huennekens (1962) reported that the NADH oxidase of submitochondrial particles prepared from *S. cerevisiae* was insensitive to Amytal. These findings were confirmed and extended by Schatz & Racker (1966). Mitochondria from *Saccharomyces carlsbergensis* also lack site 1 energy conservation and sensitivity towards Amytal and rotenone (Ohnishi & Hagihara, 1964; Ohnishi, Kawaguchi &

* Present address: Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN, U.K.

Hagihara, 1966a). However, the properties of mitochondria from *Saccharomyces* spp. are not possessed by all yeasts. Linnane, Vitols & Nowland (1962) reported that site 1 energy conservation was present in mitochondria from *Torulopsis utilis*, and similar results were obtained by Kotelnikova & Zvyagil'skaya (1963) with *Endomyces magnusi*. Ohnishi, Sottocasa & Ernster (1966b) confirmed the observations of Linnane *et al.* (1962), and also reported that *T. utilis* mitochondria were sensitive to rotenone. It was therefore possible to propose, on the basis of these comparative studies, that there was a close correlation between rotenone (and Amytal) sensitivity and the occurrence of site 1 energy conservation (Ohnishi *et al.* 1966b; Pullman & Schatz, 1967).

Further support for the conclusion that energy conservation at site 1 occurred only when electron flow from NADH to the cytochromes passed through a rotenone-sensitive route came from our own studies with iron-limited *T. utilis* (Light, Ragan, Clegg & Garland, 1968; Light & Garland, 1971), in which it was shown that both rotenone sensitivity (equated with piericidin A sensitivity) and site 1 energy conservation could be made to disappear and reappear together according to the manner in which the whole cells were grown or treated. However, more detailed studies, reported in this paper, show that site 1 energy conservation can occur in the absence of sensitivity towards rotenone or piericidin A. Preliminary accounts of part of this work have been published (Clegg *et al.* 1969; Garland, Clegg, Light & Ragan, 1969).

The observation that iron-limited growth of *T. utilis* leads to the loss of both piericidin A sensitivity and site 1 energy conservation is at best only circumstantial evidence in favour of a role for non-haem iron proteins in these two mitochondrial properties (Light *et al.* 1968; Light & Garland, 1971). In the studies described in this paper we have therefore sought a more direct means of measuring the presence or absence of mitochondrial non-haem iron proteins by making direct chemical analyses of mitochondrial non-haem iron and acid-labile sulphide concentrations. In certain cases the values for these concentrations were below the limits of detection by currently available techniques, and new or modified procedures of adequate sensitivity are described. The effect of iron-limited growth on the distribution of intracellular iron between haem and non-haem compounds and between subcellular compartments is also described.

MATERIALS AND METHODS

Reagents. Chloramphenicol was purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; cycloheximide and *NN'*-dimethyl-*p*-phenylenediamine

dioxalate was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; 1,10-phenanthroline hydrate, 4,7-diphenyl-1,10-phenanthroline, $\text{Na}_2\text{S}_2\text{O}_8 \cdot 9\text{H}_2\text{O}$, sodium acetate trihydrate, zinc acetate dihydrate, trichloroacetic acid, ammonium ferric sulphate, EDTA, citric acid, conc. HCl, H_2SO_4 and 3-methylbutan-1-ol were from British Drug Houses Ltd., Poole, Dorset, U.K. and were of AnalaR grade where available. [^{59}Fe]Ferric chloride (3–30 Ci/g of Fe) in 0.1M-HCl was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Tetracycline base was kindly given by Pfizer Ltd., Sandwich, Kent, U.K.

Microbiological apparatus. The apparatus and growth conditions for the continuous culture of *T. utilis* (N.C.Y.C. 193) were as described by Light & Garland (1971). The growth medium, however, did not contain added manganese. The sensitivity of cell growth towards cycloheximide (Kerridge, 1958), chloramphenicol, tetracycline and erythromycin was tested on solid media containing (final concentrations): glycerol (266 mM), Na_2HPO_4 (5.6 mM), NaH_2PO_4 (25.6 mM), NH_4Cl (150 mM), K_2SO_4 (23 mM), citric acid (1.0 mM), biotin (8 $\mu\text{g/l}$), and stock salts solution with iron (1 ml/l; Light & Garland, 1971). The reagents were dissolved in the sequence listed, the pH was adjusted to 5.0 with 10M-KOH, and agar was added to a final concentration of 2% (w/v). Compounds under test for growth-inhibitory properties were added to the molten solid medium before plates were poured. The compounds were in some cases also tested for inhibition of cell growth on Sabouraud's agar (Light & Garland, 1971).

Variation of the entering iron concentration of the chemostat. The iron-unsupplemented medium described by Light & Garland (1971) contained about 0.3 μM -Fe (see below), whereas the iron-supplemented medium contained 100 μM -Fe. To test the effects of iron concentrations between these extreme values, extra iron was added to the sterile unsupplemented medium as follows. The required volume of a gravimetrically standardized solution of ammonium ferric sulphate (either 10 μg of Fe/ml or 100 μg of Fe/ml) in 0.25% H_2SO_4 was added to 100 ml of twice-glass-distilled water, containing a few grains of citric acid. If [^{59}Fe]ferric chloride was also to be included, it was added after the ammonium ferric sulphate to make the growth-medium radioactivity 1–2 $\mu\text{Ci/l}$. The dilute iron solution so prepared was autoclaved at 10 lb/in² for 15 min and then added to 16 litres of unsupplemented growth medium under sterile conditions.

Preparation of mitochondria and electron-transport particles. Mitochondria were prepared as described by Light & Garland (1971). Electron-transport particles (ETP) were prepared either by ultrasonic disruption of mitochondria (referred to as preparation ETP_m*) or by

* A variety of differently treated *T. utilis* cells and their derived mitochondria is described in this paper. For convenience, the various types are referred to as follows. G-cells, G-mitochondria, G-electron-transport particles (G-ETP); all derived from glycerol-limited growth. F-cells etc.; derived from iron-limited growth. FG-cells etc.; derived from growth poised between iron- and glycerol-limitation. FR-cells etc.; derived by exposure of F-cells to an iron-recovery incubation in the presence of added iron. FR₀-cells etc.; derived by exposure of F-cells to an iron-recovery incubation in the absence of added

mechanical breakage of whole cells (referred to as preparation ETP_c), as described below. (a) Preparation of ETP_m. Mitochondria were suspended at 0°C in a 10ml polypropylene tube in tris-sulphate buffer, pH 7.4 (10mm-tris), to a final protein concentration of 10mg/ml and exposed to the maximal output of an MSE 60W ultrasonic probe for three periods each of 20s interrupted by 20s periods for cooling in a mixture of ice and NH₄Cl which surrounded the polypropylene tube during this procedure. All subsequent operations were performed at 0–4°C. The suspension of broken mitochondria was centrifuged at 9000g for 10min in the 8×50ml angle rotor of an MSE High-Speed 18 centrifuge. The supernatant was removed and centrifuged at 140000g for 45min in the 10×10ml angle rotor of an MSE Superspeed 50 centrifuge. The resultant supernatant was discarded and the pellet was washed by resuspension in tris-sulphate buffer, pH 7.4 (10mm-tris) and resedimentation at 140000g for 45min. The final pellet of preparation ETP_m was suspended in tris-sulphate buffer, pH 7.4 (10mm-tris), to a final protein concentration of either about 50mg/ml for subsequent electron-paramagnetic-resonance spectroscopy or about 30mg/ml for all other purposes. (b) Preparation of ETP_c. Washed cells were suspended to a final concentration of about 100mg dry wt./ml in a solution of sucrose (0.25M), tris-sulphate buffer, pH 7.5 (20mm-tris), and EDTA (0.5mm). Portions (25ml) of the cell suspension were mixed with 50g of glass beads (diameter 0.45–0.5mm) in a 75ml Pyrex bottle and shaken for 1min at the higher

speed of a Braun MSK mechanical cell homogenizer (Shandon Scientific Co. Ltd., Willesden, London N.W.10, U.K.). The contents of the Pyrex bottle were maintained at 0–4°C during the homogenization by cooling the revolving chamber with a jet of pressurized CO₂. All subsequent procedures were performed at 0–4°C. The contents of the Pyrex bottle from consecutive homogenizations were collected in a glass beaker and the glass beads allowed to sediment. The turbid supernatant was decanted from the beads, which were then washed with 3×25ml of the medium containing sucrose, tris-sulphate buffer and EDTA. The supernatant and washings were pooled to make a fraction referred to as the cell homogenate, and then subfractionated by differential centrifugation as described in Scheme 1.

Assays. The dry weight of yeast cell suspensions, glycerol, mitochondrial oxygen uptake, P/O ratios and inhibitor sensitivities, the fluorescence of mitochondrial flavoproteins and NAD(P)H, and mitochondrial protein, acid-extractable FAD and FMN were measured as described by Light & Garland (1971). The cytochrome content of ETP preparation was calculated as the sum of the individually assayed cytochromes *a*+*a*₃, *b*, and *c*+*c*₁, by using a wavelength-scanning split-beam spectrophotometer (Yang & Legallais, 1954) and the following millimolar extinction coefficients for the dithionite-reduced-minus-oxidized difference spectra at the wavelength pairs indicated: cytochrome *a*+*a*₃, 13mmol⁻¹·cm⁻¹ at 605–630nm (Vanneste, 1966); cytochrome *b*, 18mmol⁻¹·cm⁻¹ at 564–575nm (Ohnishi, Kröger, Heldt, Pfaff & Klingenberg, 1967), cytochrome *c*+*c*₁, 19mmol⁻¹·cm⁻¹ at 550–540nm (Ohnishi *et al.* 1967). For these measurements of cytochromes, a light-path of 1cm was used, and the suspension of ETP preparation clarified by the addition of Triton X-100 detergent to a concentration of 0.005% (w/v).

Determination of iron in the growth medium. The iron

Scheme 1. Fractionation of mechanically broken yeast cells. The buffer used was tris-sulphate, pH 7.4 (10mm-tris), and the resuspension of pellets was performed with a glass rod and, except for pellet D, with the aid of ultrasonic treatment as described for the disruption of mitochondria. The cell homogenate and resuspended pellet B were centrifuged in the 8×50 angle rotor of an MSE High-Speed 18 centrifuge, supernatant A in the 8×25ml angle rotor of an MSE Superspeed 50, and supernatant C and resuspended pellet D in the 10×10ml angle rotor of an MSE Superspeed 50.

concentration in media supporting glycerol-limited growth was assayed spectrophotometrically either by the method of Smith, McCurdy & Diehl (1952) with 4,7-diphenyl-1,10-phenanthroline, or by the less sensitive method of Mahler & Elowe (1954) with 1,10-phenanthroline. In the assay of the iron concentration of the chemostat effluents, cells were first removed by centrifugation. The iron concentration in growth medium unsupplemented with iron was low, about $0.2\text{ }\mu\text{M}$, and close to the lower limit of detection by the assay of Smith *et al.* (1952).

The maximal iron concentration arising from contamination by iron in the reagents used in the preparation of the iron-unsupplemented growth medium was calculated from the reagent specifications to be $0.7\text{ }\mu\text{M}$. Provided that the iron-unsupplemented growth medium supported iron-limited growth in a suitable yield, it was initially immaterial whether we knew accurately the iron concentration in the growth medium. Nor would such knowledge indicate the actual amount of iron available to the iron-limited cells, since additional iron could perhaps enter with the air supply or 1 M-KOH used for pH control.

Determination of non-haem iron in mitochondria and ETP preparation. Treatment of mitochondria and ETP preparation with dithionite followed by trichloroacetic acid, as described by Kurup & Brodie (1967) yielded an insoluble precipitate of protein, which contained haem iron, and an aqueous extract, which was assayed for iron (equated with non-haem iron) by the method of Mahler & Elowe (1954). The concentrations of non-haem iron occurring in iron-deficient cells were too low to assay by this method, and it was necessary to devise a more sensitive radioisotopic assay. This latter assay is based on the assumption that, when the iron-unsupplemented medium is trace-labelled with ^{59}Fe ferric chloride (which does not significantly alter the iron concentration), then the specific radioactivity of all species of iron within the culture would be the same. Given a suitable means of determining the specific radioactivity of ^{59}Fe in the culture, the amount of iron in any given subcellular fraction or extract could then be calculated from its measured radioactivity. Two methods were derived for determining the specific radioactivity of ^{59}Fe . The first method relied upon a spectrophotometric assay of the cytochrome content of the mitochondria or ETP preparation, followed by measurement of the radioactivity of haem iron in the trichloroacetic acid-insoluble fraction obtained during extraction of non-haem iron by the method of Kurup & Brodie (1967). The haem-iron concentration in the mitochondria or ETP preparation was calculated from the cytochrome content, thereby enabling the specific radioactivity of ^{59}Fe haem iron to be calculated. In practice, samples of a suspension of mitochondria or ETP preparation were plated out on planchets for radioactivity measurements (see below). A further sample ($1\text{--}2\text{ mg}$ of protein) was treated with a few grains of ferric ammonium sulphate (to act as a carrier for ^{59}Fe) and sodium dithionite and trichloroacetic acid as described by Kurup & Brodie (1967). The resultant precipitate of denatured protein (including haem iron of haem proteins) was separated by filtration with a 3.0 cm -diam. filter of $0.35\text{ }\mu\text{m}$ pore size and washed with a few ml of trichloroacetic acid (5% , w/v) containing ferric ammonium sulphate ($10\text{ }\mu\text{M}$). The filter was fixed to a cardboard disc, dried under a bench lamp and its radioactivity measured.

The radioactivity of the non-haem iron extracted into trichloroacetic acid was calculated from the difference between the radioactivities of the untreated suspension and of the acid-soluble haem iron.

The second method was simpler and made use of the fact that a chemostat culture can be used as a bioassay for the growth-limiting nutrient which, in this case, was iron. This bioassay method is described in the Results section and was used on account of its simplicity in place of the method just described. Agreement between the two methods was within 20% , which we consider to be satisfactory in view of the independence of the methods and the high sensitivity achieved (about 0.01 nmol/mg of mitochondrial protein).

Determination of acid-labile sulphide in mitochondria and ETP preparation. Acid-labile sulphide is defined as that form of sulphur appearing as H_2S when a preparation of mitochondria, ETP, or a protein is treated with strong acid, and for its assay we used the micro-diffusion method of Bray (1958) in a slightly modified manner as follows. A small roll of Whatman no. 54 filter paper (Whatman Ltd., Maidstone, Kent, U.K.) was placed in the centre well of a Warburg manometer flask and the free end of the paper moistened with $10\text{ }\mu\text{l}$ of a solution of 20% (w/v) zinc acetate and 5% (w/v) sodium acetate. The side arm of the flask contained 0.5 ml of 5 M-HCl , and the sample to be assayed (usually 0.5 ml of a suspension of mitochondria or ETP preparation containing at least 1 nmol of acid-labile sulphide) was placed in the main compartment. The flask was sealed, the acid tipped in from the side arm, and the flask then shaken at room temperature in a Warburg manometer shaker at about 100 strokes/min for 20 min . The filter paper was then rapidly transferred into 1 ml of a solution of NN' -dimethyl- p -phenylenediamine dioxalate (0.01% , w/v) in H_2SO_4 (1.5% , v/v) in a small stoppered tube, which was then vigorously shaken by hand for 1 min . Next, 0.2 ml of a solution of ferric ammonium sulphate (2% , w/v) in H_2SO_4 (1.5% , v/v) was added, care being taken to unstopper the tube for the minimum duration. The tube was again shaken vigorously by hand for 1 min , then left to stand for 5 min . Development of the blue product of the reaction between sulphide and NN' -dimethyl- p -phenylenediamine dioxalate is complete within this time. The blue solution was decanted from the filter paper (and centrifuged if it contained fragments of the paper) and its E_{670} measured in a cuvette of 2 cm light-path. A millimolar extinction coefficient of $10.6\text{ mmol}^{-1}\cdot\text{cm}^{-1}$ (King & Morris, 1967) was assumed for sulphide assayed under these conditions. Each sample of mitochondria or ETP preparation was assayed at two or three different dilutions, and a blank determination was made by using 0.5 ml of glass-distilled water in place of the sample. The assay can determine 1 nmol of sulphide and the sample size is, in theory, limited only by the capacity of the Warburg flask. Measurements of the recovery of sulphide as a fraction of the time allowed for diffusion of H_2S within the flask showed that recovery was 96% complete within 2 min . Fig. 1 demonstrates the linearity of the assay and the recovery for sulphide.

Measurements of radioactivity of ^{59}Fe . All measurements were made with a Nuclear-Chicago gas-flow counter operated in the proportional region and with a Micromil end window. All samples were prepared on planchets to a similar geometry, and self-absorption curves were con-

Fig. 1. Performance of the assay for acid-labile sulphide. The assays were carried out as described in the Materials and Methods section. (a) shows the linearity for an assay of a solution of about $40 \mu\text{M}$ - Na_2S either carried through the whole procedure of acidification, diffusion and colour development (●) or added directly to the colour-development mixture (▲). (b) shows the linearity between extinction change (i.e. acid-labile sulphide) and the sample size of a stock suspension of G-mitochondria (30 mg of protein/ml) used for assay.

structured where applicable and used to correct the observed counts to infinite thinness. All samples were duplicated and their radioactivities counted twice, to give a counting accuracy of within $\pm 4\%$.

Concentration of iron in distilled water. Water distilled twice from an all-glass apparatus has been found to contain $0.06 \mu\text{M}$ -Fe (Hewitt, 1966; Rouf, 1964). This concentration is halved if the water is passed once through

a mixed-bed-resin deionizing column (Hewitt, 1966), and such water was used in the preparation of standard iron solutions. Pyrex glass volumetric flasks used for the storage of standard ion solutions were first rinsed with dilute citric acid (10 mM) followed by four rinses with deionized water.

Recovery procedure for the restoration of site 1 energy conservation and piericidin A sensitivity in iron-limited cells (F-cells). The original procedure described by Light *et al.* (1968) consisted of an overnight incubation (12–14 h) of a suspension of F-cells (0.1 g wet wt./ml) in $200 \mu\text{M}$ - FeSO_4 at 30°C with aeration. This was modified by lowering the concentration of added iron and, in certain experiments, by including inhibitors of protein synthesis (see the Results section).

RESULTS

Effects of various entering iron concentrations on the characteristics of *T. utilis* in continuous culture. Our earlier studies of the effects on mitochondria of iron-limited growth of *T. utilis* were made at a single concentration (about $0.3 \mu\text{M}$) of entering iron, and utilized as a control glycerol-limited cells (G-cells) grown at an entering iron concentration of $100 \mu\text{M}$ (Light *et al.* 1968; Light & Garland, 1971). A number of differences were noted between F- and G-mitochondria, and it was decided to find out if any of these differences could be separated from each other by using entering iron concentrations in the range between the extreme values of 0.3 and $100 \mu\text{M}$. Fig. 2 shows the manner in which the concentration of cells and of glycerol within the chemostat depended on the entering iron concentration. At day zero shown in Fig. 2 the cells had already been grown for 10 days on an iron-unsupplemented medium. The concentrations of glycerol and cells were then measured daily and the entering iron concentration increased in a stepwise manner every 4–5 days. The increments of iron concentration made on days 4, 8 and 13 caused increases in the cell concentration and decreases in the glycerol concentration such that from day 14 onwards at least 99.7% of the glycerol entering the chemostat was consumed. A further increment of the entering iron concentration at this stage would not be expected to increase the cell concentration, since insufficient glycerol remained to support such an increase. Nevertheless, an increase of the entering iron concentration on day 17 increased the cell concentration by 27%. At this point it was difficult to state whether cell growth was limited by iron or by glycerol, for whichever nutrient was not limiting would become limiting if the concentration of the other were increased. Thus it would seem that the culture was poised in the region of the transition between iron limitation (F-cells) and glycerol limitation (G-cells), and we have therefore described cells in this state as FG-cells.

Fig. 2. Effects of changes in the entering iron concentration (b) on the cell density, and glycerol utilization (a) of iron-limited *T. utilis* growing in continuous culture. The added Fe concn. is that concentration of iron deliberately added to the culture medium, and is therefore in addition to the iron already present by contamination. The entering concentration of glycerol was 133 mM. ●, Cell density; ▲, concn. of glycerol.

During the experiment shown in Fig. 2, cells were harvested and mitochondria prepared during each of the steady states of the culture established within 48 h of the increments of iron concentration. All of the mitochondrial preparations up to day 17 had properties typical of F-mitochondria, namely that site 1 energy conservation and piericidin A sensitivity were absent (Table 1). Mitochondria prepared from day 18 onwards still lacked piericidin A sensitivity but did possess energy conservation at site 1, as assayed polarographically (Table 1) or fluorimetrically (Fig. 3).

It is evident from Fig. 2, and predictable from the theory of continuous culture (Herbert, Elsworth & Telling, 1956) that there is, in the iron-limited state, a close dependence of the concentration of cells within the chemostat and the concentration of iron entering in the growth medium. This relationship is displayed graphically in Fig. 4, which also includes measurements from other similar experiments with the chemostat. Two notable features of Fig. 4 are, first, the linear relationship between cell concentration and added iron concentration and,

secondly, the cell growth in the absence of added iron. If the cell concentration were linearly related to the entering iron concentration for all concentrations of iron from zero to that where glycerol limitation sets in (at the given dilution rate and concentration of glycerol entering the chemostat), then extrapolation of the graph of Fig. 4 to zero cell concentration would intercept the abscissa at the negative value of the 'nominal' entering iron concentration of iron-unsupplemented media. This value is nominal, since it must include all sources of iron available to the culture, such as contamination from the air supply. The value calculated in this fashion was $0.31 \mu\text{M}$ (Fig. 4), which is comparable with the value of $0.2 \mu\text{M}$ determined by assaying the entering growth medium by the method of Smith *et al.* (1952). This would suggest that the equivalent of $0.1 \mu\text{M}$ -Fe in the entering growth medium was available to the culture from other sources.

The results in Fig. 4 established a sensitive bioassay for iron which, in the context of our own experimental requirements, was exceedingly simple

and merely required determination of the cell concentration (as dry wt.) of the culture under study. The nominal concentration of iron in the entering medium was then read directly from Fig. 4. The nominal concentration determined in this manner

was then used in the calculation of the specific radioactivity of ^{59}Fe in the culture under iron-limited conditions (see the Materials and Methods section).

Minimal entering iron concentration required to establish both piericidin A sensitivity and site 1 energy conservation. At a chemostat dilution rate of 0.2h^{-1} and an entering glycerol concentration of 133mM , site 1 energy conservation was present at an entering iron concentration of $0.7\mu\text{M}$ and greater (Fig. 2 and Table 1). A further increase of the entering iron concentration to $1.0\mu\text{M}$ and study of the mitochondria from cells grown under this condition demonstrated that piericidin A sensitivity had returned. In making a study of the functional role of non-haem iron of mitochondria, there are clear advantages in working with a system containing the minimum of non-haem iron necessary for the presence of the function under study, and for this reason, we used an entering iron concentration of $2\mu\text{M}$ for all subsequent culture of G-cells. A lower concentration ($1\mu\text{M}$) could have been used for this purpose, but it seemed better to introduce a sufficient margin between the concentration actually used ($2\mu\text{M}$) and the concentration where piericidin A sensitivity disappeared ($1\mu\text{M}$). These values apply only to the growth conditions described, and must depend, *inter alia*, on the entering glycerol concentration and any other factor capable of altering the cell density within the chemostat. Table 2 summarizes the properties of mitochondria prepared from G-cells grown at entering iron concentrations of 1.3 and $100\mu\text{M}$. The cell density in the culture was identical in each case.

Table 2 shows that growth at the lower concentration of iron caused a fall in the concentration of cytochromes $a+a_3$, cytochrome b , and cytochromes c_1+c . The greatest decrease was in cytochromes c_1+c , and was exceeded in relative magnitude only by the 20-fold decrease of the non-haem iron concentration. Thus it can be concluded that, in cells grown under the usual conditions of iron

Fig. 3. Recordings of the fluorescence of NAD(P)H and flavoprotein fluorescence of mitochondria from FG-cells. Fluorescence was measured as described by Light & Garland (1971), and in both the upper two traces (a, and b, flavoprotein fluorescence) and the lower two traces [c and d, NAD(P)H fluorescence] an upward deflexion indicates reduction. The vertical bar labelled '10%' corresponds to 10% of the total photomultiplier signal at the commencement of the experiment. In each experiment the reaction cuvette contained 2ml of air-saturated mitochondrial incubation medium at 20°C , 10mg of bovine serum albumin and 2.9mg of mitochondrial protein. Other additions, abbreviations and final concentrations were: DL-glycerol 3-phosphate (G-3-P; 5mM); carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; $0.25\mu\text{M}$).

Table 1. *Properties of mitochondria from F- and FG-cells*

FG-cells were obtained in continuous culture by poisoning growth between iron- and glycerol-limitation. The methods were otherwise as described by Light & Garland (1971). The values are the means $\pm$ S.E.M.

Type of cells	No. of preparations	Oxygen uptake rate (ng-atoms of O_2 /min per mg of protein)		P/O ratio		Inhibition of pyruvate and malate oxidation (%)	
		Pyruvate+ malate	DL-Glycerol 3-phosphate	Pyruvate+ malate	DL-Glycerol 3-phosphate	Piericidin A	Antimycin A
F	7	170 ± 40	260 ± 27	1.83 ± 0.28	1.76 ± 0.17	0	>90
FG	4	182 ± 41	150 ± 82	2.5 ± 0.35	1.7 ± 0.23	0	>90

Fig. 4. Relationship between the cell density in iron-limited continuous culture and the concentration of added iron. The experimental points were obtained as in Fig. 2. Extrapolation of the line to zero cell density indicates that the cell growth supported in the absence of deliberately added iron would require the equivalent of $0.31 \mu\text{M}$ -Fe present as a contaminant of the growth medium.

supplementation ($100 \mu\text{M}$), at least 95% of the mitochondrial non-haem iron is not needed for electron flow or energy conservation.

Effects of growth inhibitors on the recovery of site 1 energy conservation and piericidin A sensitivity by F-cells incubated with iron. There are inhibitors of protein synthesis that have been claimed to act with relative specificity on either the mitochondrial or the cytoplasmic protein-synthesizing systems (Vasquez *et al.* 1969). Most studies of the effects of these inhibitors on yeasts have been concerned with *Saccharomyces* spp. and we therefore tested a number of possible inhibitors for their effects on the growth of *T. utilis*. The results are shown in Table 3, from which it is apparent that cycloheximide was a potent inhibitor of growth, that chloramphenicol was without effect, and that erythromycin and tetracycline inhibited growth only at relatively high concentrations of inhibitor. *T. utilis* has little fermentative ability, and no attempt was made to investigate the effects of these compounds on fermentative growth as opposed to oxidative growth.

The results of a study of the effects of cycloheximide, chloramphenicol, erythromycin and tetracycline on the recovery of piericidin A sensitivity and site 1 energy conservation by F-cells on aerobic incubation under non-growing conditions are shown in Table 4. The main observations are (i) that cycloheximide inhibited the return of piericidin A sensitivity but not site 1 energy conservation, (ii) that neither tetracycline (which inhibits growth) nor chloramphenicol (which does not inhibit growth) affected the recovery of site 1

Table 2. *Properties of mitochondria prepared from glycerol-limited cells grown at entering iron concentrations of 1.3 and $100 \mu\text{M}$*

The results shown are typical ones.

Property	Entering iron concentration	
	$1.3 \mu\text{M}$	$100 \mu\text{M}$
Concentration (nmol/mg of protein) of:		
Cytochrome $a+a_3$	0.17	0.44
Cytochrome b	0.13	0.30
Cytochrome $c+c_1$	0.09	0.83
Non-haem iron	0.73	15.0
Oxidation of pyruvate with L-malate:		
Oxygen uptake (ng-atoms/min per mg)	150–250	150–250
P/O ratio	2.7	2.7
Inhibition of respiration by piericidin A ($1 \mu\text{M}$)	90%	90%

Table 3. *Effects of possible inhibitors on the growth of T. utilis on solid media*

The solid media were prepared as described in the Materials and Methods section, and the results were essentially the same for both the glycerol medium and Saboraud's medium. The agar plates were inoculated and then incubated at 30°C , and inspected at 24 h intervals for growth, which was semi-quantitatively scored, the control scoring in each case being as that described in the top line of the table. No detectable growth is recorded as a negative sign, and increasing growth with positive signs.

Compound	Final concn. (mg/ml)	Time of growth		
		24 h	48 h	72 h
Cycloheximide	0	++	+++	+++
	0.1	—	—	—
Erythromycin	0.5	++	+++	+++
	1.0	+	++	+++
	4.0	—	+	++
Tetracycline	0.1	++	+++	+++
	0.5	+	++	+++
	1.0	—	+	++
	2.0	—	—	—
Chloramphenicol	2.5	++	+++	+++

energy conservation or piericidin A sensitivity, and (iii) that mitochondria prepared from erythromycin-treated cells were abnormal as judged by their low rates of oxygen uptake.

Other modifications of the recovery procedure. The original recovery procedure involved the aerobic incubation of F-cells with $200 \mu\text{M}$ -ferrous sulphate. If the recovery procedure was carried out anaerobically, the mitochondria, which were obtained in a low yield, were characterized by low rates of oxygen uptake when using the substrates of Table 1,

Table 4. *Effects of inhibitors of protein synthesis on the transition from F- to FR-cells*

The recovery procedure was carried out in the presence of FeSO_4 (see the Materials and Methods section) and inhibitors of protein synthesis as indicated. Mitochondria were then prepared and assayed for respiration rates, inhibitor sensitivities and P/O ratios as described by Light & Garland (1971). Each line of the table represents a separate mitochondrial preparation.

Additions to recovery medium		Mitochondrial properties				Inhibition of pyruvate and malate oxidation by piericidin A (%)
		Oxygen uptake rate (ng-atoms/min per mg of protein)		P/O ratio		
		Pyruvate+malate	DL-Glycerol 3-phosphate	Pyruvate+malate	DL-Glycerol 3-phosphate	
Inhibitor	FeSO ₄ (μM)					
None	200	128	235	3.06	1.84	95
	200	130	260	3.4	2.14	80
	200	270	510	2.6	2.0	65
	200	44	148	2.4	1.75	30
	1	74	140	2.6	1.5	70
	1	113	246	2.7	1.6	58
	1	86	230	2.4	1.6	81
	1	34	61	3.0	1.8	80
Cycloheximide (100 μg/ml)	200	150	135	2.3	1.7	0
	200	35	73	2.5	1.4	0
	200	120	135	2.2	1.7	0
Erythromycin (2.0 mg/ml)	200	50	100	No respiratory control		
Cycloheximide (100 μg/ml)	1	121	149	2.2	1.7	0
Chloramphenicol (2.5 mg/ml)	200	50	180	—	—	10
	200	72	242	2.3	1.8	40
	200	93	140	2.8	1.9	90
Tetracycline (2 mg/ml)	100	235	240	2.9	1.9	60
	1	129	224	2.9	1.5	60

absent respiratory control, and no detectable sensitivity towards piericidin A. If iron was omitted from the aerobic recovery procedure, the mitochondria (FR_0 -mitochondria) were indistinguishable from FR-mitochondria (Light *et al.* 1968) except that their non-haem iron and acid-labile sulphide concentrations were lower (see below).

Relationships between the manganese concentration of the iron-unsupplemented growth medium and the subsequent recovery of piericidin A sensitivity and energy conservation at site 1. The growth medium in the earlier part of this work was supplemented with 50 μM -manganous sulphate (Light *et al.* 1968). Manganese was subsequently omitted to facilitate studies with electron-paramagnetic-resonance spectroscopy. It was then found that F-cells grown in the absence of added manganese did not recover piericidin A sensitivity and site 1 energy conservation when incubated aerobically with 200 μM -ferrous sulphate. In all other respects the presence or absence of added manganese during the growth of F-cells was without effect on the properties of their

mitochondria. However, the use of 1 μM -iron (as ammonium ferric sulphate) in the incubation of the recovery procedure did not inhibit the return of piericidin A sensitivity and site 1 energy conservation in F-cells harvested from manganese-unsupplemented cultures.

Concentrations of non-haem iron and acid-labile sulphide in mitochondria and ETP preparations from various cell types. The loss from mitochondria of piericidin A sensitivity and site 1 energy conservation occurs when cell growth is limited by the availability of iron (Light *et al.* 1968) or sulphate (Clegg *et al.* 1969; Haddock & Garland, 1971) but not glycerol, magnesium, nitrogen (as NH_4^+), or phosphate (Light & Garland, 1971). In view of the association of non-haem iron protein with the region of the respiratory chain leading from NADH to the cytochromes (Hatefi, 1966; Singer, 1966), it seemed very possible that the functional alterations caused by iron- and sulphate-limited growth were due to structural modifications or even deletions of non-haem iron protein. If this were the case, it could be

Table 5. Concentrations of non-haem iron, acid-labile sulphide and cytochromes in ETP_m preparations from various cell types

All concentrations are expressed as nmol/mg of protein. Non-haem iron was assayed by the radioisotopic method except for G-cells where the spectrophotometric procedure was used.

Cell type	Non-haem iron	Acid-labile sulphide	Cytochromes		
			$a+a_3$	b	$c+c_1$
G (growth on 2 μ M-iron)	0.82	0.61	0.28	0.33	0.21
F	0.11	0.03	0.09	0.187	0.07
FR ₁	0.46	0.47	0.09	0.10	0.13
FR ₀	0.186	0.164	0.10	0.10	0.12
FR ₀ (cycloheximide)	0.119	0.065	0.08	0.08	0.07
FR ₁ (cycloheximide)	0.174	0.34	0.12	0.13	0.12

anticipated that the loss of piericidin A sensitivity and site 1 energy conservation would be accompanied by a fall in the concentration of non-haem iron and acid-labile sulphide. Table 5 summarizes the results of determinations of non-haem iron and acid-labile sulphide concentrations in ETP_m preparations from G-, F-, FG- and FR-mitochondria.

The results in Table 5 demonstrate remarkably low values for the concentrations of non-haem iron and acid-labile sulphide in ETP_m preparations from F-mitochondria, an observation that supports the hypothesis that the losses of function in this state are due to loss or modification of iron-sulphur protein. Further support is provided by the small but significant increases of non-haem iron and acid-labile sulphide found in ETP_m preparations from FR₀- and FR₀(cycloheximide)-cells (see the Discussion section).

Assay of non-haem iron in ETP preparations from FR-cells. All of the values for non-haem iron presented so far in this paper were determined either by the conventional spectrophotometric method (Smith *et al.* 1952) applied to appropriate extracts of ETP preparations from G-mitochondria, or the radioisotopic assay applied to ETP preparations from various iron-deficient cells [F, FG, FR₀ and FR₀(cycloheximide); Table 5]. However, it was necessary to devise a modification of the radioisotopic assay for application to ETP preparations from F-cells that had been submitted to the recovery procedure with added iron, for the added iron (as ferric chloride, or ferrous sulphate) could have diluted, but not necessarily equilibrated with, the ⁵⁹Fe-labelled iron of the F-cells. This problem could have been overcome by ⁵⁹Fe-labelling of the iron added to the recovery-procedure incubation mixture, and arranging that the specific radioactivity of the added iron was identical with that of the iron within the chemostat. However, the following procedure was used, since it yielded additional information. First, it was necessary to establish that the radioisotopically measured

increase in the concentration of non-haem iron in ETP_m preparations from FR₀-cells was not altered by the presence of added unlabelled iron in the recovery-procedure incubation mixture. The results in Table 6 show that this was indeed the case, and they also show that the increase of non-haem iron in ETP_m preparation from FR₀-cells was not due to the liberation of iron from breakdown of a fraction of the cells in the incubation mixture.

Next, the experimental design was altered such that the growth of iron-limited cells within the chemostat was with unlabelled iron, whereas [⁵⁹Fe]-ferric chloride of known specific radioactivity was added to the recovery-procedure incubation mixture. The radioactivity of non-haem iron in subsequently prepared ETP_m was then measured as before and used to calculate the increase in the non-haem iron concentration resulting from the addition of iron to the recovery-procedure incubation mixture. The total increase in the non-haem iron concentration was calculated as the sum of the increase arising from endogenous iron plus the increase arising from added iron. The validity of this calculation rests on the demonstration (Table 6) that the addition of iron to the recovery-procedure incubation mixture did not alter the increase in non-haem iron arising from endogenous sources. Table 7 summarizes the results of experiments involving determination of the increase in non-haem iron concentration in ETP_m preparation resulting from the addition of [⁵⁹Fe]ferric chloride to the recovery-procedure incubation mixture for unlabelled F-cells.

Table 7 shows that the addition of iron to the recovery-procedure incubation mixture did not greatly alter the cytochrome concentration of subsequently prepared ETP_m.

Binding of non-haem iron to ETP preparation. It was inevitable that, once cells had been harvested from the chemostat, they were exposed to iron contaminating the various media used for washing and enzymic digestion of the cell wall. Further

Table 6. *Redistribution of endogenous iron during the transition from F- to FR-cells*

F-cells were obtained in continuous culture with $^{59}\text{FeCl}_3$ as a trace label. The cells were then harvested, washed and submitted to the recovery procedure under the conditions indicated. ETP_m preparations were then made and assayed for [^{59}Fe]haem and non-haem iron. Where iron was added to the incubation mixture for the recovery procedure, it was non-radioactive. Values in parentheses are those observed if the recovery procedure was omitted. N.T., not tested.

Recovery inhibitors	Concn. of added Fe (μM)	Specific radioactivity of ^{59}Fe in F-cells (c.p.m./ng-atom of Fe)	Radioactivity in ETP _m (c.p.m./mg of protein)		
			Total iron	Non-haem iron	Haem iron
None	0	1117	614 (462)	208 (148)	406 (314)
None	1	348	116 (104)	43 (34)	73 (70)
None	200	317	103 (75)	N.T.	N.T.
Cycloheximide (0.1 mg/ml)	0	1117	426 (462)	150 (148)	276 (314)

Table 7. *Transition from F- to FR-cells: effects of FeSO_4 on the mitochondrial concentration of non-haem iron and cytochromes*

F-cells were subjected to the recovery procedure (see the Materials and Methods section) under the conditions indicated. The medium used for growth was non-radioactive, whereas $^{59}\text{FeCl}_3$ was used as a trace label for FeSO_4 added to the recovery medium. ETP_m preparations were then made and assayed for ^{59}Fe -labelled non-haem iron and cytochromes. The ^{59}Fe -labelled non-haem iron was therefore representative of non-haem iron derived from that added to the recovery medium and not from redistribution of pre-existing intracellular iron. N.T., not tested; N.A., not applicable.

Concn. of added Fe (μM)	Incorporation of ^{59}Fe into ETP _m (ng-atom/mg of protein)			Concn. of cytochrome (nmol/mg of protein)		
	Total iron	Non-haem iron	Haem iron	$a + a_3$	b	$c + c_1$
1	0.371	0.302	0.069	0.09	0.10	0.13
1	0.364	0.261	0.093			
1	0.143	N.T.	N.T.			
200	21.4	N.T.	N.T.	0.20	0.21	0.19
200	53.0	N.T.	N.T.			
200	25.0	N.T.	N.T.			
0	N.A.	N.A.	N.A.	0.11	0.15	0.13

exposure of the mitochondria to contaminating iron occurred during their isolation and the preparation of ETP_m. It is doubtful if any such contamination caused much error in the measured non-haem iron concentration as measured by extraction and spectrophotometry (Kurup & Brodie, 1967; Mahler & Elowe, 1954) with G-ETP_m preparation. However, the concentration of non-haem iron in F-ETP preparation is so low that contamination by iron during their preparation might well cause errors. The radioisotopic assay for non-haem iron would not be sensitive to iron contamination provided that ^{59}Fe in non-haem iron did not exchange with ^{56}Fe . Experiments were therefore performed to measure the extent to which the radioactivity of mitochondria prepared from F-cells grown on ^{59}Fe -labelled growth medium could be displaced by

incubation of the mitochondria with ferrous sulphate. The results are shown in Table 8, which shows that the ^{59}Fe of mitochondria pre-labelled during cell growth is not exchangeable under the conditions described.

Intracellular distribution of non-haem iron. Table 9 summarizes the results of determinations of the concentration of non-haem iron in mitochondria and whole cells. The cells grown at an entering iron concentration of $0.3\mu\text{M}$ were F-cells, and nearly 90% of their non-haem iron was mitochondrial. Subsequent preparation of ETP_m showed that the concentration of non-haem iron in the ETP_m was the same as that of the whole mitochondria, demonstrating that non-haem iron also occurred in the protein fraction solubilized on ultrasonic treatment. The cells grown at an entering iron

concentration of $1.3\text{ }\mu\text{M}$ were G-cells (note the lower entering glycerol concentration), and the distribution of non-haem iron was similar though the absolute amounts were about threefold greater than in the F-cells.

Sensitivity of respiration towards rotenone. Piericidin A is a highly specific inhibitor of the oxidation of intramitochondrial NADH in *T. utilis*. Rotenone is only effective at concentrations three orders of magnitude greater than those needed for inhibition by piericidin A (Light & Garland, 1971). For this

reason we have preferred the use of piericidin A in these studies. Nevertheless, the effects of rotenone ($5\text{ }\mu\text{M}$) on respiration of mitochondria and ETP preparations were also studied. It was found that the presence or absence of piericidin A sensitivity was closely paralleled by the presence or absence of rotenone sensitivity.

Time-course for the recovery of site 1 and piericidin A sensitivity by mitochondria. Fig. 5 shows the results of an experiment in which studies were made of mitochondria prepared from cells harvested at various times during aerobic incubation of F-cells (the recovery procedure). The concentration of added ferrous sulphate in the recovery medium was $1\text{ }\mu\text{M}$. After 3 h exposure to the recovery procedure, the cells yielded mitochondria with marked increases of the state 3 oxidation rate of pyruvate with malate and also of glycerol 3-phosphate. After 5 h the cells yielded mitochondria with a return of the oxidase rates to the original value or slightly less, and with an increase of piericidin A sensitivity from zero to 28%. After 7 h the P/O ratio for pyruvate and malate oxidation had risen from 1.7 to 2.6, and piericidin A sensitivity had increased to 45%. Piericidin A sensitivity continued to rise to 80% at 9 h and 86% at 16 h.

Fig. 6 shows the results of an experiment carried out similarly to that of Fig. 5, except that the $1\text{ }\mu\text{M}$ -ferrous sulphate added to the recovery incubation was trace-labelled with [^{59}Fe]ferric chloride, and ETP_m preparations were made and assayed for oxidase rates, piericidin A sensitivity, acid-labile

Table 8. *Exchange between ^{59}Fe -labelled mitochondria from F-cells and added ferrous sulphate*

A portion (1 ml) of stock suspension of pre-labelled F-mitochondria was mixed at 0°C with 2.0 ml of potassium phosphate buffer, pH 7.4 (0.1M), containing sucrose (0.5M) and FeSO_4 ($200\text{ }\mu\text{M}$), and allowed to stand for 100 min. The mitochondria were then sedimented and washed once as in the preparation procedure for them (Light *et al.* 1970). Samples of the incubation mixture, the pooled supernatant plus washing, and washed mitochondria were measured for radioactivity and protein.

Fraction	Protein (mg)	Total radioactivity (c.p.m.)
Mitochondrial incubation mixture	31	2008
Mitochondrial supernatant plus washings	Not tested	0
Washed mitochondria	30	1998

Table 9. *Effects of the entering iron concentration on the intracellular distribution of non-haem iron*

The haem iron concentration of whole cells was calculated from the sum of cytochromes $a + a_3$, b , and $c + c_1$ as determined from the reduced-minus-oxidized difference spectrum. The total iron concentration (not listed) of cells was calculated from the cell yield and iron uptake, and the non-haem iron concentration was calculated as the difference between total iron and haem iron. It was assumed that all of the cell cytochrome $a + a_3$ was mitochondrial, and the percentage of cell non-haem iron accounted for by mitochondria was calculated as:

$$\% = \frac{(\text{cell concn. of cytochrome } a + a_3) \times (\text{mitochondrial concn. of non-haem iron}) \times 100}{(\text{mitochondrial concn. of cytochrome } a + a_3) \times (\text{cell concn. of non-haem iron})}$$

Cell and mitochondrial type ...	F	G	G
Concn. of glycerol entering chemostats (mM)	133	55	133
Iron concn. (μM)			
Entering chemostat	0.3	1.3	90
Leaving chemostat	$<10^{-10}$	$<10^{-10}$	50
Cell yield (mg dry wt./ml of culture)	2.6	3.0	7.1
Cell concn. (nmol/mg dry wt.) of:			
Haem iron	0.045	0.155	0.279
Non-haem iron	0.070	0.278	5.370
Cytochrome $a + a_3$	0.017	0.057	0.083
Mitochondrial concn. (nmol/mg of protein) of:			
Non-haem iron	0.18	0.73	15
Cytochrome $a + a_3$	0.05	0.17	0.44
% of cell non-haem iron accounted for by mitochondria	87	88	53

Fig. 5. Time-course for the recovery of piericidin A sensitivity and site 1 phosphorylation during the transition from F-cells to FR-cells. F-cells were incubated aerobically with $1\mu\text{M-FeSO}_4$ at 30°C . Mitochondria were prepared from samples of cells harvested at the time-intervals indicated, and assayed for (a) oxygen uptake rates and piericidin A sensitivity and (b) P/O ratios as described by Light & Garland (1971). The concentration of piericidin A used was 1 nmol/mg of mitochondrial protein. $\circ$, With glycerol 3-phosphate; $\bullet$, with pyruvate + malate; $\blacktriangle$, inhibition by piericidin.

sulphide and the incorporation of ^{59}Fe into non-haem iron. As was the case with intact mitochondria the oxidase rates with glycerol 3-phosphate and NADH (equating the latter with pyruvate and malate oxidation in mitochondria) increased considerably over the first 3 h of the recovery procedure, and then declined to the initial value. The return of piericidin A sensitivity commenced after 3 h and was 50% complete by 5 h. The concentration of acid-labile sulphide rose steeply between 3 and 5 h from 0.04 to 0.55 ng-atom/mg of protein. By contrast, the incorporation of ^{59}Fe into non-haem iron showed a gradual rise between 1 and 7 h. These results show that the correlation of the acid-labile sulphide concentration in ETP_m preparations with the presence of piericidin A sensitivity and site 1 phosphorylation is observed not only at the end of the recovery procedure (Table 5) but also during the recovery procedure.

DISCUSSION

Radioisotopic assay for non-haem iron. There appear to be no great drawbacks to this assay, for it is specific, simple, sensitive, free from interfering influences, and applicable in principle to batch culture as well as to our own usage of continuous culture.

Transition between iron limitation and glycerol limitation. This region of growth is particularly noteworthy in that it is one where small changes of the entering iron concentration can determine the presence or absence of energy conservation at site 1. Piericidin A sensitivity was absent throughout this region of growth conditions, and it is therefore clear that significance can no longer be attached to proposals that piericidin A (or rotenone) sensitivity is necessary for energy conservation at site 1 (Ohnishi *et al.* 1966b; Pullman & Schatz, 1967). The

Fig. 6. (a) Time-course for the recovery of piericidin A sensitivity ($\blacktriangle$) and (b) concentrations of acid-labile sulphide ($\square$) and non-haem iron ($\triangle$) in ETP_m preparations during the transition from F- to FR-cells. The experiment was carried out as for Fig. 5 except that the incubation medium of $1\mu\text{M}$ -FeSO₄ was trace-labelled with $^{59}\text{FeCl}_3$. Other methods were as described in the Materials and Methods section. The concentration of ^{59}Fe -labelled non-haem iron represents that non-haem iron synthesized from added iron, and does not take account of any non-haem iron synthesized by redistribution of unlabelled intracellular iron (see the text). The concentration of piericidin A used was 1 nmol/mg of protein. $\circ$, With glycerol 3-phosphate; $\bullet$, with NADH.

span of entering iron concentrations that covers the whole region from F-cells through FG-cells to G-cells is small (a change of $0.1\mu\text{M}$ -entering iron at a glycerol concentration of 133mM), and care, with perhaps luck, is required to obtain FG-cells where mitochondria are completely devoid of piericidin A sensitivity. The cell yield can provide a rapid but simple indicator of the absence or presence of energy conservation at site 1, and further assistance

(though we have not tried it) may come from the use of a higher entering glycerol concentration in an attempt to decrease the sharpness of the transitions as the iron concentration is varied. For it should be understood that the entering iron concentration for obtaining any particular cell type is not a constant but a variable depending on the entering concentration of glycerol. For instance, an entering iron concentration of $0.7\mu\text{M}$ will result in the production

of FG-cells at a glycerol concentration of 133 mM, whereas if the glycerol concentration is lowered to 55 mM, then G-cells are obtained.

A microbiological system in which the efficiency of energy conservation can be readily controlled is clearly of interest to those concerned with the energy balance of cell growth.

Effects of inhibitors of protein synthesis on the recovery of piericidin A sensitivity and site 1 energy conservation by non-growing F-cells. It has not been demonstrated for *T. utilis*, as has been claimed for *S. cerevisiae* or *S. carlsbergensis*, that cycloheximide is an inhibitor of cytoplasmic protein synthesis whereas chloramphenicol, tetracycline and erythromycin are inhibitors of mitochondrial protein synthesis (Vasquez *et al.* 1969). It follows, therefore, that our observation that cycloheximide inhibited the recovery of piericidin A sensitivity, but not site 1 energy conservation, cannot be readily interpreted in terms of a requirement for cytoplasmic protein synthesis in the recovery of piericidin A sensitivity. Nor can any definite conclusions be drawn about the involvement or otherwise of mitochondrial protein synthesis for the recovery of site 1 energy conservation. Nevertheless it is tempting to speculate from the effects of cycloheximide and tetracycline that recovery of piericidin A sensitivity involved cytoplasmic protein synthesis, whereas recovery of site 1 energy conservation involved reconstitution of an apo-protein with non-haem iron rather than protein synthesis.

Chloramphenicol did not inhibit the growth of *T. utilis* on a solid medium, and no conclusions can be drawn about the failure of chloramphenicol to interfere with the recovery of piericidin A sensitivity or site 1 energy conservation. It is possible that the *T. utilis* used in these studies is not permeated by chloramphenicol. A definite knowledge of the involvement or otherwise of protein synthesis in the recovery of piericidin A sensitivity and site 1 energy conservation must await more direct studies than those with inhibitors and whole cells, and the real value of the effect of cycloheximide in the context of this paper is the simplicity with which piericidin A sensitivity can be dissociated from energy conservation at site 1.

Studies made with batch cultures. Ohnishi and her co-workers (Ohnishi, Schleyer & Chance, 1969; Ohnishi & Schleyer, 1969; Ohnishi & Chance, 1969) have been able to repeat a number of our observations of the effects of iron deficiency by using batch culture. There are some obvious attractions to the use of batch culture, such as its simplicity and lack of expense. However, a number of differences exist between our results and those of Ohnishi and her colleagues. Before considering these, it is as well first to consider the differences involved in

obtaining iron deficiency in a batch culture rather than a continuous culture. In the latter, a steady state is achieved within the culture and maintained for long periods. In batch culture, commencing with a low concentration of iron (say 2 μ M), the initial stages of growth would not be iron-limited for, as we have shown, glycerol limitation can occur at undetectable concentrations of iron (<0.1 nM) in the bulk phase of the culture (Table 9). Thus iron limitation must become established as the iron concentration decreases due to the growth of cells. Cell growth must ultimately stop when the minimal cell concentration of iron has been achieved, and this concentration is about 0.115 nmol/mg dry wt. (Table 9, the sum of the haem and non-haem iron concentrations for F-cells). At *n* generations before the end of growth, the cell concentration of iron could be 2^{*n*} times greater if all the available iron were utilized. Thus, at 2 generations before the end of growth, the cell concentrations of iron would be 0.46 nmol/mg dry wt., which is consistent with the cells being of the G-type (Table 9). In fact, it is highly unlikely that all of the cells in a batch culture would be of the same generation. For instance, variation within the population could result in cessation of growth of a proportion of the cells as the iron concentration decreased. This raises the possibility that the cells harvested from an iron-deficient batch culture are heterogeneous with respect to their iron concentration and those properties that depend on the intracellular iron concentration. It is from this viewpoint that we now discuss some of the ways in which the results of Ohnishi & Chance (1969), Ohnishi & Schleyer (1969) and Ohnishi *et al.* (1969) differ from ours.

Our value for the concentration of non-haem iron in ETP_m preparation from F-cells is 0.11 nmol/mg of protein, or even less, and is almost 13-fold lower than the minimum value (1.4 nmol/mg) reported by Ohnishi *et al.* (1969). Another difference concerns the magnitude of the electron-paramagnetic-resonance-spectroscopy signal at *g* = 1.94 induced by NADH. In our own preparation this signal is undetectable in ETP_m preparation from F-cells (Clegg *et al.* 1969), whereas Ohnishi *et al.* (1969) reported that the amplitude of the signal in ETP preparation from their iron-deficient cells was about 5% of that in ETP preparations from fully iron-supplemented cells. The failure of Ohnishi *et al.* (1969) to observe lower non-haem iron concentrations or complete absence of the electron paramagnetic resonance-spectroscopy signal at *g* = 1.94 may arise from the complexities of batch culture. For instance if, at the time of harvesting an iron-deficient batch culture, 5% of the cells were of the fully iron-supplemented G type (Table 9, right-hand column), whereas the remaining 95% were of the

maximally iron-deficient F type (Table 5), then the ETP_m preparation subsequently made would have properties very similar to those of the iron-deficient ETP_m preparation used by Ohnishi *et al.* (1969). Thus, some of the true properties of the ETP_m preparation from F-cells would be obscured by contamination with ETP_m preparations from G-cells, and the amount of contamination would not be detectable with any confidence or accuracy on the basis of determinations of piericidin A sensitivity.

Significance of measurements of non-haem iron and labile sulphide. With but a few exceptions, the majority of non-haem iron proteins are iron-sulphur proteins in which each atom of non-haem iron is accompanied by an atom of acid-labile sulphide (Hall & Evans, 1969). Iron-limited growth of *T. utilis* lowered the concentration of acid-labile sulphide in ETP_m preparations from 0.63 ng-atom/mg of protein (glycerol-limited) to 0.03 ng-atom/mg (iron-limited) (Table 4). The similar fall in non-haem iron concentration (0.82 to 0.11 ng-atom/mg) indicates that most of the non-haem iron in ETP_m preparations is present as iron-sulphur proteins, and that the formation of the acid-labile sulphide component is dependent on the availability of iron. When the ratio of non-haem iron to acid-labile sulphide is approximately unity, it is likely that the measurements represent iron-sulphur protein(s). When the ratio deviates markedly from unity (Haddock & Garland, 1971), then only the lower of the two measurements (one for non-haem iron, one for acid-labile sulphide) can be used as a measure of iron-sulphur protein. An excess of non-haem iron over acid-labile sulphide may represent specific ferroproteins such as ferritin or, in ETP preparation from FR-cells, non-specific adsorption of iron. Alternatively, an excess of acid-labile sulphide over non-haem iron can be obtained (Haddock & Garland, 1971) and may arise from iron-less iron-sulphur proteins.

Mechanisms for the recovery of site 1 and piericidin A sensitivity. Although iron deficiency is required for the loss of these properties, their recovery on aerobic incubation of F-cells does not require that iron be added (Table 1). This does not exclude the possibility that F-cells, which grow at free iron concentration of 0.1 nM or less, can obtain further iron during their harvesting, washing and subsequent 12 h aerated incubation.

The strongest evidence indicating that recovery of site 1 and piericidin sensitivity requires the participation of iron-sulphur proteins comes from Table 5, where it is shown that, although recovery in the absence of added iron (F to FR₀ transition) is associated with an increased concentration in ETP preparations of non-haem iron (derived from pre-existing iron), there is a far greater increase in the acid-labile sulphide concentration. The iron/

sulphur ratio consequently falls from 5.0 in ETP preparations from F-cells to 1.27 in those from FR₀-cells. These results are therefore consistent with the conclusion that the recovery of site 1 and piericidin A sensitivity are due to the recovery of iron-sulphur proteins. It is not known whether the new acid-labile sulphide is in combination with iron derived from contamination in the harvesting, washing and incubation procedures, or from a redistribution of pre-existing intracellular iron.

The partial inhibition by cycloheximide of the appearance of acid-labile sulphide in the recovery procedure [Table 5; compare ETP_m preparations from F-, FR₀- and FR₀(cycloheximide)-cells], indicates that the maximal concentration of iron-sulphur protein required for site 1 is 0.065 ng-atom/mg of ETP_m-preparation protein. For both site 1 and piericidin A sensitivity, the corresponding value is 0.132 ng-atom/mg.

Relationship between piericidin A sensitivity and site 1 energy conservation. Although comparative studies with yeasts led to the proposal that electron flow through a rotenone- (or piericidin A-) sensitive route was a requirement for site 1 energy conservation (Ohnishi *et al.* 1966b), it became apparent from the studies of Schuurmans-Steekhoven (1966) with *S. carlbergensis* and Jones & Redfearn (1967) with *Azotobacter vinelandii* that this correlation was not generally applicable. However, it could be argued that, although these two latter species were rotenone-insensitive, electron flow still proceeded through a component which, although not able to bind rotenone owing to some species difference in structure, was otherwise similar to the rotenone-sensitive component. In this context the respiratory chain of *T. utilis* is instructive for, although it is considered to be rotenone-sensitive (Ohnishi *et al.* 1966b), it is only so at concentrations of rotenone three or four orders of magnitude greater than those inhibitory to mammalian mitochondrial respiration. Nevertheless, the extreme sensitivity towards piericidin A indicates that the 'rotenone-sensitive' component is present despite being relatively rotenone-insensitive in this species (Light & Garland, 1971).

The question of species variations in the relative sensitivities of rotenone-sensitive components does not arise when comparisons are made within a single species, and the observations described in this paper indicate that the rotenone- (and piericidin A-) sensitive component need not be present for electron flow and energy conservation between NADH and the cytochromes. It follows that there is now a strong case for dropping the traditional insistence (Singer, 1966) that a solubilized NADH-coenzyme Q reductase activity is an artifact if (as is invariably the case) its sensitivity to rotenone is atypical or

even absent. This important point is discussed in greater detail by Ragan & Garland (1971).

This work was generously supported by the Medical Research Council, the Royal Society, Shell Research Ltd. and NATO Grant no. 318. We are grateful to Mrs J. Fielding for skilled technical assistance. R.A.C. was in receipt of a Medical Research Council Studentship.

REFERENCES

- Bray, R. C. (1958). *Analyst, Lond.*, **83**, 379.
- Clegg, R. A., Ragan, C. I., Haddock, B. A., Light, P. A., Garland, P. B., Swann, J. C. & Bray, R. C. (1969). *FEBS Lett.* **5**, 207.
- Ernster, L., Dallner, G. & Azzone, G. F. (1963). *J. biol. Chem.* **238**, 1124.
- Ernster, L., Jalling, O., Löw, H. & Lindberg, O. (1955). *Expt Cell Res. Suppl.* **3**, 124.
- Garland, P. B., Clegg, R. A., Light, P. A. & Ragan, C. I. (1969). *Proc. 20th Mosbach Colloq., Mosbach, Baden*, p. 217. Heidelberg: Springer-Verlag.
- Haddock, B. A. & Garland, P. B. (1971). *Biochem. J.* **124**, 155.
- Hall, C., Wu, M., Crane, F. L., Takahashi, N., Tamura, S. & Folkers, K. (1966). *Fedn Proc. Fedn Am. Soc. exp. Biol.* **25**, 530.
- Hall, D. O. & Evans, M. C. W. (1969). *Nature, Lond.*, **223**, 1342.
- Hatefi, Y. (1966). In *Comprehensive Biochemistry*, vol. 14, p. 199. Ed. by Florkin, M. & Stotz, E. H. Amsterdam: Elsevier Publishing Co.
- Herbert, D., Elsworth, R. & Telling, R. C. (1956). *J. gen. Microbiol.* **14**, 601.
- Hewitt, E. J. (1966). *Sand and Water Culture Methods Used in the Study of Plant Nutrition*, 2nd ed. Farnham Royal: Commonwealth Agricultural Bureaux.
- Jeng, M., Hall, C., Crane, F. L., Takahashi, N., Tamura, S. & Folkers, K. (1968). *Biochemistry, Easton*, **7**, 1311.
- Jones, C. W. & Redfearn, E. A. (1967). *Biochim. biophys. Acta*, **143**, 340.
- Kerridge, D. (1958). *J. gen. Microbiol.* **19**, 497.
- King, T. E. & Morris, R. O. (1967). In *Methods in Enzymology*, vol. 10, p. 634. Ed. by Estabrook, R. W. & Pullman, M. E. New York: Academic Press Inc.
- Kotelnikova, A. V. & Zvyagil'skaya, R. A. (1963). *Bio-khimiya*, **38**, 879.
- Kurup, C. K. R. & Brodie, A. F. (1967). *J. biol. Chem.* **242**, 2909.
- Light, P. A. & Garland, P. B. (1971). *Biochem. J.* **124**, 123.
- Light, P. A., Ragan, C. I., Clegg, R. A. & Garland, P. B. (1968). *FEBS Lett.* **1**, 4.
- Linnane, A. W., Vitols, E. & Nowland, P. G. (1962). *J. Cell Biol.* **13**, 345.
- Mackler, B., Collipp, P. J., Duncan, H. M., Rao, N. A. & Huennkens, F. M. (1962). *J. biol. Chem.* **237**, 2968.
- Mahler, H. R. & Elowe, D. G. (1954). *J. biol. Chem.* **210**, 165.
- Ohnishi, T. & Chance, B. (1969). *Abstr. 3rd int. Symp. Flavins and Flavoproteins, Durham, N.C., U.S.A.*, p. 37.
- Ohnishi, T. & Hagihara, B. (1964). *J. Biochem., Tokyo*, **56**, 484.
- Ohnishi, T., Kawaguohi, K. & Hagihara, B. (1966a). *J. biol. Chem.* **241**, 1797.
- Ohnishi, T., Kröger, A., Heldt, H., Pfaff, E. & Klingenberg, M. (1967). *Eur. J. Biochem.* **1**, 301.
- Ohnishi, T. & Schleyer, H. (1969). *Fedn Proc. Fedn Am. Soc. exp. Biol.* **28**, 885.
- Ohnishi, T., Schleyer, H. & Chance, B. (1969). *Biochem. biophys. Res. Commun.* **36**, 487.
- Ohnishi, T., Sottocasa, G. & Ernster, L. (1966b). *Bull. Soc. Chim. biol.* **48**, 1189.
- Pullman, M. E. & Schatz, G. (1967). *A. Rev. Biochem.* **36**, 539.
- Quastel, J. H. & Wheatley, A. H. M. (1933). *Proc. R. Soc. B*, **112**, 60.
- Ragan, C. I. & Garland, P. B. (1971). *Biochem. J.* **124**, 171.
- Rouf, M. A. (1964). *J. Bact.* **88**, 1545.
- Schatz, G. & Racker, E. (1966). *Biochem. biophys. Res. Commun.* **22**, 579.
- Schuurmans-Steckhoven, F. M. A. H. (1966). *Archs Biochem. Biophys.* **115**, 555.
- Singer, T. P. (1966). In *Comprehensive Biochemistry*, vol. 14, p. 127. Ed. by Florkin, M. & Stotz, E. H. Amsterdam: Elsevier Publishing Co.
- Smith, C. F., McCurdy, W. H. & Diehl, H. (1952). *Analyst, Lond.*, **77**, 418.
- Vanneste, W. H. (1966). *Biochim. biophys. Acta*, **113**, 175.
- Vasquez, D., Staehelin, T., Celma, M. L., Battaner, E., Fernandez-Munoz, R. & Munro, R. E. (1969). *Proc. 20th Mosbach Colloq., Mosbach, Baden*, p. 100. Heidelberg: Springer-Verlag.
- Vitols, E. & Linnane, A. W. (1961). *J. biophys. biochem. Cytol.* **9**, 701.
- Yang, C. C. & Legallais, V. (1954). *Rev. scient. Instrum.* **25**, 801.