Biochem. J. (1971) **124**, 123–134
Printed in Great Britain

A Comparison of Mitochondria from *Torulopsis utilis* Grown in Continuous Culture with Glycerol, Iron, Ammonium, Magnesium or Phosphate as the Growth-Limiting Nutrient

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(*Received* 18 *January* 1971)

1. Mitochondria prepared from *Torulopsis utilis* grown in a chemostat with iron-limited growth were found to lack energy conservation but not electron flow in that segment of the respiratory chain leading from intramitochondrial NADH to the cytochromes [i.e. the site 1 segment (Lehninger, 1964)]. 2. Site 1 energy conservation was present in mitochondria prepared from cells grown under conditions of limitation by glycerol, ammonium and magnesium. Phosphate-limited growth resulted in mitochondrial preparations without respiratory control. 3. Mitochondria from cells grown under conditions of iron limitation were insensitive to the respiratory inhibitor piericidin A, whereas sensitivity was present in mitochondria prepared from glycerol-, ammonium-, magnesium- or phosphate-limited cells. 4. These observations are considered to provide indirect evidence for a role of non-haem iron in the mechanism of energy conservation and also piericidin A sensitivity in *T. utilis* mitochondria. 5. A readily constructed and inexpensive pH-measuring and -controlling circuit is described for use with continuous-culture apparatus.

It has been known for some years that mitochondria contain considerable quantities of non-haem iron (Green, 1956; Crane, Glenn & Green, 1956). Although some of these have been solubilized and partially characterized (Rieske, Zaugg & Hansen, 1964; Hatefi & Stempel, 1967), the elucidation of their role in functionally intact mitochondria has met with some difficulties. For instance, non-haem iron proteins have relatively undistinctive absorption bands in the visible region of the spectrum, and such spectral changes as do occur in association with oxidoreductions not only have low extinction coefficients (Beinert, 1965), but are also readily obscured by changes due to cytochromes or flavoproteins (Palmer, Horgan, Tisdale, Singer & Beinert, 1968; Rajagopalan & Handler, 1964). Further, studies with reconstituted systems such as the succinate oxidase of Yamashita & Racker (1968) have failed to implicate any non-haem iron protein (e.g. that of Rieske *et al.* 1964) other than that associated directly with the succinate dehydrogenase flavoprotein. Lastly, the conclusions about a role for non-haem iron in site 1† energy conservation based on comparative electron paramagnetic resonance-spectroscopy studies with different yeasts (Ohnishi, Sottocasa & Ernster, 1966*b*; Ohnishi, Racker, Schleyer & Chance, 1968; Schatz, Racker, Tyler, Gonze & Estabrook, 1966) have been discredited by more-direct experimental methods (Imai, Asano & Sato, 1968*b*; Clegg *et al.* 1969; Ohnishi, 1970).

Of other experimental methods used to study the role of non-haem iron in the site 1 region of the respiratory chain, the use of the iron-chelating agent *o*-phenanthroline (Butow & Racker, 1965) has been criticized on the grounds that similar results were obtainable by using the non-chelating *m*-phenanthroline (Imai, Asano & Sato, 1968*a*). The use of iron-deficient growth of *Micrococcus denitrificans* did not demonstrate any interrelationship between energy-conservation mechanisms and non-haem iron (Imai *et al.* 1968*b*).

In this paper we describe the manner in which

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† There are normally three sites in the mitochondrial respiratory chain where electron flow is coupled to the synthesis of ATP from ADP and P_i. For convenience they are termed sites 1, 2 and 3 respectively (Lehninger, 1964). These three sites of phosphorylation (or, preferably, energy conservation) are associated with electron flow in three segments or regions of the respiratory chain which are between NADH and cytochrome *b* (site 1 segment) cytochrome *b* and cytochrome *c* (site 2 segment), and cytochrome *c* and oxygen (site 3 segment).

iron-limited growth of *Torulopsis utilis* results in the loss of energy conservation but not of electron flow between intramitochondrial NADH and the cytochromes. Preliminary accounts have been published (Light, Ragan, Clegg & Garland, 1968; Garland, Clegg, Light & Ragan, 1969). Further studies of the effects of iron-limited growth on mitochondrial non-haem iron (Clegg & Garland, 1971), of the mitochondrial effects of sulphate-limited growth (Haddock & Garland, 1971) and of detailed spectroscopic studies (Ragan & Garland, 1971) of *T. utilis* are presented in the accompanying papers.

MATERIALS AND METHODS

Apparatus for continuous culture. Four chemostats were constructed in the 'Porton' style as described by Herbert, Elsworth & Telling (1956) and Herbert, Phipps & Tempest (1965), with the non-ferrous modifications introduced by Ware, Thompson & Light (1970). For convenience, each chemostat was mounted on a small trolley (A.P.T. Electronic Industries Ltd., Byfleet, Surrey, U.K.) which also carried the associated electronic units, the 20-litre medium bottle, and a peristaltic pump (model MHRE from Watson–Marlow Ltd., Marlow, Bucks., U.K.). In all experiments the working volume of the chemostat was between 500 and 700 ml and the medium entered at a constant rate between 120 and 150 ml/h.

Measuring and control circuits for pH. The requirements for pH control in the present context were not exacting. This, and the availability of low-cost operational amplifiers with high input impedances ($>10^{11}\Omega$) and low offset currents ($<10^{-11}$ A) led us to design and construct our own circuits for pH measurement and control. A full account of operational amplifier usage for measuring pH is given by Goepfert (1967). In the circuit shown in Fig. 1, amplifier A1 acts as a voltage follower for the glass electrode. This electrode is steam-sterilizable, has a combined reference electrode, an impedance of $3\times10^{8}\Omega$, and an isopotential value nominally at pH 7.0 (Chemetric Ltd., Twickenham, Middx., U.K.). The screening of the electrode cable was earthed and connected to the reference electrode. At 30°C, the voltage of the glass electrode (E_c) with respect to the reference electrode is given by $E_c = E_0 - 0.60$ pH, where E_0 is the pH-independent component of electrode voltage and has a value of about +0.42 V for an electrode with an isopotential nominally at pH 7.0. The voltage at the output of amplifier A1 therefore changes by 0.06 V for a change of 1.0 pH unit at the electrode. Amplifier A2 multiplies the output voltage of A1 by $-(R2+VR2)/R1$ and sets an overall sensitivity of +1.0 V/pH unit. The output of amplifier A2 is connected to a voltmeter and recorder socket. VR2 is a voltage divider and, by providing an adjustable positive or negative current at the summing point of amplifier A2, acts as the electrode asymmetry control and sets the output of amplifier A2 to zero volts at pH 7.0.

Calibration of the electrode was effected by first immersing the electrode in buffer at pH 7.0 and 30°C; VR1 was then adjusted until the output of amplifier A2 was zero. This sets the zero point at pH 7.0. Next, the electrode was immersed in buffer at pH 4.0 and 30°C; VR2 was adjusted until the output of amplifier A2 was −3.0 V. This sets the sensitivity, i.e. +1.0 V/pH unit. It is noteworthy

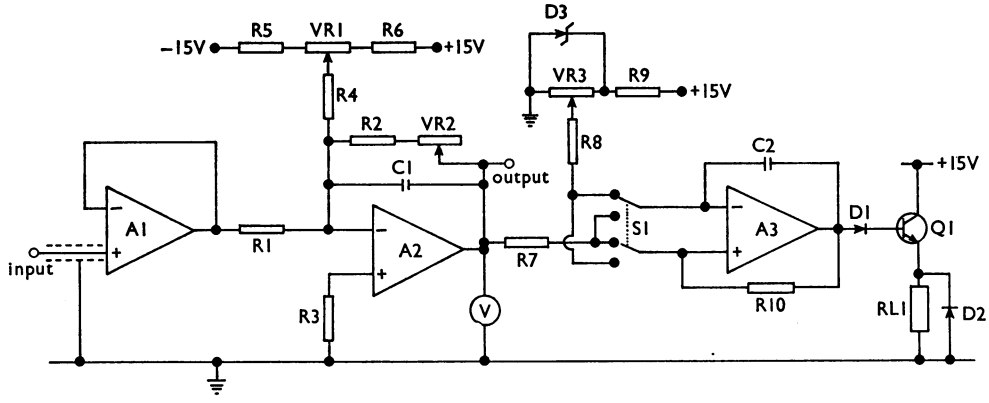

Fig. 1. Circuit for measuring and controlling pH. The components are: A1, operational amplifier P501B (Analogue Devices Inc., 59 Eden St., Kingston-on-Thames, Surrey, U.K.), A2 and A3, integrated circuit operational amplifier 2N72741N (Texas Instruments Ltd., Bedford, U.K.); R1, 2 kΩ; R2, 20 kΩ; R3, 1.5 kΩ; R4, 20 kΩ; R5 and R6, 1 kΩ; R7 and R8, 2.2 kΩ; R9, 300 kΩ; R10, 470 kΩ; (all resistors carbon film, 5% ¼ W); C1, 4.4 μF; C2, 47 pF; VR1, 5 kΩ preset; VR2, 10 kΩ preset; VR3, 10 kΩ (a ten-turn helical potentiometer fitted with a turn counting dial: the number of turns registered gives the pH level at which level-detector A3 switches); diodes D1 and D2, IS920 (Texas Instruments Ltd.); D3, IS2100A (Texas Instruments Ltd.); Q1, 2N3704 (Texas Instruments Ltd.); RL1, a reed relay with an operating voltage of 10–15 V and a winding resistance of 350Ω. Maximum switched power, 60 W, at 250V a.c. (e.g. Hercon HRE 80 relay from ITT, Edinburgh Way. Harlow, Essex, U.K.); S1, a DPDT switch. The position shown is appropriate for a culture which tends to acidify. V′ is a 5-0-5V centre-zero voltmeter reading from 2 to 12 pH.

that the asymmetry control VR1 should be adjusted only at pH7.0 (or whatever the desired zero point) when the exact gain of amplifier A2 (set by VR2) is immaterial. The electrode was autoclaved in position within the chemostat vessel, and its characteristics were not detectably altered by this procedure.

The pH-control circuit commences with amplifier A3 (Fig. 1). This acts as a voltage-level detector, which closes a reed relay whenever the measured pH (i.e. the output voltage of A2) decreased below a certain value preset by voltage divider VR3. Closure of the reed relay activates a small peristaltic pump which adds 2M-KOH to the culture until the pH rises sufficiently to turn the pump off. The peristaltic pumps used for this purpose were constructed to the design of Dr G. C. Ware. A similar but commercially available pump is the single-tube Delta Micro-Metering Pump from Watson–Marlow Ltd., Marlow, Bucks., U.K. Positive switching of the reed relay was obtained by introducing a hysteresis of about 50mV (equivalent to 0.05pH unit) in the level-detecting action of amplifier A3 by means of resistors R7 and R10. Capacitors C1 and C2 give further immunity to noise.

The pH-measuring and -control circuit is simple, reliable and easily constructed at low cost. There is also a single-ended recorder output giving +1.0V/pH unit from a low-impedance source. Calculations of the errors arising from imperfections of the amplifiers (Goepfert, 1967) indicated that the largest error arose from the ambient temperature dependence of the offset current of amplifier A1, and was not greater than 0.005pH unit/°C for the electrode and amplifier combination described. The temperature dependence of the electrode potential itself is 0.3% per °C at 30°C and can be ignored in a temperature-regulated chemostat.

For growth limited by glycerol, NH_4^+, Mg^{2+}, phosphate, iron or SO_4^{2-}, the pH of the culture tended to fall and 2M-KOH was added automatically whenever the pH fell below 5.0. When growth was limited by acetate (as the sodium salt; Fukami, Light & Garland, 1970), the pH of the culture tended to rise and the correct controlling action was achieved by reversing the connexions to the inputs of amplifier A3 and substituting 2M-HCl for KOH.

Measuring and control circuits for temperature. Since a regulated ±15V supply was required for the operational amplifiers of the pH-measuring and -control circuit, it was convenient to use cheap integrated-circuit amplifiers (see Fig. 1) for measuring and controlling the temperature of the culture. The measuring circuit of a thermistor bridge and differential amplifier was quite conventional and need not be described. The control circuit consisted of a voltage-level detector which closed a reed relay whenever the temperature of the culture fell below a preset value. Closure of the reed relay provided 250V a.c. to a flexible 36W heating tape (type HT340 from Electrothermal Engineering Ltd., London E.7, U.K.) which made a turn around the lower part of the culture vessel. Design considerations otherwise followed that for the pH control circuit, and a hysteresis of 0.2°C was introduced to prevent jitter of the reed relay.

Measurement of oxygen concentration in the culture. The oxygen concentration in the culture was measured with an autoclavable oxygen electrode as described by Johnson, Borkowski & Engblom (1967) and obtained from the New Brunswick Scientific Co. Inc., New Brunswick, N.J., U.S.A. The electrode current was measured with a 1.0kΩ load and a 0–20mV potentiometric recorder.

Reagents. ADP (trisodium salt), NADH (disodium salt), 2-oxoglutarate, pyruvate, oxaloacetate, sodium phosphoenolpyruvate, lactate dehydrogenase (EC 1.1.1.27) and pyruvate kinase (EC 2.7.1.40) were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Tris base, rotenone, antimycin A, DL-glycerol 3-phosphate and biotin were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; succinate was from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.; Oxoid mycological peptone was from Oxoid Ltd., London S.E.1, U.K.; Bacto agar, powdered yeast extract and powdered malt extract were from Difco Laboratories, Detroit, Mich., U.S.A.; antifoam FG was from Midland Silicones Ltd., Barry, Glam., U.K.; D-sorbitol, L-malate and 2-mercaptoethylamine hydrochloride were from Koch–Light Ltd., Colnbrook, Bucks., U.K.; and snail gut enzyme was from Industrie Biologique Française, Gennevilliers, Seine, France. Bovine plasma albumin was purchased from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K., and freed from fatty acids by the method of Goodman (1958). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was a gift from Dr P. G. Heytler, and piericidin A was a gift from Professor K. Folkers. All other chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, U.K., and were of the highest grade available. All aqueous solutions were made in water distilled twice from an all-glass apparatus. Stock solutions of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, rotenone, piericidin A and antimycin A were prepared in methanol and at concentrations such that about 5 or 10μl of the solution had to be added to a mitochondrial incubation of 2 or 3ml to obtain the desired final concentration of inhibitor.

Culture media. The following media were employed during these studies and, in all instances, the percentage composition is expressed as w/v. Saboraud's agar: glucose (4%), Oxoid mycological peptone (1%), agar (2%). The pH was adjusted to 5.4 with 2M-HCl. Wickerham (1951) medium: glucose (1%), malt extract (0.3%), yeast extract (0.3%), peptone (0.5%), antifoam FG (0.25ml/l). The pH was adjusted to 6.0 with 2M-HCl. Glycerol-limited medium: we are indebted to Dr D. Herbert for this recipe: glycerol (133mM), Na_2HPO_4 (2.8mM), NaH_2PO_4 (12.8mM), NH_4Cl (75mM), K_2SO_4 (11.5mM), citric acid (1.0mM), biotin (4μg/litre), antifoam FG (0.25ml/l) and a stock solution of salts (1ml/l). Stock solution of salts: this contained $MgCl_2$ (1.25M), $CaCl_2$ (0.1M), $FeCl_3$ (90mM), $MnCl_2$ (50mM), $ZnCl_2$ (25mM), $CoCl_2$ (10mM), $CuCl_2$ (5mM), H_3BO_3 (5mM) and Na_2MoO_4 (10mM) dissolved in HCl (about 3M). This solution was made by adding 250ml of conc. HCl to 250ml of water; the salts were then dissolved in the order given, and the volume was made up with water to 1 litre. Glucose medium: this resembled the glycerol medium except that glucose (55.6mM) replaced glycerol. Iron-limited medium: this was similar to the glycerol medium except that $FeCl_3$ was omitted from the salts solution. Ammonium-limited medium: this was similar to the glycerol medium except that the concentration of NH_4Cl was lowered from 75 to 10mM. Phosphate-limited medium: this was similar to the glycerol medium except that Na_2HPO_4 (0.88mM) replaced Na_2HPO_4 (2.8mM) and NaH_2PO_4 (12.8mM).

Magnesium-limited medium: the data of Tempest, Dicks & Meers (1967) were used as a basis for calculating a suitable entering concentration of Mg^{2+}, and the medium resembled the glycerol medium except that the concentration of $MgCl_2$ was lowered from 1.25 mM to 25 μM. Autoclaving: the chemostat vessels with associated tubing, filters and pH electrodes were sterilized at 5 lb/in^2 for 20 min, and the 20-litre Pyrex bottles containing medium (16 litres) were sterilized at 15 lb/in^2 for 45 min. Uniform dispersion of the antifoam was achieved by first suspending 4 ml of antifoam in 100 ml of water before adding it to the main volume (15.9 litres) of medium.

Maintenance of stock cultures and inoculation of chemostats. Stock cultures of *T. utilis* were maintained on slopes of Saboraud's agar at 2°C. At about 2-monthly intervals subcultures were made, incubated at 30°C for 48 h and then stored at 2°C. To prepare an inoculum for initiating a continuous culture, a recent slope culture was recultured on a plate of Saboraud's agar at 30°C for 48 h. A suitable colony was then removed with a sterile platinum loop and used to inoculate 100 ml of glycerol medium with or without added $FeCl_3$, as appropriate. After growth for 36 h at 30°C, 1 ml of this culture was added to 500 ml of medium in the chemostat vessel. Sterility was ensured by generously dousing the top plate of the chemostat with ethanol, and igniting it, before and after the removal of the threaded plug occupying the port through which the inoculum was added. Once inoculated, the culture in the chemostat vessel was allowed to grow for 24 h at 30°C with gentle aeration (about 100 ml/min) but without stirring, pH adjustment or further addition of medium. After this stirring and pH adjustment were started, the air flow was increased and medium pumped in at 50 ml/h. During the next 36–48 h the flow rate of entering medium was gradually increased to its final value. At least another 48 h were then allowed to elapse before any experiments were performed.

Collection of cells and preparation of spheroplasts. When cells were required for the preparation of mitochondria, the chemostat effluent was collected into a 2-litre polythene bottle surrounded by ice. An overnight collection of 15 h (with 133 mM-glycerol as the growth-limiting nutrient) gave about 50 g wet wt. of cells, which yielded about 3 mg of mitochondrial protein/g wet wt. Except where stated otherwise, all procedures in the preparation of spheroplasts and mitochondria were carried out at 0–2°C. The collected effluent was centrifuged at 3000*g* for 10 min in the 4 × 1.25-litre head of an MSE serum centrifuge, and the cells were washed twice by resuspending in water and recentrifuging. The cells were then converted into spheroplasts by the use of snail gut enzyme to digest the cell wall, and subsequently broken by a combination of osmotic and mechanical means to obtain a cell-free system from which mitochondria were prepared by differential centrifugation. The procedure, which followed that of Duell, Inoue & Utter (1964) was as follows: *n* g of washed cells was suspended in 2*n* ml of citrate–K_2HPO_4 buffer (0.1 M-citric acid and K_2HPO_4 added to pH 5.8) containing sorbitol (0.63 M), EDTA (0.4 mM), 6*n* mg of 2-mercaptoethylamine hydrochloride and 0.3*n* ml of snail gut enzyme (as obtained from the suppliers). The suspension was thoroughly mixed, placed in a 500 ml round-bottomed flask, and shaken in a water bath at 30°C for 1 h. This treatment converted 70–90% of the cells into spheroplasts, as judged microscopically. The cells were then sedimented by centrifugation at 5000*g* for 10 min in the 8 × 50 ml angle rotor of an MSE High-Speed 18 refrigerated centrifuge, and washed twice in 0.9 M-sorbitol at 0°C. Resuspension of the cells in the washing solution was performed gently with an ice-cold test-tube. This procedure for making spheroplasts was usually carried out on 20–50 g wet wt. of cells.

Preparation of mitochondria from spheroplasts. The washed spheroplasts were suspended in 300 ml of a solution of sucrose (50 mM), potassium phosphate (50 mM-KH_2PO_4 adjusted to pH 5.8 with KOH) and EDTA (1 mM), and shaken vigorously by hand for 1 min. This procedure breaks the majority of spheroplasts, and its effectiveness can be checked by microscopy. Sufficient 3.0 M-sucrose was then added to bring the final concentration of sucrose to 0.25 M, and the mixture was centrifuged at 1000*g* for 15 min. The sediment, which contained unbroken cells and nuclei, was discarded, and the supernatant was centrifuged at 9000*g* for 10 min to yield a mitochondrial pellet. This pellet was washed twice by resuspension in a solution of sucrose (0.5 M), potassium phosphate (50 mM-KH_2PO_4 adjusted to pH 6.8 with KOH) and EDTA (1 mM), and resedimentation at 9000*g* for 10 min. Finally, the mitochondria were resuspended in further washing medium to yield a final protein concentration of about 30 mg/ml. This procedure gave 2–4 mg of mitochondrial protein/g wet wt. of cells, and, from spectrophotometric measurements of the cytochrome *a* content of cells and mitochondria, it can be calculated that the recovery of mitochondria from the whole cells was about 20%. Microscopy of the mitochondrial preparation failed to detect unbroken yeast cells, and culture of the preparation on nutrient agar demonstrated only an occasional bacterial colony.

Selection of strains of T. utilis *sensitive to snail gut enzyme.* The sensitivity of yeast cell walls to the snail gut enzyme varies with the species strain and growth conditions. At the start of this work, no information was available about the sensitivity of various strains of *T. utilis* towards the snail gut enzyme, and five separate strains were obtained for testing from the National Collection of Yeast Cultures, Nutfield, Surrey, U.K. These were strain nos. 168, 193, 321, 322 and 359. After culture on Saboraud's agar to check their purity, batch cultures of 2 litres in glycerol medium were grown in 5-litre glass flasks aerated with sterile air passed through a glass sinter at 2 litres/min and maintained at 30°C in a water bath. Cells were harvested after 30–36 h and subjected to the spheroplast preparation procedure described above. The sensitivity of the cells towards the snail gut enzyme was assessed both by microscopy and by light-scattering measurements of samples (taken throughout the incubation with snail gut enzyme) diluted in water. The order of increasing sensitivity was strain no. 193, 321, 322, 168, 359. Spheroplast formation was complete with strain no. 193 after 60 min incubation with snail gut enzyme and only 50% complete for no. 359. Sensitivity also varies with the growth conditions. For instance, strain no. 193 was insensitive to the snail gut enzyme after continuous culture with ethanol as the carbon source and the omission of pH control of the culture. Nevertheless, *T. utilis* N.C.Y.C. 193 was used in all the experiments that follow.

Assay techniques. Mitochondrial protein was assayed by the method of Lowry, Rosebrough, Farr & Randall

(1951), NADH by its E_{340}, glycerol by the method of Garland & Randle (1962), ADP by the method of Adam (1963), and the dry weight of suspensions of yeast cells by the method of Tempest & Herbert (1965). Measurements of oxygen uptake by cells or mitochondria were performed in a vessel with a working volume of 3 ml, with an oxygen electrode constructed and calibrated as described by Chappell (1964*a*,*b*). The fluorescence of mitochondrial NADH and NADPH (Chance & Baltscheffsky, 1958) and flavoprotein (Chance & Schoener, 1966) was measured with a modified Eppendorf fluorimeter (Garland, Shepherd & Yates, 1965). Spectrophotometer measurements of the cytochrome content of cells and mitochondria were made with either a dual-wavelength spectrophotometer (Chance, 1951) or wavelength-scanning spectrophotometer (Yang & Legallais, 1954). Optical cuvettes of 1 cm light-path were used, and the following wavelength pairs and reduced-minus-oxidized extinction coefficients were used: cytochrome $a+a_3$, 605–630 nm, ϵ_{mM} 13 $mmol^{-1} \cdot cm^{-1}$ (Vanneste, 1966); cytochrome *b*, 563–575 nm, ϵ_{mM} 18 $mmol^{-1} \cdot cm^{-1}$ (Ohnishi, Kröger, Heldt, Pfaff & Klingenberg, 1967); cytochrome *c* and c_1, 550–540 nm, ϵ_{mM} 18 $mmol^{-1} \cdot cm^{-1}$ (Ohnishi *et al.* 1967). The wavelength pairs were then used in the dual-wavelength spectrophotometer for observing the extinction change between the oxidized state (state 2 of Chance & Williams, 1956) and the reduced state (state 5). Alternatively, a state 5 minus state 2 difference spectrum was obtained with the wavelength-scanning spectrophotometer.

Mitochondrial incubations. The medium used for all mitochondrial incubations for the measurement of oxygen uptake, fluorimetric or spectrophotometric changes was that of Ohnishi, Kawaguchi & Hagihara (1966*a*), and contained D-sorbitol (0.65 M), EDTA (0.1 mM), HCl (10 mM), potassium phosphate buffer (10 mM-KH_2PO_4 adjusted to pH 6.5 with KOH) and tris–maleate buffer (20 mM-maleic acid adjusted to pH 6.5 with tris base). All other aqueous solutions used in experiments with mitochondria were adjusted to pH 6.5 with tris base or HCl. Mitochondrial P/O ratios were measured polarographically, and the respiratory-control ratio referred to is that defined by Chance & Williams (1956) as the ratio of the rate of oxygen uptake during ADP stimulation to the rate after conversion of the ADP into ATP. Mitochondrial incubations were done at 30°C.

RESULTS

Oxygen concentration within the culture. The chemostats used in this work are so designed that high rates of oxygen transfer into the culture can be obtained. With an aeration rate of 1.0 vol. of air/vol. of culture, the rate of oxygen transfer is about 135 mmol of O_2/h per litre of culture or 4.5 μg-atom of O_2/min per ml of culture (Herbert *et al.* 1965). This anticipated oxygen transfer rate is at least 20-fold greater than the rate of oxygen consumption of the culture as estimated from oxygen-electrode measurements of the oxygen consumption by cells withdrawn from the culture. Direct measurement with an oxygen electrode immersed in the culture showed that the oxygen concentration in the culture almost equilibrated with that of incoming air (Fig. 2).

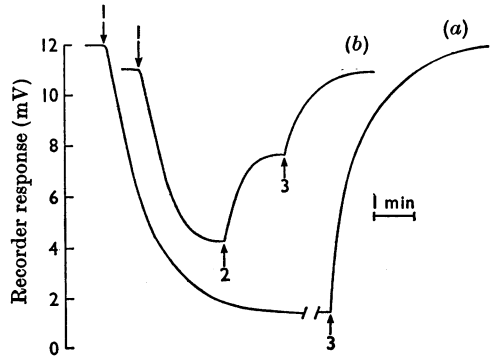

Fig. 2. Recordings from an oxygen electrode within the chemostat culture. Recording (*a*) was made when the chemostat contained 0.5 litre of water at 30°C, stirred at 1400 rev./min and aerated with an air flow of 0.5 litre/min from the laboratory air line. At the first transition (1), both stirring and aeration were stopped and a few grains of sodium dithionite were added. At (2), stirring and aeration were recommenced after a period which included a 5 min interruption of the recording. This experiment establishes the electrode output for saturation of water with air. Recording (*b*) was made when the chemostat contained 0.5 litre of a culture of *T. utilis* growing at 30°C under conditions of ammonium limitation at a cell concentration of 3.2 mg dry wt./ml. The stirring speed and aeration rate were 1400 rev./min and 0.5 litre/min respectively. At the first transition (1) the stirrer motor was turned off, and at (2) the stirrer restarted but the aeration rate decreased to 0.1 litre/min. At (3) the aeration rate increased to its original value of 0.5 litre/min.

Demonstration of the growth-limiting nutrient. Table 1 presents the concentration of cells and of glycerol within the chemostat as judged from measurements made on samples of the culture. These concentrations are affected by changes in the composition of the entering growth medium. It is apparent that in each case where growth was considered to be limited by a specific nutrient, the concentrations of cells and glycerol within the culture were in keeping with this behaviour. Thus, for glycerol-limited growth (Table 1), virtually all of the entering glycerol was utilized and the cell concentration depended on the entering glycerol concentration. For limitation by other nutrients, considerable concentrations of glycerol remained unused within the culture and the cell concentration was dependent on the entering concentration of the chosen limiting nutrient.

Respiratory characteristics of mitochondria from T. utilis *grown in continuous culture.* The extensive work of Ohnishi and her colleagues using batch

Table 1. *Effects of the composition of the entering medium on the concentration of cells and glycerol within the chemostat culture*

The experimental details are described in the Materials and Methods section.

Medium	Concn. in entering medium of: glycerol (M)	Concn. in entering medium of: limiting nutrient (mM)	Concn. in culture of: cells (mg dry wt./ml)	Concn. in culture of: glycerol (mM)
Glycerol-limited	0.133	133	7.2	<0.06
Glycerol-limited	0.055	55	3.0	<0.06
Iron-limited	0.133	0.0003	3.1	40
Ammonium-limited	0.133	10	3.18	56
Phosphate-limited	0.133	0.88	2.06	25
Magnesium-limited	0.133	0.025	1.51	100

culture has laid a foundation for the study of mitochondria from *T. utilis* (Ohnishi *et al.* 1966*b*, 1968) Nevertheless, certain aspects of that work had to be repeated in our studies to establish any possible differences that might have arisen from our use of continuous as opposed to batch culture. Additionally, information was sought about the fluorescence of mitochondrial flavoprotein and its sensitivity towards the respiratory inhibitor piericidin A (Hall *et al.* 1966).

Table 2 lists the oxygen-uptake rates, P/O ratios and respiratory-control ratios observed with mitochondria oxidizing a variety of substrates and derived from cells of different growth-limiting conditions. The P/O ratios observed with mitochondria from glycerol-limited cells are similar to those reported by Ohnishi *et al.* (1966*b*) and Kotelnikova & Zvyagilskaya (1963), and confirm that the oxidation of NAD-linked substrates via intramitochondrial NAD proceeds with a P/O ratio approaching 3.0, whereas the oxidation of extramitochondrial NADH and DL-glycerol 3-phosphate (presumably only the L-isomer was oxidized) proceeds with a P/O ratio of 2.0. Similar results were obtained with mitochondria from ammonium- and magnesium-limited cells. Mitochondria from phosphate-limited cells were probably damaged; respiratory control was not obtained and the rates of oyxgen uptake were low. Mitochondria from iron-limited cells exhibited a P/O ratio of approx. 3.0 for 2-oxoglutarate oxidation, and 2.0 for the oxidation of pyruvate with malate, added NADH and DL-glycerol 3-phosphate. From these observations it can be concluded that iron-limitation resulted in the loss of energy conservation but not electron flow in the site 1 segment of the respiratory chain.

Sensitivity of mitochondria towards respiratory inhibitors. The effects of antimycin A on the oxidation of various substrates by mitochondria from either glycerol- or iron-limited cells were similar to those reported by Ohnishi *et al.* (1966*b*) and Butow & Zeydel (1968). Oxygen uptake with any of the substrates listed in Table 2 was at least 97% inhibited by 2.0mM-potassium cyanide. The effects of rotenone on yeast mitochondria are particularly notable, since a correlation has been obtained between the presence of energy conservation at site 1 and rotenone sensitivity (Ohnishi *et al.* 1966*b*). Ernster, Dallner & Azzone (1963) demonstrated that rotenone inhibited the respiration of NAD-linked substrates by rat liver mitochondria with a stoicheiometry of 27pmol of rotenone/mg of mitochondrial protein. Takahashi, Suzuki & Tamura (1965) and Hall *et al.* (1966) have introduced a new inhibitor, piericidin A, which was considered to act similarly to rotenone. However, the conclusion that mitochondria from *T. utilis* are sensitive to rotenone is somewhat relative, for mitochondria from mammalian tissues are three or four orders of magnitude more sensitive to rotenone than are mitochondria from *T. utilis* (Ohnishi *et al.* 1966*b*). This is shown in Fig. 3, which also shows that piericidin A is a potent inhibitor of the oxidation of NAD-linked substrates by *T. utilis* mitochondria.

If it is assumed that piericidin A interacts with a component of the respiratory chain with a one-to-one stoicheiometry, and that the piericidin A is pure, then it can be calculated from the results in Fig. 3 that the mitochondrial concentration of the piericidin A-sensitive component is about 75pmol/mg of protein. The oxidation of DL-glycerol 3-phosphate or NADH was unaffected by piericidin A tested to a final concentration of 2μM. The sensitivity to piericidin A of the oxidation of pyruvate with L-malate by mitochondria from ammonium-, magnesium- or phosphate-limited cells was the same as that with mitochondria from glycerol-limited cells. The oxidation of any of the substrates in Table 2 by mitochondria from iron-limited cells was unaffected by piericidin A at concentrations from 0 to 2μM.

Fluorimetric studies of the site of action of piericidin A and of reversed electron flow at site 1. Since neither fluorimetric nor spectroscopic measurements made

Table 2. *Oxygen-uptake rates and P/O ratios of mitochondria prepared from cells with different growth-limiting nutrients*

The oxygen-uptake rates (ng-atoms of O_2/min per mg of protein) are not corrected for the endogenous rates but are the observed rates in state 3 (Chance & Williams, 1956). N.T., not tested.

Limited by ...	Glycerol		Iron		Ammonium		Phosphate		Magnesium	
Substrate and concn. (mM)	O_2 uptake	P/O ratio	O_2 uptake	P/O ratio	O_2 uptake	P/O ratio	O_2 uptake	P/O ratio	O_2 uptake	P/O ratio
Endogenous	40–90	2.18 ± 0.33 (5)	30–70	1.67 ± 0.4 (4)						
2-Oxoglutarate (5)	120–140	3.83 ± 0.42 (3)	125–270	2.9 ± 0.2 (4)	225	3.6	85	—	210	3.69
Pyruvate (2) and L-malate (2)	145–205	3.0 ± 0.21 (5)	166–275	1.95 ± 0.2 (4)	225	2.8	82	—	180	2.04
Citrate (5)	65–115	2.4 ± 0.28 (2)	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Ethanol (1)	135–160	2.6 ± 0.16 (4)	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Succinate (5)	40–90	—	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
DL-Glycerol 3-phosphate	390–480	1.77 ± 0.15 (3)	332–480	1.97 ± 0.17 (4)	90	1.2	254	—	420	1.89
NADH	370–420	1.65 ± 0.16 (4)	280–350	1.6 ± 0.3 (4)	590	1.92	286	—	N.T.	N.T.

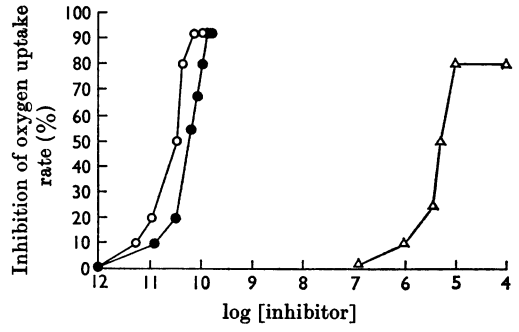

Fig. 3. Sensitivity of mitochondrial respiration towards rotenone and piericidin A. The experiments were carried out with an oxygen electrode and conditions were as described in the Materials and Methods section, with pyruvate (5mM) and L-malate (5mM) as the combination of substrates. Similar results were obtained with ethanol (1mM) or citrate (5mM). The two left-hand curves are for piericidin A at mitochondrial protein concentrations of 0.64mg/ml (○) and 1.28mg/ml (●). The right-hand curve (△) is for rotenone at a protein concentration of 1.3mg/ml.

directly on mitochondria distinguish between NADH and NADPH, the two are written together as NAD(P)H to indicate the uncertainty as to which is being studied. Likewise, $NAD(P)^+$ refers to a combination of the oxidized forms. Further uncertainty is introduced by the fact that in *T. utilis* mitochondria there is not only an NADH dehydrogenase but also an analogous NADPH dehydrogenase capable of reducing the cytochromes (Ragan & Garland, 1971). It therefore cannot be assumed, as is the case with mammalian mitochondria, that oxidoreductions occurring between NADP and the cytochromes occur via NAD and a transhydrogenase.

The addition of glycerol 3-phosphate to mitochondria from glycerol-limited cells resulted in a rapid reduction of intramitochondrial $NAD(P)^+$. The subsequent addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, an uncoupler of oxidative phosphorylation, caused a rapid reoxidation (Fig. 4). These observations indicate that electron flow from NAD(P)H to the cytochromes in these mitochondria can be reversed in an energy-requiring manner (Chance & Hollunger, 1960; Klingenberg & Slenczka, 1959). It could, however, be argued that the reduction of NAD(P) was effected by endogenous mitochondrial substrates and that the role of glycerol 3-phosphate was merely one of inhibiting the reoxidation of intramitochondrial NAD(P)H by competing for a common and rate-limiting carrier. However, this argument can be countered by the observation that, when the respiratory chain was blocked (by the

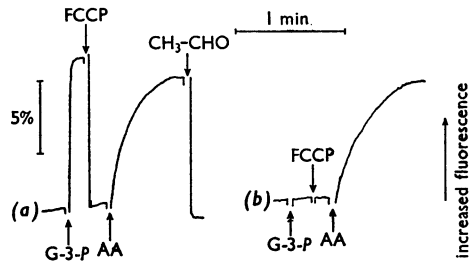

Fig. 4. Recordings of the fluorescence of mitochondrial NAD(P). The exciting wavelength was at 366nm with a band width of about 15nm, and the emitted light was measured at wavelengths above 420nm (Garland *et al.* 1965). The incubation medium was as described in the Materials and Methods section and the final volume was 2.2ml. In trace (*a*) 3.9mg of mitochondria from glycerol-limited cells was used, and in trace (*b*), 3.9mg of mitochondria from iron-limited cells. Other additions and final concentrations were 5mM-DL-glycerol 3-phosphate (G-3-*P*); 0.25μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP); 0.5μM-antimycin A (AA), and 0.25mM-acetaldehyde. The vertical bar to the left corresponds to 5% of the total photomultiplier signal. An upward deflexion of the recording corresponds to increased fluorescence or reduction of NAD(P).

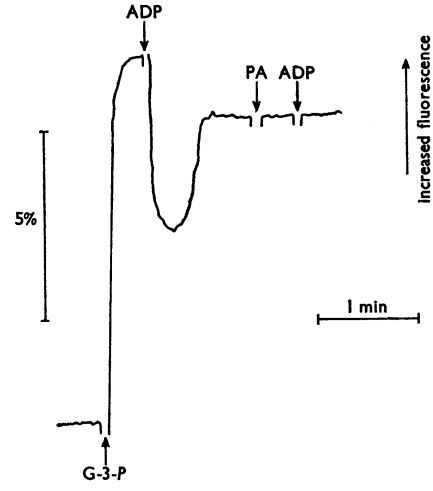

Fig. 5. Effect of piericidin A on the oxidation of intramitochondrial NAD(P)H. The recording is of NAD(P)H fluorescence and the experimental conditions were as in Fig. 4(*a*) except that 2.9mg of mitochondrial protein from glycerol-limited cells was used. The final concentration of ADP was 0.2mM for each addition, and, for piericidin A (PA), 0.1μM.

addition of antimycin A; Fig. 4), then the subsequent reduction of NAD(P) by endogenous substrates occurred with a much longer half-time (15–30s) and diminished extent (60–90%) compared with that observed with glycerol 3-phosphate oxidation in state 4.

Mitochondria from *T. utilis* oxidize ethanol (Table 2), and it could be inferred that there is an intramitochondrial alcohol dehydrogenase (EC 1.1.1.1). An alternative demonstration of intramitochondrial alcohol dehydrogenase is provided in Fig. 4, which shows that the addition of acetaldehyde reoxidized intramitochondrial NAD(P)H.

Reversal of electron flow between intramitochondrial NADH and the cytochromes requires that the energy-conservation mechanism at site 1 shall operate in reverse, utilizing energy to accomplish an otherwise thermodynamically unlikely oxidoreduction. It could therefore be anticipated that mitochondria from iron-limited cells, which lack site 1 energy conservation as assayed polarographically (Table 2), would not catalyse an energy-dependent reduction of NAD(P) by glycerol 3-phosphate. This was indeed so (Fig. 4). Mitochondria from ammonium- and magnesium-limited cells resembled those from glycerol-limited cells in that energy conservation at site 1 was demonstrable, not only polarographically (Table 2) but also by the state 4 reduction of NAD(P) by glycerol 3-phosphate. The fluorimeter readings for these latter mitochondria resembled those in Fig. 4(*a*).

The experiment recorded in Fig. 5 demonstrates the partial reoxidation of NAD(P)H during ADP-stimulated oxidation of glycerol 3-phosphate. NAD(P)H remained reduced after the addition of piericidin A, in keeping with the proposed site of action of this inhibitor on the respiratory chain (Hall *et al.* 1966).

Flavoprotein fluorescence of T. utilis *mitochondria.* Measurements of the flavoprotein fluorescence of mitochondria from glycerol-limited cells gave recordings that were remarkably similar to those observed when measuring the fluorescence of NAD(P)H, except that the direction of fluorescence change (but not reduction change) was reversed. For instance (Fig. 6), the addition of glycerol 3-phosphate caused a rapid decrease of flavoprotein fluorescence (i.e. reduction of flavoprotein) that was complete within 1–2s. This change was reversed by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and restored at a slower rate and to a lesser extent by the subsequent addition of antimycin A. Finally, the addition of acetaldehyde caused a complete reoxidation of flavoprotein, presumably indirectly via the reoxidation of NAD(P)H catalysed by intramitochondrial alcohol dehydrogenase. From this experiment it appears that most, if not all, of the fluorescent flavoprotein capable of enzymic oxidoreduction in these mitochondria is of the 'low-potential' variety (Chance *et al.* 1967; Ragan & Garland,

1969). The absence of 'high-potential' fluorescent flavoprotein, and the nature of the low-potential fluorescence, is discussed elsewhere (Ragan & Garland, 1971). The characteristics of flavoprotein fluorescence exhibited by mitochondria from glycerol-limited cells (Fig. 6) were also shown by mitochondria from ammonium- and magnesium-limited cells. Although mitochondria from iron-limited cells contained apparently undiminished amounts of low-potential fluorescent flavoprotein, an energy-dependent reduction by glycerol 3-phosphate was not demonstrable (Fig. 6).

Effects of iron-limited growth on the cytochrome content of whole cells and their mitochondria. Table 3 shows that iron limitation decreased the concentrations of cytochromes about fivefold when compared with glycerol limitation. The decrease was similar in both whole cells and mitochondria, and it can therefore be concluded that iron limitation did not affect the proportion of cell dry weight accounted for as mitochondrial protein. The effects of iron limitation were similar for cytochrome *b*, cytochrome $c+c_1$ and cytochrome $a+a_3$. No marked differential effects were observed.

DISCUSSION

Preparation of mitochondria from cells grown in continuous cultures. One possible disadvantage of growing cells in continuous culture rather than batch culture could arise from the need to harvest the effluent of the continuous culture for several hours until sufficient cells were obtained for preparing an adequate batch of mitochondria. Any changes occurring in the cells during this collection period could defeat one of the objects of using continuous culture, namely that of obtaining a highly homogeneous cell population whose properties

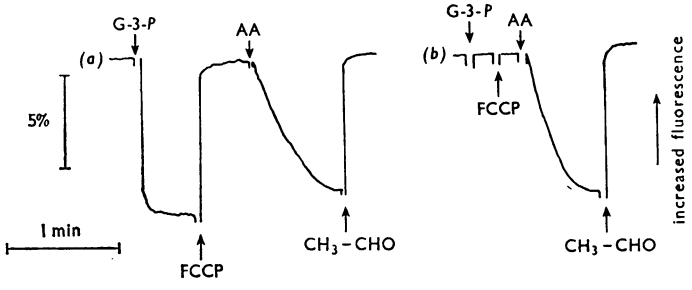

Fig. 6. Recordings of the fluorescence of mitochondrial flavoprotein. The exciting wavelength was at 456nm with a band width of 10nm, and the emitted light was measured at wavelengths above 520nm. The experimental conditions and abbreviations were otherwise as in Fig. 4 except that an upward deflexion of the recording indicates an oxidation. Trace (*a*), mitochondria from glycerol-limited cells; trace (*b*), mitochondria from iron-limited cells.

Table 3. *Effects of iron-limited growth on the concentrations of cytochromes in whole cells and their mitochondria*

The results given are for dual-wavelength spectrophotometric assays on two preparations and are similar to those obtained by wavelength-scanning spectroscopy on any other preparation derived from iron-limited growth without added iron or glycerol-limited growth with 100 μM added iron. In all cases, spectroscopic measurements were made on sufficient material to give a final concentration of cytochrome $(a+a_3)$ in the cuvette of about 0.1 μM. For whole cells, the suspension in air-saturated 50mM-potassium phosphate buffer, pH7.2, was allowed to become anoxic while the extinction difference at an appropriate wavelength pair was measured. A similar technique was used for mitochondria except that air-saturated incubation medium was used containing 0.25 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and the reduced-minus-oxidized extinction change at a given wavelength pair attributable to a cytochrome was calculated from the sum of changes occurring on (i) the addition of 5mM-DL-glycerol 3-phosphate, and (ii) the exhaustion of oxygen.

	Concn. of cytochromes in			
	Mitochondria (nmol/mg of protein)		Cells (nmol/g dry wt.)	
	Glycerol-limited	Iron-limited	Glycerol-limited	Iron-limited
Cytochrome $a+a_3$	0.21	0.038	26.8	5.6
Cytochrome *b*	0.22	0.053	21.6	7.0
Cytochrome $c+c_1$	0.50	0.073	47.3	7.2

depended on the conditions of culture rather than storage. This possible drawback does not appear to have materialized; the mitochondria from cells grown continuously with glycerol as the growth-limiting nutrient were found to have properties very similar to those described for batch culture (Ohnishi *et al.* 1966*b*). We have not met any real drawbacks in the construction or continuous operation of four chemostats. The mechanical design of the apparatus (Herbert *et al.* 1956, 1965; Ware *et al.* 1970) has proved reliable over several years of intensive use, as have the control circuits for pH and temperature. Infection of the culture has occurred only rarely, and uninterrupted cultures of 2 or 3 months are commonplace. Failures have been rare, and in decreasing order of frequency, have been due to (i) breakdown of the central compressed air supply, (ii) mechanical failure of pumps, and tubes subjected to wear in peristaltic pumps, (iii) latching of relays in the control circuits, and (iv) aberrant bottles of media. In most of these cases it has not been necessary to dismantle the chemostat; provided that sterility was preserved, sufficient cells remained within the chemostat to restart the culture.

Relative merits of batch and continuous culture. Batch culture is undoubtedly cheap and easy to establish. It is, however, in our experience, considerably inferior to continuous culture in providing a reliable, convenient and reproducible supply of cells available at all times. However, the real object of our use of continuous culture was not to provide automation of cell growth but to make accurate and repeatable alterations of the growth conditions leading to changes in mitochondrial function. This object has been obtained and, although certain parts of these studies have been repeated and extended by using batch culture (Demaille, Vignais & Vignais, 1970; Ohnishi, Schleyer & Chance, 1969), the need for continuous culture remains in the following major aspects: (i) the achievement of sulphate-limited growth (Haddock & Garland, 1971), (ii) the production of cells poised between iron- and glycerol-limitation, leading to further unusual mitochondrial properties (Clegg *et al.* 1969), (iii) the production of cells with a mitochondrial content of non-haem iron and acid-labile sulphide between one and two orders of magnitude lower than any previously reported (Clegg & Garland, 1971), (iv) the growth of *Saccharomyces carlsbergensis* under conditions where site 1 energy conservation is derepressed (P. A. Light & P. B. Garland, unpublished work), and (v) the production of a homogeneous cell population.

A further advantage of the continuous-culture technique as used by us is the high degree of aeration tion that can be achieved. This is evident from the work of Herbert *et al.* (1965), and is also demonstrated by the oxygen-electrode recordings in Fig. 2.

Interpretation of the mitochondrial effects of iron-limited growth. There are three characteristics of the respiratory chain leading from NADH to the cytochromes which may involve non-haem iron, and these are (i) electron flow, (ii) energy conservation, and (iii) piericidin A sensitivity. The first of these characteristics is presumably necessary for cell growth on a non-fermentable substrate, and it could be anticipated that cells growing with glycerol as the carbon source would exhibit electron flow from NADH to the cytochromes irrespective of whether or not non-haem iron was involved. The second of these characteristics, site 1 energy conservation, is not required for growth when there are two further energy-conservation sites in the respiratory chain. It is therefore of particular significance that iron-limited growth resulted in the loss of site 1 energy conservation whereas ammonium- or magnesium-limited growth did not. The third characteristic, piericidin A sensitivity, is presumably of biological importance only as a target for the *Streptomyces* sp. which fabricates this antibiotic, and is therefore not needed in any functional sense by *T. utilis*. This characteristic is therefore free to disappear and, that it should do so under conditions of iron-limitation, but not ammonium-, magnesium- or phosphate-limitation, indicates that the piericidin-sensitive component or pathway may involve non-haem iron (see also Bois & Estabrook, 1969).

Other interpretations of the mitochondrial effects of iron-limited growth. Katz (1970) has reported that the loss of piericidin A sensitivity and site 1 energy conservation in mitochondria prepared from *T. utilis* grown in iron-deficient batch culture occurred only at restricted rates of aeration of the culture. This is undeniably not the case under our own conditions of culture and aeration (see Fig. 2), and the results of Katz (1970) can be attributed to one or both of the following possibilities: first, that increasing the air flow to a culture may increase the amount of contaminating iron entering with the air, the iron arising variously from the environment, the compressor, or lines; or secondly, that the effect of diminished aeration of an iron-deficient medium is to lower the oxygen concentration of the culture sufficiently to stimulate cytochrome synthesis (Moss, Rickard, Beech & Bush, 1969) with the result that a marginally limiting concentration of iron becomes truly limiting.

Ohnishi (1970) has suggested that the effects of iron limitation on site 1 energy conservation are not directly due to the lack of iron but rather arise from the resultant accumulation of carbon source (glycerol) within the culture. That this is not the case was already shown (Clegg *et al.* 1969) by a study of the transition between iron-limited and glycerol-limited growth; iron limitation could be relaxed to the point where the glycerol concentration within

the medium was about 100 μM without the return of site 1 energy conservation. Further evidence that glycerol accumulation does not cause the disappearance of site 1 energy conservation is also provided by the studies with ammonium- and magnesium-limited growth (Tables 1 and 2).

Pathways of NADH oxidation. The ability of piericidin A to inhibit the oxidation of NADH generated intramitochondrially but not added extramitochondrially provides further support for the conclusion that there are two distinct pathways of electron transfer from NADH to the cytochromes (Ohnishi *et al.* 1966*b*; Light *et al.* 1968; Von Jagow & Klingenberg, 1970). One pathway utilizes intramitochondrial NADH in a rotenone- and piericidin A-sensitive route that is also coupled to energy conservation; the other pathway utilizes extramitochondrial NADH and is neither sensitive to the inhibitors nor coupled to energy conservation at site 1. For convenience, these two pathways and their NADH dehydrogenases may be called 'internal' and 'external' respectively. Clearly it is the internal pathway which exhibits altered characteristics in mitochondria from iron-limited cells.

Reversible dissociation of energy conservation and electron flow. The effects of iron-limited growth could have been due to selection within the chemostat of a new genotype lacking the first energy-conservation site. However, demonstration that aeration of iron-limited cells under non-growing conditions (Light *et al.* 1968; Clegg & Garland, 1971) results in recovery of energy conservation at site 1 leads to the conclusion that the original loss was phenotypic, not genotypic.

The development of a biological system whereby one of the three mitochondrial energy-conservation sites could be made to disappear and to return in a manner controlled by the cell environment was clearly a considerable novelty in the study of mitochondrial energy-conservation mechanisms. Further, the possibility that energy conservation can be biologically and reversibly dissociated from electron flow between NADH and the cytochromes can be extended to other organisms. In *S. carlsbergensis*, site 1 energy conservation is apparently de-repressed by growth on certain carbon sources (P. A. Light & P. B. Garland, unpublished work) or by aeration of repressed but non-growing cells (Ohnishi, 1970). In *Azotobacter vinelandii*, oxygen-limited growth de-represses energy conservation at site 1 (Ackrell & Jones, 1971). It remains to be determined whether a similar site-specific dissociation of electron flow and energy conservation can be obtained at sites 2 and 3.

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 D. Bees and Mrs J. Fielding for skilled technical assistance, and to Dr G. C. Ware for his great help with continuous-culture techniques. Part of this work was completed during the tenure (by P.A.L.) of grants from the Science Research Council and the Wellcome Trust.

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