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Biochemical Applications of Continuous Culture: Energy-Conservation Mechanisms in *Torulopsis utilis*

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Introduction

The experimental advantages of working with a microbial system are beautifully illustrated by this excerpt from Keilin (1966) describing experiments performed some 40 years earlier (Keilin, 1925): 'One day, while I was examining a suspension of yeast, freshly prepared from a few bits of compressed yeast shaken vigorously with a little water in a test-tube, I failed to find the characteristic four-banded spectrum, but before I had time to remove the suspension from the field of vision of the microspectroscope, the four absorption bands suddenly reappeared. This experiment was repeated time after time, and always with the same result: the absorption bands disappeared on shaking the suspension with air and reappeared within a few seconds on standing. I must admit this first visual perception of an intracellular respiratory process was one of the most impressive spectacles I have witnessed in the course of my work. Now I had no doubt that cytochrome is not only widely distributed in nature . . . but that it is an intracellular respiratory pigment which is more important than haemoglobin.'

A more recent view of the mitochondrial respiratory chain of yeasts and higher animals is shown in Scheme 1, and, in addition to the relatively well-

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characterized cytochromes, flavoproteins and ubiquinone, there are iron-sulphur proteins whose functional significance is problematical (Hall & Evans, 1969). Apart from iron and sulphur present as part of the flavoprotein dehydrogenases particular to the immediate oxidation of NADH and succinate, distinctive iron-sulphur proteins have been implicated in the mechanism of electron transfer from NADH to the cytochromes (Hatefi & Stempel, 1967) and from cytochrome *b* to cytochrome *c*₁ (Rieske, MacLennan & Coleman, 1964). However, there is as yet no general agreement about the role of iron-sulphur proteins: that of Hatefi has been considered non-proven by Gutman, Singer & Casida (1969) and Albracht & Slater (1969), whereas Rieske's iron-sulphur protein is apparently not required for a reconstituted succinate oxidase system (Yamashita & Racker, 1969). The study of iron-sulphur proteins in complex systems such as mitochondria is hindered by the absence of sharply defined absorption bands in the visible region, and such spectral characteristics that are available overlap those of flavoproteins (Rajagopalan & Handler, 1964). Other physicochemical techniques are either too insensitive at present (e.g. Mossbauer spectroscopy) or provide information on only a small fraction of the non-haem iron of mitochondria (e.g. electron paramagnetic resonance; Beinert & Lee, 1961).

The sequence of respiratory carriers shown in

Scheme 1. Respiratory chain and the site of action of piericidin A. Abbreviations: Fp_D, NADH dehydrogenase; Fe-S, iron-sulphur protein; UQ, ubiquinone; cyt *b*, *c*, *a* and *a*₃, the respective cytochromes. The sequence of carriers in parentheses is undecided.

Scheme 1 illustrates perhaps the least interesting aspect of their function—namely the reduction of oxygen to water. The standard oxidoreduction potentials (mid-potentials) at pH 7.0 of NADH and oxygen are separated by a span of 1.135 V, equivalent to a free-energy change of 52 kcal in the reduction of 1 g-atom of oxygen by NADH and therefore sufficient for the synthesis of not more than 5 mol of ATP from ADP and P_i . The actual amount synthesized is 3 mol at the most, and for thermodynamic and other reasons the primary processes of energy conservation whereby ATP synthesis is ultimately coupled to electron flow are subdivided to be associated with three segments of the respiratory chain (one per molecule of ATP) that correspond to three so-called coupling or energy-conservation sites: site I between NADH and the cytochromes, site II between the carriers at the level of cytochrome *b* and cytochrome c_1 , and site III between cytochrome *c* and oxygen. It is axiomatic that energy can be conserved only where it would otherwise have been dissipated (as heat). For our present purposes only four more points need be made: (1) the primary form in which energy is conserved is not as ATP but as a high-energy state whose nature is disputed; (2) the high-energy state can be recognized by its ability to perform work such as the movement of electrons against an otherwise unfavourable potential gradient (e.g. from cytochrome *b* to NADH; Chance & Hollunger, 1960); (3) although there are three sites of energy coupling there is only one set of enzymes required for the synthesis of ATP driven by the energy from each site (Racker, 1965); (4) the energy-conservation mechanism, like the respiratory chain, is bound to and part of the inner mitochondrial membrane. Energy conservation but not electron flow is rapidly lost as fractionation of the membrane into its components proceeds. The subject of mitochondrial energy conservation has been reviewed most recently by Greville (1969).

It is apparent that the typical biochemical approach of dismantle and reconstitute has so far

had only a limited success in the study of functions that are properties of complex and presumably highly organized membrane systems. In only some cases can the membranous components be detected spectroscopically (Keilin, 1925; Chance & Williams, 1956) or by other non-destructive physicochemical means.

Biological techniques in the study of the respiratory chain

With few exceptions, most of our present-day knowledge of the organization of the mitochondrial respiratory chain and energy-conservation processes is based on experiments with preparations from higher animals, notably rat liver and ox heart. These starting materials have the advantage of readily yielding adequate quantities of mitochondria that have energy conservation coupled to electron flow, and the disadvantage of being virtually incapable of previous genotypic or phenotypic manipulation. Microbial systems are freely open to biological experimentation, but, regrettably, the energy-conservation mechanisms of cell-free preparations have proved to be somewhat labile towards the cell-disruption processes. Perhaps the ease with which large yields of highly coupled mitochondria can be quickly prepared from rat liver has been a disservice to the subject, for it has set a norm that is discouragingly not achieved with microbial systems. However, there are now procedures available for the preparation of mitochondria or membrane fractions from an increasing range of eucaryotic and procaryotic micro-organisms, and these preparations have acceptably intact energy-conservation mechanisms.

Comparative studies of the NADH-to-cytochrome segment of the respiratory chain

Table 1 lists some characteristics of this segment as found in a variety of yeasts. The interesting point that emerges from this comparative study is

Table 1. *Differences between the NADH-to-cytochrome segments of the respiratory chain of various yeasts*

References: ¹Linnane, Vitols & Nowland (1962); ²Ohnishi, Kawaguchi & Hagihara (1966); ³Vitols & Linnane (1961); ⁴Ernster, Dallner & Azzone (1963); ⁵Ohnishi, Sottocasa & Ernster (1966); ⁶Mahler, Mackler, Slonimski & Grandchamp (1964); ⁷Mackler, Collipp, Duncan, Rao & Huennkens (1962); ⁸Schatz & Racker (1966); ⁹Hall *et al.* (1966); ¹⁰Light *et al.* (1968); ¹¹Sharp, Mackler, Douglas, Palmer & Felton (1967); ¹²Duncan & Mackler (1966); ¹³Crane (1961); ¹⁴Glover, Isler, Morton, Luegg & Wiss (1958); ¹⁵Ohnishi, Racker, Schleyer & Chance (1967); ¹⁶Schatz *et al.* (1966).

	<i>T. utilis</i>	<i>S. carlsbergensis</i>	<i>S. cerevisiae</i>
Site I energy conservation	Present ¹	Absent ²	Absent ³
Rotenone ⁴ -sensitivity	Sensitive ⁵	Insensitive ²	Insensitive ^{6,7,8}
Piericidin A ⁹ -sensitivity	Sensitive ¹⁰	Insensitive ¹⁰	—
Flavin of NADH oxidase	FMN and FAD ¹¹	—	FAD ^{7,12}
Ubiquinone species	UQ ₇ ¹³	UQ ₆ ⁵	UQ ₆ ¹⁴
NADH reduced <i>g</i> =1.94 signal	Present ^{11,15}	—	Absent ^{12,16}

that there is a correlation between the presence of coupling at site I and the presence of rotenone-sensitivity. A further correlation exists between these two properties and the presence of an e.p.r. (electron paramagnetic resonance) signal at $g=1.94$ that is attributable to the NADH dehydrogenase. From such observations have arisen the proposals that electron flow through a rotenone-sensitive site and also an NADH dehydrogenase giving an e.p.r. signal at $g=1.94$ is essential for site I energy conservation (Schatz & Racker, 1966; Pullman & Schatz, 1967; Schatz, Racker, Tyler, Gonze & Estabrook, 1966).

That non-haem iron was involved in energy conservation at site I was indicated not only by the e.p.r. spectroscopy of yeast mitochondria but also by some observations by Butow & Racker (1965), who described inhibitory effects of the iron-chelating agent *o*-phenanthroline on this segment of the respiratory chain. The effects of *m*-phenanthroline (which does not chelate iron) were not described, although Imai, Asano & Sato (1968a) have observed inhibitory effects of this compound on the NADH-to-cytochrome segment of *Micrococcus denitrificans*. These latter workers also reported (Imai, Asano & Sato, 1968b) that iron-deficient growth of *M. denitrificans* did not abolish coupling at site I whereas the signal at $g=1.94$ was no longer detectable. Thus the evidence in favour of a function for non-haem iron in energy conservation at site I was, at best, slight. The comments of Keilin (1966) on the pitfalls of the comparative approach are relevant: 'However, with all these advantages, the study of micro-organisms is not without pitfalls, especially when, disregarding their great variability, attempts are made to form generalizations based upon the study of only a few isolated strains or species'.

Continuous culture

In the hope of using microbial techniques for approaching mitochondrial problems, we started work in 1965 with yeasts and used a continuous-culture apparatus for growing the cells (Light & Garland, 1966). Initially the main advantage of continuous culture lay in its convenience: the cells were literally on tap. Another advantage lay in the day-to-day constancy of the growth conditions and therefore of the cells and their mitochondria. Further and more unique advantages became apparent with time, and to understand these a brief account of continuous-culture techniques is in place.

Fig. 1 illustrates the essentials of a continuous-culture apparatus used as a 'chemostat', and the design is essentially that of Herbert, Phipps & Tempest (1965) with the inclusion of modifications

Fig. 1. Functional diagram of a chemostat. For structural details see Herbert *et al.* (1965) and Ware *et al.* (1970).

by Ware, Thompson & Light (1970) for the use of non-ferrous materials in the culture vessel and stirrer, and our own use of integrated circuits for sensing and controlling the pH and temperature of the culture. The kinetics of chemostat behaviour have been analysed by Monod (1950), Novick & Szilard (1950) and Herbert, Elsworth & Telling (1956).

In these analyses it is assumed that there is homogeneity of the culture, that the flow rate of medium into the vessel is constant and that the volume of culture is constant. It is further assumed that all of the growth nutrients but one, the limiting substrate, are in excess for growth, and that all cells are equally viable. The following variables are defined: F , flow rate (flow rate of medium into the vessel = flow rate of culture out of the vessel); V , volume of the culture (in the vessel); D , dilution rate, i.e. F/V ; s_0 , concentration of growth-limiting substrate in the entering medium; s , concentration of growth-limiting substrate in the culture; x , concentration of cells in the culture; μ , specific growth rate, i.e. $(dx/x)/dt$.

Monod (1949) found that the specific growth rate μ was related to the concentration of limiting substrate in a fashion fitted by eqn. (1):

$$\mu = \mu_{\max} \cdot \left(\frac{s}{K_s + s} \right) \quad (1)$$

where $\mu_{\max}$ is the maximum growth rate and K_s is the substrate concentration required for $\mu = \mu_{\max}/2$.

Monod (1949) also introduced the growth-yield constant, y , which under suitably high rates of growth relates the appearance of new cell mass to the disappearance of substrate:

$$\frac{dx}{dt} = -y \cdot \frac{ds}{dt} \quad (2)$$

A chemostat culture can be said to be in a steady state when the concentrations of cells within the culture are constant. It follows that the rate at which cell mass is lost from the vessel is equal to the rate at which new cell mass is formed, i.e.:

$$Fx = \frac{dx}{dt} \cdot V \text{ or } \frac{dx/x}{dt} = \frac{F}{V}$$

i.e. $\mu = D$ (3)

Attainment of the steady state also requires that the substrate concentration within the culture be constant, i.e.:

Substrate accumulation = 0 = input - output
- substrate used for growth

or $\frac{ds}{dt} = 0 = F_{s0} - F_s - \frac{\mu x}{y} \cdot V$ (4)

It should be noted that:

$$x = y(s_0 - s) \quad (5)$$

and that when $s_0 \gg s$:

$$x = ys_0 \quad (5a)$$

The theoretical steady-state behaviour of a chemostat is given by eqns. (1), (3) (4) and (5), and various degrees of agreement between the theoretical and actual steady-state behaviour have been reported (Herbert *et al.* 1956). Two interesting and not immediately self-evident consequences follow from theoretical behaviour. First, the steady-state cell concentration within the culture depends only on the yield constant and entering substrate concentration s_0 provided that $s_0 \gg s$. Secondly, the steady-state concentration s of limiting substrate within the culture is independent of the entering concentration s_0 , as is shown by manipulating eqns. (1) and (3):

$$s = K_s \left(\frac{D}{\mu_{\max} - D} \right) \quad (6)$$

Both the steady-state and transient behaviour of a continuous culture can be simulated with an analogue computer (Koga & Humphrey, 1967; Bungay, 1968). Fig. 2 demonstrates the theoretical behaviour of a chemostat. Initially the culture vessel is filled to its working volume with medium, then inoculated with cells. A phase of exponential growth follows and ceases as the concentration of growth-limiting substrate falls towards zero. On starting a flow of medium into what until now had been a batch culture, the substrate concentration rises and then, as the cell mass increases, falls to the steady-state value characteristic of eqn. (6). Next the concentration of entering substrate is doubled: the concentration of substrate in the

Fig. 2. Analogue computer simulation of chemostat behaviour. The computer was built in collaboration with Dr C. I. Ragan. The ordinates are in volts but otherwise arbitrary. The operating conditions were $K_s = 0.2V$, $\mu_{\max} = 0.5h^{-1}$. Initially the model was operated in the mode of a batch culture, and continuous culture was started at 'pump on' with $s_0 = 30V$ and $D = 0.18h^{-1}$. At the 'substrate jump' s_0 was doubled, at the first flow jump D was increased to $0.26h^{-1}$ and at the second flow jump D was increased to $0.47h^{-1}$.

culture rises until it is pulled back to the previous steady-state concentration by the increasing concentration of cells. The next transition involves an increase of dilution rate from $0.18h^{-1}$ to $0.26h^{-1}$: there is a rapid jump in substrate concentration and little change in the cell concentration. Finally, a larger increase in dilution rate up to $0.47h^{-1}$ brings the culture nearer to the wash-out point where D equals $\mu_{\max}$ ($0.5h^{-1}$).

Continuous culture and mitochondria

From this point we can now see how continuous culture in the chemostat can be used to alter mitochondria in response to the extracellular environment. For by selecting any one of numerous possible growth substrates as the one that limits growth, and by biasing the choice in favour of a substrate that has a function which involves at least a mitochondrial component, then it is possible to anticipate alterations of mitochondrial function in response to the environmental conditions. Table 2 lists some possible applications of this approach, and extends the list of 'normal' growth substrates to include those additional requirements of possible auxotrophic mutants. It is, of course, possible to effect growth limitation by some particular substrate in batch culture and in fact our earliest experiments on iron deficiency of *Torulopsis utilis* were performed in such a manner (P. A.

Table 2. *Growth-limitation by specific substrates and the modification of mitochondrial function*

Substrate	Organism	Reference	Function and comment
Iron	<i>T. utilis</i>	Light, Ragan, Clegg & Haddock (1968)	Loss of site I; low cytochrome content; low non-haem iron content
Sulphate	<i>T. utilis</i>	Ohnishi & Schleyer (1969)	Loss of site I; cytochrome content normal; low non-haem iron content
Copper	<i>Saccharomyces cerevisiae</i>	B. A. Haddock (personal communication)	Low cytochrome oxidase content
Threonine	<i>S. cerevisiae</i> (auxotroph)	Elvehjem (1931)	Low cytochrome oxidase content
p-Hydroxybenzoate	<i>Escherichia coli</i> mutant	Surdin, de Robichon-Szulmajster, Lachowicz & Slonimski (1969)	Lacks ubiquinone; yeast mutants should be obtainable
Riboflavin	Riboflavin auxotroph required	Cox, Snoswell & Gibson (1968)	Minimal flavin content of mitochondria; ? formation of flavin apoproteins
Pantothenic acid	<i>Saccharomyces carlsbergensis</i>	A.T.C.C. 9080 } A.T.C.C. 9371 }	Possible study of mitochondrial CoA and control of its synthesis
β -Alanine	<i>S. cerevisiae</i>		

Light, personal communication). However, the batch culture is never in a steady state and the concentration of the substrate is continuously changing during growth. By contrast, continuous culture affords an exact and reproducible control of the all-important growth condition, and must surely be the method of choice for this purpose.

Iron-limited growth of T. utilis

The concentration of iron in a growth medium composed of A.R.-grade chemicals and water distilled twice from an all-glass apparatus was about $0.3 \mu\text{g-atom/l}$, a value that proved to be virtually ideal for limiting the chemostat growth of *T. utilis* grown on 130mm-glycerol as the carbon source. Fig. 3 shows the effect of increasing the concentration of entering iron on the steady-state concentration of cells in the culture. The linearity of this relationship indicated that eqn. (5a) rather than eqn. (5) was being followed, i.e. $s_0 \gg s$. The addition of carrier-free ^{59}Fe to the entering medium and subsequent radioactive analysis of the bulk phase of the culture showed that less than $0.1 \text{ ng-atom of Fe/l}$ remained unassociated with the cells.

The assay of low concentrations of iron in the entering medium was difficult in that there were problems of sensitivity, of contamination by other reagents and apparatus, and of the likelihood that the culture was obtaining traces of iron from the glass vessel, the air supply, the potassium hydroxide supply for pH control and so on. Fortunately a continuous culture acts as a bioassay for its own limiting substrate, as Fig. 3 so clearly shows. From Fig. 3 it could be calculated that the iron reaching the cells other than that deliberately added amounted to $0.3 \mu\text{g-atom/l}$, a value in close agree-

Fig. 3. Relationship between cell concentration and added entering iron concentration. The intercept on the abscissa corresponds to the amount of iron obtained by the cells in the absence of any added iron. (Experiment of R. A. Clegg.)

ment with that obtained by extraction, concentration and spectrophotometric assay. Given a simple and sensitive assay for iron entering the culture (i.e. wash, dry and weigh the cells from a few millilitres of culture, then use Fig. 3) it was a simple matter to add ^{59}Fe of high specific radioactivity, and any subsequent cell component could be assayed for non-haem iron on the basis of its radioactivity and spectrophotometrically assayed cytochrome content.

Non-haem iron apart, other measurements of interest in mitochondria from iron-limited *T. utilis* were those of cytochrome content, energy conservation at each of the three sites, sensitivity to various inhibitors, labile sulphide content and e.p.r. spectroscopy. Some of the results are shown in Table 3 and are contrasted with measurements from glycerol-limited cells and their mitochondria.

Not surprisingly, iron-limited growth caused

Table 3. *Properties of mitochondria derived from phenotypically modified T. utilis*For methods see Light *et al.* (1968) and Clegg *et al.* (1969). +, Normal; —, absent; N.T., not tested.

Cell treatment	Property			
	Respiration	Piericidin inhibition	Site I	Signal at $g=1.94$
Glycerol-limited growth	+	+	+	+
Iron-limited growth	+	—	—	—
Iron-recovered cells	+	+	+	+
Iron-recovered cells + cycloheximide	+	—	+	N.T.
Iron- to glycerol-limited transition	+	—	+	—
Sulphate-limited growth	+	—	—	—
Sulphate-recovered cells (low sulphate)	+	+	+	N.T.

Fig. 4. Energy-dependent reduction of NAD(P)⁺ by glycerol 3-phosphate oxidation. Fluorescence excitation at 366nm, emission at 420nm upwards. F mitochondria were from iron-limited *T. utilis*, FR mitochondria from iron-recovered cells. Mitochondrial incubation conditions were as in Light *et al.* (1968). Abbreviations: YM, yeast mitochondria, 0.40mg/ml; G3P, glycerol 3-phosphate, 5mM; FCCP, carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone, 0.1μM; AA, antimycin A, 0.5μM. An upward deflexion represents NAD(P)⁺ reduction. The apparent fluorescence of the suspension was defined as 100%. [From Light (1968).]

marked falls in the mitochondrial contents of cytochromes and non-haem iron. Presumably none of these components was rate-limiting for respiration in the whole mitochondria, since the rates were not greatly changed. The most remarkable finding was the absence of energy conservation coupled to still-competent electron flow between NADH and the cytochromes. The parallel absence of rotenone- and piericidin A-sensitivity, and of a detectable e.p.r. signal at $g=1.94$, supported the idea based on comparative studies, that electron flow through a rotenone-sensitive pathway exhibiting a signal at $g=1.94$ was necessary for energy conservation at site I. As will be shown below, such a conclusion was premature and incorrect.

Fig. 5. Polarographic assay of P/O ratios of *T. utilis* mitochondria. F mitochondria were from iron-limited *T. utilis*, FR mitochondria from iron-recovered cells. Mitochondria incubation conditions were as in Light *et al.* (1968). Abbreviations: YM, yeast mitochondria, 0.40mg/ml; P+M, pyruvate and L-malate, 5mM final concentration; PA, piericidin A, 0.1μM; G3P, glycerol 3-phosphate, 5mM. ADP cycles used 0.092mM-ADP. [From Light (1968).]

Reversal of the effects of iron-limited growth

Had iron-limited growth selected a strain or mutant of *T. utilis* that, like the *Saccharomyces* species, was genetically incompetent to form a functional energy-conservation mechanism at site I? This question was answered by an experiment where iron-limited cells were harvested, washed several times with water, suspended in water and then aerated overnight at 30°C in the presence of a low concentration of iron. The cells were then re-harvested and mitochondria prepared. Both site I energy conservation and piericidin A-sensitivity had returned. Fig. 4 shows fluorimeter tracings of the energy-dependent reduction of mitochondrial NAD by glycerophosphate oxidation, and provides a more striking (though less quantitative) assay of energy conservation at site I than that given by polarographic recordings of oxygen-uptake throughout ADP cycles. The latter are shown in Fig. 5.

Thus the conclusion to be drawn from experiments of this type is that, since site I energy conservation and piericidin A-sensitivity were recoverable in the absence of cell growth, then their initial disappearance was a phenotypic rather than a genotypic phenomenon. Some other properties of the mitochondria from iron-recovered cells are listed in Table 3.

Dissociation of site I energy conservation from piericidin A-sensitivity

Repetition of the iron-recovery experiment just described in the presence of cycloheximide (50 µg/ml), an inhibitor of cytoplasmic rather than of mitochondrial protein synthesis (Kerridge, 1958; Clark-Walker & Linnane, 1966), resulted in cells whose mitochondria lacked sensitivity towards piericidin A but possessed energy conservation at site I (Clegg *et al.* 1969, 1970; Garland, Clegg, Light & Ragan, 1969). From this observation it can be concluded (the comparative data of Table 2 notwithstanding) that electron flow at site I need not pass through a piericidin A- or rotenone-sensitive component for energy conservation to occur. The other but more tentative conclusion is that the piericidin A-sensitive component is a cytoplasmically synthesized protein.

Transition from iron-limited to glycerol-limited growth

Since site I energy conservation and piericidin A-sensitivity were now clearly established as being two distinct and dissociable properties, it seemed possible that manipulation of the iron concentration within the culture vessel might also separate these two properties. There were two ways in which we could set about this. One was to increase the dilution rate of the iron-limited growth conditions, when initially the iron concentration would increase slowly and then much more rapidly as the dilution rate approached μ_{max} . The other way, which we chose, was to increase the concentration of entering iron until growth switched from iron limitation to glycerol limitation. The theoretical behaviour of the iron concentration within the culture would be this: the steady-state iron concentration would remain constant until the glycerol concentration became rate-limiting for growth, whereupon the iron concentration would rise, perhaps sharply, to a new steady-state value that was now a function of the entering iron concentration.

At the start of such an experiment the culture was iron-limited: a considerable amount of glycerol remained unused in the medium, and on in-

creasing the entering iron concentration in a step-wise manner we observed corresponding increases in the steady-state cell concentration that were associated with increases in the utilization of the glycerol carbon source. The apparent K_s value for glycerol as a growth substrate is low. During glycerol-limited growth at a dilution rate of 0.2h^{-1} the concentration of glycerol within the culture was less than 0.1 mM, a value that it is interesting to compare with the K_m of glycerol kinase (EC 2.7.1.30) of *T. utilis*, which is about $6 \times 10^{-5}\text{M}$. As was to be expected, therefore, the glycerol concentration fell to a low concentration (less than 1 mM) as the entering iron concentration was increased in small steps. Rather surprisingly, a further increment of entering iron concentration occurring when the carbon source was at least 99% utilized resulted in a 20–30% increase in the concentration of cells in the culture. The most reasonable explanation was that site I energy conservation had returned (as was in fact the case) with the result that the growth-yield constant y (eqn. 5) had increased (Clegg *et al.* 1970). Piericidin A-sensitivity did not return until the entering iron concentration was still further increased to about 2 µg-atoms/l.

This particular transition would be interesting to investigate in greater detail with the aid of measurements of oxygen uptake and heat output, in the expectation of learning how the presence or absence of one of the three energy-conservation sites affects the overall energy metabolism of the cell.

Sulphate-limited growth of T. utilis

The foregoing experiments seemed to establish quite firmly that there were at least two involvements of non-haem iron in the segment from NADH to the cytochromes: one for piericidin A-sensitivity and one for energy conservation. The majority of non-haem iron proteins contain equal proportions of iron and labile sulphide (Hall & Evans, 1969), and it was therefore pertinent to investigate the effects of sulphate-limited growth. At a dilution rate of 0.2h^{-1} , and entering concentrations of glycerol and sulphate of 130 mM and 100 µM respectively, site I energy conservation and piericidin A-sensitivity were absent. Increasing the sulphate concentration in steps caused a parallel return of the two missing properties until their recovery was complete at an entering sulphate concentration of 300 µM. A sharp transition as observed with the iron-limited system did not occur. Overnight recovery of sulphate-limited cells in the presence of low concentrations (about 50 µM) of sulphate with or without cycloheximide behaved similarly to the corresponding iron-recovery experiments (B. A. Haddock, personal communication).

Table 4. *Effects of growth conditions on mitochondrial flavin and non-haem iron/labile sulphide ratio*

The FMN and FAD contents refer to the acid-extractable species. For methods see Light *et al.* (1968) and Clegg *et al.* (1969, 1970). N.T., Not tested.

Cells	Content in submitochondrial particles (nmol or ng-atoms/mg of protein)				
	FMN	FAD	Non-haem iron	Sulphide	Fe/S ratio
Glycerol-limited (100 μ M-Fe)	0.07	0.2–0.35	8.2	N.T.	N.T.
Glycerol-limited (2 μ M-Fe)	0.07	0.2–0.35	0.75	0.65	1.15
Iron-limited	0.07	0.2–0.35	0.1	0.013	7.7
Iron-recovered	0.07	0.2–0.35	0.36	0.42	0.82
Iron-recovered with cycloheximide	0.07	0.2–0.35	0.17	0.34	0.30

Fig. 6. E.p.r. spectra obtained on addition of NADH to submitochondrial particles from *T. utilis* grown in media of differing iron contents. The media were as described in Clegg *et al.* (1970) except for the following: (a) 0.47 μ g-atom of Fe/l, 130 mm-glycerol, no added MnCl_2 ; (b) 1.2 μ g-atom of Fe/l, 55 mm-glycerol, no added MnCl_2 ; (c) 0.37 μ g-atom of Fe/l, 55 mm-glycerol, no added MnCl_2 . Samples were frozen in 3 mm-internal-diameter quartz tubes 10 min after the addition of 1 μ mol of NADH at 0–4°C. The e.p.r. spectra (9.3 GHz) were obtained at about 98°K and 10 mW power. Instrument settings were identical and protein concentrations were all about 50 mg/ml. The features in the region of $g=2.0$ and above arise largely from traces of cupric ion and free radical in the samples. [From Clegg *et al.* (1969).]

Iron-sulphur proteins and e.p.r. spectroscopy of submitochondrial particles

The non-haem iron of complex systems such as mitochondria is assayed as just that, i.e. the iron unaccountable for as haem. This value cannot readily be equated with the iron of iron-sulphur proteins unless it is equalled by the value for labile sulphide. Table 4 shows that the absolute values of non-haem iron and labile sulphide varied considerably, and only under certain conditions did the

iron/sulphide ratio approach unity. The mitochondrial concentrations of non-haem iron obtained with iron-limited growth were remarkably low, and indicated that over 90% of the non-haem iron of 'normal' mitochondria is functionally redundant. A further interesting point is that there was a barely detectable increase in mitochondrial non-haem iron associated with the reappearance of site I energy conservation. Assuming no redistribution of iron, the maximum amount of a putative iron-sulphur protein needed for site I energy conservation corresponds to 0.07 μ g-atom of Fe/mg of protein.

These low concentrations of non-haem iron seem analytically sound, since they are based on the specific radioactivity of ^{59}Fe in the culture. The ^{59}Fe of harvested cells and their subsequently prepared mitochondria and particles did not exchange with an excess of added non-radioactive iron (R. A. Clegg, personal communication). There is therefore no risk that the assayed concentration of non-haem iron is influenced by the adsorption of iron from apparatus and reagents during the preparative and analytical procedures, as is very possibly the case with the tenfold higher contents of chemically assayed non-haem iron reported by Ohnishi, Schleyer & Chance (1969) for *T. utilis* grown in iron-deficient batch culture.

Ohnishi, in an extension of her comprehensive studies with yeast mitochondria and e.p.r. spectroscopy, has confirmed a number of our observations of the effects of iron deficiency on *T. utilis*, and has concluded that (i) site I phosphorylation parallels piericidin A-sensitivity (Ohnishi & Schleyer, 1969), (ii) the piericidin A-sensitive site and the signal at $g=1.94$ associated with NADH dehydrogenase are not directly related to each other (Ohnishi *et al.* 1969) and (iii) a very small fraction of the signal at $g=1.94$ is closely related to site I phosphorylation (Ohnishi & Chance, 1969). Of these three not entirely compatible conclusions, the first is clearly wrong (see above), the second is

based on a highly dubious correlation and the third is impossible of disproof since a small signal at $g=1.94$ could be obscured by noise or other signals. Fig. 6 shows e.p.r. spectra obtained by Dr C. I. Ragan in collaboration with Dr J. C. Swann and Dr R. C. Bray, and demonstrates that submitochondrial particles from iron-limited *T. utilis* lack detectable signals in the $g=1.90$ region. On increasing the entering iron concentration of the culture to the point where site I phosphorylation but not piericidin A-sensitivity returned, the e.p.r. spectra again failed to show a signal at $g=1.94$, but instead showed a broader signal at $g=1.93$ that was elicited by either NADH or glycerophosphate. Finally, the return of piericidin A-sensitivity at an even higher entering iron concentration was associated with the return of the signal at $g=1.94$ (Clegg *et al.* 1969).

Inhibitor-resistant mutants of T. utilis

Butow & Zeydel (1968) isolated a mutant of *T. utilis* that was relatively resistant to the respiratory inhibitor antimycin A. Mutants with mitochondrial resistance to piericidin A would clearly be of value in further definition of the inhibitor-sensitive site and its properties. Unfortunately *T. utilis* grew normally on solid media containing rotenone or piericidin A at high concentrations and with glycerol as the carbon source. The reasons for this insensitivity have been discussed elsewhere (Garland *et al.* 1969), and were thought to involve the presence of an externally orientated NADH dehydrogenase that catalysed a rotenone-insensitive oxidation of extramitochondrial NADH (Ohnishi *et al.* 1966). Recently Dr M. Fukami has shown in our laboratory that growth of *T. utilis* on acetate represses the external NADH dehydrogenase of mitochondria (Fukami, Light & Garland, 1970). Both respiration and growth of whole cells were highly sensitive to rotenone and piericidin A provided that the carbon source was acetate, and screening for resistant mutants now poses no difficulty (P. A. Light, personal communication).

Novel respiratory pathway in mitochondria from T. utilis

B. A. Haddock (personal communication) has manipulated the conditions of sulphate recovery of sulphate-limited cells to obtain cells whose mitochondria have the following unusual properties: (i) the oxidation of pyruvate and malate is sensitive to piericidin A, and exhibits a P/O ratio approaching 2.0; (ii) cyanide or antimycin A did not inhibit the oxidation of pyruvate or malate, but caused the P/O ratio to fall to approx. 1.0; (iii) the oxidation of ascorbate was cyanide-sensitive. These observa-

tions indicate that a new pathway of respiration was present between the region of cytochrome *b* and oxygen. Submitochondrial particles exhibited normal sensitivity towards antimycin A and cyanide.

Conclusions from studies with iron- and sulphate-limited growth of T. utilis

These studies provide strong evidence in favour of a role for an iron-sulphur protein in energy conservation at site I, and also dispel two incorrect hypotheses concerning the requirements for energy conservation at this site. Whether or not iron deficiency alters the function of a respiratory carrier or some accessory protein is not clear from these studies, since arguments can be advanced in favour of either possibility and critical experiments have not yet been devised. The use of ^{59}Fe in transitions from iron deficiency to iron recovery should, it is hoped, enable us to identify the proteins that are altered in the recovery. Likewise the use of cycloheximide on this recovery, and of mutants, should enable us to make an exact identification of the piericidin A-sensitive component. Finally, there remains the matter of obtaining a reconstitution of piericidin A-sensitivity and of site I energy conservation *in vitro*. Reconstitution might involve isolating and adding to mitochondria a complete iron-sulphur protein. Alternatively, an apoprotein might already be present in the mitochondria, and could conceivably be reconstituted by incubation with sulphide and an iron source. In the case of piericidin A-sensitivity, the results of recovery in the presence of cycloheximide *in vivo* indicated that protein synthesis was involved. In keeping with this, C. I. Ragan (personal communication) has demonstrated that not more than 25–30% restoration of piericidin A-sensitivity can be restored by anaerobic incubation of submitochondrial particles with NADH, sulphide and an iron source. All of these ingredients were needed for the reconstitution, and the experiment provides a striking and quite direct demonstration of the role of an iron-sulphur protein in piericidin A-sensitivity.

The use of biologically determined lesions of the respiratory-chain and energy-conservation mechanisms, both phenotypic and genotypic, would seem to offer a powerful means of probing the complexities of these difficult systems. The genetic approach is being actively pursued in many laboratories, and is particularly appropriate for the *Saccharomyces* species. *T. utilis*, by contrast, is diploid and asexual, and it is fortunate that site I energy conservation, absent from the *Saccharomyces* species, should be capable of phenotypic investigation in the genetically inconvenient *T. utilis*.

The list of applications for growth-conditioned lesions (Table 2) can be greatly extended, and the real value of continuous culture would lie in the controlled production of large quantities of cells that are deficient in some particular component or function. The subsequent recovery of the growth-limiting factor under non-growing conditions may then allow the synthesis or reconstitution of the component to be studied under conditions of minimal interference from other cellular processes. Such usage of a chemostat perhaps illustrates a biochemist's view of continuous culture—it is a means to an end.

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