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Effect of Sulphate-Limited Growth on Mitochondrial Electron Transfer and Energy Conservation between Reduced Nicotinamide–Adenine Dinucleotide and the Cytochromes in *Torulopsis utilis*

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1. Conditions have been established for the sulphate-limited growth of *Torulopsis utilis* in continuous culture. 2. Mitochondria prepared from sulphate-limited cells lack both piericidin A sensitivity and the first energy-conservation site (site 1). Sensitivity to antimycin A or cyanide and the second and third energy-conservation sites were apparently unaffected by sulphate-limited growth. 3. Aerobic incubation for 8h of sulphate-limited cells with a low concentration of sulphate (50μM or less) resulted in the recovery of mitochondrial piericidin A sensitivity and site 1. The use of higher concentrations of sulphate (250μM or more) still resulted in the recovery of mitochondrial piericidin A sensitivity and site 1, but also resulted in the appearance of a non-phosphorylating oxidase, which mediated oxidation of the respiratory chain at about the level of cytochrome *b* in an antimycin A- and cyanide-insensitive manner. Both this alternative route and the conventional normal route of respiration were shown to coexist and to intercommunicate at the level of cytochrome *b*. 4. Low-temperature spectroscopy failed to identify any new respiratory component to explain the alternative route. 5. The apparent affinity of the alternative route for oxygen was similar to that for the conventional route through cytochrome oxidase, namely half-maximal activity at 0.1μM-oxygen or less. 6. The non-haem iron concentration of submitochondrial particles was unaffected by sulphate limitation, whereas the acid-labile sulphide concentration was lowered tenfold. Marked increases (between four- and 30-fold) in the acid-labile sulphide concentration of submitochondrial particles were observed in sulphate-limited cells after aerobic incubation with various concentrations of sulphate. The lowest increase (fourfold) was observed without added sulphate, the highest (30-fold) with 1.0mM added sulphate. 7. The ratio of non-haem iron to acid-labile sulphide in submitochondrial particles varied with different growth conditions from a maximum of 15.0 to a minimum of 0.72. It is suggested that analytical measurements of non-haem iron are an inadequate guide to the concentration of iron–sulphur protein in complex systems. 8. The effects of sulphate-limited growth on site 1 and piericidin sensitivity are interpreted to indicate a role for iron–sulphur protein in these properties. 9. The aerobic incubation of sulphate-limited cells with cycloheximide resulted in the recovery by mitochondria of site 1 but not of piericidin sensitivity. 10. The appearance of the alternative route for cyanide- and antimycin-A (but not piericidin A-) insensitive respiration on incubating sulphate-limited cells with sulphate concentrations higher than 250μM indicates that the alternative route involves an iron–sulphur protein.

Iron-limited growth of *Torulopsis utilis* has been found to cause functional alterations of the respiratory chain between NADH and the cytochromes (Light, Ragan, Clegg & Garland, 1968; Ohnishi, Schleyer & Chance, 1969; Light & Garland, 1971; Clegg & Garland, 1971). It was concluded that these alterations rose from changes in mitochondrial non-haem iron protein(s) (Light *et al.* 1968). Since the majority of non-haem iron proteins are iron–sulphur proteins, containing equivalent amounts of non-haem iron and labile sulphide

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(Hall & Evans, 1969), it was decided to study the effect of sulphate-limited growth of *T. utilis* on mitochondria. Preliminary reports of part of this work have been published (Clegg *et al.* 1969; Garland *et al.* 1970; Garland, 1970). In the present paper we also describe the properties of mitochondria from *T. utilis* cells that had been incubated with sulphate under non-growing conditions after previous sulphate-limited growth, and the emergence of a new mitochondrial respiratory pathway which by-passed cytochromes *c* and *a*, the second and third energy-conservation sites, and the sites of respiratory inhibition by antimycin A and cyanide.

MATERIALS AND METHODS

The apparatus for continuous culture of *T. utilis*, the preparation of mitochondria, and techniques for measuring glycerol, the dry weight of cells, protein concentration, oxygen uptake and the fluorescence of mitochondrial flavoproteins and NADH have been reported (Light & Garland, 1971). The medium used in all mitochondrial incubations for the measurement of oxygen uptake and fluorimetric changes and for the measurement of oxygen uptake by electron-transport particles was that described by Ohnishi, Kawaguchi & Hagihara (1966) and contained: D-sorbitol (0.65M); EDTA (0.1mM); KCl (10mM); potassium phosphate buffer (10 mM-KH_2PO_4 adjusted to pH6.5 with KOH); and tris–maleate buffer (20mM-maleic acid adjusted to pH6.5 with tris). Electron-transport particles were prepared by mechanical breakage of whole cells as described by Clegg & Garland (1971) except that tris buffers were adjusted to pH7.4 with HCl rather than H_2SO_4. The concentrations of cytochromes, acid-labile sulphide, non-haem iron and acid-extractable flavin in electron-transport particles were measured as described by Clegg & Garland (1971).

The culture medium used for obtaining sulphate-limited growth contained: glycerol (133mM); NaH_2PO_4 (12.8mM); Na_2HPO_4 (2.8mM); NH_4Cl (75mM); KCl (10mM); citric acid (1.0mM); $MgCl_2$ (1.25mM); $CaCl_2$ (100μM); $CoCl_2$ (10μM); $CuCl_2$ (5μM); H_3BO_3 (5μM); Na_2MoO_4 (10μM); 4μg of biotin/l; 0.24ml of antifoam GF (Midland Silicones Ltd., Barry, Glam., U.K.)/l; and, except where indicated otherwise, K_2SO_4 (100μM). Water twice-distilled from all-glass apparatus was used throughout. The working volume of the culture vessel was 650ml, and the dilution rate 0.17h^{-1}.

Potassium [^{35}S]sulphate, specific radioactivity about 10mCi/mmol, was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and, when used, was added to the growth medium to give a final concentration of about 3μCi/litre. The final specific radioactivity was not constant but varied with the sulphate concentration of the medium. Samples were prepared for radioactivity measurements by diluting the sample (either growth medium, or chemostat effluent freed of cells by centrifugation) to 0.5ml with water, and then adding 5.0ml of a solution of naphthalene (6%, w/v), 2,5-diphenyloxazole (0.4%, w/v) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.02%, w/v) in 1,4-dioxan. Radioactivity was measured in a Nuclear–Chicago three-channel liquid-scintillation counter at a constant, but undetermined, efficiency for all samples, and the observed count rates were not converted into Ci but used as such for subsequent calculations. Since the experiments with [^{35}S]sulphate were designed to measure the uptake by cells of sulphate from the growth medium, it was sufficient to measure the radioactivity of the growth medium (as c.p.m./nmol of sulphate) and the radioactivity of the chemostat effluent after removal of cells. All samples (growth medium and effluent) were measured for radioactivity at virtually the same time, thereby correcting for the half-life (87.2 days) of ^{35}S.

Dual-wavelength spectrophotometric measurements were made with an instrument constructed according to the principles of Chance & Legallais (1951). The spectral band width was approximately 1nm and the optical cuvette had a light-path of 1cm. Wavelength-scanning spectrophotometric measurements were made with an instrument constructed according to the principles of Yang & Legallais (1954). The spectral band width was 1nm, and optical cuvettes of 1cm light-path were used. The scanning speed was approx. 5nm/s and the time-constant of the measuring circuit was set at 0.1s.

RESULTS

Conditions for sulphate-limited growth. At the outset of this work it was necessary to establish culture conditions where growth was limited by the supply of sulphate rather than any other nutrient. In preliminary experiments with batch culture, the cell yield after 36h growth was independent of the presence or absence of added sulphate (1mM). This result probably arose from an adequate sulphur source being introduced into the culture with the inoculum, and the use of batch culture was not further investigated.

Fig. 1 shows the results of an experiment in which the concentration of sulphate entering the culture was increased incrementally every 5 days. Initially the culture was grown in the vessel for 48h before the flow of growth medium was started. In the absence of added sulphate and at a dilution rate of 0.17h^{-1}, washed-out of the culture occurred. Measurements of the cell yield (as dry wt.) and glycerol in the chemostat effluent, and their dependence on the entering sulphate concentration, demonstrated that sulphate-limited growth was achieved at entering sulphate concentrations of less than 160μM (Fig. 1).

The cell density and glycerol uptake showed a linear dependence on the entering sulphate concentration from 20μM to at least 120μM. Extrapolation of these lines to their respective origins show that even in the absence of added sulphate, the growth medium contained approx. 20μM-sulphate or its metabolic equivalent (Fig. 1). This is consistent with the known contamination calculated from reagent specifications. Somewhat surprisingly, the uptake of sulphate by the culture exhibited a linear dependence on the entering sul-

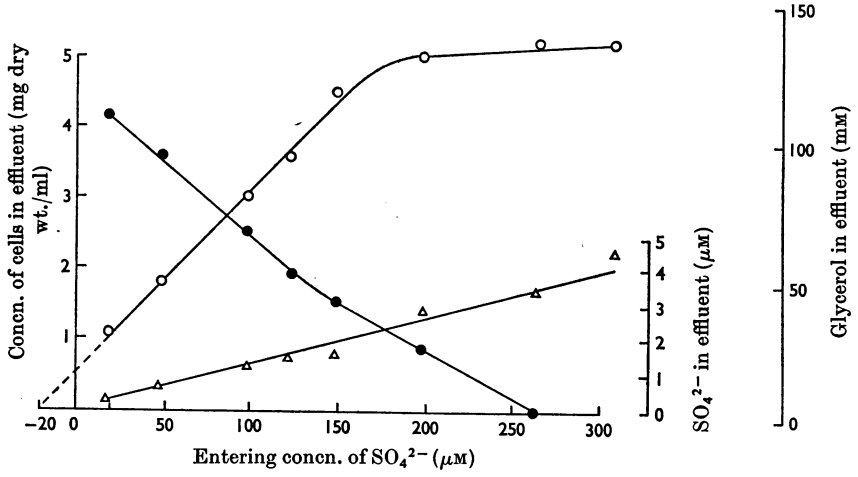


Fig. 1. Behaviour of *T. utilis* in continuous culture at various entering concentrations of sulphate. The assays were carried out as described in the Materials and Methods section. ○, Dry wt. of cells in effluent; △, sulphate (assayed as ^{35}S) in effluent; ●, glycerol in effluent. Each point on the graphs corresponds to measurements made on samples collected 5 days after an increment in the entering concentration of sulphate.

Table 1. *Cytochrome contents of glycerol- and sulphate-limited cells and their mitochondria*

Spectrophotometric measurements of cytochrome concentrations were made as described by Light & Garland (1971).

	Whole cells (nmol/g dry wt.)		Isolated mitochondria (nmol/mg of protein)	
	Glycerol-limited	Sulphate-limited	Glycerol-limited	Sulphate-limited
Cytochrome $a+a_3$	34	20	0.10	0.09
Cytochrome b	25	15	0.13	0.11
Cytochrome $c+c_1$	63	25	0.11	0.10

phate concentration even when growth was glycerol-limited. Between 98 and 99% of the sulphate entering the culture was incorporated into cells and the chemical identity of the ^{35}S remaining outside the cells is not known. Maw (1963) has suggested that part of the sulphur taken up by yeast cells during growth (<5%) is released back into the medium in a non-utilizable organic form; however, if it were sulphate, then the concentration of sulphate in the culture required for half-maximal growth under these conditions would be about 1 μM. A further unexpected feature of the experiment of Fig. 1 is the manner in which the utilization of glycerol was incomplete at a stage when the cell yield was independent of the entering sulphate concentration.

Properties of whole cells. Sulphate-limited cells were found to have the same shape as glycerol-limited cells when examined with a light-microscope, and the alteration of shape to a rod-like form caused by iron-limited growth did not occur with sulphate limitation (Light & Garland, 1971). Measurements of the concentrations of cytochromes in sulphate-limited cells and their mitochondria are shown in Table 1, and are compared with measurements made on glycerol-limited cells and mitochondria. The concentrations of cytochromes b, $c+c_1$ and $a+a_3$ in the mitochondria are the same for both glycerol and sulphate limitation, whereas the concentrations of these cytochromes in the whole cells are lowered (Table 1). On the basis of the measurements for cytochrome $a+a_3$ it can be calculated that the concentration of mitochondria within the cells, expressed as mg of mitochondrial protein/g dry wt., is decreased from 340 in glycerol limitation to 220 in sulphate limitation.

Properties of mitochondria from sulphate-limited cells. In initial experiments it became apparent that the oxidation of NAD-linked substrates by mitochondria prepared from cells grown at an entering sulphate concentration of 150 μM or less was insensitive to piericidin A, whereas at an entering

sulphate concentration of 200 μM of more, piericidin A sensitivity was similar to that observed with mitochondria from glycerol-limited cells (Light *et al.* 1968; Light & Garland, 1971). All subsequent sulphate-limited cultures were made with an entering sulphate concentration of 100 μM.

Fig. 2 shows polarographic recordings of oxygen uptake by mitochondria from sulphate-limited cells, and Table 2 lists the results of numerous such experiments with alternative substrates. The results in Table 2 show that the P/O ratios for the mitochondrial oxidation of 2-oxoglutarate, pyruvate+L-malate, added NADH, and glycerol 3-phosphate are all about 2.0. It can be concluded, therefore, that mitochondria prepared from sulphate-limited cells lack not only the first energy-conservation site (as assayed polarographically) but also the substrate-level phosphorylation normally associated with 2-oxoglutarate oxidation. However, as the respiratory rate with this substrate is so low, this polarographic assay technique may not be sensitive enough to detect such substrate-level phosphorylation.

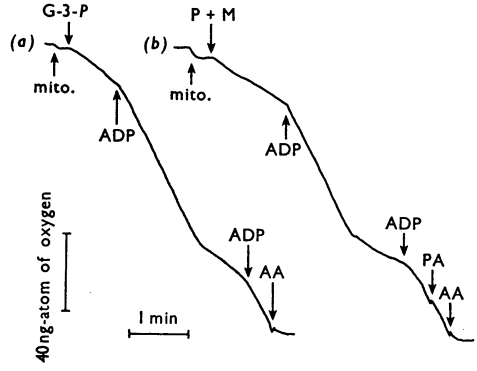


Fig. 2. Polarographic recordings of oxygen uptake by mitochondria from sulphate-limited cells. The experiments were carried out in a water-jacketed chamber at 30°C. Initially, 0.51 mg of mitochondrial protein (mito.) was added to 3.0 ml of air-saturated incubation medium. Further additions as shown in the figure were: DL-glycerol 3-phosphate (G-3-*P*, 15 μmol); ADP (0.3 μmol); antimycin A (AA, 0.5 nmol); pyruvate+L-malate (P+M, 15 μmol of each); and piericidin A (PA, 0.1 nmol). Two experiments (*a* and *b*) are shown.

A further notable feature of these mitochondria was the failure of piericidin A (200 pmol/mg of mitochondrial protein) or rotenone (5 μM) to inhibit the oxidation of any of these substrates. Antimycin A (0.2 μg/ml) or cyanide (3.3 mM) inhibited respiration by at least 97%.

Reversal of electron transport at site 1. The energy-dependent reduction of intra-mitochondrial NAD(P) and flavoprotein of a comparably low oxidoreduction potential (about −320 mV at pH 7.0) by glycerol 3-phosphate was used as a qualitative assay for energy conservation at site 1 in mitochondria from glycerol- and iron-limited cells (Light & Garland, 1971), and the results obtained with this assay were in agreement with those obtained with the polarographic method of Chance & Williams (1956). Fig. 3 shows recordings of fluorimetric measurements made on mitochondria from sulphate-limited cells, and these show that the addition of glycerol 3-phosphate to respiring mitochondria did not cause any detectable reduction of NAD(P) or fluorescent flavoprotein. These observations are therefore in agreement with the conclusion drawn from polarographic measurements of P/O ratios, namely that energy conservation at site 1 was absent from mitochondria prepared from sulphate-limited cells.

Effects of incubating sulphate-limited cells with sulphate. In view of the fact that the loss of site 1

Table 2. *Respiration rates, respiratory control ratios and P/O ratios for mitochondria from sulphate-limited cells (S-cells)*

The experimental conditions were as described in the legend of Fig. 2, except that the only additions apart from mitochondria and bovine plasma albumin were the substrate under test and ADP (0.2 mM). The respiration rates listed are the rates observed in the presence of ADP and substrate and are uncorrected for any contribution by endogenous substrates. Respiratory-control ratios and P/O ratios were calculated as described by Light & Garland (1971). The experiments were repeated with several different preparations of mitochondria, and the results are expressed as the mean ± S.E.M., with the number of observations in parentheses.

Substrate	Respiration rate (ng-atoms of oxygen/min per mg of protein)	Respiratory-control ratio	P/O ratio
Pyruvate (5 mM)+L-malate (5 mM)	134±33 (6)	2.6±0.3 (3)	1.9±0.1 (6)
DL-Glycerol 3-phosphate (5 mM)	196±81 (6)	2.3±0.3 (6)	1.9±0.2 (6)
2-Oxoglutarate (5 mM)	38± 7 (4)	2.1±0.3 (4)	2.1±0.2 (4)
NADH (0.5 mM)	195±14 (2)	2.4±0.2 (2)	1.8±0.1 (2)
Endogenous	7± 4 (6)	1.0 (6)	—

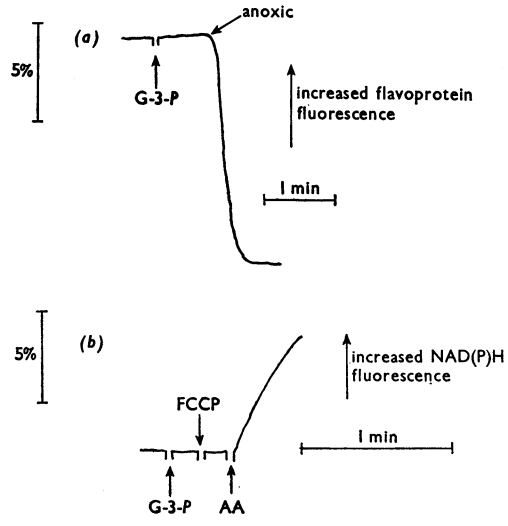


Fig. 3. Fluorimetric recordings of mitochondrial NAD(P) and flavoprotein. Fluorescence is expressed as a percentage of the total fluorescence of the sample, and in both the upper trace (*a*) (flavoprotein) and lower trace (*b*) [NAD(P)], an upward deflexion corresponds to reduction of the carrier. The primary and secondary filters and fluorimeter were as described by Light & Garland (1971) and the experiments were carried out at 20°C in 3.0 ml of an air-saturated incubation medium containing 6.1 mg of mitochondrial protein (sulphate-limited) and 9 mg of defatted bovine plasma albumin. In each experiment the mitochondria were incubated for 1 min before the addition of DL-glycerol 3-phosphate (G-3-*P*, 15 μmol). During the 1 min preincubation mitochondrial NAD(P) and flavoprotein fluorescence settled to their maximally oxidized condition. Other additions were carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 0.75 nmol; Heytler & Pritchard, 1962) and antimycin A (AA, 0.15 nmol).

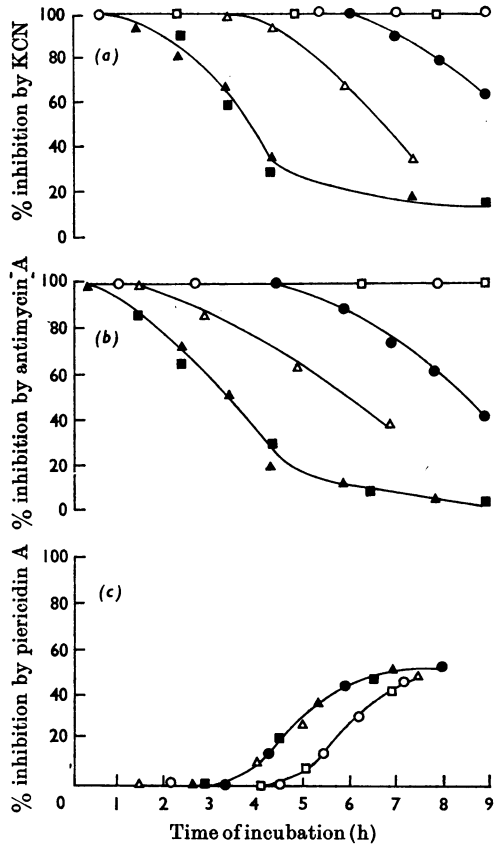


Fig. 4. Effect of respiratory inhibitors on the endogenous respiration rates of S-cells incubated with various concentrations of potassium sulphate. Harvested S-cells were suspended in water to a final concentration of 0.1 g wet wt./ml and volume of 250 ml containing antifoam FG (0.1 mg/l) and K_2SO_4 at various concentrations: ○, no added sulphate; □, 50 μM; ●, 250 μM; △, 500 μM; ■, 1 mM; and ▲, 5 mM. The resultant suspensions were incubated aerobically at 30°C as described by Clegg *et al.* (1969). At known time-intervals, a sample (5 ml) was removed from the incubation flasks, spun in a MSE bench centrifuge and the resultant supernatant discarded. The pellet was resuspended in water (0.5 ml), and the endogenous respiration rate of samples (0.2 ml) assayed by using an oxygen electrode. These measurements were performed in a water-jacketed chamber at 30°C, and the apparatus was similar to that described by Chappell (1964). The degree of sensitivity of the endogenous respiration rate to various inhibitors was tested: (*a*) KCN (2 mM); (*b*), antimycin A (5 μM); and (*c*), piericidin A (1 μM). The results are expressed as the percentage inhibition of the endogenous respiration rate at a given time after the start of the incubation and were calculated as described in the legend to Table 4. During the time-course of the incubation, the endogenous respiration rate decreased to 60–70% of the original rate; no correction for any such decrease was made in calculating values for the percentage inhibition.

energy conservation and piericidin A sensitivity from iron-limited *T. utilis* could be restored by incubation of the cells with low concentrations of iron salts under conditions when cell growth could not occur except cryptically (Light *et al.* 1968), it was decided to attempt a similar recovery with sulphate-limited cells and sulphate. For convenience, it was simplest to carry out initial studies of the effects of piericidin A on whole-cell respiration during exposure of sulphate-limited cells to various concentrations of sulphate. These studies are summarized in Fig. 4, and also include the effects of antimycin A and cyanide on cell respiration.

The following points are established by the results in Fig. 4. (1) Piericidin A sensitivity of whole-cell respiration was restored to a maximum of 55% in 8 h of incubation with 0 or 50 μM-sulphate added and to a similar maximum in 6 h with 1 or 5 mM-sulphate.

(2) Antimycin A sensitivity of whole-cell respiration was 100%, and was unaffected by incubation with 0 or 50 μM-added sulphate, but fell to low values as the concentration of added sulphate was increased through 0.25, 0.5, 1.0 and 5.0mM. For instance, whole-cell respiration was only 8% inhibited by antimycin A after 8h incubation with 1 or 5mM-sulphate. (3) Cyanide sensitivity of whole-cell respiration behaved very similarly to antimycin A sensitivity. (4) The dependence of the recovery of piericidin A sensitivity on both time and the concentration of added sulphate differed markedly from that of the loss of antimycin A or cyanide sensitivity. The recovery of piericidin A sensitivity was not unexpected, but the other effects were. It seemed that at least two independent modifications had occurred, one the recovery of piericidin A sensitivity and the other the loss of antimycin A and of cyanide sensitivity. Since piericidin A sensitivity could return in the absence of added sulphate whereas the loss of antimycin A and of cyanide sensitivity required at least 0.25mM added sulphate, two conditions only were used for all subsequent experiments; either no added sulphate, when the cells were described as SR_0, or 1mM added sulphate, giving cells described as SR_1. The effects of 16h incubation were the same as 8h incubation under these conditions, and for convenience the 16h incubation was subsequently used. The incubation of S-cells (i.e. sulphate-limited cells) with various concentrations of sulphate was also carried out in the presence of cycloheximide (0.1mg/ml), and the resultant cells are referred to as SR_0(cylcoheximide) and SR_1(cycloheximide). Neither of these latter two cell types respired with endogenous substrates or added ethanol (5mM) or D-glucose (5mM), and it was not possible to test their sensitivity towards respiratory inhibitors.

Properties of mitochondria from SR_0- and SR_0-(cycloheximide)-cells. Table 3 summarizes the respiratory rates, respiratory-control ratios and P/O ratios of mitochondria from SR_0- and SR_0(cycloheximide)-cells, and Table 4 lists the sensitivities of these mitochondria towards various respiratory inhibitors. The results in Tables 3 and 4 show that incubation of S-cells without added sulphate resulted in the return of both piericidin A sensitivity and of energy-conservation at site 1. Further, if cycloheximide was included in the cell incubation, then energy conservation at site 1 returned whereas piericidin A sensitivity did not. Additional evidence for the return of energy conservation at site 1 was sought by measuring the reduction of intramitochondrial NAD(P) and low-potential flavoprotein by the oxidation of glycerol 3-phosphate in state 4 (Chance & Williams, 1956; Light *et al.* 1968; Light & Garland, 1971). The results of such experiments are shown in Fig. 5, and they show that the addition

Table 3. *Respiration rates, respiratory-control ratios and P/O ratios for mitochondria from SR_0 and SR_0(cycloheximide)-cells*

The experiments were performed as described in the legend to Table 2.

Substrate	Mitochondria from SR_0-cells			Mitochondria from SR_0(cycloheximide)-cells		
	Respiration rate (ng-atoms of oxygen/min per mg of protein)	Respiratory-control ratio	P/O ratio	Respiration rate (ng-atoms of oxygen/min per mg of protein)	Respiratory-control ratio	P/O ratio
Pyruvate (5mM)+L-malate (5mM)	234 ± 26 (4)	3.4 ± 0.4 (4)	2.6 ± 0.2 (4)	220 ± 30 (2)	2.5 ± 0.3 (2)	2.7 ± 0.1 (2)
DL-Glycerol 3-phosphate (5mM)	349 ± 52 (4)	2.7 ± 0.3 (4)	1.9 ± 0.2 (4)	283 ± 37 (2)	2.7 ± 0.3 (2)	1.7 ± 0.1 (2)
2-Oxoglutarate (5mM)	134 ± 31 (4)	2.9 ± 0.3 (4)	2.9 ± 0.4 (4)	89 ± 11 (2)	2.2 ± 0.2 (2)	2.9 ± 0.3 (2)
NADH (0.5mM)	340 ± 25 (2)	2.0 ± 0.3 (2)	1.9 ± 0.2 (2)	276 ± 31 (2)	2.0 ± 0.2 (2)	1.8 ± 0.2 (2)
Endogenous	107 ± 34 (4)	2.3 ± 0.5 (4)	2.8 ± 0.5 (4)	47 ± 23 (2)	1.0	—

Table 4. *Effects of respiratory inhibitors on the oxygen uptake by mitochondria from SR_0- and SR_0(cycloheximide)-cells*

The experiments were performed essentially as described in Fig. 2, except that the concentration of ADP was 0.4mM and the order of additions was mitochondria, substrate, ADP and finally inhibitor during the ADP-stimulated phase of respiration. The final concentrations of inhibitors were piericidin A, 0.2nmol/mg of mitochondrial protein; antimycin A, 2nmol/mg of mitochondrial protein; KCN, 2.0mM. Percentage inhibition was calculated as (oxygen uptake rate before inhibitor−rate after inhibitor)×100/rate before inhibitor. These results were obtained from at least four separate preparations of mitochondria.

	Inhibition of respiration (%)					
	Mitochondria from SR_0-cells			Mitochondria from SR_0(cycloheximide)-cells		
Substrate	Piericidin A	Antimycin A	KCN	Piericidin A	Antimycin A	KCN
Pyruvate (5mM)+L-malate (5mM)	>90	>90	100	<10	>90	100
DL-Glycerol 3-phosphate (5mM)	<5	>90	100	<5	>90	100
NADH (0.5mM)	<5	>90	100	<5	>90	100

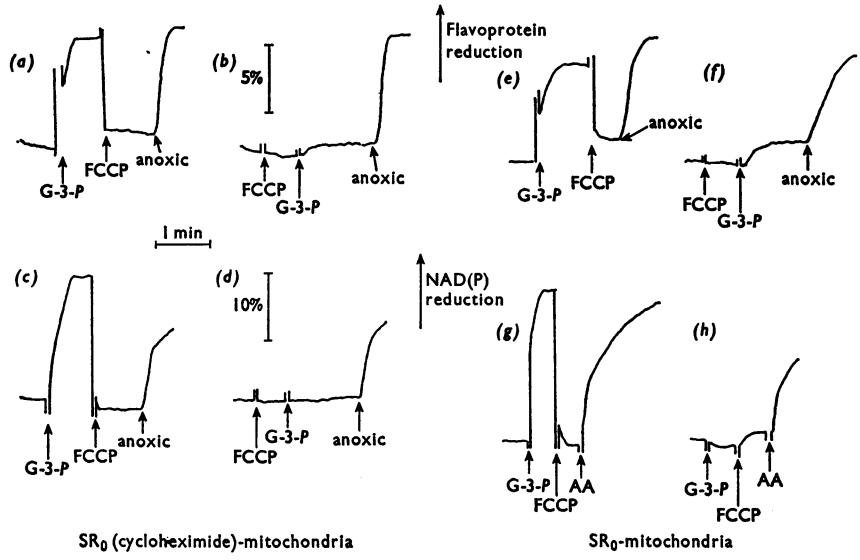


Fig. 5. Fluorimetric recordings of mitochondrial NAD(P) and flavoprotein observed with SR_0- and SR_0-(cycloheximide)-mitochondria. Fluorescence is expressed as a percentage of the total fluorescence of the sample, and in both the upper traces (*a*), (*b*), (*e*) and (*f*) (flavoprotein) and lower traces (*c*), (*d*), (*g*) and (*h*) [NAD(P)], an upward deflexion corresponds to reduction of the carrier. The concentration of mitochondrial protein in the reaction cuvette was 1.2mg/ml [SR_0(cycloheximide)-mitochondria] in traces (*a*), (*b*), (*c*) and (*d*) or 1.3mg/ml (SR_0-mitochondria) in traces (*e*), (*f*), (*g*) and (*h*). All other conditions and abbreviations were as described in the legend to Fig. 3.

of glycerol 3-phosphate to mitochondria from SR_0- and SR_0(cycloheximide)-cells causes a rapid and extensive reduction of mitochondrial NAD(P) and low-potential flavoprotein that is abolished by the uncoupling agent carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Heytler & Pritchard, 1962).

Properties of mitochondria from SR_1-cells. These mitochondria were unusual in that their respiration with all substrates (except the combination of ascorbate with *NNN'N'*-tetramethylphenylenediamine) was relatively insensitive to either antimycin (2nmol/mg of mitochondrial protein) or potassium cyanide (2mM). Fig. 6 presents polarographic recordings of oxygen uptake by mitochondria from SR_1-cells, and Table 5 lists the respiratory rates,

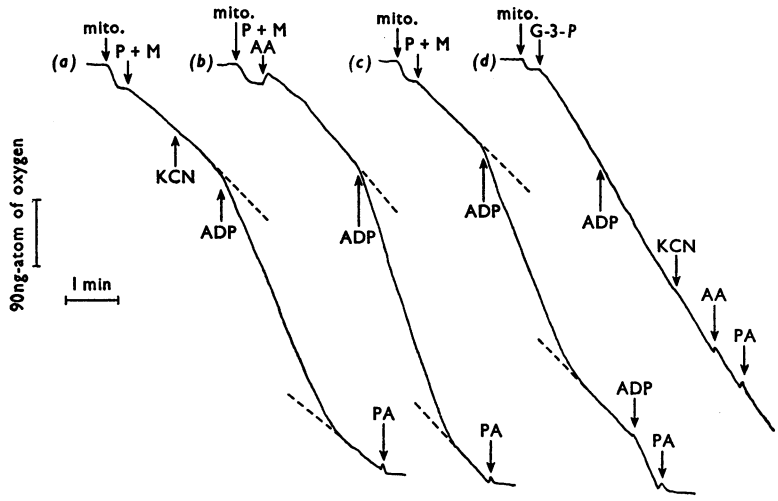


Fig. 6. Polarographic recordings of oxygen uptake by mitochondria from SR_1-cells. The experimental conditions were as described for Fig. 1, and the additions to a 3.0 ml incubation medium containing 3.0 mg of defatted bovine plasma albumin were mitochondria (mito., 1.08 mg of protein), pyruvate+L-malate (P+M, 15 μmol of each), KCN (15 μmol), ADP (0.4 μmol), antimycin A (AA, 15 nmol), piericidin A (PA, 0.6 nmol) and DL-glycerol 3-phosphate (G-3-*P*, 15 μmol). The P/O ratios for the cycles of ADP-stimulated respiration in traces (*a*), (*b*) and (*c*) were 1.2, 1.1 and 1.4 respectively. Traces (*a*), (*b*), (*c*) and (*d*) are separate experiments.

respiratory-control ratios, P/O ratios and inhibitor sensitivities.

The results in Fig. 6 and Table 5 indicate that, although the segment of the respiratory chain from intramitochondrial NADH to the level of cytochrome *b* was normal, in that both piericidin A sensitivity and site 1 energy conservation were present, the remainder of the respiratory chain (between the antimycin A-sensitive site and oxygen) was probably being by-passed with a non-phosphorylating route of electron transport. This we will call the 'alternative route'. However, this simple conclusion must be qualified to take account of the fact that the respiration of pyruvate+malate or of glycerol 3-phosphate could be inhibited by up to 20% (according to the mitochondrial preparation) with antimycin A or cyanide (Table 5). Scheme 1 illustrates the possible pathways of electron transfer and sites of energy conservation in mitochondria from SR_1-cells, and further evidence described below demonstrated that, in the absence of added respiratory inhibitors, both the normal respiratory chain and the alternative route were functional.

Effects of respiratory inhibitors on the P/O ratios of mitochondria from SR_1-cells. Table 6 lists the respiratory rates, respiratory-control ratios and P/O ratios of mitochondria from SR_1-cells in the presence and absence of antimycin A and cyanide. The first point to emerge from these results is that the P/O ratio in the absence of respiratory inhibitors is significantly greater than unity. Secondly, the addition of antimycin A or cyanide caused the P/O ratio to fall to a value (1.1) that was not significantly different from unity. These results, though perhaps not absolutely convincing due to the experimental errors, do indicate that, in the absence of the respiratory inhibitors, phosphorylation of ADP was occurring at more than one energy-conservation site, although most of the ADP phosphorylation presumably occurred at site 1.

Energy-dependent reduction of NAD(P) by glycerol 3-phosphate oxidation in mitochondria from SR_1-cells. The reduction of NAD(P) by glycerol 3-phosphate oxidation requires not only an intact energy-conservation mechanism at site 1 but also the conservation of energy at sites 2 and/or 3 to reverse electron flow from the cytochromes to NAD. Fig. 7 shows that the addition of glycerol 3-phosphate to mitochondria (from SR_1-cells) in the absence of ADP caused a rapid and extensive reduction of NAD(P) that was abolished by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. From this it can be concluded that in mitochondria from SR_1-cells energy conservation occurred at not only site 1 but also at a site between glycerol 3-phosphate and oxygen.

Steady-state reduction of cytochromes in mitochondria from SR_1-cells. Fig. 8 shows difference spectra at room temperature for the cytochrome α-band region for mitochondria from SR_1-cells. The

Table 5. *Respiration rates, respiratory-control ratios, P/O ratios and inhibitor sensitivities of mitochondria from SR_1-cells*

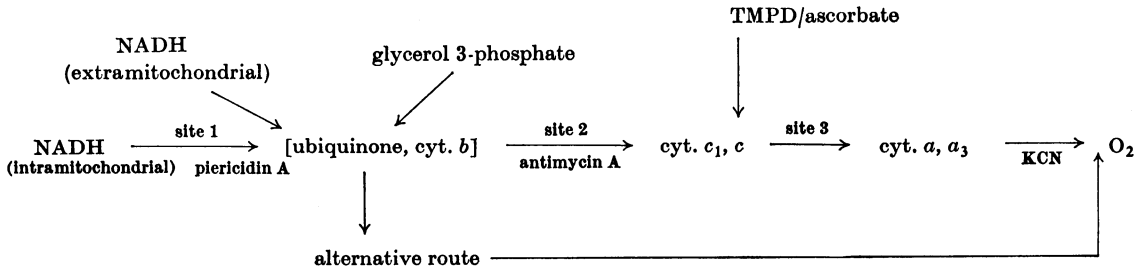
The experimental conditions were as described in Tables 2 and 4 and in Fig. 6. N.T., not tested.

Substrate	Respiration rate (ng-atoms of oxygen/min per mg of protein)	Respiratory-control ratio	P/O ratio	Inhibition of respiration (%)		
				Piericidin A	Antimycin A	KCN
Pyruvate (5 mM)+L-malate (5 mM)	176±60 (8)	2.7±0.4 (8)	1.4±0.2 (8)	92±5 (8)	9±6 (8)	13±8 (8)
DL-Glycerol 3-phosphate (5 mM)	155±25 (6)	1.0 (6)	—	3±2 (6)	10±5 (6)	12±9 (6)
2-Oxoglutarate (5 mM)	89±28 (4)	2.7±0.4 (4)	1.3±0.2 (4)	91±6 (4)	9±6 (4)	11±7 (4)
NADH (0.5 mM)	178±23 (3)	1.0 (3)	—	33±6 (3)	N.T.	N.T.
NN'N'-Tetramethylphenylene-diamine (50 μM)+L-ascorbate (5 mM)	128±21 (5)	1.0 (5)	—	N.T.	N.T.	71±8 (5)
Endogenous	40±11 (6)	1.0 (6)	—	N.T.	N.T.	N.T.

most notable feature is that the transition from state 4 to state 3 with pyruvate+malate as the substrate is associated with a marked reduction of cytochrome *a* measured at 605–630 nm. For instance, if it were assumed that cytochrome *a* were completely oxidized in state 2 (and we do not think that it is in these mitochondria: see below), and completely reduced in state 5, then the percentage reduction of cytochrome *a* shown in Fig. 8 is 40% in state 3 when stimulated with ADP and 58% when stimulated with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. These are remarkably high state 3 reductions of cytochrome *a* when compared with those in mitochondria from S-cells (<5%) or rat liver (<5%; Chance & Williams, 1956), and they lead us to conclude that the cytochrome oxidase of mitochondria from SR_1-cells is partially inhibited even in the absence of experimentally added inhibitors.

It could be predicted on the basis of Scheme 1 that an energy-dependent electron flow from cytochrome *c* to cytochrome *b* (and thence to oxygen via the alternative route) could result in the oxidation of cytochrome *c* in the presence of cyanide. The dual-wavelength spectrophotometric measurements shown in Fig. 9 demonstrate the validity of this prediction. The first experiment (trace *a*) of Fig. 9 shows that, as expected, the addition of cyanide to mitochondria from SR_1-cells in state 2 resulted in the reduction of cytochrome *c* as measured at 550–540 nm. The addition of pyruvate+malate then caused a marked reoxidation of cytochrome *c*, an observation that is not readily explained except in terms of Scheme 1, where energy generated at site 1 is used to drive the energy-dependent reoxidation of cytochrome *c* by cytochrome *b* and the alternative route. Further observations consistent with this interpretation were that the reoxidation of cytochrome *c* was reversed by anoxia (trace *a*) or antimycin A (trace *b*), and that in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone the addition of pyruvate+malate caused a reduction rather than oxidation of cytochrome *c* (trace *c*). Table 7 lists the steady-state reduction of cytochrome *c* in S- and SR_1-mitochondria under a variety of conditions, and extends the observations of Fig. 9 on the energy-dependent reoxidation of cytochrome *c* by the alternative route.

Low-temperature spectroscopy of mitochondria from S- and SR_1-cells. Fig. 10 shows state 5 minus state 2 (i.e. reduced-minus-oxidized) difference spectra at 77°K for G-, S- and SR_1-mitochondria. In each case the α-peaks at 600 nm (cytochrome $a+a_3$), 559 nm (cytochrome *b*), 554 nm (cytochrome c_1) and 547 nm (cytochrome *c*) are well characterized, as are the γ-bands at 446 nm (cytochrome *a*) and 429 nm (cytochrome *b*). Apart from the apparent fall in the concentration of cytochrome $a+a_3$ in the SR_1-mitochondria (which we attribute to a high reduction of



Scheme 1. Normal and alternative routes of electron transport in mitochondria from SR_1-cells. Abbreviations: TMPD, *NNN'N'*-tetramethylphenylenediamine; cyt. *b*, c_1, *c*, *a* and a_3, the respective cytochromes.

Table 6. *Respiration rates, respiratory-control ratios and P/O ratios observed with SR_1-mitochondria oxidizing pyruvate* + L-*malate in the presence of respiratory inhibitors*

The experimental conditions were as indicated in the legend to Table 5. The order of additions was mitochondria, pyruvate (5 mM) + L-malate (5 mM), inhibitor and ADP (0.2 mM).

Inhibitor	Respiration rate (ng-atoms of oxygen/min per mg of protein)	Respiratory-control ratio	P/O ratio
None	176 ± 60 (8)	2.7 ± 0.4 (8)	1.4 ± 0.2 (8)
Antimycin A (5 μM)	159 ± 57 (4)	2.7 ± 0.3 (4)	1.1 ± 0.1 (4)
Cyanide (5 mM)	156 ± 52 (6)	2.8 ± 0.3 (6)	1.1 ± 0.2 (6)
Antimycin A (5 μM) + cyanide (5 mM)	163 ± 55 (5)	2.7 ± 0.3 (5)	1.1 ± 0.1 (5)

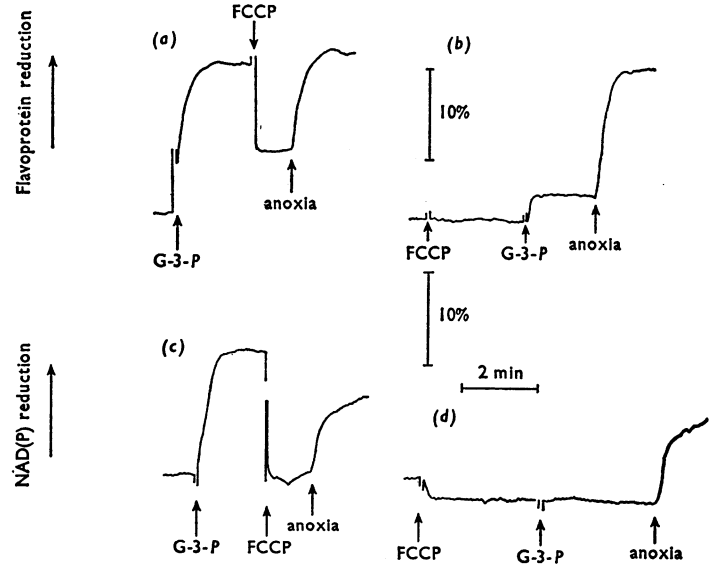


Fig. 7. Fluorimetric recordings of mitochondrial NAD(P) and flavoprotein observed with SR_1-mitochondria. Fluorescence is expressed as a percentage of the total fluorescence of the sample, and in both the upper traces (flavoprotein) and lower traces [NAD(P)], an upward deflexion corresponds to reduction of the carriers. The concentration of SR_1-mitochondrial protein in the reaction cuvette was 1.4 mg/ml and all other conditions and abbreviations were as described in the legend to Fig. 3. Traces (*a*), (*b*), (*c*) and (*d*) are separate experiments.

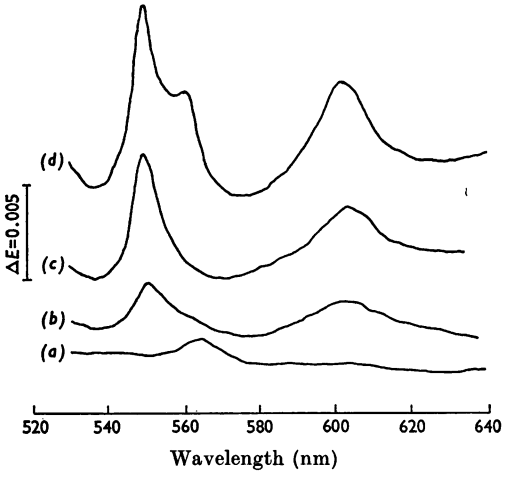


Fig. 8. Difference spectra in the cytochrome α-band region for mitochondria derived from SR_1-cells. Spectra are presented as the difference in extinction of a test minus a reference cuvette. Spectra (*a*)–(*c*) are steady-state spectra and were followed by the changes characteristic of anoxia (*d*). The suspensions for spectroscopy were prepared by mixing 0.4ml of mitochondrial suspension (48mg of protein/ml) into 3.6ml of incubation medium, air-saturated at 25°C, containing bovine plasma albumin (2.5mg/ml). The resulting suspension was then divided between the test and reference cuvettes, and a base line recorded (not shown). The appropriate addition was made to the test cuvette and the spectrum recorded: (*a*) state 4 minus state 2, pyruvate (5mM)+L-malate (5mM); (*b*) state 3 minus state 2, pyruvate (5mM), L-malate (5mM) and ADP (2mM); (*c*) state 3 uncoupled minus state 2, pyruvate (5mM), L-malate (5mM), and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (0.25mM); and (*d*) state 5 minus state 2 obtained from the experiment of trace (*c*) after exhaustion of oxygen. All spectra have been corrected for anomalous behaviour of the base line from the state 2 minus state 2 difference spectrum.

cytochrome oxidase owing to partial inhibition of its activity even in state 2), there is little difference between the three spectra. Certainly it is not possible to identify any new carrier that might have been responsible for the alternative route in SR_1-mitochondria.

Fig. 11 shows the effects of cyanide and antimycin A on the low-temperature spectra of SR_1-mitochondria frozen in state 2, and also presents analogous spectra for S-mitochondria. In traces (*a*) and (*b*) of Fig. 11 where the inhibitor was cyanide, there is in each instance a clearly defined reduction of cytochrome oxidase, as shown by the α-peak at 599nm, with reduction of cytochromes *b*, c_1 and *c* as shown by their respective α-peaks. Thus, although the respiration of SR_1-mitochondria is not more than 20% sensitive to cyanide, the spectroscopic effects of cyanide were much the same in both S- and SR_1-mitochondria. The other two traces in Fig. 11(*c* and *d*) show the effects of antimycin A on S- and SR_1-mitochondria, and there is a clearly defined 'cross-over' (Chance & Williams, 1956) in trace (*d*) between cytochrome *b* and cytochrome *c*. If, as we suggested above, electron flow from endogenous substrate to a partially inhibited cytochrome oxidase resulted in a high steady-state reduction of cytochrome $a+a_3$ in SR_1-mitochondria in states 2 and 3, then inhibition of the electron flow by antimycin A should cause an extensive reoxidation. This prediction is fulfilled by traces (*c*) and (*d*) in Fig. 11 (which are directly comparable on the basis of the spectrophotometer sensitivities and protein concentrations) where it is shown that the addition of antimycin A to SR_1-mitochondria caused a fourfold deeper trough at 600nm than that observed with S-mitochondria.

Effects of other possible respiratory inhibitors on mitochondria from SR_1-cells. It would have been useful to have found a respiratory inhibitor for the

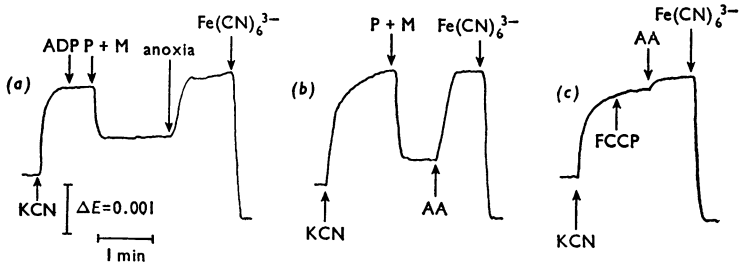


Fig. 9. Changes in the steady-state redox level of endogenous cytochrome *c* in SR_1-mitochondria oxidizing pyruvate with L-malate in the presence of cyanide. The experimental techniques used to follow these spectrophotometric changes were as described in the legend to Table 7; an upward deflexion corresponds to reduction of endogenous cytochrome *c*. Additions to the reaction cuvette, expressed as final concentrations, were: SR_1-mitochondria (1.2mg/ml), bovine plasma albumin (1mg/ml), KCN (5mM), ADP (2mM), pyruvate (P, 5mM)+L-malate (M, 5mM), antimycin A (AA, 5μM), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 0.25μM); and a few grains of potassium ferricyanide [$Fe(CN)_6^{3-}$]. Traces (*a*), (*b*) and (*c*) are separate experiments.

Table 7. *Steady-state redox levels of endogenous cytochrome* c *in S- and SR_1-mitochondria oxidizing pyruvate +*L*-malate in the presence of respiratory inhibitors*

The values below indicate the percentage reduction of cytochrome *c* in S- and SR_1-mitochondria in various metabolic states. Mitochondria were suspended in the incubation medium, air-saturated at 25°C, containing bovine plasma albumin (1 mg/ml) to a final mitochondrial protein concentration of 1.2 mg/ml (SR_1) or 1.1 mg/ml (S) and volume of 2.0 ml. Changes in the redox level of cytochrome *c* were followed at 550 nm minus 540 nm with a dual-wavelength spectrophotometer. The value for 100% reduction of cytochrome *c* was calculated from the change in extinction after transitions from anaerobically reduced (state 5) to potassium ferricyanide-oxidized, and corresponded to 0.13 nmol of cytochrome *c*/mg of SR_1-mitochondrial protein or 0.12 nmol/mg of S-mitochondrial protein. Additions to the reaction cuvette, expressed as final concentrations, were: pyruvate (5 mM)+L-malate (5 mM), ADP (2 mM), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 0.25 μM), KCN (5 mM), and antimycin A (5 μM). N.T., not tested.

	Reduction of cytochrome *c* (%)					
Inhibitor ...	None		+KCN		+KCN+antimycin A	
Additions	SR_1	S	SR_1	S	SR_1	S
None	30	25	85	90	85	90
Pyruvate with malate	20	30	40	90	80	90
ADP	55	30	85	90	85	90
Pyruvate, malate and ADP	50	40	60	90	85	90
FCCP	67	30	90	90	N.T.	90
Pyruvate, malate and FCCP	67	35	85	90	N.T.	90

alternative route. A number of compounds were tested (Table 8), but none caused a specific inhibition of the alternative route. Nor did the spectroscopic effects of carbon monoxide on G-mitochondria differ in any way from those on SR_1-mitochondria, and the ± carbon monoxide difference spectra shown in Fig. 12 demonstrate only the formation of the complex between carbon monoxide and cytochrome *a* (Chance, 1953*a*,*b*). There is no evidence in Fig. 12 for the presence of cytochrome *o* which would be characterized by a γ-peak at 418 nm in the presence of carbon monoxide (Castor & Chance, 1955).

Apparent K_m of the alternative pathway for oxygen. This value was measured very approximately by allowing a mitochondrial suspension to oxidase substrate until the polarographic recording indicated that anoxia was being approached. The recorder sensitivity was then increased in two steps of ten-fold each, giving a scale expansion of 100-fold over that normally used. Recordings were made with SR_1-mitochondria both in the presence and absence of cyanide. In each case the apparent K_m for oxygen was about 0.1 μM or less.

Properties of electron-transport particles (ETP_c) derived from various cell types. Table 9 summarizes the properties of ETP_c preparations from G-, S-, SR_0-, SR_0(cycloheximide)- and SR_1-cells. The points that we wish to make from these results are as follows. (1) The piericidin A sensitivity of the electron-transport particles follows that already described for intact mitochondria (Tables 4 and 5; Fig. 2; Light & Garland, 1971). (2) Respiration by ETP_c preparations from SR_1-cells was 90% or more sensitive to antimycin A or cyanide, unlike the behaviour of SR_1-mitochondria and SR_1-cells (Tables 4 and 5; Fig. 4). (3) The concentration and ratios of cytochromes was essentially the same in all ETP_c preparations, and the apparent decrease in cytochrome $a+a_3$ concentration observed in SR_1-mitochondria (Fig. 10) was not observed in ETP_c preparations from SR_1-cells. (4) The non-haem iron concentration was the same in all cases except in ETP_c preparations from SR_1-cells, where the concentration was increased by 50% or more compared with the other types. (5) The concentration of acid-labile sulphide in ETP_c preparations was diminished nearly tenfold in S-cells compared with G-cells, partially restored in SR_0-cells but rather less so in SR_0(cycloheximide)-cells, and increased threefold in SR_1-cells. (6) The ratio of non-haem iron to labile sulphide varied from a maximum of 15 in ETP_c preparations from S-cells to a minimum of 0.72 in SR_1-cells.

DISCUSSION

Loss of piericidin A sensitivity and site 1 *energy conservation from mitochondria prepared from sulphate-limited cells.* These effects are remarkably similar to those found in mitochondria from iron-limited cells, and provide independent support for the conclusion that non-haem iron proteins (iron–sulphur proteins) are involved in the mechanisms of piericidin A sensitivity and of energy conservation at site 1 (Light *et al.* 1968; Light & Garland, 1971). The recovery of piericidin A sensitivity and site 1 energy conservation on aeration of S-cells for 16 h in the absence of added growth nutrients demon-

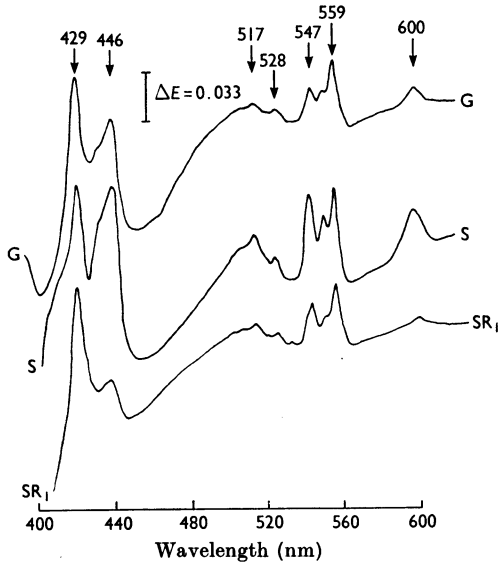


Fig. 10. Glycerol 3-phosphate reduced (state 5) minus oxidized (state 2) difference spectra recorded at 77°K obtained with S-, G- and SR_1-mitochondria. Wavelength-scanning spectrophotometric measurements were performed with a laboratory-built machine similar to that used by Yang & Legallais (1954). The entrance and exit slit widths were 0.5 mm corresponding to a spectral band width of approx. 1.35 nm. For spectra at the temperature of liquid N_2, a cell holder and a vacuum-flask assembly were constructed as described by French & Lawrence (1968). The time-constant of the measuring apparatus was 0.1 s, the scanning speed was approx. 15 nm/min, and the cuvettes had a light-path of 0.2 cm. Spectra are presented as the difference in extinction between a test and a reference cuvette. Mitochondria were suspended in the incubation medium, air-saturated at 20°C, in a test-tube to a final volume of 1.5 ml so as to give a final protein concentration of: S-mitochondria (15.4 mg/ml), G-mitochondria (6.7 mg/ml) or SR_1-mitochondria (9.3 mg/ml). Half of this suspension was transferred to the reference cuvette, and glycerol 3-phosphate (5 mM) was added to the remainder in the test-tube. After sufficient time had been allowed for the latter to become anaerobic, as calculated from polarographic measurements, the contents of the test-tube were transferred to the test cuvette, and the cuvette assembly was plunged into a Dewar flask containing liquid N_2. After about 1 min the cuvette assembly was quickly transferred to the vacuum assembly on the spectrophotometer and a difference spectrum recorded.

strates that the changes under study were of phenotypic origin, and did not arise as a result of selection in continuous culture of a strain of *T. utilis* that lacked piericidin A sensitivity and site 1 energy conservation. One observation, however, does cast some doubt on these conclusions, and that is the finding that sulphate need not be added to an aerated suspension of S-cells to restore piericidin A sensitivity and site 1 energy conservation (Tables 4 and 5). Although we do not wish to make light of this objection to our conclusions about a role for iron–sulphur proteins, we can nevertheless point out that the transition from S- to SR_0-cells could involve a redistribution of intracellular sulphur or even the gain of sulphur by some cells at the expense of others during the 16 h incubation. In addition, the transition from S- to SR_0-cells involves a fourfold increase in the acid-labile sulphide concentration of electron-transport particles (Table 9). It should also be noted that the reappearance of piericidin A sensitivity during the transition from S- to SR_0-cells is accelerated if sulphate is added (Fig. 4), and that both visible (Ragan, Clegg, Haddock, Light & Garland, 1970) and electron paramagnetic resonance–spectroscopy (C. I. Ragan, B. A. Haddock, J. C. Swann & R. C. Bray, unpublished work) demonstrate the reappearance of iron–sulphur proteins in ETP preparations from SR_0-mitochondria.

The use of cycloheximide to produce SR_0(cycloheximide)-cells demonstrated that energy conservation at site 1 could be dissociated from piericidin A sensitivity, in agreement with our findings with iron-limited cells (Clegg *et al.* 1969). A similar dissociation was achieved by careful manipulation in continuous culture of the transition from iron limitation to glycerol limitation (Clegg *et al.* 1969; Clegg & Garland, 1971), but we were not able in the present study to separate piericidin A sensitivity from site 1 energy conservation by manipulation of the entering sulphate concentration (Fig. 1).

Alternative route of mitochondrial electron transport. Many plant tissues yield mitochondria, the respiration of which is partially insensitive to inhibition by cyanide (James & Beevers, 1950; Yocum & Hackett, 1957). The most notable of such tissues are the spadices of the flowers of *Arum maculatum* (Bendall, 1957; Bendall & Hill, 1956), and the skunk cabbage *Symplocarpus foetidus* (Hackett & Haas, 1958; Bonner, Bendall & Plesnicar, 1967). Mitochondria isolated from these tissues are often totally insensitive to respiratory inhibition by cyanide or antimycin A, and it has been suggested that such mitochondria contain two terminal oxidases, one a mammalian-like cytochrome oxidase sensitive to inhibition by cyanide, the other an enzyme of unknown structure insensitive to cyanide inhibition (Bonner, 1961; Yocum & Hackett, 1957). There is now considerable evidence that the alternative oxidase is located at or near the level of the *b*-type cytochromes (Storey & Bahr, 1969*a*,*b*); however, no component identifiable by differential spectrophotometric measurements qualified for the role of the alternative oxidase on kinetic grounds (Storey & Bahr, 1969*a*), and it was concluded that

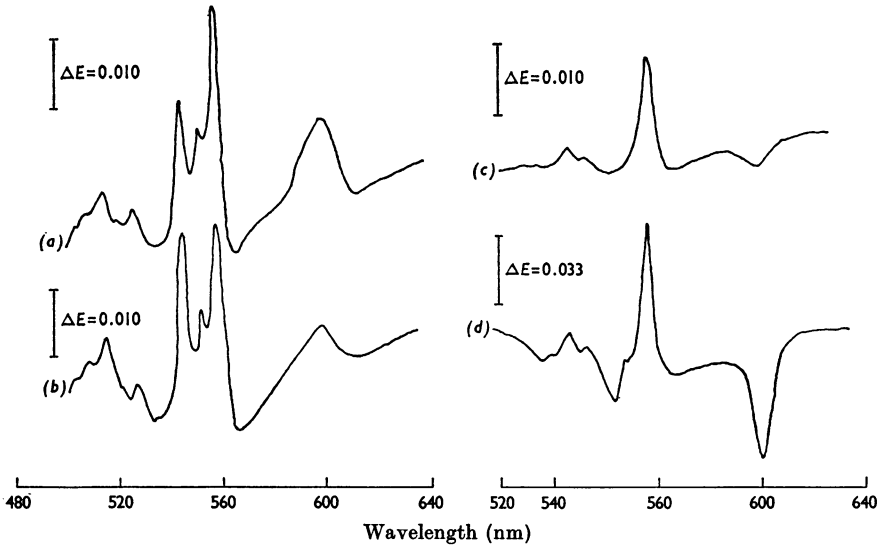


Fig. 11. Potassium cyanide- or antimycin A-inhibited-minus-oxidized difference spectra of S- and SR_1-mitochondria recorded at 77°K. Difference spectra were obtained as described in the legend to Fig. 10. Cyanide (5mM)-inhibited-minus-oxidized difference spectra are shown in traces (*a*) and (*b*), and antimycin A (5μM)-inhibited-minus-oxidized difference spectra in traces (*c*) and (*d*). Mitochondria were suspended in incubation medium, air-saturated at 20°C, in a test-tube so as to give a final protein concentration of: trace (*a*), S-mitochondria (6.7mg/ml); trace (*b*), SR_1-mitochondria (9.3mg/ml); trace (*c*), S-mitochondria (15.4mg/ml); trace (*d*), SR_1-mitochondria (5.0mg/ml). In each case half of this mitochondrial suspension was transferred to the reference cuvette and inhibitor was added to the remainder, which was then transferred immediately to the test cuvette, and the cuvette assembly plunged into a Dewar flask containing liquid N_2. Difference spectra were then recorded as indicated in the legend to Fig. 10.

Table 8. *Effect of various electron-transport inhibitors on glycerol 3-phosphate oxidation by SR_1-mitochondria*

The experimental conditions were as described in the legend of Table 2. The order of additions was mitochondria (0.50mg/ml), bovine plasma albumin (3mg/ml), glycerol 3-phosphate (5mM), ±cyanide (3mM), ADP (0.4mM) and the inhibitor under study. To measure the percentage inhibition by carbon monoxide, the contents of the incubation vessel were allowed to go anaerobic, the oxygen electrode was removed, and the mitochondrial suspension bubbled with a thin stream of coal gas for about 10s. After this time a known volume of mitochondrial incubation medium, air-saturated at 30°C, was added to the incubation vessel, the oxygen electrode was replaced and the rate of oxygen uptake recorded.

	Inhibition of glycerol 3-phosphate respiration (%)	
	Control (%)	+Cyanide (3mM) (%)
Carbon monoxide (saturated)	10–20	0–5
Sodium azide (3mM)	10–20	0–5
Sodium sulphide (1mM)	10–20	0–5
p-Hydroxymercuribenzoate (1mM)	75	75
Mersalyl (1mM)	75	75
1,10-Phenanthroline (2mM)	35	35
2,2′-Bipyridyl (0.4mM)	35	35

an iron–sulphur protein might function as an alternative oxidase in these mitochondria, as had been proposed by Bonner *et al.* (1967). Cyanide-insensitive respiration of this type has also been reported in mitochondria from *Euglena gracilis* grown under defined conditions (Sharpless & Butow, 1970), from

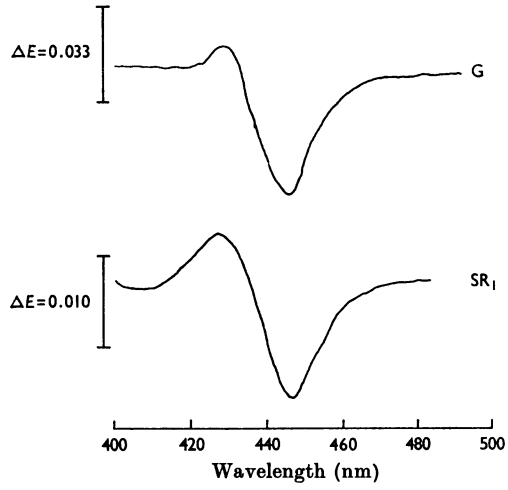


Fig. 12. Carbon monoxide difference spectra obtained with G- and SR_1-mitochondria. Mitochondria were suspended in the incubation medium, air-saturated at 25°C, to give a final protein concentration of 7.2mg/ml (G) or 3.7mg/ml (SR_1), and volume of 4.0ml, in a test-tube. A few grains of sodium dithionite were added to the mitochondrial suspension, which was then divided equally between the test and reference cuvettes. A reduced-minus-reduced base-line was recorded, as described in the legend to Fig. 8 (not shown), to allow for subsequent correction of any base-line anomaly. The contents of the test cuvette were then bubbled with a thin stream of coal gas for about 5s, and after waiting for a further 5min, to allow time for any slow-reacting species to combine with carbon monoxide, a carbon monoxide–reduced-minus-reduced difference spectrum was recorded. The spectra reproduced above have been corrected for alterations in the base line as indicated.

a mutant strain of *Candida albicans* (Ward & Nickerson, 1958),. and from *Candida floreri* (S. Austin, personal communication).

The nature of the component(s) responsible for the alternative route of electron transport in SR_1-mitochondria is as yet unknown. The critical dependence on the added potassium sulphate concentration for the emergence of this novel terminal oxidase activity implicates some aspects of sulphur metabolism in this process. As the apparent K_m for oxygen was similar for SR_1-mitochondria oxidizing pyruvate with L-malate in the presence or absence of cyanide, it seems likely that the novel terminal oxidase activity is due to an enzymic rather than a non-enzymic reaction.

The properties of SR_1-mitochondria reported above indicate that the alternative terminal oxidase accepts reducing equivalents from the respiratory chain at the level of cytochrome *b*. It has been proposed that *b*-type cytochromes may function as terminal oxidases in the cyanide-insensitive respiration of plant mitochondria (Schichi & Hackett, 1966). However, the kinetic results of Storey & Bahr (1969*a*) indicate that, although a *b*-type cytochrome is involved in such respiration, some other component must act as the terminal oxidase itself. Difference spectra recorded at 77°K offered no evidence for more than one *b*-type cytochrome in SR_1-mitochondria.

It has been reported that under suitable growth conditions *T. utilis* mitochondria possess cytochrome *o* (Mok, Rickard & Moss, 1969), which functions as a terminal oxidase in several bacteria (Bartsch, 1968) and in the tapeworm *Monezia expansa* (Cheah, 1968). The spectroscopic effects of

Table 9. *Inhibitor sensitivities and the concentrations of non-haem iron and acid-labile sulphide of electron-transport particles derived from G-, S-, SR_0(cycloheximide)- and SR_1-cells*

Electron-transport particles (ETP_c) were prepared from whole cells by a modification of the method of Clegg & Garland (1971) as described in the Materials and Methods section. Oxygen uptake measurements and the incubation medium were as described in the Materials and Methods section, and the final concentrations and order of additions were: ETP_c (0.5–1.0mg of protein/ml), cytochrome *c* (5μM), NADH (50μM) and inhibitor, either piericidin A (0.2nmol/mg of protein), or antimycin A (2nmol/mg of protein) or KCN (5mM). The initial respiration rates in the absence of inhibitor were similar for ETP_c preparations from all cell types and the percentage inhibitions were calculated as described in the legend to Table 4. These results were obtained from at least four separate ETP_c preparations. The concentrations of cytochromes of the ETP_c preparations were also measured and did not differ significantly in any of the preparations from the values observed with ETP_c preparations from G-cells where the values were (as nmol/mg of protein): cytochrome $a+a_3$, 0.06 ± 0.02; cytochrome *b*, 0.09 ± 0.01; cytochrome $c+c_1$, 0.08 ± 0.02.

	ETP_c preparation				
% inhibition of NADH oxidation by:	G	S	SR_0	SR_0(cycloheximide)	SR_1
Piericidin A	65–85	5–10	80–90	5–15	80–85
Antimycin A	85–95	90–95	80–90	80–90	80–90
KCN	100	100	100	100	100
Concn. (nmol/mg of protein) of					
Non-haem iron	0.65 ± 0.10	0.60 ± 0.15	0.58 ± 0.04	0.67 ± 0.15	0.95 ± 0.05
Acid-labile sulphide	0.38 ± 0.04	0.04 ± 0.01	0.15 ± 0.03	0.09 ± 0.02	1.32 ± 0.05

carbon monoxide on SR_1-mitochondria offer no evidence for the presence of cytochrome *o* in *T. utilis* grown under the conditions described here.

Characterization of the properties of electron-transport particles derived from SR_1-cells indicated that they contain elevated concentrations of acid-labile sulphide and non-haem iron, thus implicating iron–sulphur protein(s) in this alternative oxidase activity. However, respiration by electron-transport particles derived from SR_1-cells is as sensitive to inhibition by cyanide or antimycin A as is respiration by electron-transport particles from S- or G-cells. From this it would appear that the novel oxidase activity is a property of the intact mitochondrion that is lost if the integrity of the mitochondrion is destroyed.

Although the nature of the component(s) responsible for this alternative oxidase activity remains obscure, from the information available the mechanism of cyanide-insensitive respiration by *T. utilis* appears to be similar to that described for plants and *E. gracilis*. However, this system affords distinct advantages for the study of such respiration when compared with these species. The fact that cyanide-insensitive respiration in *T. utilis* is inducible offers a powerful tool for studying the mechanism of such respiration and the control systems governing its induction. Other advantages include the lack of seasonal dependence controlling the supply of plant material, the comparative simplicity of the respiratory chain of yeast, and the ease with which mitochondria with intact energy conservation at site 1 can be prepared.

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