Biochem. J. (1973) **134**, 1051–1061
Printed in Great Britain

An Antimycin A- and Cyanide-Resistant Variant of *Candida utilis* Arising during Copper-Limited Growth

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(*Received* 9 *April* 1973)

1. During copper-limited growth of *Candida utilis* in continuous culture on a non-fermentable carbon and energy source there is a selective pressure favouring the emergence of variants that are less dependent on copper. 2. We describe the properties of such a variant that by-passes cytochrome oxidase (EC 1.9.3.1) by utilizing an alternative oxidase communicating with the respiratory chain at about the level of cytochrome *b*. 3. Both direct studies of isolated mitochondria and calculations based on growth parameters showed that only one of the normal three phosphorylation sites was active. This site was localized between NADH and the cytochromes. 4. Growth of the variant with copper-supplemented media resulted in the return of cytochrome oxidase but not the loss of the alternative oxidase. 5. The alternative oxidase is inhibited by substituted benzhydroxamic acids. 6. Submitochondrial particles from the variant did not exhibit any novel electron-paramagnetic-resonance-spectroscopy features at about $g = 2.0$ either at 80°K or 12°K.

It has been known for many years that copper deficiency limits the growth of yeasts (Elvehjem, 1931; Wohlrab & Jacobs, 1967*a*,*b*; Keyhani & Chance, 1971; Light, 1972), and that the cellular and mitochondrial content of the copper-containing enzyme cytochrome oxidase was diminished under these conditions. However, it was not known whether growth limitation arose from the deficiency of cytochrome oxidase or from the failure of some other, possibly unknown, copper-dependent function. In the present paper we describe the properties of a variant of *Candida utilis* that is considerably less dependent on copper for growth than is the wild-type. This lessened dependence is due to an alternative terminal oxidase that by-passes cytochrome oxidase and cytochrome *c*. The alternative pathway is insensitive to both antimycin A and CN^-. A preliminary report has been published (Downie & Garland, 1972).

Materials and Methods

The apparatus for continuous culture of *C. utilis* (N.C.Y.C. 193), the maintenance of cultures, the collection of cells and the preparation of spheroplasts, the isolation of mitochondria, the measurement of O_2 uptake, the measurement of cytochromes at room temperature with a wavelength-scanning spectrophotometer, the assays of glycerol, protein and ADP, and the medium used for mitochondrial incubations were as described by Light & Garland (1971). Submitochondrial particles were prepared from mitochondria by the method of Clegg & Garland (1971). Spectra of mitochondria at 77°K were obtained as described by Haddock & Garland (1971), except that the suspending medium was 0.8M-mannitol. The value of Y_{oxygen} (g of dry cell mass produced/g-atom of O consumed) was calculated by the methods of Johnson (1964) and von Meyenburg (1969) (see also Clegg & Light, 1971).

Preparation of copper-deficient growth medium

A 16-fold concentrate (8 litres) of the glycerol-limiting medium of Light & Garland (1971) was prepared, except that $CuCl_2$ and antifoam were omitted and the pH was not adjusted. Copper was extracted from this concentrated medium in 1 litre batches as described by Giorgio *et al.* (1963), by using 0.015% (w/v) dibenzyldithiocarbamate in carbon tetrachloride. The 1 litre batches of copper-extracted medium were combined to minimize variation in copper concentration of the medium on subsequent dilution and usage. In practice the combined 8 litres were divided again into 1-litre batches which were then stored at −20°C. A batch was thawed as required, diluted to 16 litres, antifoam was added [0.025% (v/v) Antifoam FG, from Midland Silicones Ltd., Barry, Glam., U.K.], and the pH adjusted to 5.0 with 10M-KOH. The medium was then sterilized by autoclaving at 6.9kPa (10lbf/in²) for 1h after steaming at 100°C for 1h. We are grateful to Dr. P. A. Light for details of this method of copper extraction.

Preparation of solid media containing antimycin A

The glycerol-limiting medium of Light & Garland (1971) was used as the nutrient base to which was added 1.5% (w/v) Oxoid no. 3 agar. Antimycin A was added as an ethanolic solution to a final concentration of 2 μM just before pouring the plates.

Recovery procedures

Incubations of copper-deficient cells under a variety of conditions with added copper are known as recovery procedures (Light, 1972). Details of the conditions are shown in Table 2, but in general, recovery procedures could be under conditions that do, or do not permit net cell growth (i.e. an increase in the cell mass as opposed to cryptic growth of a fraction of the culture at the expense of the remainder). Large-scale recovery of cells under growing conditions was effected by inoculating 200ml of glycerol-limiting medium with a single colony from a culture on a solid medium. This culture, after 36h of aerobic growth at 30°C, was used to inoculate 7 litres of glycerol-limiting medium. The cells were harvested after 10h of aerobic growth at 30°C and pH 5.0. At this stage the cells are still in the exponential phase of growth, and are sensitive to digestion by snail gut enzyme. The vessel for large-scale batch-growth was a scaled-up version of the culture vessel described by Ware *et al.* (1970), with a vessel volume of 10 litres. The temperature was controlled at 30°C by circulating water at 28°C through internal coils.

Preparation of samples for electron-paramagnetic-resonance (e.p.r.) spectroscopy

A suspension of submitochondrial particles (40–60mg of protein/ml of 20mM-Tris–HCl buffer, pH 7.4) stored at 77°K was warmed to room temperature. A portion (0.2ml) of this suspension was then placed in the bottom of a test-tube with 0.05ml of 0.1M-NADH, 0.05ml of air-saturated 20mM-Tris–HCl buffer, pH 7.4, and, in some experiments, 0.01ml of 0.1M-*m*-chlorobenzhydroxamic acid. The contents of the tube were mixed by agitation for 5s with a vibrating mixer (Whirlimixer from Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.), drawn with a syringe into a Teflon tube of 2mm internal diameter, and gently extruded into the bottom of a quartz e.p.r. tube of 3.0mm internal diameter (Varian Associates Ltd., Walton-on-Thames, Surrey, U.K.). The e.p.r. tube was then slowly introduced into liquid N_2. The whole procedure from mixing to freezing took 40–60s.

Electron-paramagnetic-resonance spectroscopy

Spectra were measured by Dr. D. Lowe and Dr. R. C. Bray at the University of Sussex, by using a Varian E9 spectrometer. Instrumental settings are given in the legend to Fig. 8. Temperature measurements of the sample were made as described by Lowe *et al.* (1972).

Results

Isolation of a variant with diminished copper requirements for growth

During continuous culture of *C. utilis* under conditions of copper limitation at a dilution rate of $0.2h^{-1}$ and an entering glycerol concentration of 133mM, the concentration of cells within the chemostat was within the range 0.66–1.2mg dry wt./ml. Most of the glycerol was unused, the concentration in the culture effluent being about 110–120mM. These values correspond to the most copper-deficient cultures described by Light (1972). Somewhat surprisingly, it was found that during a culture that had been running for 7 days, the cell concentration had risen to 4.4mg dry wt./ml, whereas the glycerol concentration had dropped to 3mM. The trivial explanation was that the medium was contaminated with copper. However, three reasons suggested that this was not so. (1) Provision of freshly extracted copper-deficient media did not alter the unusual values for cell concentration and glycerol utilization. (2) The $Y_{glycerol}$ value was atypical, having dropped from the value of 54mg dry wt. of cell/mmol of glycerol characteristic of glycerol- or copper-limited growth to a lower value of 35mg dry wt./mmol of glycerol (Table 1). (3) The concentration of cytochrome $a+a_3$ in the cells would not be expected to fall if the concentration of entering copper increased. In fact, the concentration of cytochrome $a+a_3$ fell by at least a factor of five to the limits of sensitivity of the assay (Table 1).

From these data (Table 1) it is clear that the organism in this culture differed from the starting *C. utilis* in exhibiting a greatly diminished copper requirement for growth, and a lower $Y_{glycerol}$ value. The question therefore arose as to whether this new organism was a variant of the parent strain, or a contaminant. Either possibility could be anticipated in view of the selective pressures within a chemostat (Novick & Szilard, 1950). The new organism was identical to *C. utilis* in the following respects: (1) both phase-contrast microscopy of whole cells and electron microscopy of whole-cell sections showed a budding yeast of oval shape and small size (longer diameter about 3.5 μm); (2) similar colony morphology when grown on solid glycerol medium; (3) similar cytochrome composition when grown in the presence of added copper (see below); (4) similar rotenone and piericidin A sensitivity of the NADH to cytochrome segment of the respiratory chain (see below); (5) failure to form spores. Thus the new organism can be

Table 1. *Properties of normal and variant cells from continuous culture*

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

	Copper-limited normal cells	Glycerol-limited normal cells	Copper-limited variant cells
Cell density (mg dry wt./ml) in effluent	0.7	7.2	4.4
Glycerol in effluent (mM)	120	<0.1	3
$Y_{glycerol}$ (mg dry wt. of cells/mmol of glycerol)	53.8	54.1	34.6
Cytochrome concentration (nmol/g dry wt.)			
Cytochrome $a+a_3$	9.2	48.3	<0.5
Cytochrome b	34.1	29.0	36.4
Cytochrome $c+c_1$	54.6	57.4	65.5

described as a variant of *C. utilis*. Whether or not this variant arose from a mutational event we cannot say, since genetic markers and a sexual cycle are absent from the wild-type. However, the emergence of the variant is uncommon, and did not occur in the extensive studies of Light (1972) or in several independent copper-limited cultures lasting over 2 months in our laboratory.

For the purpose of the present paper we will refer to the variant with a low copper requirement as 'variant cells', and the strain of *C. utilis* (N.C.Y.C. 193) with the higher copper requirement as 'normal cells', irrespective of their growth conditions.

Cytochrome composition of the variant cells

The concentration of cytochrome $a+a_3$ in copper-limited *C. utilis* is about 7 or 8 nmol/g dry wt. (Light, 1972). This represents a sixfold decrease from the value observed with glycerol limitation. Our own values confirm these findings (Table 1). The concentration of cytochrome $a+a_3$ in the copper-limited variant cells was so low as to be undetectable by measurements of reduced minus oxidized difference spectra at either 22°C or 77°K. The limit of sensitivity with these techniques corresponded to 0.5 nmol of cytochrome $a+a_3$/g dry wt. of cells. Thus the concentration of cytochrome $a+a_3$ in the variant under copper-limited growth conditions was less than 1% of that for glycerol-limited normal *C. utilis* (Table 1).

Fig. 1 shows reduced minus oxidized difference spectra of whole cells at 22°C. The following conclusions may be drawn. (1) The glycerol-limited cells have well-defined peaks at 605, 562, 550, 444, 430 and 418 nm, corresponding in that order to the α bands of cytochromes $a+a_3$, b and $c+c_1$, and their respective Soret bands. (2) The α band (605 nm) and Soret band (444 nm) of cytochrome $a+a_3$ is diminished but clearly visible in copper-limited normal cells. (3) Neither the α nor Soret bands of cytochrome $a+a_3$ were detectable in spectra of the copper-limited variant cells. No other novel characteristics were observed, the spectrum corresponding closely to that of glycerol-limited normal cells with the deletion of the known reduced minus oxidized difference spectrum of cytochrome $a+a_3$. (4) Recovery of copper-limited normal cells under non-growing conditions resulted in an increase of the concentration of cytochrome $a+a_3$, in confirmation of the findings of Light (1972), but in contrast with those of Keyhani & Chance (1971). (5) Recovery of copper-limited variant cells under non-growing conditions did not result in the return of cytochrome $a+a_3$ (spectrum not shown). However, recovery under growing conditions for 36h (see below) caused the return of cytochrome $a+a_3$ to normal concentrations.

Cytochrome a_3 was also sought by means of its spectrally unique combination with CO (Chance, 1953). Fig. 2 shows the characteristic difference spectrum in glycerol-limited normal cells of the CO complex of cytochrome a_3; there is a peak at 431 nm and a trough at 445 nm. The comparable spectra of copper-limited variant cells showed a peak at 418 nm, and this could be attributed to cytochrome o or yeast haemoglobin (Mok *et al.*, 1969). Only a suspicion of a shoulder was present at 430 nm, in confirmation of the virtual absence of cytochrome a_3 as shown by reduced minus oxidized difference spectra.

Low-temperature spectra of mitochondria isolated from variant cells

Fig. 3 shows reduced minus oxidized difference spectra at 77°K for the α-band region of the absorption spectrum of mitochondria from glycerol-limited normal cells and copper-limited variant cells. It is clear that the concentration of cytochrome $a+a_3$ was decreased to undetectable values in the copper-limited variant. No new peaks were seen.

Recovery of cytochrome $a+a_3$ during copper-supplemented growth of variant cells

Incubation of copper-limited variant cells at 30°C for 16–36h with aeration and 40 μM-$CuSO_4$ did not result in the appearance of cytochrome $a+a_3$. This

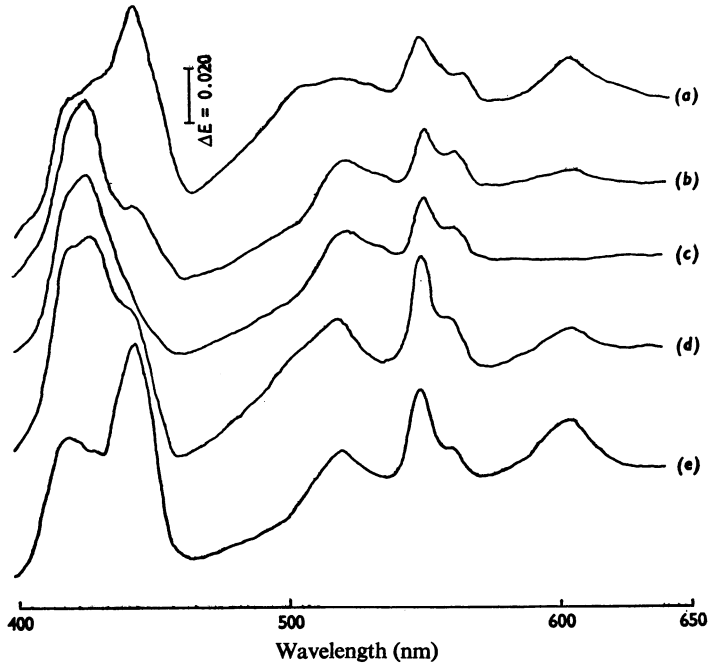

Fig. 1. *Reduced minus oxidized difference spectra of whole cells*

The cell suspensions for spectroscopy were prepared by diluting washed and harvested cells in distilled water at the concentrations indicated. Samples (3.0ml) of the suspensions were placed in the reference and test cuvettes of light path 1.0cm, and a baseline was recorded at a scanning speed of about 100nm/min and at a spectral band width of 2.6nm. During this time the suspensions became anaerobic. H_2O_2 (5μl of 1.7M) was then added to the reference cuvette, and the spectrum recorded. The curves drawn in Fig. 1 have been corrected for the baseline, and there is a consistent isosbestic point at about 500nm. The cell types and dry weight concentrations used were: (*a*) glycerol-limited normal cells, 22.9mg/ml; (*b*) copper-limited normal cells, 21.5mg/ml; (*c*) copper-limited variant cells, 23.0mg/ml; (*d*) copper-limited normal cells recovered in 40μM-$CuSO_4$ for 16h in the absence of growth, 24.9mg/ml; (*e*) variant cells batch-cultured in complete medium for 16h, 42mg/ml.

differs from the behaviour reported by Light (1972) for copper-limited normal cells. An investigation of the effect of medium composition on the recovery of cytochrome $a+a_3$ by copper-limited variant cells was therefore made (Table 2). It was found that recovery of cytochrome $a+a_3$ occurred only if a complete medium for growth was provided. It was confirmed that copper-limited normal cells recovered their cytochrome $a+a_3$ in the absence of growth (Light, 1972). Fig. 4 shows the time-course of the recovery of cytochrome $a+a_3$ in previously copper-limited variant cells on transfer to a complete growth medium. Cell growth and the concentration of cytochrome $a+a_3$/unit cell mass (as measured by extinction) proceeded approximately in phase, there being an initial lag period of 1h before increases were observed. The concentration of cytochrome *c*/unit cell mass did not change significantly.

Respiratory properties of mitochondria isolated from variant cells

Table 3 summarizes measurements of the respiratory rates, inhibitor sensitivities and P/O ratios for a variety of substrates oxidized by mitochondria from glycerol- or copper-limited normal cells, copper-limited variant cells and variant cells batch-cultured in complete medium. The most significant findings were these. (1) The respiration of mitochondria from variant cells was insensitive to CN^- or antimycin A, irrespective of whether cytochrome $a+a_3$ was absent (copper-limited variant cells) or present (variant cells batch-cultured in complete medium). (2) Mitochondria from copper-limited variant cells did not oxidize the combination of *NNN'N'*-tetramethylphenylenediamine and L-ascorbate, whereas the other varieties of mitochondria did. (3) The P/O ratios for mitochondria from copper-limited variant cells showed

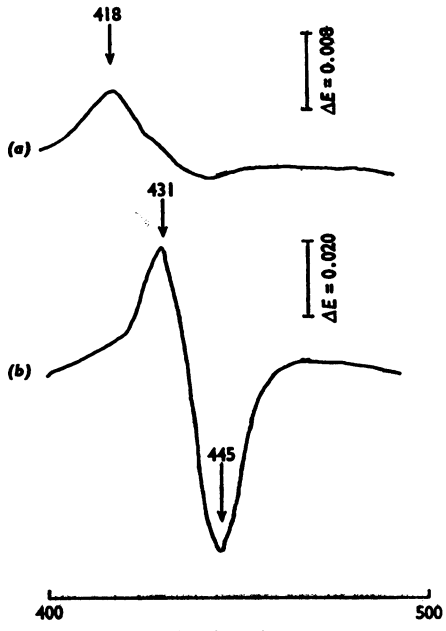

Fig. 2. *CO difference spectra of whole cells*

The cell suspensions in distilled water were prepared as in Fig. 1, and a baseline was recorded. The contents of the test cuvette were then gently bubbled with CO for 10min, and the spectrum was recorded. The spectra shown are corrected for the baseline, and correspond to (*a*) copper-limited variant cells, 22.9mg dry wt./ml, and (*b*) glycerol-limited normal cells, 21.0mg dry wt./ml. The spectroscopic conditions were otherwise as for Fig. 1.

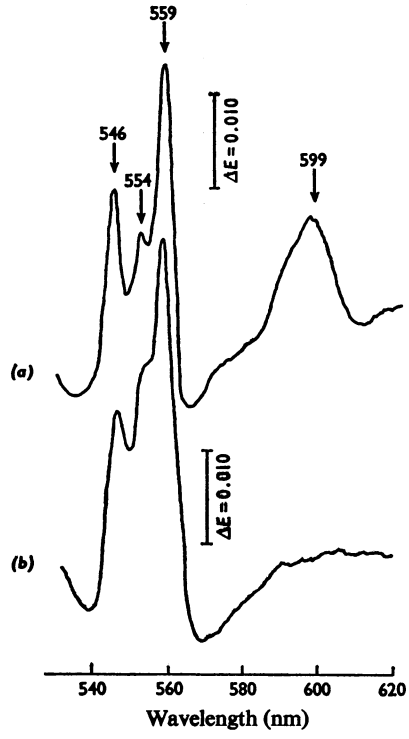

Fig. 3. *Reduced minus oxidized spectra of mitochondria at 77°K*

The spectroscopic conditions were as described by Haddock & Garland (1971), with glycerol 3-phosphate as the reductant for the test cuvette. Curve (*a*), mitochondria (6.7mg of protein/ml) from glycerol-limited normal cells; curve (*b*), mitochondria (8.6mg of protein/ml) from copper-limited variant cells.

that only one phosphorylation site, that between intramitochondrial NADH and the cytochromes, is operative (site 1, Lehninger, 1966). (4) The P/O ratio for pyruvate and malate oxidation by mitochondria from variant cells batch-cultured in complete copper-supplemented medium was relatively low at a value of 1.5, increasing to 2.2 if the CN^--insensitive alternative oxidase was inhibited with *m*-chlorobenzhydroxamic acid (see below). With these mitochondria in the presence of CN^- or antimycin A the P/O ratio for pyruvate and malate oxidation by the alternative oxidase was 0.8. The P/O ratio for glycerol 3-phosphate oxidation was 1.2 in the presence of *m*-chlorobenzhydroxamic acid, and this oxidation rate was CN^- sensitive. The P/O ratio for glycerol 3-phosphate in the absence of *m*-chlorobenzhydroxamic acid was not measurable polarographically owing to the lack of respiratory control (Chance & Williams, 1956).

The O_2-electrode recordings in Fig. 5 demonstrate that the respiratory control of mitochondria from copper-limited variant cells was sufficiently high to permit confident use of the polarographic assay of P/O ratios when only a single phosphorylation site was rate-limiting for respiration in state 4 (Chance & Williams, 1956). Also shown in Fig. 5 is the inhibition by piericidin A of the oxidation of pyruvate and malate by mitochondria from copper-limited variant cells. By contrast, Light (1972) showed that the respiration of mitochondria from copper-limited normal cells was insensitive to piericidin, and we have confirmed that observation. Keyhani & Chance (1971) found that under their growth conditions, copper-limited growth did not cause loss of piericidin sensitivity. It is therefore apparent that piericidin sensitivity can vary with the growth conditions in a

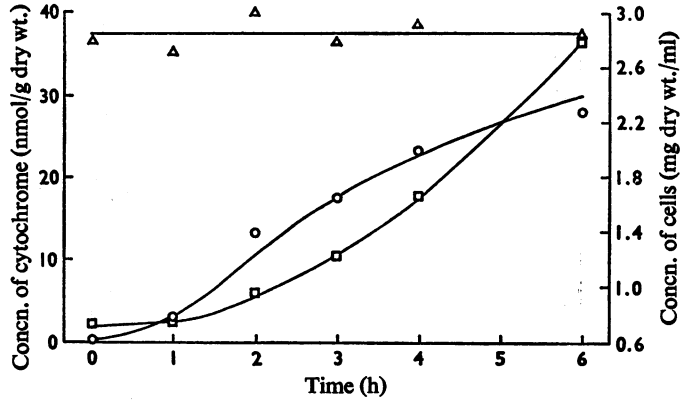

Fig. 4. *Time-course for the recovery of copper-limited variant cells on transfer to complete growth medium*

Cells from 40ml of a copper-limited continuous culture of the variant were harvested, washed and suspended at zero time in 250ml of complete medium at 30°C. The suspension was aerated by mechanical shaking, and sampled at hourly intervals. Samples of 1.0ml were diluted tenfold in water before reading their extinction at 500nm. The extinction values were converted into cell concentration (mg dry wt./ml) by using a previously constructed calibration curve. Cells from samples of 40ml were harvested by centrifugation, resuspended in 5.0ml of water, and used for difference-spectra measurements as described in the legend for Fig. 1. Cytochrome concentrations were then calculated as nmol/g dry wt. The curves are: △, cytochrome $c+c_1$ concentration; ○, cytochrome $a+a_3$ concentration; □, cell concentration.

Table 2. *Relationship between composition of recovery medium and the appearance of cytochrome $a+a_3$ in previously copper-limited variant cells*

Batches (50ml) of variant *C. utilis* cells were collected from the copper-limited culture, washed in twice-distilled water, and resuspended in the media described below. The pH of the media was corrected to 5.0 with 1M-KOH and 1M-HCl. 'Complete medium' refers to the glycerol-limited medium described by Light & Garland (1971); nutrients from this medium were omitted as listed. The cell density at zero time was 0.66mg dry wt./ml, and the concentration of cytochrome $a+a_3$ was less than 0.2nmol/g dry wt.

Medium composition	Cell density after 6h (mg dry wt./ml)	Concn. of cytochrome $a+a_3$ after 6h (nmol/g dry wt.)
40μM-$CuSO_4$	0.62	<0.2
Complete medium	2.8	26
Complete medium deficient in glycerol	0.7	<0.2
Complete medium deficient in NH_4Cl	1.4	<0.2
Complete medium deficient in phosphate but buffered with 0.1M-citrate in place of phosphate	1.4	0.5

manner that is neither entirely understood nor, in new situations, predictable (see also Katz, 1971).

Properties of submitochondrial particles from copper-limited variant cells

The oxidation of NADH (0.5mM) or glycerol 3-phosphate (5mM) by submitochondrial particles from copper-limited variant cells was not inhibited by antimycin A (0.3μg/ml) or KCN (1mM). The conditions were otherwise as described by Haddock & Garland (1971), who reported that the antimycin- and cyanide-resistant respiration of mitochondria prepared from sulphate-recovered cells of *C. utilis* was lost on conversion of mitochondria into submitochondrial particles.

Table 3. *O_2-uptake rates, P/O ratios and effects of respiratory inhibitors for mitochondria prepared from normal and variant cells grown with or without copper deprivation*

The incubation conditions were as described by Light & Garland (1971). Concentration of substrates and inhibitors were 5mM for pyruvate, L-malate, L-ascorbate and DL-glycerol 3-phosphate, 0.5mM for NADH, 1.6mM for *m*-chlorobenzhydroxamic acid, 1.0mM for KCN, 0.5μM for piericidin A, 0.5μg of antimycin A/ml, and 50μM for *NNN'N'*-tetramethyl-*p*-phenylenediamine. The O_2-uptake rates are those observed in state 3 (Chance & Williams, 1956) and refer to a single mitochondrial preparation. P/O ratios are given in parentheses. No respiratory control is indicated by n.r.c. in parentheses in place of the P/O ratio. Not tested is given as n.t. All results were repeated to within 10% with at least two further preparations of mitochondria.

Substrates and inhibitors	Rate of O_2 uptake (ng-atom/min per mg of protein) and P/O ratio for mitochondria from: Normal cells: Glycerol-limited	Normal cells: Copper-limited	Variant cells: Copper-limited	Variant cells: Batch-culture with complete medium
Pyruvate+malate with:				
No inhibitors	170 (2.5)	85 (2.4)	460 (0.7)	450 (1.5)
Antimycin A	<5	<5	460 (0.7)	450 (0.8)
Antimycin A+KCN	<5	<5	460 (0.6)	450 (0.8)
m-Chlorobenzhydroxamate	170 (2.5)	n.t.	20	450 (2.2)
m-Chlorobenzhydroxamate+KCN	n.t.	n.t.	10	25
Piericidin A	10	80	20	25
DL-Glycerol 3-phosphate and piericidin A with:				
No additional inhibitor	214 (1.6)	84 (1.6)	310 (n.r.c.)	330 (n.r.c.)
Antimycin A	<15	<10	310 (n.r.c.)	330 (n.r.c.)
KCN	<5	<5	310 (n.r.c.)	330 (n.r.c.)
m-Chlorobenzhydroxamate	210 (1.6)	n.t.	100 (n.r.c.)	330 (1.2)
m-Chlorobenzhydroxamate+KCN	n.t.	n.t.	30	<20
NNN'N'-Tetramethyl-*p*-phenylenediamine+ascorbate with:				
No inhibitor	340 (n.r.c.)	86 (n.r.c.)	35 (n.r.c.)	460 (n.r.c.)
KCN	<15	<15	35	35
NADH and piericidin A with:				
No additional inhibitor	195 (1.6)	80 (1.4)	315 (n.r.c.)	275 (0.7)
Antimycin A	<15	<15	315 (n.r.c.)	275 (n.r.c.)
KCN	<15	<15	315 (n.r.c.)	275 (n.r.c.)
m-Chlorobenzhydroxamate	190	n.t.	85 (n.r.c.)	275 (1.4)
m-Chlorobenzhydroxamate+KCN	n.t.	n.t.	25	10

Sensitivity of cell growth to antimycin A

When normal cells were inoculated on to solid nutrient agar plate containing antimycin A (2μg/ml) and glycerol as a non-fermentable energy source, cell growth observed at 36h was severely inhibited. The growth of variant cells under these conditions was not visibly inhibited by antimycin A.

Reaction product of the alternative oxidase

The possibility arose that the product of the antimycin A-insensitive alternative oxidase of mitochondria or submitochondrial particles from variant cells was not water but H_2O_2 as is the case with muscle mitochondria from *Ascaris lumbricoides* (Cheah & Chance, 1970). This possibility was excluded by showing that the rate of O_2 uptake in the presence of antimycin A was unaffected by a concentration of CN^- that was sufficient to decrease the catalase activity (E.C. 1.11.1.6) of the preparation to 17% of the rate of O_2 uptake (Fig. 6). The reasoning behind the experiment was this; if reaction (1):

$$SH_2 + O_2 \rightarrow S + H_2O_2 \quad (1)$$

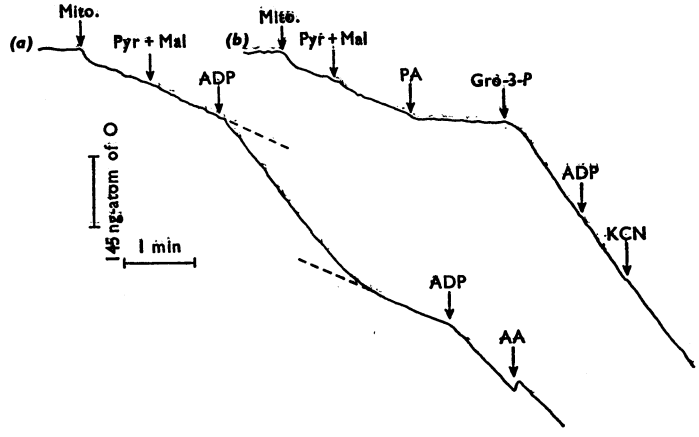

Fig. 5. *Recordings of O_2 uptake by mitochondria from copper-limited variant cells*

Mitochondria (0.8mg of protein) were suspended in 3.0ml of air-saturated incubation medium at 30°C (Light & Garland, 1971), containing 10mg of bovine serum albumin. In trace (*a*), the sequence of additions was mitochondria (Mito.), pyruvate+L-malate (Pyr+Mal, 15μmol of each), ADP (0.24μmol), again ADP (0.24μmol) and finally antimycin A (AA, 1μg). In trace (*b*), the sequence was mitochondria (Mito.), pyruvate+L-malate (Pyr+Mal, 15μmol of each), piericidin A (PA, 150pmol), DL-glycerol 3-phosphate (Gro-3-*P*, 15μmol), ADP (0.24μmol) and KCN (3μmol). The P/O ratio for the cycle of ADP phosphorylation in trace (*a*) was 0.7.

represented the alternative oxidase with SH_2 representing its reduced substrate, then the combined action of catalase, reaction (2):

$$H_2O_2 \rightarrow H_2O + \tfrac{1}{2}O_2 \qquad (2)$$

would give an overall water-producing oxidase reaction (3) that is the sum of reactions (1) and (2):

$$SH_2 + \tfrac{1}{2}O_2 \rightarrow S + H_2O \qquad (3)$$

It is clear that the rate of reaction (3) cannot be faster than the rate of either of the two part reactions, if it arises from their sum. Since CN^- decreased catalase activity to 17% of the alternative oxidase rate without affecting the alternative oxidase rate, it follows that at least 83% of the alternative oxidase must proceed through a pathway not involving catalase. The product of the alternative oxidase is therefore presumed to be water.

Sensitivity of the alternative oxidase to substituted benzhydroxamates

The CN^--resistant respiration of plant mitochondria is inhibited by substituted benzhydroxamic acids (Schonbaum *et al.*, 1971). Fig. 7 shows that the alternative oxidase of mitochondria from variant *C. utilis* cells is also inhibited by substituted benzhydroxamic acids. *m*-Chlorobenzhydroxamic acid was the most effective, although the concentration of 1mM needed for 50% inhibition of the CN^--resistant oxidase was 33-fold higher than that reported for mung bean (*Phaseolus aureus*) mitochondria and six-fold higher than that for skunk cabbage mitochondria (Schonbaum *et al.*, 1971).

Growth yields for the copper-limited variant cells

Clegg & Light (1971) showed that when one of the three phosphorylation sites associated with the respiratory chain is lost as a result of iron-limited growth, the Y_{oxygen} (defined as g of dry cell mass produced/g-atom of O consumed) fell by 37% or 29% depending whether the calculation was made by the method of Johnson (1964) or von Meyenburg (1969) respectively. The actual value of Y_{oxygen} calculated for copper-limited variant cells by the method of Johnson (1964) was 7.9 (g of dry cell wt./g-atom of O consumed), which compares with values of 15.0 and 23.8 for iron- and glycerol-limited growth respectively (Clegg & Light, 1971). Thus the Y_{oxygen} values were in the proportion 1.0:1.9:3.0 for cells where isolated mitochondria were demonstrated to have respectively one, two or three phosphorylation sites. Similar ratios were calculated if the method of von Meyenburg (1969) was used to calculate Y_{oxygen}. Thus it is clear that in this case at least, the Y_{oxygen} values are dependent linearly on the overall efficiency of oxidative phosphorylation.

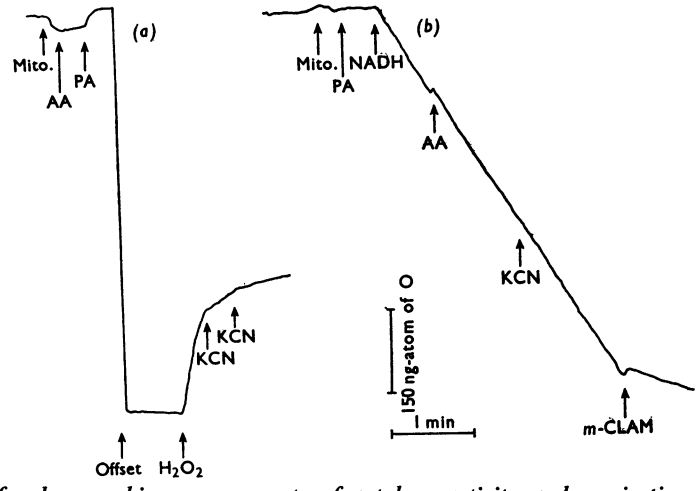

Fig. 6. *Recordings of polarographic measurements of catalase activity and respiration of mitochondria from variant cells batch-grown in complete medium*

Mitochondria were suspended as described in the legend to Fig. 5. In trace (*a*) the sequence of additions was mitochondria (Mito.), antimycin A (AA, 1 μg), piericidin A (PA, 150 pmol), H_2O_2 (H_2O_2, 3 mM) and two additions of KCN (1.5 μmol each). The recorder zero was shifted at the point marked 'Offset'. In trace (*b*) the sequence of additions was mitochondria (Mito.), piericidin A (PA, 150 pmol), NADH (5 μmol), antimycin A (AA, 1 μg), KCN (3 μmol) and *m*-chlorobenzhydroxamic acid (*m*-CLAM, 1 mmol).

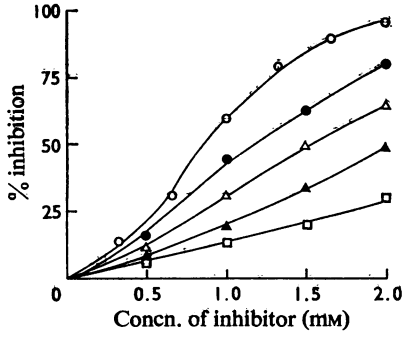

Fig. 7. *Inhibition of alternative oxidase by substituted benzhydroxamic acids*

Mitochondria (0.8 mg of protein) were incubated as described in the legend to Fig. 5, except that 0.5 mM-KCN was also present. The substrate used in all incubations was DL-glycerol 3-phosphate (5 mM). The curves represent inhibition by: ○, *m*-chlorobenzhydroxamic acid; ●, *m*-iodobenzhydroxamic acid; △, *p*-chlorobenzhydroxamic acid; ▲, *p*-fluorobenzhydroxamic acid; □, *p*-toluylbenzhydroxamic acid.

Electron-paramagnetic-resonance-spectroscopy studies of submitochondrial particles

Bonner *et al.* (1967) observed that CN^-- insensitive respiration of skunk cabbage mitochondria is sensitive to iron chelating agents, and they suggested that there was an alternative oxidase containing non-haem iron responsible for the CN^--insensitive pathway. More recently Schonbaum *et al.* (1971) observed enhancement of e.p.r. signals at $g = 2.0$ and $g = 1.94$ arising from reduced skunk cabbage submitochondrial particles on the addition of *m*-iodobenzhydroxamic acid, the most effective of the substituted benzhydroxamic acid inhibitors. We therefore obtained e.p.r. spectra of copper-deficient variant *C. utilis* submitochondrial particles in the absence and in the presence of *m*-chlorobenzhydroxamic acid, which is the most inhibitory of the substituted benzhydroxamates in the *C. utilis* system (see Fig. 7).

Fig. 8 shows e.p.r. spectra of NADH-reduced submitochondrial particles from copper-deficient variant cells measured at 12°K. The predominant features are those of the NADH dehydrogenase, as described by Orme-Johnson *et al.* (1971) for ox heart and by Ohnishi *et al.* (1972) for *C. utilis* submitochondrial particles. By using the designation of Orme-Johnson *et al.* (1971), the resonances owing to iron–sulphur centres 3+4 at approximate *g* values of 2.09, 1.88 and 1.85 are clearly resolved, as is the low-field peak of centre 2 at $g = 2.05$. The peaks at $g = 2.02$, 1.94 and 1.92 correspond to centre 1, and include contributions from the high-field peak of centre 2 ($g = 1.92$) and presumably free radical at $g = 2.00$. No novel features that might be attributed to the

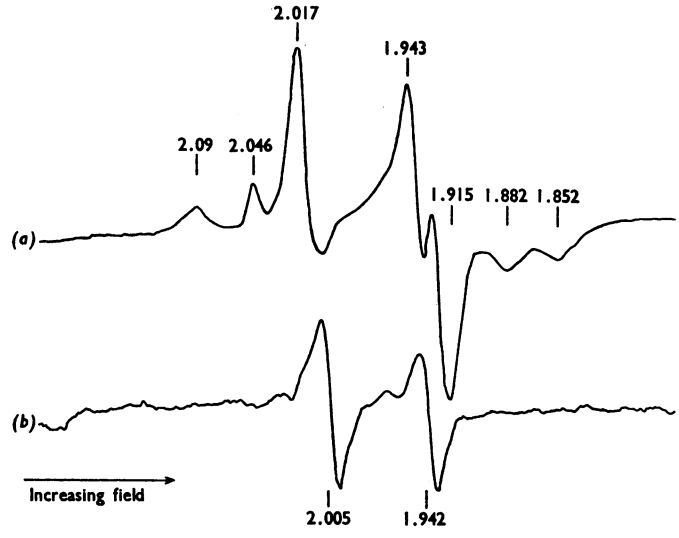

Fig. 8. *Electron-paramagnetic-resonance spectra of submitochondrial particles from copper-deficient variant C. utilis*

The particles were reduced with NADH as described in the Materials and Methods section. The protein concentration was 40mg/ml, and the time-delay between adding NADH and freezing in liquid N_2 was sufficient for anaerobiosis to be established. The upper spectrum (*a*) was measured at a sample temperature of 12°K. The modulation amplitude was 10G, the time-constant 0.3s, the scanning rate $500\,G\cdot min^{-1}$, and the microwave power 20mW. The lower spectrum (*b*) was measured at a sample temperature of 80°K. The modulation amplitude was 10G, the time-constant 1.0s, the scanning rate $250\,G\cdot min^{-1}$, and the microwave power 0.1mW. In each spectrum the microwave frequency was approximately 9.1GHz. The abscissa indicates magnetic field increasing linearly from left to right, the ordinate the first derivative of the microwave absorption in arbitrary units. The *g* values numbered in the figure correspond to the field positions given by the vertical bars.

alternative oxidase can be seen, nor did the inclusion of 3.3mM-*m*-chlorobenzhydroxamic acid alter the spectrum. The spectrum at 80°K exhibits resonances at $g = 1.94$ and $g = 2.00$ (Fig. 8). We do not observe fine structure of the $g = 2.00$ signal similar to that described for submitochondrial particles from skunk cabbage, nor was the signal increased in amplitude by the presence of 3.3mM-*m*-chlorobenzhydroxamic acid (see Schonbaum *et al.*, 1971).

Discussion

CN^--resistant respiration in yeasts has been known for many years (Pett, 1935; Stier & Castor, 1941; Eppright & Williams, 1947), although more detailed pathways of electron flow could not be elucidated in these early studies. The ability of *C. utilis* to develop a mitochondrial CN^--resistant NADH oxidase was demonstrated by Haddock & Garland (1971), and the pathway described involved a rotenone-sensitive site and also the first energy-conservation site. In these respects the NADH oxidase described in the present paper is similar, but otherwise differs in being constitutive and in retaining its activity after ultrasonic disintegration of the mitochondria. We lack genetic evidence that the appearance in our chemostat of a *C. utilis* with a constitutive alternative oxidase was due to a mutation followed by selection. Interestingly, von Jagow & Klingenberg (1972) have described a mutant of *Neurospora crassa* with an alternative oxidase similar to that of the variant *C. utilis*.

Exposure of copper-limited variant cells to copper under growth conditions resulted in the return of a CN^-- and antimycin-sensitive oxidase without loss of the insensitive alternative oxidase. When present together either oxidase alone was sufficient to sustain the maximal rate of mitochondrial O_2 uptake, from which it can be concluded that the two oxidases intercommunicate at a common branch-point, and cannot be separately allocated to two different mitochondrial

populations. On the basis of inhibitor sensitivities, P/O ratios and the usual rationale (Bendall & Bonner, 1971; Haddock & Garland, 1971), it can be concluded that the branch-point is located in the respiratory chain at about the level of cytochrome *b* or ubiquinone. Electron flow from the branch-point to O_2 via the antimycin- and CN^--resistant oxidase is not coupled to ATP synthesis.

When the variant is grown without added copper, the conventional cytochrome chain becomes a dead-end owing to absence of cytochrome oxidase. Mitochondrial respiration then depends exclusively on the alternative oxidase, and respiration-driven ATP synthesis occurs only at site 1. This much can be inferred from our observations with isolated mitochondria (see the Results section), and is demonstrated to be valid for the situation *in vivo* by the fact that the cell growth yield for O_2 of the copper-limited variant is one-half that observed when the respiratory chain has two energy-conservation sites (iron-limited normal cells), and one-third that for three sites (glycerol-limited normal cells).

The identity of the alternative oxidase is unknown. Neither visible nor e.p.r. spectroscopy demonstrated any unusual features. The unusual e.p.r.-spectral features of skunk cabbage submitochondrial particles noted by Schonbaum *et al.* (1971) do not appear to apply to *C. utilis*, despite the similarities in sensitivity towards substituted benzhydroxamic acids. Even though the alternative oxidase is unidentified there are some particular advantages offered by this variant of *C. utilis*. These are (1) an active NADH oxidase that utilizes only the first energy-conservation site, and provides a means of studying respiration-driven proton translocation at this site (Downie & Garland, 1973), (2) a readily available and non-seasonal source of material suitable for attempts at identification and isolation of the alternative oxidase, (3) a convenient system for studying the interplay between copper availability and the formation of cytochrome oxidase, and (4) the role of copper in determining the size and number of mitochondria in a cell (Dallman & Goodman, 1970; Davison *et al.*, 1972).

This work was generously supported by the Medical Research Council. J. A. D. holds a Science Research Council studentship. We are grateful to Professor E. C. Slater for providing references to earlier work on CN^--resistant respiration in yeasts, to Dr. P. A. Light for her advice on copper-limited continuous culture, to Dr. D. Evans, Dr. R. Brown and Dr. B. Storey for gifts of substituted benzhydroxamic acids, to Dr. K. Folkers for piericidin A, to Mr. T. A. Gray for preparing samples for e.p.r. spectroscopy, and to Dr. D. Lowe and Dr. R. C. Bray for the e.p.r. spectra. The wavelength scanning spectrophotometer was constructed with the aid of grant number 318 from the North Atlantic Treaty Organization. Skilled technical assistance was given by Mr. D. Coutie and Mr. A. Tucker.

References

Bendall, D. S. & Bonner, W. D. (1971) *Plant Physiol.* **47**, 236–245
Bonner, W. D., Bendall, D. S. & Plesnicar, M. (1967) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **26**, 731
Chance, B. (1953) *J. Biol. Chem.* **202**, 383–396
Chance, B. & Williams, G. R. (1956) *Advan. Enzymol.* **17**, 65–134
Cheah, K. S. & Chance, B. (1970) *Biochim. Biophys. Acta* **233**, 55–60
Clegg, R. A. & Garland, P. B. (1971) *Biochem. J.* **124**, 135–151
Clegg, R. A. & Light, P. A. (1971) *Biochem. J.* **124**, 152–154
Dallman, P. R. & Goodman, J. R. (1970) *Blood* **35**, 496–505
Davison, M. T., Downie, J. A. & Garland, P. B. (1972) *Biochem. J.* **129**, 46P–47P
Downie, J. A. & Garland, P. B. (1972) *Biochem. J.* **129**, 47P
Downie, J. A. & Garland, P. B. (1973) **134**, 1045–1049
Elvehjem, C. A. (1931) *J. Biol. Chem.* **90**, 111–132
Eppright, M. A. & Williams, R. J. (1947) *Proc. Soc. Exp. Biol. Med.* **64**, 123–125
Giorgio, A. J., Cartwright, G. E. & Wintrobe, M. M. (1963) *J. Bacteriol.* **86**, 1037–1046
Haddock, B. A. & Garland, P. B. (1971) *Biochem. J.* **124**, 155–170
Johnson, M. J. (1964) *Chem. Ind. (London)*, 1532
Katz, R. (1971) *FEBS Lett.* **12**, 153–156
Keyhani, E. & Chance, B. (1971) *FEBS Lett.* **17**, 127–132
Lehninger, A. L. (1966) *The Mitochondrion*, W. A. Benjamin Inc., New York
Light, P. A. (1972) *FEBS Lett.* **19**, 319–322
Light, P. A. & Garland, P. B. (1971) *Biochem. J.* **124**, 123–134
Lowe, D. J., Lynden-Bell, R. M. & Bray, R. C. (1972) *Biochem. J.* **130**, 239–249
Mok, T. C. K., Rickard, P. A. D. & Mass, F. J. (1969) *Biochim. Biophys. Acta* **172**, 438–449
Novick, A. & Szilard, L. (1950) *Proc. Nat. Acad. Sci. U.S.* **36**, 708–719
Ohnishi, T., Asakura, T., Wilson, D. F. & Chance, B. (1972) *FEBS Lett.* **21**, 59–62
Orme-Johnson, N. R., Orme-Johnson, W. R., Hansen, R. E., Beinert, H. & Hatefi, Y. (1971) *Biochem. Biophys. Res. Commun.* **44**, 446–452
Pett, L. B. (1935) *Biochem. J.* **29**, 937–944
Schonbaum, G. R., Bonner, W. D., Storey, B. T. & Bahr, J. T. (1971) *Plant Physiol.* **47**, 124–128
Stier, T. J. B. & Castor, J. G. B. (1941) *J. Gen. Physiol.* **25**, 229–233
von Jagow, G. & Klingenberg, M. (1972) *FEBS Lett.* **24**, 278–282
von Meyenburg, K. (1969) in *Continuous Culture of Microorganisms* (Malek, I., Beran, K., Fenel, Z., Munk, V., Ricica, J. & Smekova, H., eds.), pp. 129–140, Academia, Prague
Ware, G. C., Thomson, A. S. & Light, P. A. (1970) *Lab. Pract.* **19**, 181–182
Wohlrab, H. & Jacobs, E. (1967*a*) *Biochem. Biophys. Res. Commun.* **28**, 991–997
Wohlrab, H. & Jacobs, E. (1967*b*) *Biochem. Biophys. Res. Commun.* **28**, 998–1102