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Influence of Specific Growth Limitation and Dilution Rate on the Phosphorylation Efficiency and Cytochrome Content of Mitochondria of *Candida utilis* NCYC 321

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Abstract. With *Candida utilis* cells that had been removed directly from a 6 l chemostat culture, in steady state, well-coupled mitochondria generally could be isolated. This required a modified snail-gut enzyme procedure that allowed the total processing time to be decreased to 3 h, or less. Examination of these mitochondria in an oxygraph showed the presence of 3 sites of energy conservation when the cells were grown at various dilution rates between 0.1 and 0.45 h^{-1} in environments that were, successively, glucose-, ammonia-, magnesium-, phosphate- and sulphate-limited. Potassium-limited cells also apparently possessed 3 sites of oxidative phosphorylation when growing at dilution rates greater than 0.2 h^{-1}, but only 2 sites when growing at lower dilution rates. Analysis of cytochrome spectra obtained with these intact mitochondria revealed large quantitative (but not qualitative) differences, depending on the environmental conditions under which the yeast had been cultured. In particular, comparison of the ratio of cytochrome *b* to cytochrome *a* showed a pattern of change with dilution rate in mitochondria from potassium-limited cells that was distinctly different from those evident in mitochondria from cells that had been limited in their growth by the availability of other nutrients.

Key words: Candida utilis – Continuous culture – Mitochondria – Oxidative phosphorylation – Cytochromes – Respiratory chain – Potassium-limitation – Sulphate-limitation – Phosphate-limitation – Magnesium-limitation.

Although potassium is seemingly a major constituent of all living cells, the precise physiological considerations underlying an organisms' quantitative requirement for this cation are not known with certainty. Potassium is, of course, known to be involved in a number of cellular functions among which appears to be an involvement in the energy-generating reactions of oxidative phosphorylation (Blond and Whittam, 1965; Gómez-Puyou et al., 1972). This led us to investigate the efficiency with which oxygen and glucose were used for cell synthesis when *Candida utilis* was grown in a potassium-limited chemostat culture. Interestingly, we observed an almost linear relationship between the cellular potassium content (which varied with growth rate) and the yield values for both glucose (Y_g) and oxygen (Y_O) which suggested that potassium was, in some way, stoicheiometrically related to the efficiency with which ATP was generated and/or utilized for cell synthesis (Aiking and Tempest, 1976). However, the relationship between Y_O and growth rate (D) was not uniformly linear, and when plotted as their reciprocals showed a sharp inflection at the lower growth rates that could be interpreted as showing the loss of one site of oxidative phosphorylation at the lower growth rates. Hence we were led to examine mitochondria that had been isolated from cells grown in a potassium-limited chemostat culture at a low dilution rate (0.1 h^{-1}) and to compare them with mitochondria from correspondingly slowly-grown glucose-limited cells. This indeed showed the loss of either site 2 or site 3 of oxidative phosphorylation (Aiking and Tempest, 1976).

From these preliminary observations, a number of questions arose: in particular, (i) whether the loss of one site of oxidative phosphorylation was due to potassium-limitation per se or to catabolite repression imposed by the presence of excess unutilized glucose in the culture, and (ii) whether this site of oxidative phosphorylation was regained at the higher growth rates, as the Y_O data suggested. Thus it became necessary to isolate mitochondria from cells that had been cultivated in a chemostat under a wide range of conditions, i.e., several different dilution rates between 0.1 and 0.45 h^{-1} with, as growth-limiting substrate, the culture content of glucose, ammonia,

Table 1. Composition of the media

Component	Growth-limitation						Units (final conc.)
	C	K	S	N	P	Mg	
Glucose	25	150	150	150	150	150	mM
NaH_2PO_4	5	5	5	5	0.25	5	mM
NH_4Cl	45	45	50	3	45	45	mM
$(NH_4)_2SO_4$	2.5	2.5	0.05	2.5	2.5	2.5	mM
KCl	5	0.075[a]	5	5	5	5	mM
NaCl	–	–	–	42	5	–	mM
Citric acid	1	1	1	1	1	1	mM
$MgCl_2$	0.63	0.63	0.63	0.63	0.63	0.05	mM
$CaCl_2$	0.02	0.02	0.02	0.02	0.02	0.02	mM

Trace-elements / Vitamins were added throughout to final concentrations of: 0.05 mM $MnCl_2$, 0.025 mM $ZnCl_2$, 0.01 mM $CoCl_2$, 0.005 mM $CuCl_2$, 0.1 mM $FeCl_3$, 0.004 mM H_3BO_3, 0.1 µM $NaMoO_4$, 0.55 mM inositol, 0.16 mM nicotinic acid, 0.02 mM calcium pantothenate, 0.013 mM pyridoxine-HCl, 0.006 mM thiamin-HCl, and 0.02 µM biotin.

The 150 mM glucose might be interchanged with 350 mM ethanol or 300 mM glycerol; the NaCl was added for osmotic constancy of the media

[a] at $D = 0.1\ h^{-1}$ only; 0.3 mM at $D = 0.2\ h^{-1}$ and $0.3\ h^{-1}$ and 0.6 mM at $D = 0.4\ h^{-1}$ or higher

Note: The symbols C, K, S, N, P, and Mg were used throughout and stand for, respectively, glucose-, potassium-, sulphate-, ammonia-, phosphate- and magnesium-limitation

potassium, magnesium, phosphate or sulphate, and as the carbon source, either glucose, glycerol or ethanol. Examination of mitochondria from these variously-grown organisms allowed conclusions to be drawn that contrast sharply with those of other workers (Light and Garland, 1971; Haddock and Garland, 1971) but which support our previous finding that a low cellular potassium content dramatically affects the functioning of the respiratory chain.

In our earlier experiments we routinely cultured *C. utilis* in a 0.5 l Porton-type chemostat (Herbert et al., 1965), and in order to obtain a sufficiently large quantity of cells, required for the preparation of mitochondria, it was necessary to collect the effluent culture (in an ice-cooled receiver) over many hours. Invariably these cells possessed walls that were resistant to digestion by Helicase, and therefore needed pretreatment with either mercaptoethanol or thioglycollate in order to render them susceptible. Moreover, with cultures growing at a low dilution rate, the mitochondria that were then isolated tended to be of a poor quality. Hence we changed to using a larger fermentor (of 6 l working volume) from which sufficient starting material (about 40 g wet weight of cells) could be obtained by draining the whole content of the culture vessel into a cooled receiver prior to the start of each experiment. This not only resulted in a marked improvement in the quality of the mitochondria, but also allowed the isolation procedure to be simplified since these "fresh" cells were considerably more susceptible to the Helicase treatment: indeed, so much so that the pretreatment step with a reducing agent was rendered unnecessary – even undesirable. Thus, the total preparation time was decreased to 3 h, or less, and active, well-coupled, mitochondria were obtained consistently. This modified procedure is detailed in the Methods section.

MATERIALS AND METHODS

Organism. Candida utilis NCYC 321 was maintained by monthly subculture on a yeast extract-peptone-glucose medium (pH 5.5) solidified with 2% (w/v) agar.

Media. The media were composed as described in Table 1. Double glass distilled water was used throughout. The glucose (a 40%, w/v, solution) and the vitamins were separately heat sterilized at 110° C for 20 min, and added to the bulk solution of salts which had been heated at 120° C for 1 h.

Culture Conditions. The organisms were grown in a P.E.C. fermentor (Chemap AG, Männedorf, Switzerland) with a working volume of 6 l. In all cases the temperature was controlled at 30° C and the pH value at 5.5. The titrant was a 4 N NaOH solution (1 N in the case of phosphate-limitation) of "Suprapur" quality (containing less than 0.0002% K^+) and was added automatically to maintain the culture pH value within ±0.05 of the set value. All media were designed to give a steady state equivalent dry weight of 2.0 ± 0.5 mg organisms per ml of culture fluid.

Mitochondria. These were prepared by a modification of the method of Kováč et al. (1968), using the following buffer solutions. Solution A contained: 0.7 M sorbitol, 0.3 M mannitol, 1 mM EDTA, 10 mM citrate, 20 mM KH_2PO_4. The pH was brought to 5.8 with NaOH. Solution B contained 1.5 M sorbitol and 2 mM EDTA (pH 7.6). Solution C contained 0.44 M mannitol, 1 mM EDTA and 1 g/l bovine serum albumin – essentially fatty acid free (pH 7.6). Solution D contained 0.65 M mannitol and 0.5 mM EDTA (pH 6.5).

When the culture had reached a steady state (50 h at $D = 0.1\ h^{-1}$ or 24 h when at $D \geqslant 0.2\ h^{-1}$), its whole 6 l were harvested rapidly (within 5 min) into a pre-cooled bottle and 6 pre-cooled centrifuge tubes (500 ml each). In 2 runs (2.5 min at $1500 \times g$) in a Sorvall RC-2B centrifuge (Sorvall, Connecticut, U.S.A.), which was held at 0° C, throughout, the cells (a mean of 40 g wet weight) were spun down. From that moment on, an SS-34 rotor (8×50 ml) was used. The cells were washed once with water (2.5 min at $3000 \times g$), once with solution A (2.5 min at $6000 \times g$) and then suspended in a 50 ml solution of snail-gut enzyme (Helicase, Industrie Biologique Française S.A., Gennevilliers, France) in solution A. The original concentration of Helicase was 40 mg/ml, but the same solution could be used repeatedly by storing it at −20° C and adding an additional 300 mg of Helicase (to the 50 ml batch) each time it was re-used, checking whether the pH was 5.8, still. The cells were then incubated at 30° C, and every 2 min a 0.2 ml sample was taken, of wich 0.1 ml was added to 2.5 ml solution A and 0.1 − 2.5 ml solution C (low osmotic value). Both were thoroughly mixed and a drop of each was inspected microscopically (magnification 100 ×). As soon as the sample in solution C contained 50% less whole cells than the one in solution A, the incubation was stopped (6 − 20 min, depending on the history of the Helicase-solution and on the nature of the growth-limitation; ammonia- and potassium-limited cells breaking easily, phosphate-limited ones difficultly). From now on the centrifuge was used without brake. The protoplasts were centrifuged for 10 min at $3000 \times g$; the supernatant Helicase solution was kept in the deep freeze for the next time. The protoplasts were next washed 3 times with solution B (10 min at $1500 \times g$), and then were suspended in 150 ml of solution C and mixed for 20 s in a Braun type MX 3 mixer (Braun, Frankfurt, W.-Germany) at maximum speed. The debris were removed by centrifuging for 10 min at $1500 \times g$, and the mitochondria were separated by centrifuging the supernatant for 10 min at $8000 \times g$. The pellet was suspended in 10 ml solution D, homogenized in a small glass Potter-Elvehjem homogenizer with teflon pestle and centrifuged for 5 min at $1200 \times g$. The supernatant was centrifuged for 12 min at $18000 \times g$. Then the supernatant fluid was removed and the surface of the purified mitochondrial pellet was carefully washed with 1 − 2 ml solution D. This pellet was finally suspended in 1 − 2 ml solution D, homogenized and a sample was taken for a protein determination by the method of Lowry et al. (1951). The complete procedure – but for the incubation – was performed at 0° C, as much as possible. It generally yielded well-coupled mitochondria in less than 3 h; the quantity being about 1 mg of mitochondrial protein per g wet weight of cells, except with organisms grown at low dilution rates, where the yield was less.

Polarographic Experiments. P/O ratios were determined polarographically as described by Chance and Williams (1956). The 1.5 ml oxygraph vessel contained 0.6 M sorbitol, 10 mM TRIS, 10 mM maleic acid, 10 mM KCl, 10 mM K_2HPO_4, 1 mM EDTA, 10 mM substrate, 2.5% (w/v) bovine serum albumin (essentially fatty acid free) and mitochondria (pH 6.5). To determine the P/O ratio 5 µl 50 mM ADP was added. The ADP concentration was checked each time by the method of Adam (1962).

Cytochrome Spectra. These were recorded using an Aminco Chance DW-2 Spectrophotometer (American Instrument Co., Silver Spring, Maryland, U.S.A.). To one of two identical cuvettes, containing about 1 mg of mitochondrial protein in 2 ml of the same medium as described above for polarographic measurements (minus substrate and serum albumin), sodium dithionite was added as a reductant and a 500 − 650 nm spectrum was recorded in duplicate at 5 nm/s. Cytochrome contents were calculated from their molar extinction coefficients (van Gelder, 1966; Berden and Slater, 1970; Ohnishi et al., 1966a), after correction for the baseline.

RESULTS

Previous experiments (Aiking and Tempest, 1976) showed that mitochondria from potassium-limited *Candida utilis*, that had been grown at a low dilution rate ($0.1\ h^{-1}$), lacked one site of oxidative phosphorylation as compared with mitochondria from glucose-limited organisms that had been grown at a correspondingly low rate. Thus, assuming a recovery per site of 0.6 (molecules ADP phosphorylated per functional site per atom of oxygen used; see Discussion), the P/O values found with, respectively, 2-oxoglutarate (+ malonate), ethanol, pyruvate plus malate, and NADH, were close to 4:3:3:2 with mitochondria from glucose-limited cells, and 3:2:2:1 with mitochondria from potassium-limited cells. And since exogenous NADH is oxidized in yeast via an external NADH-dehydrogenase that transfers the electrons to ubiquinone (that is, past site 1; Ohnishi et al., 1966b), it must be concluded that this missing site is either site 2 or 3. With these potassium-limited cultures, progressively increasing the growth rate caused the P/O ratios found with the above-mentioned four substrates to increase (Table 2). More significantly, however, the relationship of these ratios to one another also changed, and at growth rates above $0.2\ h^{-1}$ were close to 4:3:3:2, indicating the presence of three sites of oxidative phosphorylation. This finding accords completely with the prediction, based on Y_O data (Aiking and Tempest, 1976) that a shift from two to three sites of oxidative phosphorylation occurred in the mitochondria of potassium-limited *C. utilis* at, or about, a dilution rate of $0.2\ h^{-1}$.

Since it was possible that, in these potassium-limited *C. utilis* organisms, deletion of one site of oxidative phosphorylation was caused by catabolite repression, and not by potassium-limitation per se, mitochondria were isolated from organisms that were, successively, glucose-, ammonia-, and magnesium-limited, and the P/O ratios with respect to six substrates compared (Table 3). As expected, the number of sites present in the mitochondria of glucose-limited organisms was invariably three, irrespective of the growth rate. However, the same was true for ammonia-limited organisms, although mitochondria from cells grown at a dilution rate of $0.1\ h^{-1}$ were generally of a poor quality. In this latter connection, it was found that ammonia-limited organisms, grown at a low dilution rate, were extremely sensitive to Helicase treatment, and it is probably for this reason that we failed repeatedly to obtain mitochondria from such cells that were more than slightly coupled – and even these deteriorated within a few minutes.

Well-coupled mitochondria could, however, be obtained from slowly-growing magnesium-limited

Table 2. *P/O* ratios obtained with mitochondria that had been isolated from potassium-limited *Candida utilis* NCYC 321, grown in a chemostat at several dilution rates (30° C; pH 5.5)

Exp.	D (h^{-1})	2-Oxoglutarate (+ malonate)	Pyruvate + malate	Ethanol	NADH	α-Glycerol-phosphate	Succinate
1	0.093	1.74 (2.00)	1.16 (3.57)	0.84 (2.80)			
2	0.097	1.77 (2.00)	1.20 (3.00)	1.20 (2.20)	0.57 (1.10)		
3	0.098	1.86 (1.80)	1.19 (1.90)	1.39 (1.43)	0.89 (1.20)	1.16 (1.21)	
4	0.103	1.67 (1.70)	1.01 (1.83)	1.24 (1.27)	0.79 (1.72)	0.81 (1.24)	– (1.00)
5	0.108	1.87 (1.37)	1.65 (1.51)	1.62 (1.37)	0.97 (1.20)	0.65 (1.13)	
6	0.207	2.36 (1.80)	1.89 (1.81)	1.97 (1.25)	1.35 (1.58)	1.29 (1.29)	1.28 (1.13)
7	0.213	1.93 (2.02)	1.40 (2.41)	1.20 (1.55)	1.14 (1.58)	1.05 (1.43)	1.05 (1.09)
8	0.229	2.58 (1.57)	1.81 (2.39)	1.75 (1.67)	1.20 (1.64)	1.29 (1.55)	– (1.00)
9	0.289	2.63 (1.71)	1.95 (2.22)	1.82 (1.52)	1.46 (1.31)	1.39 (1.37)	1.10 (1.12)
10	0.296	2.23 (1.50)	1.80 (1.92)	2.12 (1.26)	1.34 (1.37)	1.34 (1.14)	– (1.00)
11	0.423	2.66 (1.57)	1.77 (2.47)	1.97 (1.48)	0.96 (1.55)	1.14 (1.28)	1.37 (1.13)

Between brackets is given the respiratory control value (RC), defined by Chance and Williams (1956) as the ratio of the rate of oxygen consumption in state 3 relative to state 4

Table 3. *P/O* ratios obtained with mitochondria that had been isolated from either glucose- (C), ammonia- (N) or magnesium-limited (Mg) *Candida utilis* NCYC 321, grown in a chemostat

Limitation	D (h^{-1})	2-Oxoglutarate (+ malonate)	Pyruvate + malate	Ethanol	NADH	α-Glycerol-phosphate	Succinate
C	0.094	2.05 (1.90)	1.83 (2.00)	1.80 (2.40)	1.20 (1.10)	1.00 (1.20)	0.83 (1.20)
	0.093	2.31 (1.80)	1.77 (1.90)	1.80 (2.40)			
	0.092	2.11 (2.28)	1.97 (2.52)	2.11 (1.73)	1.05 (2.26)	1.05 (1.32)	
	0.284	2.97 (2.31)	2.17 (2.08)	2.09 (2.26)	1.57 (1.60)	1.41 (1.53)	1.18 (1.44)
	0.394	2.68 (2.00)	2.11 (1.50)				
N	0.195	2.54 (2.24)	1.86 (1.93)	1.66 (1.44)	1.21 (1.77)	1.18 (1.43)	0.78 (1.93)
	0.295	2.40 (2.03)	1.94 (2.49)	– (1.00)	1.17 (2.29)	1.17 (1.88)	– (1.00)
Mg	0.108	1.92 (1.70)	1.74 (2.70)	1.92 (1.93)	1.17 (1.42)	– (1.00)	– (1.00)

The value between brackets is the RC (compare Table 2)

C. utilis, and from the results obtained with ethanol, pyruvate + malate and NADH (Table 3) it is clear that a high level of glucose in the environment does not, in itself, provoke suppression of a site of oxidative phosphorylation. A low value was, however, found with 2-oxoglutarate (+ malonate) suggesting that a low level of magnesium in the culture may affect the efficiency of substrate-level phosphorylation (which is strongly Mg^{2+} dependent).

In order to further extend our observations concerning the physiology of *C. utilis* when growing in glucose sufficient chemostat culture, phosphate- and sulphate-limitations also were examined since other workers (see Light, 1972) had found that mitochondria from the former were virtually uncoupled, and those from the latter lacked site 1. From the *P/O* ratios observed with different substrates (Table 4) it can be seen that phosphate-limited *C. utilis* contained well-coupled mitochondria with three sites of oxidative phosphorylation. More surprisingly, the mitochondria from sulphate-limited cells also seemingly possessed three sites, even when grown at a dilution rate of 0.1 h^{-1}. However, Haddock and Garland (1971), who, in their classic study, showed site 1 to be missing from sulphate-limited *C. utilis* NCYC 193 grown at a dilution rate of 0.17 h^{-1}, used glycerol, and not glucose, as the carbon and energy source. Hence we repeated our experiments with *C. utilis* NCYC 321, replacing glucose in the growth medium by glycerol. Nevertheless, three sites still were present (Table 4).

It is of interest to note that the mitochondria from sulphate-limited cells, as well as those from phosphate-limited cells, showed almost no respiratory control when incubated with either succinate or ethanol, and this correlated with extremely low oxygen uptake rates (less than 100 ng atoms oxygen/min × mg protein; uncorrected for endogenous metabolism) in the presence of these substrates. This may not be uncommon for succinate, but the ethanol dehydrogenase system routinely was found to have a high activity when the organisms were otherwise

Table 4. *P/O* ratios obtained with mitochondria that had been isolated from either phosphate- (P) or sulphate-limited (S) *Candida utilis* NCYC, grown in a chemostat

Limitation	D (h^{-1})	2-Oxoglutarate (+ malonate)	Pyruvate + malate	Ethanol	NADH	α-Glycerol-phosphate	Succinate
P	0.218	2.31 (3.02)	1.74 (1.79)	– (1.00)	0.96 (1.60)	0.99 (1.40)	– (1.00)
	0.205	2.04 (1.87)	1.67 (2.55)		– (1.00)		
	0.195		2.31 (1.67)			1.44 (1.19)	
S	0.106	2.23 (2.40)	2.49 (2.97)	– (1.00)	0.91 (2.10)	1.11 (1.95)	
	0.104	2.32 (1.95)	1.87 (2.73)		1.35 (2.14)		
	0.098	2.55 (3.04)	1.84 (2.62)		1.12 (1.84)	1.15 (1.52)	– (1.00)
	gly 0.128	2.56 (2.07)	1.95 (2.40)	– (1.00)	1.13 (1.76)		
	gly 0.100	2.51 (2.24)	1.94 (2.96)		1.22 (1.87)	1.19 (1.67)	– (1.00)
S	0.201	2.39 (1.97)	1.82 (2.21)	– (1.00)	1.14 (1.90)	– (1.00)	1.05 (1.19)
	0.192	2.36 (2.20)	1.71 (3.00)	– (1.00)	1.18 (1.72)	1.18 (1.44)	– (1.00)
	0.204	2.82 (1.97)	1.51 (1.53)	2.12 (1.33)	1.11 (1.38)	1.26 (1.18)	– (1.00)
	gly 0.202	2.59 (1.83)	2.24 (2.17)	1.76 (1.24)	0.68 (1.10)	0.72 (1.19)	
	gly 0.203	2.86 (2.57)	2.12 (2.81)	– (1.00)	0.90 (1.57)	0.95 (1.47)	

gly refers to experiments in which glucose as a carbon source had been replaced by glycerol; also compare Table 3

Table 5. The cytochrome content of coupled mitochondria from *Candida utilis* NCYC 321 grown under a variety of conditions in chemostat culture (30°C; pH 5.5)

Limitation	C-source	D (h^{-1})	Cyt *a*	Cyt *b*	Cyt *c*	Cyt *b*/cyt *a*
			(nmoles/mg protein)			
K	glucose	0.098	0.075	0.194	0.147	2.58
	glucose	0.108	0.084	0.301	0.198	3.58
	ethanol	0.118	0.174	0.424	0.307	2.44
	glucose	0.213	0.116	0.517	0.194	4.46
	glucose	0.207	0.133	0.454	0.311	3.41
	glucose	0.289	0.176	0.445	0.335	2.52
	glucose	0.296	0.202	0.445	0.291	2.20
	glucose	0.423	0.102	0.494	0.298	4.84
C	glucose	0.093	0.135	0.243	0.145	1.80
	glucose	0.284	0.119	0.470	0.287	3.95
	glucose	0.394	0.111	0.549	0.227	4.96
N	glucose	0.105[a]	0.042	0.223	0.105	5.33
	glucose	0.105[a]	0.056	0.279	0.166	4.95
	glucose	0.195	0.076	0.392	0.255	5.14
	glucose	0.295	0.062	0.287	0.245	4.67
S	glucose	0.098	0.106	0.170	0.099	1.60
	glucose	0.192	0.082	0.262	0.176	3.18
	glycerol	0.203	0.068	0.136	0.152	2.00
P	glucose	0.218	0.222	0.292	0.303	1.31
	glucose	0.195	0.176	0.234	0.165	1.33
Mg	glucose	0.093	0.134	0.374	0.223	2.79

All mitochondria were well-coupled and the same as from Tables 2–4, except the ones indicated with [a], that were only slightly coupled

growth-limited (usually 300, but occasionally up to 800 ng atoms oxygen/min × mg protein), and its level is commonly used as a measure for intactness of a mitochondrial preparation. Thus, Beck et al. (1968) proposed a minimum ethanol oxidizing activity of 100 ng atoms oxygen/min × mg mitochondrial protein as an obligatory (though not definitive) condition for intactness of mitochondria, as the enzyme is soluble and readily washed from the mitochondria during the preparation procedure if they are damaged. Such an explanation could not, however, account for the low activities in our preparations which were

found to be extremely well coupled when tested with other substrates (Table 4). Therefore we must conclude that intramitochondrial ethanol dehydrogenase synthesis is suppressed in organisms growing under phosphate- or sulphate-limiting conditions.

In order to decide whether it is site 2 or 3 that is suppressed in potassium-limited *C. utilis*, when growing at a low rate, it is necessary to determine *P/O* ratios using electron donor systems that react with components of the respiratory chain beyond site 2. Such can be provided by TMPD/ascorbate: however, even with mitochondria from glucose-limited organisms ($D = 0.1\ h^{-1}$) we could find no coupling to phosphorylation. Thus we turned to examining components of the respiratory chain to see whether there were any quantitative or qualitative differences that would allow one to decide where potassium-limitation was exerting its main effect. Previously (Aiking and Tempest, 1976) we had found the mitochondria of potassium-limited *C. utilis* to contain each of the three types of cytochromes (*a*, *b*, *c*) that were present in mitochondria from glucose-limited cells, but that the quantity of *b*, relative to *a*, was different (ratios of 2.5 and 1.8 for potassium- and glucose-limited organisms, respectively, grown at a dilution rate of $0.1\ h^{-1}$). In the present investigation, these findings were extended to other growth limitations, and higher growth rates (Table 5). The figures quoted here were, with the exception of ammonia limitation at a low growth rate (see above), all obtained with mitochondrial preparations that showed good coupling, and reveal that the amounts of the different cytochromes present are highly dependent on the nature of the growth-limiting substrate, the carbon source, and the expressed growth rate. However, there is seemingly no correlation between cytochrome contents of these different mitochondrial preparations and the number of sites of oxidative phosphorylation. On the other hand, a comparison of the cytochrome *b* to cytochrome *a* ratios in these isolated mitochondria reveals a pattern of change, in potassium-limited cultures, that is strikingly different from either glucose-limited or ammonia-limited organisms (Fig. 1). Thus, whereas at dilution rates below $0.2\ h^{-1}$, the cytochrome *b*/cytochrome *a* ratio is significantly greater in mitochondria from potassium-limited cells than that ratio in mitochondria from glucose-limited cells, it is either similar or lower at dilution rates greater than $0.2\ h^{-1}$. But a high ratio of cytochrome *b*/cytochrome *a* is not in itself an indicator of the number of sites of energy conservation since mitochondria from ammonia-limited cells and glucose-limited cells possessed higher and lower ratios, respectively, when grown at a dilution rate of $0.2\ h^{-1}$ or less, than was found with mitochondria from potassium-limited cells, and yet both possessed three sites of oxidative phosphorylation. Nevertheless, the sharp discontinuity found between the cytochrome *b*/cytochrome *a* ratio of potassium-limited cells growing at dilution rates of 0.2 and $0.3\ h^{-1}$, respectively, can hardly be fortuitous since the differences were consistently expressed with four separate preparations from cells grown at each dilution rate.

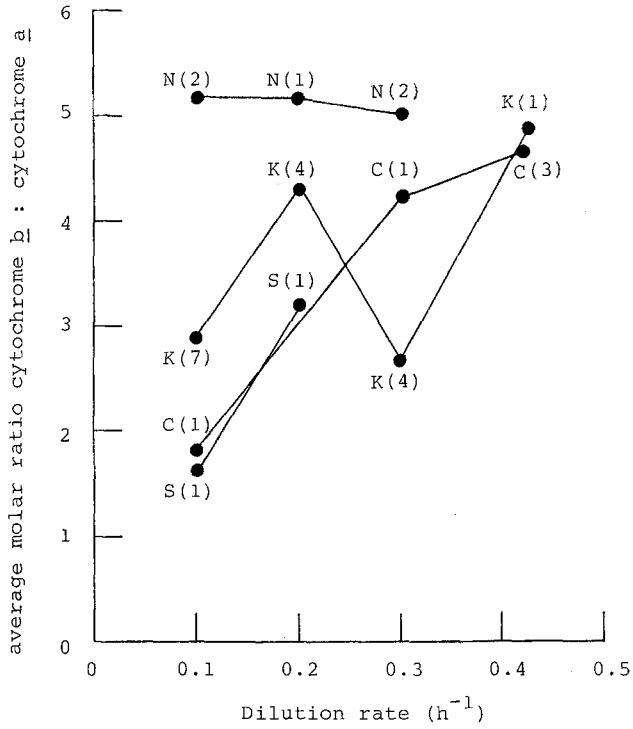

Fig. 1. The average molar ratio of cytochrome *b* to cytochrome *a* determined from mitochondrial preparations (as described in Table 5) in relation to the dilution rate (*D*). The letters indicate the growth-limitation used, whereas the numbers refer to the number of experiments used to determine this average

DISCUSSION

Since the oxidation of NADH by mitochondrial preparations of yeast can lead to the phosphorylation of, maximally, 2 molecules of ADP per atom of oxygen consumed (Ohnishi et al., 1966b), then assuming both sites 2 and 3 of energy conservation to be present in our glucose-limited organisms, the "recovery per site" was, in our experiments, about 60–70% of the theoretical maximum (Table 3). Although this figure is considerably lower than that reported by Garland's group (Light and Garland, 1971; Haddock and Garland, 1971), and by Ohnishi et al. (1966b), it is similar to that found by Kováč et al. (1968) and Beck et al. (1968). We should add here that all our calculations were based upon initial ADP concentrations

that were determined enzymically whereas spectrophotometric assays at 259 nm yielded values that were consistently 25% higher. Anyhow, using a recovery factor of 0.6 per site, it is clear that the corrected *P/O* values found with both ethanol and pyruvate plus malate were close to 3 and with 2-oxoglutarate (+ malonate) close to 4 – the values expected for these substrates (Table 3), at least for glucose-limitation. But whether or not one uses this correction factor to assess the number of sites of energy conservation present in our mitochondrial preparations, it is clear that the values found with the above four substrates were consistently lower when mitochondria from slowly-growing potassium-limited organisms were examined. Thus, when oxidizing 2-oxoglutarate (+ malonate) the values were 1.78 (±0.08) versus 2.16 (±0.14) for preparations from potassium-limited and glucose-limited organisms, respectively: and other corresponding ratios were 1.24 (±0.24) versus 1.86 (±0.10) for pyruvate plus malate, 1.26 (±0.29) versus 1.90 (±0.18) for ethanol, and 0.81 (±0.17) versus 1.13 (±0.11) for NADH. These results are compatible with our previous report (Aiking and Tempest, 1976) that the respiratory chain of slowly-growing potassium-limited *Candida utilis* lacks one site of oxidative phosphorylation.

Although these slowly-growing potassium-limited organisms clearly possess a defective respiratory chain, this is not the case with similarly-limited organisms growing at a dilution rate of 0.2 h^{-1}, or greater (Table 2). This again accords with predictions made previously, based on Y_O data (Aiking and Tempest, 1976) that a loss of energy conservation only occurs at low dilution rates. Further, it strongly suggests that deletion of one site of oxidative phosphorylation is a specific consequence of potassium-limitation and not due to catabolite repression exerted by the unconsumed glucose present in the culture.

In this latter connection, the data contained in Tables 3 and 4 show that mitochondria from several other glucose-sufficient cultures (ammonia-, magnesium-, phosphate-, or sulphate-limited) also oxidize various substrates with *P/O* ratios similar to those of mitochondria from glucose-limited organisms, confirming that sites of oxidative phosphorylation are not repressed solely by a high level of glucose in the culture. However, a mitochondrial preparation from slowly-grown potassium-limited organisms, cultured in a medium in which glucose was replaced by ethanol, gave *P/O* ratios with 2-oxoglutarate (+ malonate) and pyruvate plus malate that suggested the presence of three sites of energy conservation. Thus glucose possibly may act in conjunction with potassium limitation to suppress formation of either site 2 or 3 of energy conservation.

We were surprised to find that the mitochondria from sulphate-limited *C. utilis* showed no evidence of deletion of site 1 activity since such had been convincingly demonstrated by Haddock and Garland (1971) with a different strain (NCYC 193) of this organism. Initially we thought that this discrepancy between our results could be due to our using different carbon sources (glucose versus glycerol) in our culture media. However, changing our medium from one containing glucose to one containing glycerol did not effect a change in phosphorylating efficiency of the isolated mitochondria: three sites seemingly were still present (Table 4). The only remaining differences between the medium which we used and that used by Haddock and Garland (1971) is the presence of a number of vitamins (in our standard medium) and the absence of Mn^{2+} and Zn^{2+} (in their medium). Commenting upon the omission of manganese from the medium (which was necessary in order to carry out e.p.r. studies), Light (1972) stated that "Although the absence of manganese in no way affected any of the properties of the cells or the subsequently derived mitochondria or sub-mitochondrial particles which are described here, it should nevertheless be stressed that a complete investigation into the effects of manganese deficiency has not been undertaken". Our results suggest that such an investigation is, indeed, highly desirable, if not essential.

The use of continuous culture techniques for studying the physiology of microorganisms, and subcellular components derived from them, has many distinct advantages (Tempest, 1970; Light and Garland, 1971; Light, 1972), but one possible disadvantage arises from the fact that substantial quantities of biomass, often required for biochemical investigation, can only be accumulated by collecting the effluent culture over several hours. And this is particularly the case when chemostats of moderate size (say, 0.5 l working volume) are operated at low dilution rates (say, 0.1 h^{-1}). Thus, one of the objects of using continuous culture, namely that of obtaining a highly homogeneous population whose physiological properties depend solely on the environmental conditions imposed in the chemostat, could be negated by changes during storage. Light and Garland (1971) state that (in their work) "This possible drawback does not appear to have materialized": however, our experience is different. For not only was it difficult to prepare mitochondria from slowly-growing organisms that had been held up to 20 h in an ice-cooled receiver, but these preparations were invariably of a poor quality. This may, of course, have been due to our initial inexperience in carrying out this complex procedure; nevertheless, we were consistently able to isolate mitochondria from batch-grown *C. utilis* and also

from organisms that had been drained directly from a larger (6 l) chemostat. Moreover, the walls of these "fresh" continuously-grown organisms were considerably more sensitive to Helicase; so much so that treatment with reduced sulphur-containing compounds was no longer necessary. Thus, with these cells, the time required to effect a greater than 50% conversion of cells into spheroplasts varied from as little as 6 min up to, maximally, 20 min, depending largely on the nature of the growth-limitation. This contrasts strikingly with the 30–60 min enzyme treatment used by Kováč et al. (1968), and which required pretreatment of the cells for 30 min with thioglycollate, and the procedure of Light and Garland (1971) whose cell suspensions required a 60 min treatment of snail-gut enzyme (in the presence of 2-mercaptoethylamine) in order to achieve a 70–90% conversion of cells into spheroplasts. In this connection, a low pH value in the growth medium, such as commonly occurs towards the end of the exponential phase of a batch culture, is known to render cells resistant to digestion by snail-gut enzyme (Light and Garland, 1971). And, in our experience, the pH value of the effluent culture collected into an ice-cooled receiver overnight (16 h) was frequently found to have fallen from 5.5 (the control value in the chemostat) to 3.5. Clearly, this could be the reason for our own inability to isolate well-coupled mitochondria from an overnight collection of effluent culture and the difficulty which Light and Garland (1971) experienced in obtaining coupled mitochondria from phosphate-limited (and therefore unbuffered) cultures. If so, this supports our contention that caution is needed in too readily assuming that the physiological properties of organisms do not vary significantly during storage in an ice-cooled receiver for prolonged periods.

The results contained in this paper fail to reveal the nature of the basic physiological considerations underlying this organisms' quantitative requirement for potassium; they even seem to suggest that potassium is operating incredibly specifically, exerting an effect on one site (cytochrome) only. And because only a *general* effect of potassium on coupling of oxidative phosphorylation (to ATP synthesis) is known (Blond and Whittam, 1965; Gómez-Puyou et al., 1972), we are forced to conclude that, under conditions of strict potassium-limitation, as were applied in our experiments, subtle differences in the affinity constant for K^+ of the three respective sites of phosphorylation may be responsible for this unforeseen specificity. Nevertheless, the dramatic effects that potassium-depletion exerts on the organization of the respiratory chain point the direction in which future investigations should proceed.

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