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Properties of Mitochondria Isolated from Cyanide-Sensitive and Cyanide-Stimulated Cultures of *Acanthamoeba castellanii*

By STEVEN W. EDWARDS and DAVID LLOYD
Department of Microbiology, University College, Newport Road, Cardiff CF2 1TA, Wales, U.K.

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1. Mitochondria isolated from cultures of *Acanthamoeba castellanii* exhibit respiratory control and oxidize α-oxoglutarate, succinate and NADH with ADP : O ratios of about 2.4, 1.4 and 1.25 respectively. 2. Mitochondria from cultures of which the respiration was stimulated up to 50% by 1mM-cyanide (type-A mitochondria) and from cyanide-sensitive cultures (type-B mitochondria) had similar respiratory-control ratios and ADP: O ratios. 3. State-3 rates of respiration were generally more cyanide-sensitive than State-4 rates, and the respiration of type-A mitochondria was more cyanide-resistant than that of type-B mitochondria. 4. Salicylhydroxamic acid alone had little effect on respiratory activities of either type of mitochondria, but when added together with cyanide, irrespective of the order of addition, inhibition was almost complete. 5. Oxidation of externally added NADH by type-A mitochondria was mainly via an oxidase with a low affinity for oxygen ($K_m \simeq 15 \mu M$), which was largely cyanide-sensitive and partially antimycin A-sensitive; this electron-transport pathway was inhibited by ADP. 6. Cyanide-insensitive but salicylhydroxamic acid-sensitive respiration was stimulated by AMP and ADP, and by ATP after incubation in the presence of $MgCl_2$. 7. Addition of rotenone to mitochondria oxidizing α-oxoglutarate lowered the ADP: O ratios by about one-third and rendered inhibition by cyanide more complete. 8. The results suggest that mitochondria of *A. castellanii* possess branched pathways of electron transport which terminate in three separate oxidases; the proportions of electron fluxes via these pathways vary at different stages of growth.

Cyanide-insensitive respiration, originally observed in *Chlorella* (Warburg, 1919), has been the subject of much attention over recent years. The phenomenon is of widespread occurrence, particularly among higher plants (Solomos, 1977) but is also found in animals and micro-organisms (Lloyd, 1974; Henry & Nyns, 1975; Knowles, 1976). It was first shown that cyanide-insensitive respiration was localized within mitochondria of *Arum maculatum* (James & Elliott, 1955) and is mediated via an oxidase which is not a cytochrome; this pathway branches from the main phosphorylating chain at the substrate side of the antimycin-A site (Storey & Bahr, 1969; Bendall & Bonner, 1971). Specific inhibition of this alternative oxidase in plant mitochondria by substituted hydroxamic acids (Schonbaum *et al.*, 1971) has since been demonstrated in many other examples, and it is generally assumed that all mitochondrial cyanide-insensitive but hydroxamate-sensitive oxidases are identical. The physiological role of this alternative oxidase is obscure, but in many species, particularly micro-organisms, whose respiratory components can be manipulated environmentally or genetically (Lloyd, 1974; Lloyd & Edwards, 1978), its activity is enhanced when the conventional cytochrome chain is damaged or impaired.

Whole-cell respiration of the soil amoeba *Acanthamoeba castellanii* shows marked variations in sensitivity to cyanide at different stages of exponential growth (Edwards & Lloyd, 1977*b*). In early exponential-phase cultures, respiration is stimulated up to 50% by 1mM-cyanide, whereas that of late-exponential-phase cultures is extremely cyanide-sensitive. Although salicylhydroxamic acid alone has little inhibitory or stimulatory effect, when added together with cyanide, irrespective of the order of addition, inhibition is always more complete. Although there are variations in the ratios of individual cytochromes throughout the transition from cyanide-insensitive to cyanide-sensitive respiration (Edwards & Lloyd, 1977*a*), the nature of these components remains unchanged throughout growth.

Spectral evidence suggests the presence of *b*-, *c*- and *a*-type cytochromes in mitochondria of *A. castellanii* (Lloyd & Griffiths, 1968; Edwards *et al.*, 1977). In difference spectra, there is evidence for two cyanide-reacting cytochromes (Edwards *et al.*, 1977), one of which may be cytochrome a_3. Also, there are a

number of CO-reacting pigments which participate in whole-cell respiration, and there is evidence for an oxidase with a low affinity for oxygen (Edwards *et al.*, 1977).

The present study was undertaken to determine whether the observations of the changing cyanide-sensitivity of whole cells are associated with altered mitochondrial properties, with the aim of establishing a better understanding of the nature of the components and processes involved in mitochondrial electron transport in *A. castellanii*. A preliminary account of this work has been presented (Edwards & Lloyd, 1977*c*).

Experimental

Maintenance, growth and harvesting of the organisms

Acanthamoeba castellanii was maintained and grown axenically, with shaking at 30°C, exactly as described previously (Edwards *et al.*, 1977). Organisms were counted in a Fuchs–Rosenthal haemocytometer slide (Baird and Tatlock, Chadwell Heath, Romford, Essex, U.K.) after a suitable dilution with either fresh growth medium or 50mM-$MgCl_2$. Harvesting, at 4°C, was by centrifugation at 2000rev./min for 8min in the 6×1-litre head of an MSE Mistral centrifuge.

Disruption of the organism and isolation of mitochondria

Cells were washed once with 10vol. of 50mM-$MgCl_2$, pH7.4, and resedimented by centrifugation at 3000rev./min for 1.5min in the 6×250ml head of an MSE 18 centrifuge. The temperature of this and all subsequent operations was 4°C. The cell pellet was then suspended in 2vol. of disruption buffer containing 0.32M-sucrose, 10mM-Tris/H_2SO_4, 0.2mM-EGTA, pH8.0, and transferred to a chilled hand homogenizer (Jencons Scientific Ltd., Hemel Hempstead, Herts., U.K.) and homogenized to 50% breakage (estimated microscopically). The suspension was then centrifuged at 3000rev./min (1000*g*; $r_{av.}$ 7.6cm) for 8min in the 8×50ml head of an MSE 18 centrifuge, to sediment intact cells and nuclei. The supernatant was carefully decanted and then centrifuged at 10000rev./min (10000*g*; $r_{av.}$ 7.6cm) for 10min and the supernatant discarded. The crude mitochondrial pellet was then washed with 5vol. of disruption buffer, pH7.4, and resedimented. The washed pellet was then resuspended in 2vol. of disruption buffer, pH7.4, and used as the mitochondrial suspension.

Analytical methods

Measurements of oxygen uptake by mitochondrial suspensions, in disruption buffer supplemented with 10mM-KCl, 10mM-KH_2PO_4, 5mM-$MgCl_2$ and 0.15% (w/v) bovine serum albumin, pH7.4 (respiratory medium), were made at 30°C in a Clark oxygen electrode (Lloyd & Brookman, 1967). The total volume was 1ml. Difference spectra (Chance, 1957) were recorded at 77K in a Unicam SP.1800 spectrophotometer (Edwards & Lloyd, 1973; Edwards *et al.*, 1977); reduction of the sample cuvette was by sodium dithionite and oxidation of the reference cuvette by potassium ferricyanide. Steady-state reduction of cytochromes (Chance & Williams, 1956), in the presence or absence of inhibitors, was measured on mitochondrial preparations suspended in respiratory medium. After addition of substrate, inhibitor etc., the contents of the cuvette were vigorously aerated before immersion in liquid N_2. The spectral bandwidth was 1nm, the scanning speed 1nm/s and the path length of the cuvettes 2mm. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Substrates were used as aqueous solutions adjusted to pH7.4 with Tris. KCN (pH7.4) was added as an aqueous solution, but other inhibitors were used as ethanolic or methanolic solutions, at concentrations of solvent that were without effect.

Chemicals

Rotenone, α-oxoglutaric acid, ATP (disodium salt), ADP (sodium salt), AMP (sodium salt) and NADH (grade III, yeast) were from Sigma, Kingston upon Thames, Surrey, U.K., salicylhydroxamic acid was from Koch–Light Laboratories, Colnbrook, Bucks., U.K., antimycin A was from Boehringer, Lewes, Sussex, U.K., and bovine serum albumin was from Armour Pharmaceuticals Co., Eastbourne, Sussex, U.K. All other chemicals were of analytical grade.

Results

Respiratory properties of isolated mitochondria

Previous work has shown that the effect of 1mM-cyanide on whole-cell respiration of *Acanthamoeba castellanii* varies markedly at different stages of exponential growth (Edwards & Lloyd, 1977*b*). The respiratory properties of type-A mitochondria, isolated from 17–24h cultures (respiration stimulated up to 50% by 1mM-cyanide), and type-B mitochondria, isolated from 50–55h cultures (respiration inhibited 70–80% by 1mM-cyanide), are shown in Table 1. For oxidation of succinate and α-oxoglutarate by both types of mitochondria, and oxidation of NADH by type-B mitochondria, small amounts of ADP stimulated respiratory rates (State 3) until ADP became exhausted (State 4), enabling ADP:O and respiratory-control ratios to be calculated (Chance & Williams, 1956). Respiratory-control ratios are given as oxygen-uptake rate in the presence of ADP (State 3)/oxygen-uptake rate after the exhaustion of ADP (State 4). With α-oxoglutarate as respiratory

Table 1. *Respiratory properties of mitochondria isolated from cyanide-stimulated and cyanide-sensitive cultures of A. castellanii*

Rates of respiration in the presence (State 3) or absence (State 4) of ADP are expressed as nmol of O_2/min per mg of protein. Mean values ±S.D. are given from *n* determinations on at least five preparations of each type of mitochondria. Concentrations of α-oxoglutarate and succinate were 10 mM, and that of NADH was 1 mM.

	Type-A mitochondria				Type-B mitochondria			
Substrate	State 3	State 4	ADP:O ratio	Respiratory-control ratio	State 3	State 4	ADP:O ratio	Respiratory-control ratio
α-Oxoglutarate (n=8)	46.6 ±8.4	15.3 ±2.9	2.37 ±0.16	2.99 ±1.06	62.6 ±14.1	14.8 ±4.2	2.43 ±0.21	4.18 ±0.81
Succinate (*n*=6)	63.2 ±15.5	34.1 ±4.97	1.37 ±0.27	1.42 ±0.35	66.7 ±17.6	38.9 ±8.76	1.38 ±0.2	1.9 ±0.27
NADH (*n*=6)	—	74.3 ±11.9	—	—	121 ±23	81.4 ±12.9	1.24 ±0.13	1.82 ±0.32

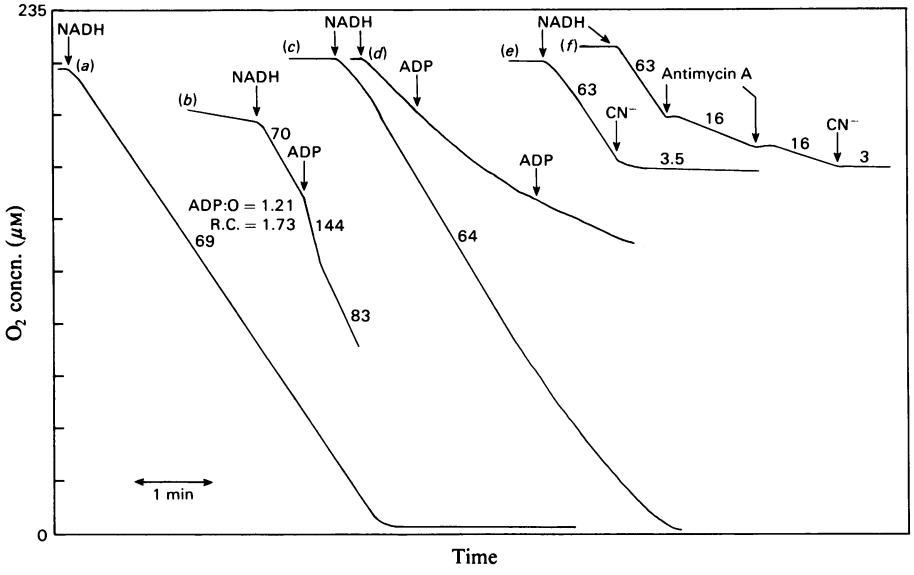


Fig. 1. *Oxidation of NADH by mitochondria of A. castellanii*

Polarographic traces (*a*) and (*b*) are for mitochondria isolated from cyanide-sensitive cultures (type B) (mitochondrial protein concentration in the reaction vessel: 0.91 mg/ml); traces (*c*)–(*f*) are of mitochondria isolated from cyanide-stimulated cultures (type A) (mitochondrial protein concentration in the reaction vessel: 0.97 mg/ml). Concentrations of additions at arrows were: NADH, 1 mM; ADP, 80 nmol; cyanide, 1 mM; antimycin A, 9 nmol. ADP:O and respiratory-control (R.C.) ratios are indicated and rates are in nmol of O_2/min per mg of protein.

substrate, ADP:O ratios were about 2.4 in both types of mitochondria, but mean values of respiratory-control ratios (and State-3 rates) were higher in type-B mitochondria. ADP:O ratios of approx. 1.4 were obtained for succinate oxidation, but again respiratory-control ratios were higher in type-B mitochondria.

Oxidation of NADH by type-A mitochondria was unusual in that ADP did not stimulate but inhibited the rate of respiration (Fig. 1*d*). Also, polarographic traces (Fig. 1*c*) suggest that oxidation of this substrate by these mitochondria proceeded largely via an oxidase with a low affinity for oxygen ($K_m \simeq 15\,\mu M$). Type-B mitochondria oxidizing NADH by way of an oxidase with a high affinity for oxygen ($K_m \simeq 7\,\mu M$, Fig. 1*a*) exhibited respiratory control (Fig. 1*b*), and ADP:O ratios of approximately 1.25 were obtained (Table 1).

Table 2. *Effect of inhibitors on the respiratory activities of mitochondria of A. castellanii*
Additions of inhibitors were made in the presence (State 3) or absence (State 4) of ADP. Values given are a percentage decrease of that rate in the absence of inhibitors, and the range of values observed in at least five preparations of each type of mitochondria is given; + indicates stimulation of respiration. Concentrations of substrates were as shown in the legend to Table 1; concentrations of cyanide and salicylhydroxamic acid (SHAM) were 1 mM and antimycin A was 10–15 nmol/mg of mitochondrial protein.

		Type-A mitochondria				Type-B mitochondria			
Substrate	State	Cyanide	SHAM	Cyanide+ SHAM	Antimycin A	Cyanide	SHAM	Cyanide+ SHAM	Antimycin A
α-Oxoglutarate	3	60–64	5–15	95–100	65–71	64–80	+8–10	95–100	100
	4	17–50	+10–11	95–100	35–62	70–87	+16–10	95–100	72–80
Succinate	3	100	3–10	100	100	100	+17	100	100
	4	57–75	3–16	100	64	78–82	+11–5	98–100	64
NADH	3	—	—	—	—	100	10	100	100
	4	80–94	3–6	91–100	60–76	81–97	+7–10	100	58

Table 3. *Effects of respiratory inhibitors on ADP:O and respiratory-control ratios of α-oxoglutarate oxidation*
Values indicated are percentage decreases of the values obtained in the absence of inhibitor. Concentration of rotenone was 12 nmol/mg of protein; other additions were as in the legend to Table 2.

Mitochondria		Cyanide	SHAM	Cyanide+ SHAM	Rotenone	Antimycin A
Type A	ADP:O	53–66	8–10	100	20–30	62
	Respiratory-control ratio	16–40	+14–15	100	11–30	60
Type B	ADP:O	69	+13	100	35	100
	Respiratory-control ratio	35	6	100	45	100

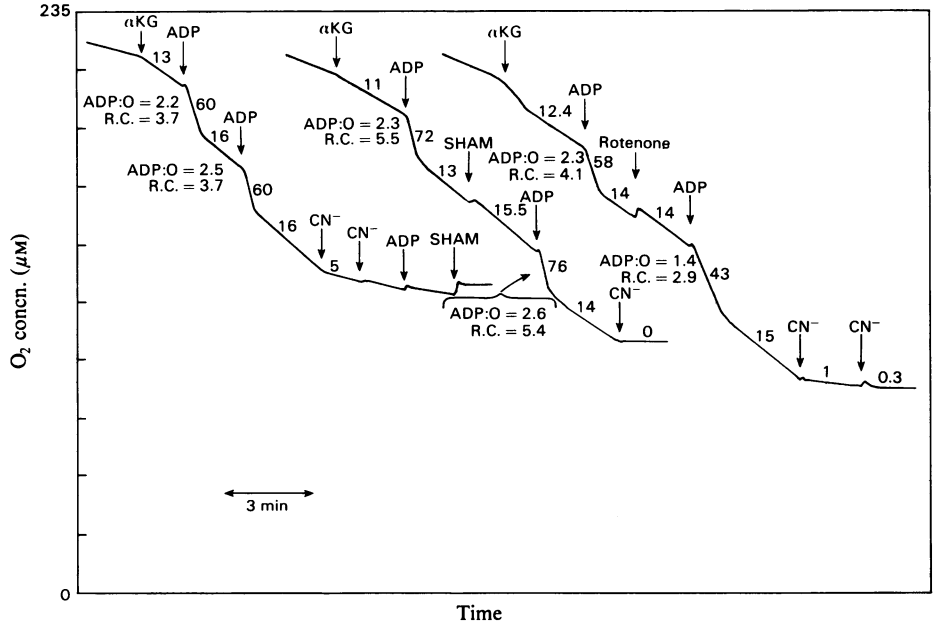


Fig. 2. *Oxidation of α-oxoglutarate by type-B mitochondria*
Concentrations of additions were: α-oxoglutarate (αKG), 10 mM; ADP, 80 nmol; cyanide, salicylhydroxamic acid (SHAM), 1 mM; rotenone, 5 nmol. The mitochondrial protein concentration was 0.36 mg/ml. ADP:O and respiratory control (R.C.) ratios are indicated and rates are in nmol of O_2/min per mg of protein.

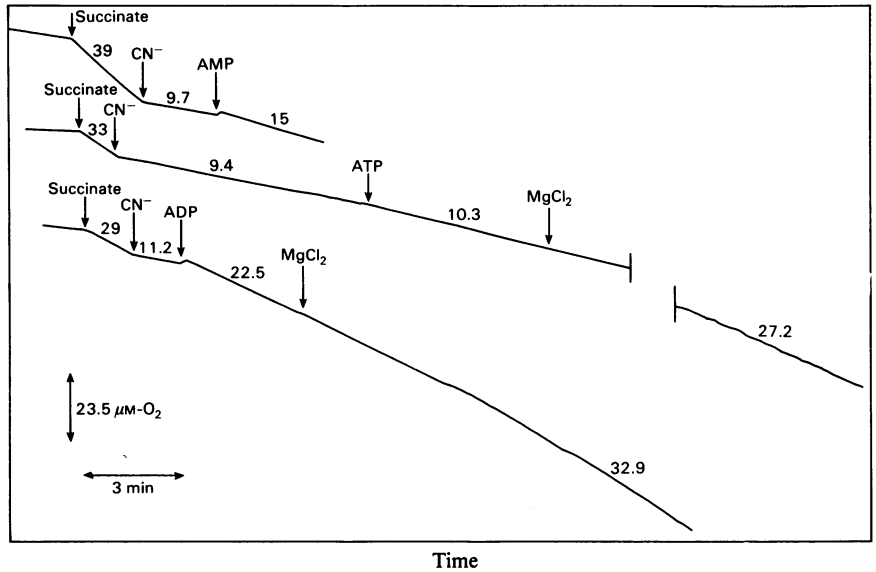


Fig. 3. *Stimulation of cyanide-insensitive respiration by adenine nucleotides*
Protein concentration (type-A mitochondria) was 0.25 mg/ml. Additions were as follows: succinate, 10 mM; cyanide, 1 mM; AMP, 250 μM; ADP, 200 μM; ATP, 500 μM; $MgCl_2$, 2.5 mM. Values indicated are nmol of O_2/min per mg of protein.

Table 4. *Effect of inhibitors on the steady-rate reduction of mitochondrial cytochromes of A. castellanii*
Mitochondria were suspended in respiratory medium and spectra recorded as described in the Experimental section. Values indicated are percentages of the values for dithionite-reduced–ferricyanide-oxidized, which was designated 100% reduction. Concentrations of inhibitors and substrates are as in Tables 1 and 2.

Mitochondria from organisms ...		Reduction (%)							
		Type A (insensitive to inhibition by cyanide)				Type B (sensitive to inhibition by cyanide)			
Substrate	Cytochrome	Steady-state	+Antimycin A	+Salicylhydroxamic acid	+Antimycin A +salicylhydroxamic acid	Steady-state	+Antimycin A	+Salicylhydroxamic acid	+Antimycin A +salicylhydroxamic acid
Succinate	*b*-555	35	38	40	38	51	73	66	
	b-562	33	45	46	53	47	85	77	
	b-565	62	53	31	14	65	47	58	
	c	17	15	6	7	54	28	14	
	a	10	25	13	30	9	9	3	
α-Oxoglutarate	*b*-555	35		48	45	17	58	51	71
	b-562	36		53	49	16	68	69	90
	b-565	24		34	28	36	68	58	74
	c	7		5	5	45	40	7	11
	a	5		9	6	14	9	0	4
NADH	*b*-555	38	48			73	76	96	84
	b-562	32	60			56	84	106	97
	b-565	29	43			82	74	73	84
	c	20	19			85	21	27	24
	a	13	37			14	9	5	9

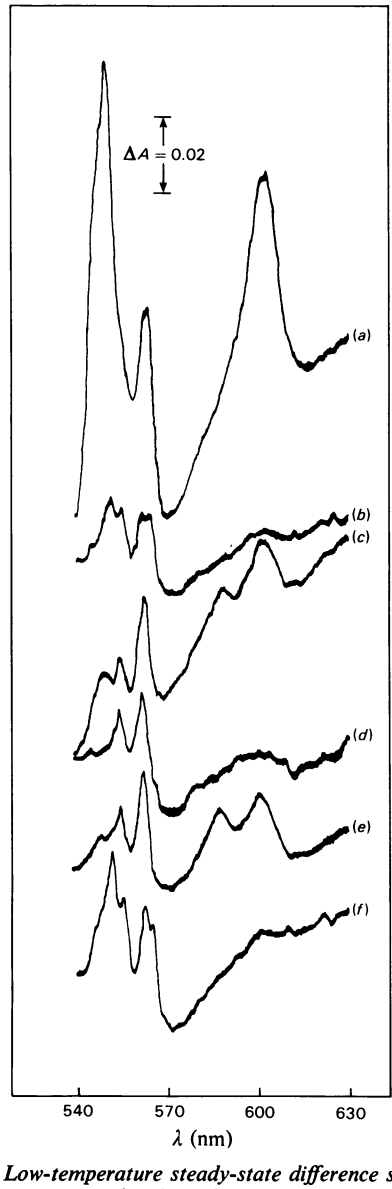


Fig. 4. *Low-temperature steady-state difference spectra of mitochondria of A. castellanii*

Mitochondria were suspended in respiration buffer in the absence of ADP (State 4). Spectra (*a*)–(*e*) are of type-A mitochondria, protein concentration 6.2mg/ml. (*a*) Difference spectrum with dithionite as reductant of the sample cuvette and ferricyanide as oxidant of the reference cuvette. (*b*) The sample cuvette was reduced by the addition of 10mM-sodium succinate and then aerated before immersing in liquid N_2. (*c*) As (*b*), except that 15nmol of antimycin A/mg of protein was added to the sample cuvette before addition of succinate. (*d*) As (*b*), but 1mM-salicylhydroxamic acid was added before addition of succinate. (*e*) As (*b*) but 15nmol of antimycin A and 1mM-salicylhydroxamic acid were added before the addition of succinate. (*f*) As (*b*), but for type-B mitochondria (13mg of protein/ml). In (*b*)–(*e*) the contents of the reference cuvette were oxidized by aeration.

Effect of inhibitors on mitochondrial respiratory rates

Table 2 summarizes the effects of cyanide, antimycin A and salicylhydroxamic acid on mitochondrial respiration. Except for α-oxoglutarate oxidation by type-B mitochondria, State-3 rates were always more cyanide- and antimycin A-sensitive than were State-4 rates. Salicylhydroxamic acid alone never inhibited or stimulated respiration by more than 16%, but when added together with cyanide, irrespective of the order of addition, inhibition was always virtually complete (91–100%). For oxidations of α-oxoglutarate and succinate, State-4 rates were less cyanide-sensitive in type-A than in type-B mitochondria, but in both types of mitochondria, State-3 rates of succinate oxidation were always completely cyanide- and antimycin A-sensitive. Malonate (up to 20mM) always completely inhibited the oxidation of 10mM-succinate. In type-B mitochondria, State-3 rates of α-oxoglutarate oxidation were more antimycin A- and cyanide-sensitive than those of type-A mitochondria. State-3 rates of NADH oxidation by type-B mitochondria were completely cyanide- and antimycin A-sensitive, but State-4 rates were again less sensitive to inhibition by these inhibitors. In type-A mitochondria, State-4 rates of oxidation of NADH were extremely cyanide-sensitive, but only partially antimycin A-sensitive (Figs. 1*e*, 1*f*).

Effect of respiratory inhibitors on ADP:O and respiratory-control ratios of α-oxoglutarate oxidation

Cyanide lowered the ADP:O ratios for α-oxoglutarate oxidation in both types of mitochondria by approximately two-thirds, i.e. in the presence of 1mM-cyanide (Table 3) ADP:O ratios of about 1 were obtained. Salicylhydroxamic acid alone did not affect ADP:O or respiratory-control ratios to any great extent, but when added together with cyanide, inhibition of respiration was virtually complete (Fig. 2*b*). Rotenone had little effect on α-oxoglutarate oxidation by type-B mitochondria in State 4, but decreased State-3 rates by about 30% (Fig. 2*c*). In type-A mitochondria corresponding decreases in State-3 and State-4 rates were approx. 60 and 45% respectively. In both types of mitochondria ADP:O ratios in the presence of rotenone were lowered by about one-third; also, in the presence of rotenone, inhibition by cyanide was more complete.

Stimulation of cyanide-insensitive respiration by adenine nucleotides

Cyanide (1mM) inhibited the oxidation of succinate in State 4 by type-A mitochondria by 57–75% (Table

2). The residual cyanide-insensitive (but salicylhydroxamic acid-sensitive) respiration was stimulated by up to 100% on the addition of AMP or ADP (Figs. 3*a*, 3*c*). ATP alone had little stimulatory effect, but when incubated in the presence of $MgCl_2$ for about 10min, a 3-fold increase in the rate was observed; a similar effect was noted for ADP+$MgCl_2$.

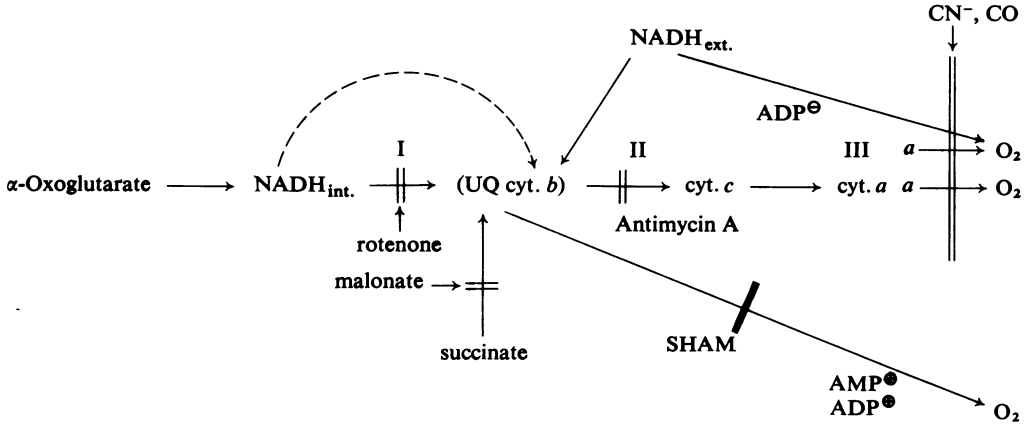
Steady-state reduction of cytochromes of mitochondria

Table 4 shows the effects of inhibitors on the steady-state reduction of cytochromes of mitochondria from cyanide-stimulated and cyanide-insensitive cultures. Previous work has revealed the presence of two mitochondrial *b*-type cytochromes, *b*-555 and *b*-562 (Edwards *et al.*, 1977), but in steady-state spectra a third cytochrome, *b*-565, was apparent (Fig. 4). In both types of mitochondria, with succinate as reductant, the addition of salicylhydroxamic acid, antimycin A, or both in combination, increased the reduction of cytochromes *b*-555 and *b*-562, whereas cytochrome *b*-565 became more oxidized in the presence of these inhibitors. However, with α-oxoglutarate as reductant, addition of these inhibitors increased the reduction of all three *b*-type cytochromes. In type-A mitochondria, the addition of inhibitors also increased the reduction of the *a*-type cytochromes with NADH or succinate as reductants; this phenomenon was not found in type-B mitochondria.

Discussion

From the results presented here, it appears that the pathways of electron transport in mitochondria of *Acanthamoeba castellanii* are superficially similar to those reported for many systems, particularly higher plants and *Neurospora crassa*, which exhibit mitochondrial cyanide-insensitive respiration (Lambowitz & Slayman, 1971; Bahr & Bonner, 1973*b*; Von Jagow *et al.*, 1973). The experimental data can be accommodated by Scheme 1, in which the pathways via the salicylhydroxamic acid-sensitive oxidase and for the oxidation of externally added NADH are more pronounced in type-A mitochondria. The previous observations of changing cyanide-sensitivity of whole cells during exponential growth (Edwards & Lloyd, 1977*b*) are reflected in the properties of isolated mitochondria (Table 2). State-3 rates of respiration are more cyanide-sensitive than those during State-4 respiration, suggesting that under conditions of ADP limitation (State 4) a greater proportion of electron flux may proceed via the alternative salicylhydroxamic acid-sensitive oxidase, as in higher plants which exhibit cyanide-insensitive respiration (Bahr & Bonner, 1973*a*). From measurements of cytochromes (Edwards & Lloyd, 1977*a*) and ADP:O ratios (Table 1) it appears that the phosphorylating cytochrome chain is present at all times and the observations on changing cyanide-sensitivity result from variations in electron flux which the alternative pathway can accommodate. Whether the transition to cyanide-sensitivity results from progressive loss of the alternative pathway as mitochondria become cyanide-sensitive, or there is a change in the parameters at the control point which determines the apportionment of electron flux, remains to be ascertained.

The nature of the salicylhydroxamic acid-sensitive



Scheme 1. *Proposed pathways of electron transport in A. castellanii*

I, II and III represent sites I, II and III of oxidative phosphorylation; $NADH_{int.}$ and $NADH_{ext.}$, internally generated and externally added NADH respectively; SHAM, salicylhydroxamic acid; UQ, ubiquinone; cyt., cytochrome. Sites of inhibition by rotenone, malonate, antimycin A, SHAM, cyanide (CN^-) and CO are indicated: ⊕, stimulation, ⊖, inhibition. The Scheme includes data from Edwards *et al.* (1977).

oxidase and the precise mechanisms occurring at the branch-point of the two pathways has been the subject of much interest over recent years. Storey (1976) has proposed that ubiquinone is linked to the alternative pathway by a flavoprotein of midpoint potential 50mV more negative than the quinone with which it is in equilibrium. It has also been proposed that the branch-point of electron flow to the alternative pathway is at the reversal step of reduction of semiubiquinone to the fully reduced form by succinate dehydrogenase (Rich & Moore, 1976), a scheme which accommodates the protonmotive ubiquinone cycle (Mitchell, 1975). The involvement of iron and/or iron–sulphur protein(s) in the alternative oxidase has been implicated (Haddock & Garland, 1971; Schonbaum *et al.*, 1971; Henry *et al.*, 1977), but more recent approaches involving e.p.r. measurements (Moore *et al.*, 1976) have led to the finding, in plant mitochondria, that the alternative oxidase is not an iron–sulphur centre itself, but is an, as yet, optically and e.p.r.-invisible component, possibly a quinone species (Rich & Bonner, 1978). Whether these hypotheses are true for *A. castellanii*, and indeed for all cases of salicylhydroxamic acid-sensitive oxidases, requires further investigation. Stimulation of cyanide-insensitive respiration of mitochondria by AMP has been reported in *Euglena gracilis* (Sharpless & Butow, 1970) and in *Moniliella tomentosa* (Hanssens & Verachtert, 1976). Hanssens & Verachtert (1976) also observed a stimulatory effect on the alternative oxidase produced by the addition of ADP or ATP, after a preincubation with $MgCl_2$, and suggested that these nucleotides are rapidly converted into AMP, which itself is responsible for the stimulatory effect. The mechanism of AMP stimulation is unknown.

The identity of the mitochondrial cyanide-reacting oxidase which is responsible for the oxidation of externally added NADH is uncertain, but the possibility that this component is a cytochrome cannot be excluded. In difference spectra, the region 580–605nm is complex, and there are a number of unidentified CO-reacting oxidases which participate in the reversal by light of CO inhibition of respiration of whole cells (Edwards *et al.*, 1977). Respiration in a lowered oxygen atmosphere (N_2/O_2, 19:1) decreased by 50%, suggesting the presence of a low-affinity oxidase; whether this oxidase is the same as that for oxidation of externally added NADH requires further investigations. Two mitochondrial *b*-type cytochromes have been previously identified in *A. castellanii* (Lloyd & Griffiths, 1968; Edwards *et al.*, 1977), which have absorption maxima at 77K at 555 and 562nm. Steady-state measurements of cytochromes have shown the presence of a third *b*-type cytochrome (*b*-565) and it may be that cytochromes *b*-562 and *b*-565 are analogous to cytochromes b_K and b_T (Chance, 1972).

Although the respiration of early-exponential-phase cultures was stimulated up to 50% by 1mM-cyanide, no cyanide-stimulated respiration was observed with mitochondrial suspensions. Measurements of respiration of cells in growth medium are complicated, however, by the nature of the growth medium, which has a number of potentially oxidizable substrates (e.g. amino acids) which may be utilized only under certain physiological conditions. Cyanide-stimulated respiration of whole cells has been suggested as being an increased electron flux through a rotenone-sensitive site, which is by-passed in the absence of cyanide (Edwards & Lloyd, 1977*b*). Indeed, the detection of the first of the three sites of energy conservation in the present investigation is not in agreement with previous reports. When *A. castellanii* was grown in 4% mycological peptone, oxidation of NAD^+-linked substrates by mitochondria isolated from exponentially dividing cultures was via a rotenone- and piericidin A-insensitive pathway, which was coupled to only two phosphorylating sites (Lloyd & Griffiths, 1968; Evans, 1973); only in mitochondria from stationary-phase cultures could site 1 be demonstrated (Evans, 1973). These observations are similar to those with *Candida utilis* (Katz, 1971; Katz *et al.*, 1971; Ohnishi, 1972); it was proposed that site 1 is present in mitochondria at all times during growth, but by-passed when cells are growing under optimal conditions (Ohnishi, 1972). It seems likely that a similar by-pass mechanism of site 1 may be functional in *A. castellanii* under the present conditions of growth, and the absence of rotenone-sensitivity is not necessarily indicative of the absence of site 1.

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