

## The Effect of Inhibitors on the Oxygen Kinetics of Terminal Oxidases of *Acanthamoeba castellanii*

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1. Respiration of growing cultures of *Acanthamoeba castellanii* is inhibited less than 60% by azide (35 mM); the respiration of early-exponential-phase cultures differs from that of late-exponential-phase cultures in being stimulated by up to 120% by low concentrations (<1 mM) of this inhibitor. Azide (0.5 mM) plus 1 mM-salicylhydroxamic acid gives 80% inhibition of respiration in early- or late-exponential-phase cultures. 2. Lineweaver–Burk plots of  $1/v$  against  $1/[O_2]$  for growing and stationary-phase cultures give values of <1  $\mu\text{M}$  for the apparent  $K_m$  for oxygen. 3. These values are not significantly altered when determined in the presence of 1 mM-salicylhydroxamic acid. 4. Higher values (>7  $\mu\text{M}$ ) for apparent  $K_m$  values for oxygen were obtained in the presence of azide, which gives non-linear Lineweaver–Burk plots. 5. Competitive inhibition of respiration by CO occurs with  $K_i$  2.4  $\mu\text{M}$ . 6. The results are discussed in terms of the presence of three terminal oxidases in this organism, namely two oxidases with high affinities for oxygen (cytochrome *c* oxidase of the main phosphorylating electron-transport chain and the salicylhydroxamic acid-sensitive oxidase) and a third oxidase with a low affinity for oxygen, sensitive to inhibition by cyanide but not by azide or salicylhydroxamic acid. The relative contributions to oxygen utilization by these oxidases change during the growth of a batch culture.

Studies of the effects of different respiratory inhibitors on mitochondrial and whole-cell respiration have revealed the existence in many organisms of alternative terminal oxidases (Degn *et al.*, 1978).

Whole-cell respiration of the soil amoeba *Acanthamoeba castellanii* shows variations in cyanide-sensitivity at different stages of growth (Edwards & Lloyd, 1977). Whereas in early-exponential-phase cultures 1 mM-cyanide gives 50% stimulation, in the late exponential phase of growth or in early-stationary-phase cultures, inhibition by cyanide (up to 95% at 1 mM) is observed. Salicylhydroxamic acid alone has little effect, but increases the effectiveness of cyanide as a respiratory inhibitor.

Results with mitochondria isolated from *A. castellanii* suggested the presence of at least three terminal oxidases (Edwards & Lloyd, 1978*a,b*); in addition to the major cytochrome *a*-type oxidase of the main phosphorylating electron-transport chain and the salicylhydroxamic acid-sensitive alternative oxidase, evidence was presented for an oxidase with a low affinity for oxygen responsible for the oxidation of NADH added externally to the mitochondria.

The present paper describes the effects of azide as a respiratory inhibitor, and further characterizes three

terminal oxidases of *A. castellanii* with respect to their affinities for oxygen.

### Experimental

#### *Maintenance and growth 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 suitable dilution with 50 mM-MgCl<sub>2</sub>.

#### *Measurements of oxygen-consumption rates*

Measurements of respiration were made at 30°C by two methods: in a closed reaction vessel fitted with an oxygen electrode (Lloyd & Brookman, 1967) or in the open system of Degn & Wohlrab (1971) modified as described by Petersen *et al.* (1974).

The open system consists of a stainless-steel reaction vessel of 4.5 ml working volume fitted with a membrane-covered oxygen electrode (Radiometer A/S, Embrupvej 72, Copenhagen N.V., Denmark). Gas mixtures are fed through an inlet in the lid, over the surface of the stirred liquid, and then to a second

electrode. The respiration rate ( $V_r$ ) of a cell suspension is given by:

$$V_r = K(T_G - T_L)$$

where  $K$  is the  $O_2$ -transfer constant,  $T_G$  is the concentration of  $O_2$  in the gas phase, and  $T_L$  is the concentration of  $O_2$  in the liquid phase.

The value of  $K$  is determined from the  $t_{1/2}$  for increasing  $T_L$ , after changing the gas phase from  $N_2$  to air, in the absence of organisms.

A linear decrease with time in the steady-state oxygen concentration in the liquid was achieved by using an electronic gas mixer (Lundsgaard & Degn, 1973) controlled by an on-line computer. Calculations and double-reciprocal plotting of respiration rate as a function of oxygen concentration were performed automatically. In experiments with  $CO$ , a constant partial pressure of this inhibitor was maintained in the gas stream by means of an extra gas mixer as described by Petersen (1977).

## Results

### Effects of azide on respiration

When 1 mM-cyanide is added to samples taken from cultures in early exponential phase of growth, up to 50% stimulation of respiration occurs; subsequent addition of 1 mM-salicylhydroxamic acid gives up to 90% inhibition (Edwards & Lloyd, 1977). Azide (0.1 mM) stimulated respiration more markedly (up to 120% stimulation) than did cyanide; addition of 1 mM-salicylhydroxamic acid then gave 82% inhibition. The dependence of these effects on azide

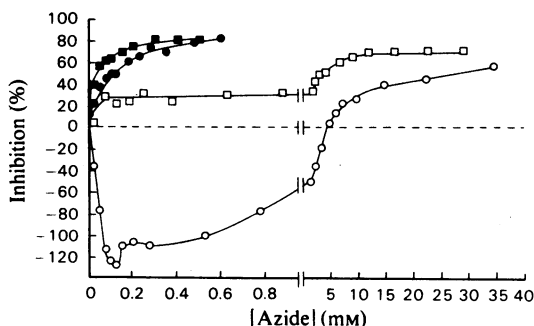


Fig. 1. Effects of azide on the respiration of cultures of *Acanthamoeba castellanii*

Inhibition (or stimulation, -values) of respiration (%) is expressed as a function of azide concentration, the control (0%) being the respiration rate in the absence of any inhibitor.  $\circ$ ,  $\bullet$ , Early-exponential-phase culture,  $3 \times 10^5$  organisms/ml;  $\square$ ,  $\blacksquare$ , late-exponential-phase culture,  $3 \times 10^6$  organisms/ml; effect of azide alone ( $\circ$ ,  $\square$ ) or in the presence of 1 mM-salicylhydroxamic acid ( $\bullet$ ,  $\blacksquare$ ).

concentration is shown in Fig. 1. Inhibition by azide in the absence of salicylhydroxamic acid required concentrations in excess of 5 mM, and even at 30 mM no more than 60% respiratory inhibition was observed. The residual respiration was not cyanide-sensitive, but more than 90% inhibition was produced when cyanide (1 mM) was added together with salicylhydroxamic acid (1 mM). Addition of these inhibitors in any order gave overall virtually complete cessation of oxygen consumption. The rate of the residual respiration in the presence of azide declined as oxygen concentration decreased in the closed electrode system, suggesting that the terminal oxidase involved is one with a low affinity for oxygen; this effect was accentuated when salicylhydroxamic acid was also present (Fig. 2a).

Stimulation of respiration by azide was not evident in late-exponential-phase cultures (Fig. 1). Again, no more than 70% inhibition by azide alone occurred, even at concentrations greater than 30 mM. In the presence of salicylhydroxamic acid (1 mM), 82% inhibition of respiration was produced by 0.3 mM-azide. The residual respiration with low concentrations of azide in the absence or presence of salicylhydroxamic acid again exhibited the characteristics of an oxidase of low oxygen affinity (Fig. 2b), i.e. the relative respiration rates decreased below a value of 1.0 at high  $O_2$  concentration.

In samples of cultures that had attained the stationary phase of growth, sensitivity to azide and salicyl-

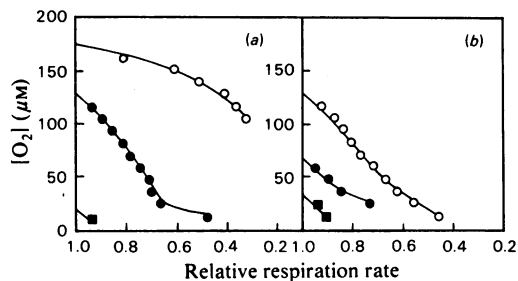


Fig. 2. Effect of oxygen concentration on respiration rate of *Acanthamoeba castellanii* in the closed electrode system. A suspension of cells in growth medium in the absence or presence of inhibitors was placed in a closed electrode system and the rate of oxygen uptake measured down to zero oxygen concentration. Re-aeration of samples and repeating this procedure over several cycles ensured that changing respiration rates were genuinely associated with altering oxygen tensions. (a) Early-exponential-phase cultures ( $3.5 \times 10^5$  cells/ml); (b) late-exponential-phase cultures ( $3 \times 10^6$  cells/ml $^{-1}$ ) in the absence of inhibitors ( $\blacksquare$ ), in the presence of 1 mM-azide ( $\bullet$ ) and 1 mM-salicylhydroxamic acid ( $\circ$ ).

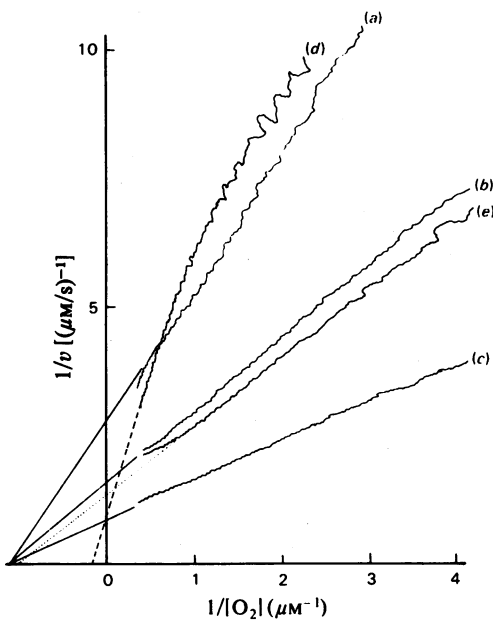


Fig. 3. Reciprocal plots of respiration rate against  $[O_2]$  in the presence of inhibitors for early-exponential-phase cultures of *Acanthamoeba castellanii*

Cultures were harvested at cell densities of (a)  $3.75 \times 10^5$  cells/ml; (b), (e)  $4.7 \times 10^5$  cells/ml; (c), (d)  $10^6$  cells/ml, and resuspended at ten times these cell concentrations in the conditioned growth medium. Curves (a), (b) and (c) obtained in absence of inhibitors, (d) 1 mM-azide, (e) 0.4 mM-salicylhydroxamic acid.

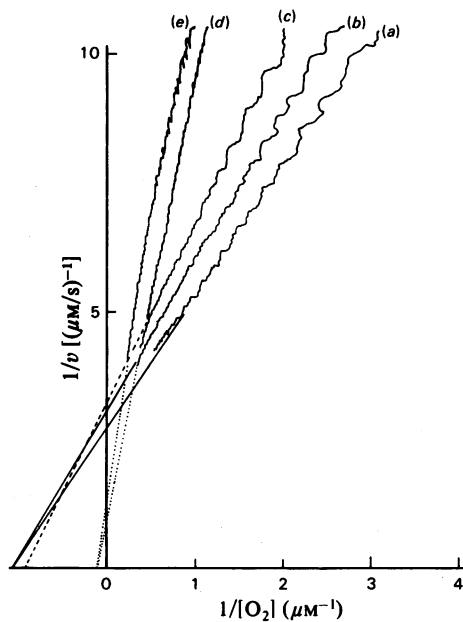


Fig. 4. Reciprocal plots of respiration rate against  $[O_2]$  in the presence of inhibitors for late-exponential-phase cultures of *Acanthamoeba castellanii*

Curves (a) and (b) obtained in the absence of inhibitors with cultures grown to  $3.1 \times 10^6$  and  $2.2 \times 10^6$  cells/ml respectively. Curve (c) was obtained after adding 1 mM-salicylhydroxamic acid ( $2.2 \times 10^6$  cells/ml); (d) and (e) were with 1 mM-azide and  $3.1 \times 10^6$  and  $2.2 \times 10^6$  cells/ml respectively.

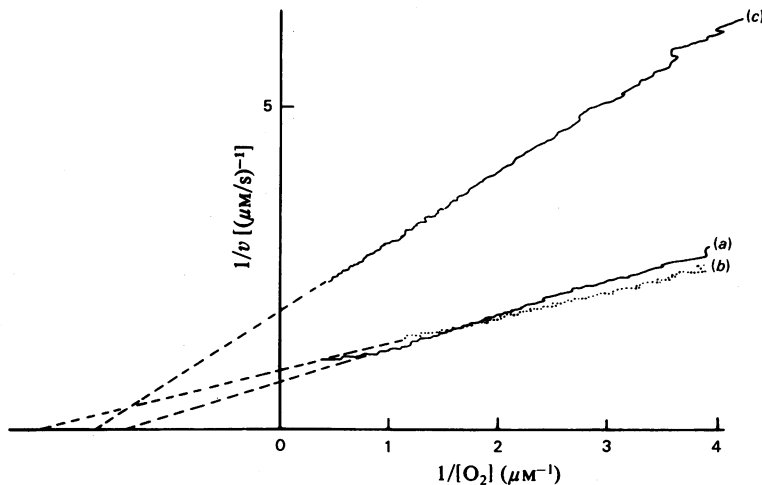


Fig. 5. Reciprocal plots of respiration rate against  $[O_2]$  in the presence of inhibitors for a stationary-phase culture of *Acanthamoeba castellanii*

Curve (a), uninhibited culture; curve (b), 1 mM-salicylhydroxamic acid; curve (c), 1 mM-azide. The culture density was  $7.5 \times 10^6$  cells/ml.

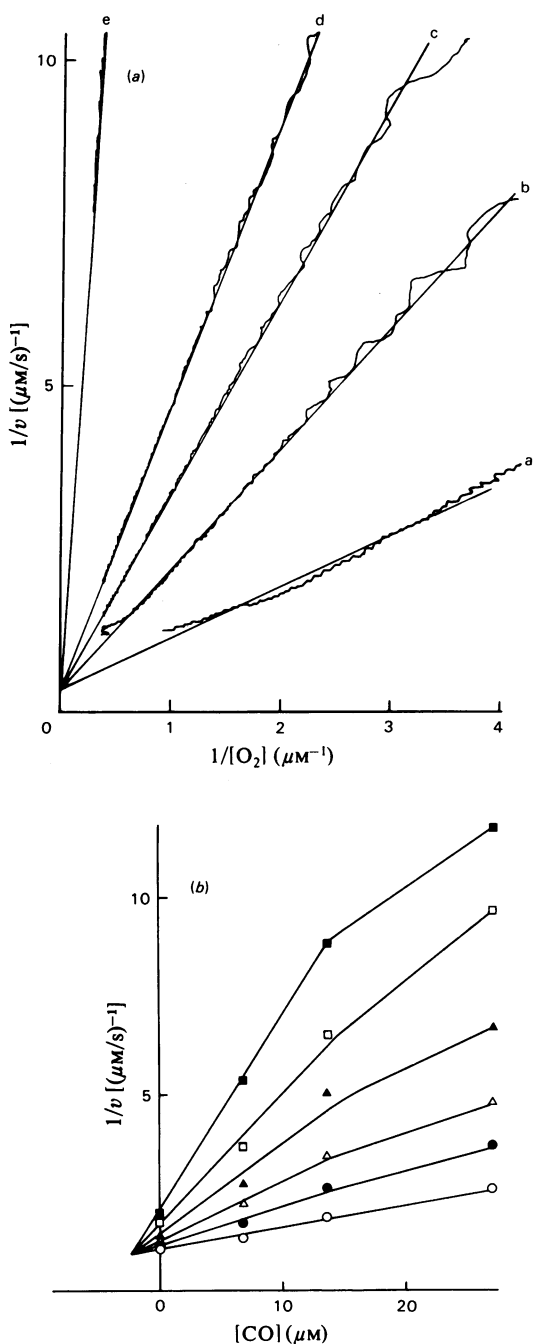


Fig. 6. (a) Reciprocal plots of respiration rate against  $[O_2]$  in the presence of varying fixed CO concentrations for a stationary phase culture of *A. castellanii*, and (b) plot of the rate of oxygen uptake against CO concentration at various fixed oxygen concentrations

(a) Curve a, uninhibited culture; b,  $6.7 \mu\text{M}$ -; c,  $13.5 \mu\text{M}$ -; d,  $27 \mu\text{M}$ -; and e,  $270 \mu\text{M}$ -CO. The culture

hydroxamic acid together was still noted, but the possible presence of a low-affinity oxidase was less evident.

#### Steady-state oxygen kinetics of terminal oxidases

*Exponentially growing cultures (early phase).* Time gradients of oxygen over a concentration range of  $0\text{--}4 \mu\text{M-O}_2$  were used to determine the dependence of respiration rate on oxygen tension in the liquid phase. For cyanide-stimulated organisms (i.e. in cultures grown to  $<10^6$  organisms/ml) extrapolation of Lineweaver-Burk plots of  $1/v$  against  $1/[O_2]$  (Fig. 3) at different cell densities (adjusted by using conditioned growth medium) gave a value for the apparent  $K_m$  for oxygen of  $0.92 \pm 0.09 \mu\text{M}$  (mean  $\pm$  s.d., four determinations). In the presence of salicylhydroxamic acid ( $0.4\text{--}1 \text{ mM}$ ), stimulation of respiration occurred, and the  $K_m$  for oxygen was  $0.88 \pm 0.12 \mu\text{M}$  (mean  $\pm$  s.d., five determinations). In cultures with added  $1 \text{ mM}$ -azide, curved reciprocal plots were obtained, and the apparent  $K_m$  for oxygen was raised to about  $7 \mu\text{M}$ .

*Exponentially growing cultures (late phase).* For cultures grown to between  $2 \times 10^6$  and  $6 \times 10^6$  cells/ml (cyanide-sensitive organisms), the apparent  $K_m$  for oxygen obtained from reciprocal plots (Fig. 4) was  $0.81 \pm 0.16 \mu\text{M}$  (mean  $\pm$  s.d., six determinations). In the presence of  $1 \text{ mM}$ -salicylhydroxamic acid, this value was  $1.14$ . Azide ( $1 \text{ mM}$ ) again produced non-linear Lineweaver-Burk plots, especially when determinations were made with cell suspensions at low cell densities. Departure from linearity was less pronounced than with early-exponential-phase cultures.

*Cultures in the stationary phase of growth.* Lineweaver-Burk plots of  $1/v$  against  $1/[O_2]$  for stationary-phase cultures (cyanide-sensitive, cell density  $7 \times 10^6$  cells/ml) gave a value of  $0.69 \pm 0.27 \mu\text{M}$  (mean  $\pm$  s.d., four determinations) for the apparent  $K_m$  for oxygen (Fig. 5). In the presence of  $1 \text{ mM}$ -salicylhydroxamic acid this value decreased to  $0.4 \mu\text{M}$ . Addition of  $1 \text{ mM}$ -azide did not produce non-linear plots (as was the case with growing organisms), and the apparent  $K_m$  for oxygen was not significantly lowered ( $0.6 \mu\text{M}$ ) as compared with control experiments in the absence of this inhibitor (i.e. the inhibition was non-competitive). Fig. 6(a) shows that inhibition of respiration of stationary-phase cultures by CO is strictly competitive, altering the slope of the double-reciprocal plots while leaving the intercept on the ordinate unchanged. Secondary plots give a value of  $K_i$   $2.4 \mu\text{M}$  for inhibition by CO (Fig. 6b).

density was  $7.7 \times 10^6$  cells/ml. (b) Data are taken from (a).  $\blacksquare$ ,  $0.44 \mu\text{M}$ ;  $\square$ ,  $0.5 \mu\text{M}$ ;  $\blacktriangle$ ,  $0.8 \mu\text{M}$ ;  $\triangle$ ,  $1 \mu\text{M}$ ;  $\bullet$ ,  $1.34 \mu\text{M}$ ; and  $\circ$ ,  $2 \mu\text{M-O}_2$ .

**Discussion**

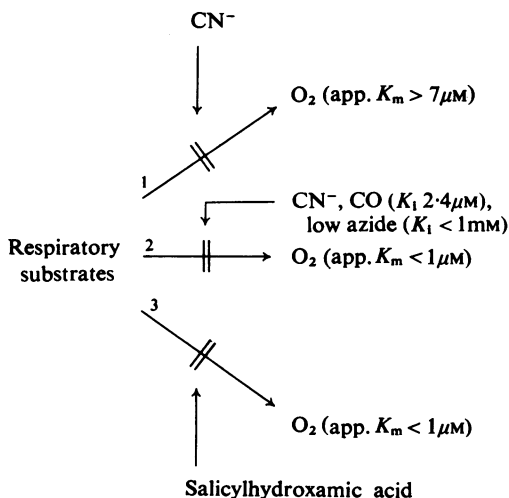
The characteristics of azide inhibition of respiration of *A. castellanii* resemble those reported previously for cyanide (Edwards & Lloyd, 1977), but, whereas cyanide inhibits the oxidase with a low affinity for oxygen and cytochrome *c* oxidase (Edwards & Lloyd, 1978*a,b*), low concentrations of azide specifically inhibit cytochrome *c* oxidase. The mechanisms of stimulation of respiration of early-exponential-phase cultures by both these inhibitors requires further study, but may not be a primarily mitochondrial phenomenon (Edwards & Lloyd, 1978*a,b*).

The addition of inhibitors of respiration to cell suspensions alters the apportionment of electron flux via the alternative oxidases. Scheme 1 summarizes the properties of three terminal oxidases in *A. castellanii*. Thus determination of  $K_m$  values in the absence of inhibitors (or in the presence of salicylhydroxamic acid) gives an apparent  $K_m$  value for  $O_2$  of  $<1 \mu M$ . This oxidase (the terminal component of pathway 2) is presumably the cytochrome *c* oxidase of the main phosphorylating electron-transport chain and is inhibited both by CO ( $K_i = 2-3 \mu M$ ) and low concentrations of azide ( $K_i < 1 mM$ ). Addition of azide results in oxygen consumption proceeding mainly via the azide-insensitive (but cyanide-sensitive) oxidase, which has a low affinity for  $O_2$  ( $K_m < 7 \mu M$ ). Activity of this oxidase (via pathway 1)

is greatest in early-exponential-phase cultures. The apparent  $K_m$  for  $O_2$  of the salicylhydroxamic acid-sensitive oxidase, which is not inhibited by cyanide, CO or azide, is  $<1 \mu M$ . The affinity of this oxidase for  $O_2$  (via pathway 3) can be estimated in the presence of azide when the activity of the low-affinity oxidase (via pathway 1) is diminished as the cultures enter the stationary phase.

The inhibitor-sensitivities of these oxidases are similar to those of the three terminal oxidases of trypanosomes (Hill, 1978). The apportionment of electron flux between the oxidases of *A. castellanii* varies at different stages of growth of a batch culture. In early exponential phase, all three oxidases are potentially active; later in growth, electron transport occurs mainly by way of the main phosphorylating chain, but still with the possibility of some flux between the alternative oxidases. In stationary-phase cultures the oxidase with the low affinity for oxygen is not detectable at low oxygen tensions. The low apparent  $K_m$  values for oxygen determined in the absence of inhibitors for all three types of culture confirms that, at low oxygen tensions, electrons must flow through cytochrome *c* oxidase and/or the salicylhydroxamic acid-sensitive oxidase. The identity of the oxidase with a low affinity for azide requires further investigation.

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Scheme 1. Inhibitor-sensitivities and oxygen affinities of terminal oxidases in *Acanthamoeba castellanii*

All three pathways of electron transport contribute in early-exponential-phase cultures, whereas in late-exponential-phase cultures electron transport is mainly via route 2.

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