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A benzoxazole inhibitor of NADH dehydrogenase in *Paracoccus denitrificans*

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1. INTRODUCTION

Tinopal AN, (1,1-bis(3,*N*-5-dimethyl benzoxazol-2-yl)-methine *p*-toluene sulphonate) (Fig. 1), is a cationic benzoxazole derivative present in the commercial product Uvitex AN (Ciba-Geigy Ltd., UK), which has been used as a fluorescent optical brightener in ultra violet light microscopy as a tool for the non-specific differentiation of bacteria and fungi from background plant material [1,2]. It was later realised that Tinopal AN was, in some cases, a potent bactericidal agent and more recently its selective toxicity was investigated [3] with particular reference to phytopathogenic pseudomonad and xanthomonad bacteria. It was found [3] that the compound was much more toxic to potentially sensitive bacteria in aerobic nutrient broth media containing 1% glucose than in the same medium lacking added glucose, suggesting that toxicity might be associated with aerobic respiratory metabolism. Thus it seemed appropriate to assess in greater detail the mode of inhibition of aerobic bacterial growth by Tinopal AN. For this purpose, the respiratory bacterium *Paracoccus denitrificans*, which was also shown (MKP, unpublished) to be very sensitive to Tinopal AN, was chosen as a test system, since this organism has been extensively characterised in terms of its electron transport and energy-coupling processes [e.g. 4–6]. We report here that low concentrations of Tinopal AN inhibited the aerobic respiration of washed cell suspensions of *P. denitrificans*. Further experiments with suspensions of cytoplasmic membrane vesicles derived from this organism revealed that this inhibition was localised in the NADH dehydrogenase region of the respiratory chain.

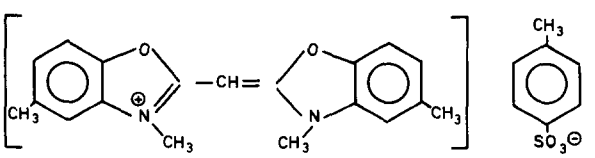

Fig. 1. Tinopal AN.

2. MATERIALS AND METHODS

Organism: For experiments with intact cells *P. denitrificans* NCIB8944 was grown and maintained aerobically on the succinate-nitrate medium [7]. Inverted cytoplasmic membrane vesicles were prepared from anaerobically grown organisms [8] and assayed for their protein content as described [8]. Respiration by suspensions of intact cells or cytoplasmic membrane vesicles was monitored in a Clark-type oxygen electrode [8].

Tinopal AN was kindly donated by Dr. A.M. Paton, Division of Agricultural Bacteriology, School of Agriculture, University of Aberdeen, Aberdeen, Scotland; a 20 mM stock solution in absolute ethanol was prepared.

3. RESULTS

Fig. 2 shows a typical trace of oxygen uptake by a washed cell suspension of *P. denitrificans*. It is apparent that successive additions of modest concentrations of Tinopal AN resulted in a decrease in the rate of oxygen uptake, indicative of an inhibition of aerobic respiration. The inhibition of respiration by increasing concentrations of Tinopal AN is shown in Fig. 3a. A rapid decrease in respiratory activity occurred when concentrations of Tinopal AN between 3 and 10 μM were present; higher concentrations elicited a more gentle decrease in respiration rate. When the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) at 1 μM, a concentration which gives maximal respiratory stimulation in intact cells (DBK, unpublished), was present in the reaction mixture, the concentration of Tinopal AN necessary for a 50% inhibition of respiration (ID_{50}) occurred at a similar concentration (Fig.3b) to that found in the absence of FCCP (Fig. 3a).

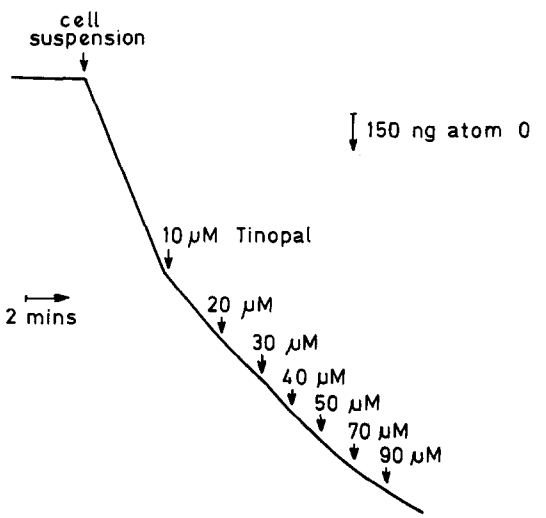

Fig. 2. Inhibition of respiration of intact cells of *Paracoccus denitrificans* by Tinopal AN. Cells from a mid-exponential culture of *P. denitrificans* were washed twice in 0.1 M Na_2HPO_4 pH 7.3 containing 1 mg/ml bovine serum albumin and resuspended in the same buffer. Cells (2.5 mg dry weight) from this suspension were added as indicated to a 3 ml reaction mixture in the oxygen electrode containing 0.1 M Na_2HPO_4 pH 7.3/10 mM disodium succinate. Tinopal AN was added to give the final concentrations shown and respiration was monitored as described in Methods.

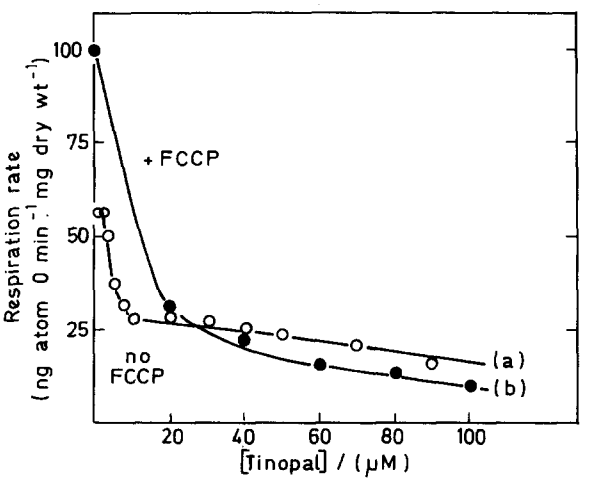

Fig. 3. Inhibition of respiration of intact cells of *Paracoccus denitrificans* by Tinopal AN in the presence and absence of uncoupler. Respiration was assayed as described in the legend to Fig. 2, except that 1 μM FCCP was either (a) absent from (○——○) or (b) present in (●——●) the reaction mixture.

To localise the site of inhibition of respiration, and to determine whether it was a consequence of a direct block in electron transfer, membrane vesicles of *P. denitrificans* were prepared. Fig. 4 shows the changes in respiration rate for the three energy-coupling segments of the aerobic respiratory chain. The rate of electron transfer either through segment III (using ascorbate plus *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) as the electron-donating system, Fig. 4c) or through segments II plus III (using succinate as electron donor, Fig. 4b), was unchanged throughout the wide range of Tinopal concentrations tested. However, the rate of electron transfer through all three segments (using NADH as electron donor) was markedly inhibited (Fig. 4a), indicating that Tinopal AN was a selective inhibitor of respiration in the NADH dehydrogenase region of the aerobic respiratory chain of *P. denitrificans*. Further, when two different concentrations of the membrane vesicle suspension were used, there was no significant difference in the amount of inhibition by Tinopal AN (Fig. 4a), indicating a relatively loose binding of Tinopal AN to its inhibitory site in the visicle membrane.

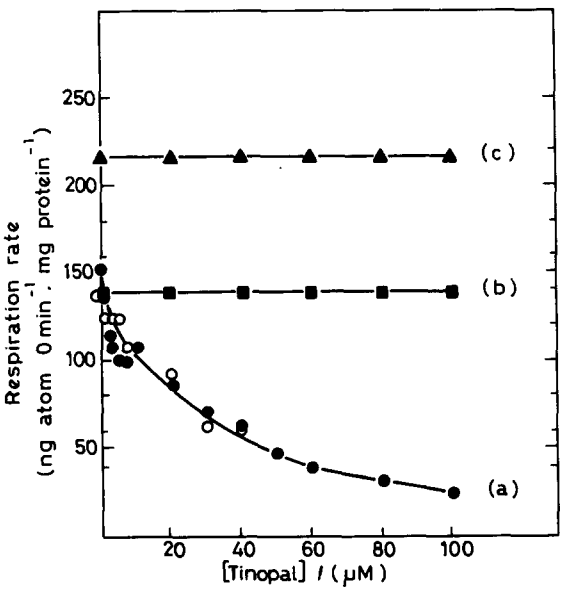

Fig. 4. Effect of Tinopal AN on respiration rate for three segments of the respiratory chain of cytoplasmic membrane vesicles of *Paracoccus denitrificans*. Respiration was monitored as described in the legend to Fig. 2 in a 3 ml reaction mixture containing 0.1 M Na_2HPO_4 pH 7.3. When NADH was the substrate (a), 1% v/v ethanol, 50 μg alcohol dehydrogenase and 0.6 mM NAD^+ together with 0.5 mg (O——O) or 1 mg (●——●) membrane vesicle protein were present. Alternatively (b) 10 mM disodium succinate and 1 μM rotenone or (c) 5 mM sodium D-*iso*ascorbate plus 0.1 mM TMPD hydrochloride and Antimycin A (1 μM) were added to the basal reaction mixture with 1 mg vesicle protein.

4. DISCUSSION

The growth inhibition of *P. denitrificans* by Tinopal AN observed previously was shown to be associated with an inhibition of respiration in washed cell suspensions in the presence of concentrations of the inhibitor as low as 5 μM. Because the addition of the protonophorous uncoupler FCCP to whole-cell suspensions had no significant effect on the ID_{50} value for Tinopal AN in the oxygen uptake assay (Fig. 3), it would appear that Tinopal AN exerted its action as a respiratory inhibitor at the outer face of the cytoplasmic membrane in vivo, for otherwise membrane de-energisation would be expected to result in a decreased sensitivity of cells to the cationic inhibitor. Indeed the approximate ID_{50} for respiration with NADH in inverted cytoplasmic membrane vesicles (Fig. 4) was found to be significantly larger than that observed in intact cells (Fig. 3).

To determine which site of electron transfer was affected, respiration was assayed in membrane-vesicle preparations (Fig. 4). The results showed that, whereas respiration through segments II and III of the aerobic electron transport chain was unaffected by the addition of rather high concentrations of Tinopal AN, respiration through segment I was strongly inhibited. Since a similar inhibition occurred for both vesicle concentrations used, it appears that Tinopal AN is not very tightly bound to its inhibitory site in the membrane. It may be concluded from the work described above that Tinopal AN acts as a potent inhibitor of electron transport in the NADH dehydrogenase region of the respiratory chain of *P. denitrificans*. However, the lethal effects of this compound may be exerted by an entirely independent mechanism, since its ability to bind selectively to nuclear material in intact polymorphonuclear leukocytes has been known for some time [1].

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