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THE EFFECTS OF MICONAZOLE ON RAT LIVER MITOCHONDRIA

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Abstract—The antifungal agent Miconazole nitrate has been shown to cause uncoupling and at higher concentrations an inhibition of oxidative phosphorylation in isolated rat liver mitochondria, with concurrent damage to the mitochondrial membranes.

Miconazole nitrate (1-(2,4-dichloro-β-(2,4-dichlorobenzyloxy)phenethyl)imidazole nitrate) has a broad spectrum of *in vitro* activity against most pathogenic fungi and Gram-positive bacteria. It is effective as a topical applicant in treating skin and nail infections and vaginal candidiasis [1]. Electron microscopic studies of *Candida albicans* exposed to low concentrations of miconazole [2] showed destructive changes in the cell wall and plasmalemma. Van den Bossche [3] demonstrated an inhibition of purine and glutamine uptake and enhanced nucleoside transport. Radioactive studies indicated that most of the drug was located in the cell wall and plasmalemma of log phase *Candida albicans*. Swamy, Sirsi and Rao [4] showed alterations in cell permeability and leakage of inorganic ions, amino acids, 260 nm absorbing materials and proteins from *Candida albicans*. Since miconazole apparently interferred with membrane function in *Candida*, its effect on mitochondrial oxidative phosphorylation and structural integrity were investigated. Since it is difficult to prepare intact mitochondria from *Candida*, rat liver mitochondria were used in this study.

Rats fed *ad lib.* were killed by decapitation, the livers removed and mitochondria prepared by the method of Chappell and Hartford [5], except that 0.25 M sucrose/5 mM Tris/1 mM EDTA pH 7.4 was used as the suspension buffer. Rates were measured in a Rank oxygen electrode maintained at 29°, connected to a Vitatron chart recorder. The electrode vessel contained a suspension of mitochondria (1–5 mg protein) in 20 mM Tris/80 mM KCl/5 mM $MgCl_2$/12.5 mM sodium phosphate buffer, pH 7.4, total vol 4.1 ml. 0.1 ml of a substrate solution was added, these were: 0.385 M sodium glutamate/0.115 M malic acid: 0.5 M sodium succinate and 1 M ascorbate/10 mM TMPD (Tetramethyl-*p*-phenylenediamine).

25 μl of 40 mM ADP was added to give state 3 mitochondria (defined by Chance [6]), and 2 min after returning to state 4, miconazole was added as a 5 mg/ml ethanolic solution and the rates of oxygen consumption determined. The rat liver mitochondria prepared gave P:O ratios of 2.5, 1.7 and respiratory control ratios of 2.5, 2.2 for glutamate–malate and succinate respectively. Protein was determined by the method of Lowry [7] using bovine serum albumin as standard. Miconazole was a gift from Janssen Pharmaceutical.

The effects of miconazole on the oxidation of glutamate–malate and succinate are shown in Fig. 1. Here the percentage value of the state 4/state 3 rates of oxygen consumption (i.e. the reciprocal respiratory control ratio) is taken as a measure of the rate of oxidation and its coupling to phosphorylation. Miconazole showed the same uncoupling/inhibition pattern for both substrates, the inhibition of glutamate–malate oxidation occurring at lower miconazole concentrations than succinate oxidation. Additions of succinate to mitochondria in which glutamate–malate oxidation had been inhibited by miconazole stimulated oxygen consumption. Ascorbate/TMPD oxidation was uncoupled by miconazole but inhibition only occurred at saturating concentrations of miconazole and was not studied further. Ethanol at the concentrations used had no effect on coupling. The uncoupling was also dependent upon the mitochondrial suspension density and there was a linear relationship between the amount of miconazole required for maximum uncoupling and the amount of mitochondrial protein used (Fig. 2).

Possible explanations for this pattern of uncoupling/inhibition include (i) an effect on the mitochondrial ATPase to give a rapid turnover of ATP to ADP. (ii) An uncoupling effect analogous to that

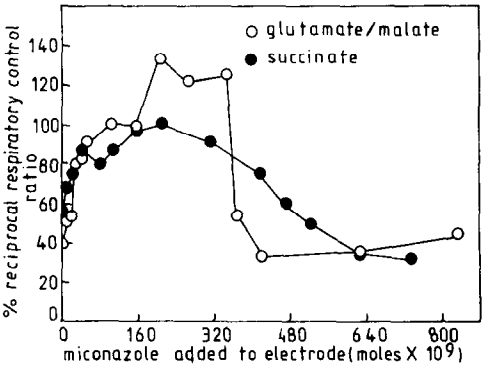

Fig. 1. The effect of miconazole on oxygen consumption by rat liver mitochondria. Total mitochondrial protein 2.5 mg. Standard error ± 0.09 × plotted %.

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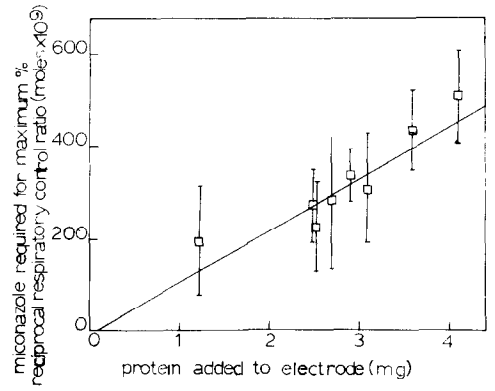

Fig. 2. The relationship between the level of miconazole required for maximum reciprocal respiratory control and the total protein used for both glutamate/malate and succinate. Correlation coefficient 0.88.

of DNP or of tyrocidin and gramicidin [8–11], which show this pattern of uncoupling and inhibition, and further, ascorbate/TMPD oxidation is only poorly inhibited [8,10]. (iii) Miconazole could cause physical changes in the inner membrane, rendering it permeable to protons.

It is unlikely that miconazole reacts directly with the ATPase, since addition of oligomycin after miconazole had no significant effect upon the rate of oxygen consumption, whilst miconazole stimulated oxygen consumption from 44% to 128% state 4/state 3 (glutamate–malate) in mitochondria which had been inhibited by oligomycin. DNP did not relieve the inhibitory effects of miconazole. The gross integrity of the inner membrane was studied by measuring the release of the matrix enzyme citrate synthase. The release was followed in the same incubation mixture as that used in following oxygen uptake. Two min after the addition of miconazole the mitochondria were removed by centrifugation and samples of the supernatant fluid were added to the following mixture: 2.7 ml 0.075 M Tris–HCl buffer pH 7.0, 0.1 ml 1 mM Acetyl CoA, 0.1 ml 5 mM DTNB (sodium dithionitrobenzoate). After equilibration 0.1 ml of 0.05 M sodium oxaloacetate was added and the reaction followed by measuring the O.D. at 412 nm using a Unicam SP 600 spectrophotometer.

Figure 3 compares the effects of miconazole concentration upon the release of citrate synthase with the effect on succinate oxidation. In the range of miconazole concentrations causing maximum succinate oxidation there was an increase in enzyme release up to a maximum value, which was comparable to that obtained by destruction of the mitochondria with 0.03% Triton X100. Ethanol at the concentrations used caused no release of citrate synthase.

The results can be explained by proposing a destruction of the inner membrane analogous to that observed from the plasmalemma of *Candida albicans*. At low miconazole/mitochondria ratios a small amount of damage would be caused, rendering the membrane permeable to protons, thereby uncoupling the mitochondria. At higher ratios more extensive damage with the release of matrix proteins, and inhibition of oxygen consumption occurs. The latter could be due to an extensive loss of membrane integrity, or alternatively a direct inhibition of the respiratory chain components. Recently [12] it has been shown that miconazole causes hemolysis of erythrocytes, and forms complexes with membrane lipoproteins. In the present study, the destruction of the mitochondrial membrane prevented any distinction being made between a specific and a non-specific uncoupler action.

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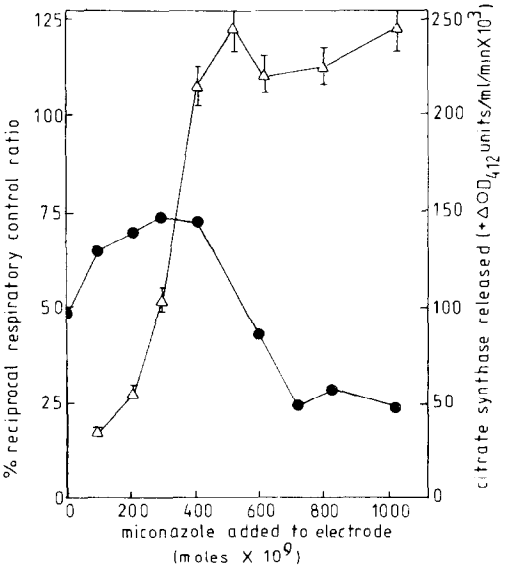

Fig. 3. The miconazole-induced release of citrate synthase from rat liver mitochondria. Succinate substrate, 2.5 mg protein.

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