pathological behavioral changes. There was, however, a slight decrease in general swimming activity.

Discussion. This is the 1st demonstration of the efficacy of an anorectic drug to reduce food intake in fish. From experiments on rats it is known that diethylpropion decreases dose-dependently the excitability of the hunger area in the lateral hypothalamus[10]. It may also be possible that it acts upon the satiation center of the ventromedial hypothalamus[11]. In fish, as in mammals, there is evidence for a hypothalamic feeding area (HFA)[12,13]. This HFA is situated near the lateral recess of the 3rd ventricle in the inferior lobe of the hypothalamus. It represents the general region from which low-threshold feeding responses can be evoked by electrical stimulation and the area in which bilateral lesions cause drastic reductions in feeding[14–16]. It may be possible that diethylpropion acts in fish in a way analogous to that in mammals by reducing the excitability of the HFA.

1 This work was carried out at the Institut für Biologie III der Universität Tübingen. Abt. physiologische Verhaltensforschung, Tübingen (FRG). I want to thank Prof. Dr H.M. Peters for suggesting this study, the Temmler-Werke, Marburg for providing the substance and Dr B. Schulz for improving the English.

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Effect of ketoconazole and miconazole on skeletal muscle mitochondrial calcium transport system

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Summary. Both ketoconazole and miconazole inhibit the state 3 respiration induced by Ca^{2+}, stimulate the state 4 respiration during succinate oxidation, inhibit the uptake of Ca^{2+} and also induce Ca^{2+} release in the aerobic steady state of skeletal muscle mitochondria. Miconazole is twice as effective as ketoconazole.

Ketoconazole, cis-1-acetyl-4-(4-((2,4-dichlorophenyl)-2-(1H-imidazole-1-ylmethyl)-1,3-dioxalan-4-yl)methoxy)-phenyl)piperazine, and miconazole nitrate, 1-(2-(2,4-dichlorophenyl)2-((2,4-dichlorophenyl)methoxy ethyl)-1H-imidazole nitrate, are chemically related imidazole antimycotic agents reported to interfere with the biosynthesis of ergosterol in fungal[1,2] and yeast[3] cells. Several observations have indicated that these imidazole compounds interfere with the cellular permeability of yeast[4–6] by binding to the cell membranes and selectively inhibiting[6] the uptake of precursors of RNA and DNA and mucopolysaccharide. It is suggested that miconazole competes with divalent cations for the membrane binding sites, which results in alteration in cell membrane permeability and ultimately leads to leakage of amino acids, protein and cations[5]. Miconazole, at high concentrations, also affects the exchange of intracellular K^+ for extracellular H^+ in yeast[7] and competitively inhibits both the yeast plasma membrane-bound and lipid-reconstituted purified plasma membrane ATPase activity[8]. Yeast mitochondrial ATPase activity is inhibited 50% at pH 6.0 with 40 μM miconazole[9].

Ketoconazole, at high concentrations, has recently been shown[10] to inhibit calcium binding and accumulation, and to induce calcium release in sarcoplasmic reticulum. The Mg^{2+}-ATPase and the $(Ca^{2+} + Mg^{2+})$-ATPase activities are stimulated at low but inhibited at high concentrations of ketoconazole. This paper reports studies of the imidazole antimycotic agents specifically showing the effect of both ketoconazole and miconazole on the Ca^{2+} transport system of isolated skeletal muscle mitochondria.

Materials and methods. ATP, antimycin A, bovine serum albumin, murexide, oligomycin, rotenone and sodium succinate were purchased from Sigma Chemical Corp.; carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP) from Boehringer Mannheim; crystalline *Bacillus subtilis* (Nagarse) proteinase from Teikoku Chemical Co.; ketoconazole and miconazole were kindly supplied by Janssen Pharmaceutica, Beerse; all other reagents were of analytical grade.

Mitochondria were isolated from porcine longissimus dorsi muscle immediately post-mortem with *B. subtilis* proteinase[11]. Oxygen uptake was determined with a Clark oxygen electrode (Yellow Spring Oxygen Monitor (Model 53)) in a total volume of 2.60 ml at 25 °C. The Ca^{2+}-stimulated respiration for succinate oxidation was carried out in a reaction medium (pH 7.20) containing 225 mM mannitol, 75 mM sucrose and 15 mM Tris-HCl in the presence of 5 mM P_i. The rates of Ca^{2+} uptake and efflux of skeletal muscle mitochondria were monitored in a magnetically-stirred cuvette (10 mm light-path) with murexide (92 μM) at 21 °C with an Aminco-Chance (DW 2A) dual-wavelength/split-beam spectrophotometer operating in the dual-wavelength mode at 540–507 nm. The reaction medium was identical to that used for measuring oxygen

uptake except that 2.0 mM P_i was used instead of 5.0 mM P_i. Protein was determined according to the method of Lowry et al.[12] using bovine serum albumin as standard.

Electron microscopy was carried out as described by Allmann et al.[13] but without using acrolein. Thin sections of the various mitochondrial preparations, embedded in Epon 812, were cut with a glass knife and stained with 2.0% uranyl acetate in methanol[14] and lead citrate[15] before examination with an AEI (Model EM6-B) electron microscope. For examinations of the ultrastructural configurations of mitochondria in the presence and absence of ketoconazole, the mitochondrial suspension at the end of each experiment was treated with an equal volume of 2.5% glutaraldehyde in 50 mM sodium cacodylate and 180 mM sucrose buffer (pH 7.4) for 2 min, and then centrifuged for 1.5 min with an Eppendorf (Model 3200) microcentrifuge. The mitochondrial pellet was fixed for 1 h with 2.5% glutaraldehyde in 50 mM sodium cacodylate and 180 mM sucrose buffer (pH 7.4) at 0 °C, post-fixed with 1% osmium tetroxide in 50 mM sodium cacodylate and 300 mM sucrose buffer (pH 7.4) for 1 h at 0 °C, and then dehydrated in ethanol before being embedded in Epon 812.

Results and discussion. Mitochondria isolated from porcine longissimus dorsi muscle were tightly coupled. The respiratory control index for the Ca^{2+}-stimulated respiration during succinate oxidation of our routine mitochondrial preparation showed a value of 6.91 ± 0.39 (n = 7) at 25 °C.

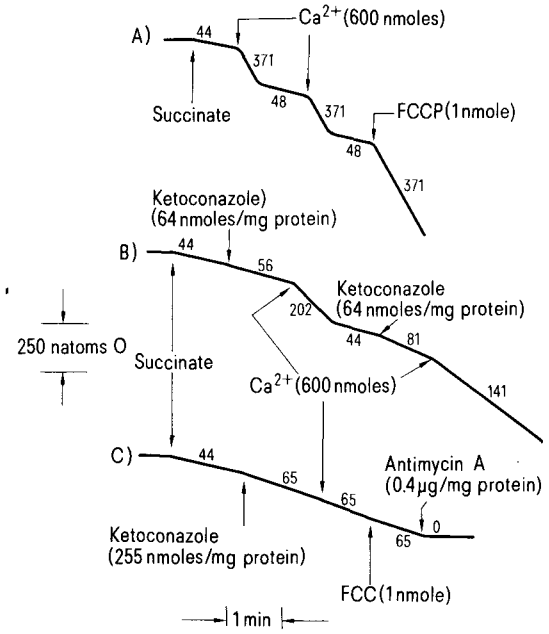

Figure 1. Effect of ketoconazole on Ca^{2+}-stimulated respiration during succinate oxidation by longissimus dorsi muscle mitochondria. *A* illustrates a typical polarographic control experiment showing the state 3-state 4 transition induced by Ca^{2+} during succinate oxidation, and the stimulation of respiration by the uncoupler FCCP. *B* illustrates a typical experiment showing the stimulation of succinate oxidation by ketoconazole and the effect of ketoconazole on Ca^{2+}-stimulated respiration. *C* shows a typical experiment of the complete inhibition of Ca^{2+}-stimulated respiration during succinate oxidation by ketoconazole, the failure of FCCP to relieve the inhibited respiration in the presence of ketoconazole, and the subsequent complete inhibition of respiration by antimycin A. – In all the polarographic experiments rotenone (2 µM) was added prior to sodium succinate (10 mM). The numbers alongside the electrode traces (A–C) represent the rates of oxygen uptake expressed in natoms O per min per mg protein at 25 °C. 2.51 mg mitochondrial protein was used in each experiment. Other experimental details are given in 'Materials and methods'.

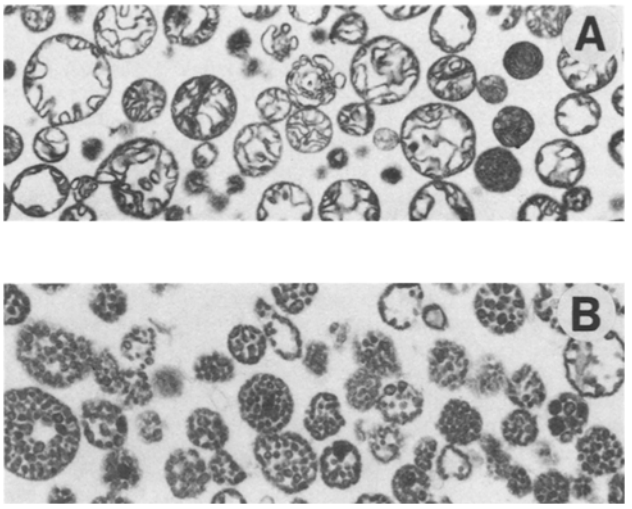

Figure 2. Thin sections of mitochondria from longissimus dorsi muscle showing the ultrastructural changes induced by ketoconazole during succinate oxidation in the presence of exogenous Ca^{2+}. *A* was prepared after 2 state 3-state 4 transition induced by 2 additions of 600 nmoles Ca^{2+} during succinate oxidation as illustrated by trace A in figure 1 except that FCCP was omitted. Total Ca^{2+} added was 478 nmoles per mg protein and this amount of exogenous Ca^{2+} was insufficient to uncouple the longissimus dorsi muscle mitochondria[11]. *B* was prepared after the addition of 255 nmoles ketoconazole per mg protein to inhibit the state 3 respiration induced by Ca^{2+} during succinate oxidation as shown in trace C of figure 1 except that FCCP and antimycin A were omitted. Other experimental details are given in 'Materials and methods'.

Figure 1 illustrates direct oxygen electrode tracings of typical experiments showing the effect of ketoconazole on mitochondrial respiration with succinate as substrate. The first addition of 64 nmoles ketoconazole per mg protein before Ca^{2+} results in stimulation of succinate oxidation from 44 to 56 natoms O per min per mg protein (trace B), and inhibition of the state 3 respiration induced by Ca^{2+} from 371 (trace A) to 202 natoms O per min per mg protein (trace B). Under these conditions the respiratory control index was reduced from 7.73 (trace A) to 4.59 (trace B). A further addition of a similar amount of ketoconazole resulted in an increase of state 4 respiration by about 1-fold and a further inhibition of the state 3 respiration with a complete loss of respiratory control. Trace C suggests that the site of action of ketoconazole is between the respiratory chain system and the formation of the high energy state since the inhibition of the state 3 respiration induced by Ca^{2+} with 255 nmoles ketoconazole per mg protein was not relieved by the classical uncoupler FCCP, and respiration was inhibited completely following the subsequent addition of antimycin A.

The state 3 and state 4 respiration induced by Ca^{2+} during succinate oxidation was affected by both ketoconazole and miconazole. State 4 respiration was stimulated by ketoconazole at concentrations greater than 40 nmol per mg protein, with maximum stimulation observed at 160 nmoles per mg protein. A further increase in the concentration of ketoconazole above this value resulted in inhibition of the state 4 respiration. State 3 respiration was inhibited by ketoconazole, and the respiratory control index was reduced to a value of 1.0 with 130 nmoles ketoconazole per mg protein. Like ketoconazole, miconazole also inhibited the state 3 respiration and stimulated the state 4 respiration, and this was accompanied by a reduction in both the respiratory control index and the Ca^{2+}/O ratio. Micona-

zole, however, was a more potent inhibitor than ketoconazole in that state 3 respiration induced by Ca^{2+} was inhibited 50% with 28 nmoles miconazole per mg protein as compared with 75 nmoles ketoconazole per mg protein.

Thin sections of mitochondria prepared at the end of the polarographic experiments using Ca^{2+} to induce the state 3 to state 4 transitions during succinate oxidation in the control experiment (see fig. 1 (trace A) except that FCCP was omitted) showed that the mitochondria were intact and in the aggregated configuration[16] after a total addition of 720 nmoles Ca^{2+} per mg protein (fig. 2, A). In the presence of 255 nmoles ketoconazole per mg protein, sufficient to inhibit the Ca^{2+}-stimulated respiration during succinate oxidation (see fig. 1 (trace C) except that FCCP and antimycin A were omitted), the mitochondria were transformed from the aggregated (fig. 2, A) to the orthodox (fig. 2, B) configuration[16]. No large amplitude swollen or broken mitochondria were observed in the presence of ketoconazole.

Both miconazole and ketoconazole inhibited the rate of Ca^{2+} uptake and induced Ca^{2+} release in longissimus dorsi muscle mitochondria. As with the oxygen electrode experiments miconazole was more potent than ketoconazole. Figure 3 illustrates typical spectroscopic experiments with murexide, showing the effect of miconazole on the rate of Ca^{2+} uptake and release. Addition of 110 nmoles miconazole per mg protein induced Ca^{2+} release from mitochondria previously loaded with Ca^{2+} (fig. 3, ---) and if added prior to Ca^{2+} plus succinate inhibited Ca^{2+} uptake (fig. 3, ...). Like miconazole, ketoconazole also inhibited Ca^{2+} uptake and induced Ca^{2+} release but 210 nmoles of ketoconazole per mg protein were required to produce a similar effect as miconazole. The concentration of antimycotic agents used in these experiments did not inhibit electron transport. The rate of Ca^{2+} uptake by mitochondria was more sensitive to miconazole. The rate of Ca^{2+} uptake by longissimus dorsi muscle mitochondria was inhibited 50% with 39 nmoles miconazole per mg protein but 84 nmoles ketoconazole per mg protein was required to give a similar extent of inhibition.

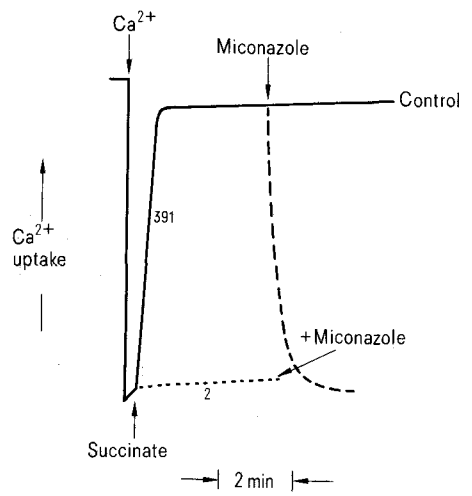

Figure 3. Inhibition of Ca^{2+} uptake and induction of Ca^{2+} release from longissimus dorsi muscle mitochondria by miconazole. The figure represents typical spectroscopic experiments showing the inhibition of Ca^{2+} uptake (···) and induction of Ca^{2+} release (---) by miconazole (110 nmoles per mg protein). The experiments were started by addition of 250 nmoles Ca^{2+} per mg protein and 10 mM sodium succinate. The amount of Ca^{2+} added was insufficient to uncouple the longissimus dorsi muscle mitochondria[11].

Experiments using polarographic and spectroscopic techniques showed that both ketoconazole and miconazole inhibited the state 3 respiration but stimulated the state 4 respiration of succinate oxidation, inhibited Ca^{2+} uptake and induced Ca^{2+} release in skeletal muscle mitochondria. Miconazole was far more effective than ketoconazole. The stimulation of state 4 respiration, the inhibition of Ca^{2+} uptake and the promotion of Ca^{2+} release suggest that these antimycotic compounds have an uncoupling effect on skeletal muscle mitochondria. However, the inhibition of respiration in the presence of Ca^{2+} tends to suggest that these compounds could have a mixed effect, combining uncoupling plus ruthenium red-like action, on skeletal muscle mitochondria. The latter suggestion is currently being investigated. The site of action of these antimycotic imidazole compounds was demonstrated to be in the inner mitochondrial membrane, acting between the electron transport chain and the formation of the high energy state. Electron microscopy showed that the cristae of skeletal muscle mitochondria were transformed from aggregated to orthodox configuration by ketoconazole at a concentration sufficient to completely inhibit the state 3 respiration induced by exogenous Ca^{2+} during succinate oxidation in the presence of rotenone. It is well documented that isolated mitochondria undergo configuration changes concomitant with changes in the energy state of the mitochondria[17-21], and that the transformation to and stabilisation of the orthodox configuration results in the loss of both the capacity of the mitochondria to carry out coupled reactions and to undergo energised configuration transitions[16]. The coupled reactions include ATP synthesis, divalent cation translocation, monovalent cation translocation and reversed electron transfer[16]. The configuration changes induced by ketoconazole thus support the combined polarographic and spectroscopic data in demonstrating that both the transport of Ca^{2+} and the formation of the high energy state of skeletal muscle mitochondria were inhibited by these antimycotic imidazole compounds.

Preliminary experiments showed that both miconazole and ketoconazole stimulated the skeletal muscle mitochondrial Mg^{2+}-ATPase activity but inhibited the $(Ca^{2+} + Mg^{2+})$-ATPase activity with miconazole being at least 2.5 times more effective than ketoconazole. The stimulation of the mitochondrial Mg^{2+}-ATPase activity could be explained by an increase in mitochondrial permeability by both ketoconazole and miconazole, as in yeast[5], resulting in an activation of the latent mitochondrial enzyme. The effective concentrations of these antimycotic agents are very much higher than those required to inhibit sterol synthesis in yeast. For example, the inhibition of state 3 respiration induced by Ca^{2+} required 90 nmoles miconazole per mg protein i.e. about 9×10^{-5} M as compared with a concentration of between 10^{-10} and 10^{-8} M for inhibition of sterol synthesis in yeast[2,3]. Thus, the antimycotic agents could be useful for in vitro studies on skeletal muscle mitochondria and sarcoplasmic reticulum[10], and are unlikely to have any effect on these organelles in vivo.

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Elimination of selenium compounds by mice through formation of different volatile selenides

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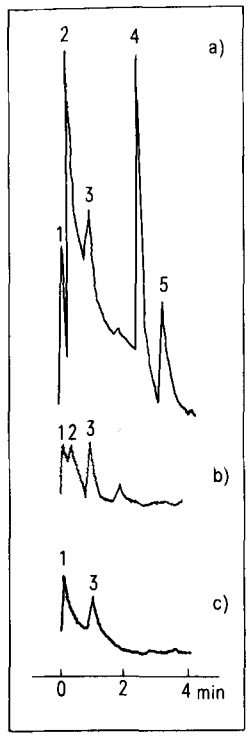
Summary. The chemical form of added selenium determines the amount and the species of selenide metabolites in the breath of mice. Exhalation seems to be a minor form of selenium elimination.

Animals, especially rats, given large doses of different selenium compounds, exhale volatile selenides, primarily dimethylselenide. Prochazkova et al.[2] trapped volatile selenium species, exhaled by rats after addition of selenite, on a synthetic adsorbent and separated them by gas chromatography. Dimethylselenide proved to be the major compounds, while a smaller amount of another selenium species was detected, which behaved on chromatography in a similar way to dimethyldiselenide. It has been shown for rats that the chemical form of selenium administered[3-5], the method of administration[5,6], the quantity[4,5], the purity of the added compound[7] and some properties of the diet[3,7-11] are determining factors in the quantity of volatile selenium formed, and therefore different experimental conditions were tested out on mice. By combining a cryogenic sampling procedure with a simple gas chromatographic-graphite furnace atomic absorption system as described by Jiang et al.[12] and applied for the detection and determination of alkylselenides in environmental air[13], we were able to determine different selenides in the breath of mice, down to 0.3 ng/100 g b.wt.

Addition of sodium selenite, D,L-selenomethionine or D,L-selenocystine to the drinking water of mice resulted in the formation of dimethylselenide and also dimethyldiselenide. The same amount of dimethylselenide was observed in the breath at different sampling times in both experiments with the seleno-amino acids added to the drinking water. For the selenomethionine a 3rd unidentified selenium metabolite was observed in the breath, which contributed to a large extent to the elimination of selenium by the lungs. Selenocystine and selenite addition to drinking water resulted in the production mainly of dimethylselenide, while for selenomethionine the main compounds were the dimethyldiselenide and an unidentified 3rd species as shown in the figure. These findings extend the observations of Thomson et al.[14] on the metabolism of selenocystine, selenomethionine and selenite in rats. They stated that the bioconversion of selenocystine ressembled that of selenite rather than that of selenomethionine.

Also, Greeder and Milner[15] proved that the effectiveness of selenite, selenate and D,L-selenocystine in limiting tumor growth in mice differs considerably from that of selenomethionine. Literature data on the different bioconversion of selenite and selenomethionine in rats has been summarized by Cary et al.[16].

D,L-selenocystine, added to the drinking water or i.p. injected into mice, is predominantly metabolized to the dimethylselenide species. Injection of a 8.4 times higher selenium concentration as D,L-selenocystine resulted in a



Selenium metabolites in the breath of mice after administration for 14 days of DL-selenomethionine (a), DL-selenocystine (b) and selenite (c) via drinking water. Peaks: 1 (air), 2 (water), 3 (dimethylselenide), 4 (unidentified compound) and 5 (dimethyldiselenide).