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Effects of Equisetin on Rat Liver Mitochondria: Evidence for Inhibition of Substrate Anion Carriers of the Inner Membrane

Tamás König,[1] András Kapus,[2] and Balázs Sarkadi[3]

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The effect of equisetin, an antibiotic produced by *Fusarium equiseti*, has been studied on mitochondrial functions (respiration, ATPase, ion transport). Equisetin inhibits the DNP-stimulated ATPase activity of rat liver mitochondria and mitoplasts in a concentration-dependent manner; 50% inhibition is caused by about 8 nmol equisetin/mg protein. The antibiotic is without effect either on the ATPase activity of submitochondrial particles or on the purified F_1-ATPase. It inhibits both the ADP- or DNP-activated oxygen uptake by mitochondria in the presence of glutamate + malate or succinate as substrates, but only the ADP-stimulated respiration is inhibited if the electron donors are TMPD + ascorbate. It does not affect the NADH or succinate oxidation of submitochondrial particles. Equisetin inhibits in a concentration-dependent manner the active Ca^{2+}-uptake of mitochondria energized both by ATP or succinate without affecting the Ca^{2+}-uniporter itself. The antibiotic inhibits the ATP-uptake by mitochondria (50% inhibition at about 8 nmol equisetin/mg protein) and the P_i and dicarboxylate carrier. It does not lower the membrane potential at least up to 200 nmol/mg protein concentration. The data presented in this paper indicate that equisetin specifically inhibits the substrate anion carriers of the mitochondrial inner membrane.

KEY WORDS: Respiratory chain; ATPase; substrate anion carriers; equisetin; rat liver mitochondria.
ABBREVIATIONS: EGTA, ethyleneglycol bis/-aminoethylether/-N, N-tetraacetic acid; DNP, 2, 4-dinitrophenol; TMPD, N,N,N',N',tetramethyl-p-phenylenediamine; CCP, carbonylcyanide-m-chlorophenyl hydrazone; TPP, tetraphenyl-phosphonium; Hepes, /4,(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid/.

INTRODUCTION

Equisetin, an antibiotic produced by *Fusarium equiseti* (Corda) Saccardo has been isolated and purified by Burmeister *et al.* (1974). It is active against several strains of Gram-positive bacteria; however, it does not inhibit Gram-negative bacteria and fungi (Burmeister *et al.*, 1974; Vesonder *et al.*, 1979). The structure of equisetin has been elucidated by Vesonder *et al.* (1979) and identified as a derivative of *N*-methyl-2,4-pyrollidone with a molecular mass of 373. In the course of testing antibiotics regarding their possible interaction with mitochondria, we have found that equisetin has an unusual ensemble of effects on rat liver mitochondria. These effects are described in this paper with the conclusion that the mitochondrial mechanism of equisetin action is the inhibition of substrate anion carriers of the inner membrane. Parts of the results have already been published in short form (König *et al.*, 1986; König and Kapus, 1990), though in the first abstract (König *et al.*, 1986) the results were misinterpreted, suggesting that equisetin was a proton pump inhibitor.

[1] Department of Biochemistry II, Semmelweis University of Medicine, Budapest, Hungary.
[2] Department of Physiology, Semmelweis University of Medicine, Budapest, Hungary.
[3] National Institute of Haematology, Blood Transfusion, and Immunology, Budapest, Hungary.

MATERIALS AND METHODS

Chemicals: [U-^{14}C] succinate was purchased from Radiochemical Centre (Amersham, England). [U-^{14}C]ATP was a product of UVVVR (Institute for

0145-479X/93/1000-0537$0.7.00/0

Research, Production, and Application of Radioisotopes) Prague, Czechoslovakia and their radiochemical purity was checked as described earlier König *et al.* (1982). Equisetin was a kind gift from Dr. Ronald F. Vesonder (Northern Regional Research Laboratory, Agricultural Research Service, Peoria, Illinois) and was used as an ethanol solution. In all experiments appropriate controls were made with ethanol. All substrates were added in the form of either Tris or sodium salt, pH 7.2. The chemicals used were the purest commercially available.

Preparation of Mitochondria, Mitoplasts, and Submitochondrial Particles

Rat liver mitochondria were isolated as described earlier (König *et al.*, 1977) except that EGTA was omitted from the medium used for washing the mitochondria. Mitoplasts were prepared from rat liver mitochondria according to Schnaitman *et al.* (1967); (Schnaitman and Greenawalt, 1968) using 1.2 mg digitonin/10 mg mitochondrial protein. The mitoplasts showed less than 3% of the mitochondrial adenylate kinase activity and their ATPase was completely carboxyatractylate sensitive. Submitochondrial particles were prepared according to Gregg (1963) as described in König *et al.* (1977).

Measurement of O_2 Uptake

Oxygen uptake was measured with a Clark-type oxygen electrode at 37°C in a medium (1.4 ml) containing 90 mM sucrose, 80 mM KCl, 20 mM Tris-HCl, pH 7.2 100 μM EGTA, and 5 mM potassium phosphate. Other experimental details are given in the legend to the figures.

Determination of ATPase Activity

The ATPase activity of mitochondria and mitoplasts was measured as described earlier (König *et al.*, 1977) at 37°C in a medium (1 ml) containing 90 mM sucrose, 80 mM KCl, 20 mM Tris-HCl, pH 7.2, and 100 μM EGTA. The concentration of ATP was 6 mM and, where indicated, 3 mM $MgCl_2$ was also added. When the ATPase activity of submitochondrial particles was measured, the incubation medium contained 120 mM KCl, 20 mM Tris-HCl, pH 7.2, 1 mM EGTA, and 4 mM $MgCl_2$. The concentration of ATP was 6 mM. Further experimental details are given in the legend to the figures.

Determination of Ca^{2+} Uptake

Active calcium uptake by mitochondria was measured by rapid vacuum filtration through Sartorius membrane filters (0.45 μm pore size). The incubation media contained 80 mM KCl, 90 mM sucrose, 20 mM Tris-HCl, pH 7.2, 1 μM rotenone, 0.5 mM ATP or 10 mM Na-succinate, and 200 μg/ml mitochondrial protein. The reaction was started by the addition of $CaCl_2$ (50 μM final concentration, including ^{45}Ca tracer). Samples were taken after 1 min incubation. Incubation temperature was 22°C. Calcium uptake without added substrate (rotenone present) was less than 2 nmol/mg protein. It was antimycin sensitive if the energy source was succinate oxidation, oligomycin sensitive if energy was generated by ATP hydrolysis, and uncoupler sensitive in both cases.

Determination of ATP Uptake

Mitochondrial ATP uptake was measured by the method of Wojtczak and Zaluska (1967), with the modifications described earlier (König *et al.*, 1982) using 20 μM [U-^{14}C]ATP as substrate, in a medium (1 ml) containing 100 mM KCl, 1 mM EGTA, 20 mM HEPES-Tris, pH 7.2, 10 μg oligomycin, 1 μM CCCP, mitochondria (2.5 mg protein), and equisetin at the concentrations indicated. Incubation temperature was 0°C. The uptake was started by the addition of 20 μM [U-^{14}C]ATP and after 10 sec stopped by adding 50 μM carboxyatractylate to the reaction mixture. After rapid centrifugation the pellet was washed twice and extracted with perchloric acid. An aliquot of the protein-free extract was assayed for radioactivity. Corrections were made by running parallel samples in which carboxyatractylate was added to the incubation medium 10 sec before the labeled substrate. Other experimental details are described in the legend to the figure.

Measurement of P_i Transport

P_i transport across the inner membrane of liver mitochondria was followed under two different conditions, either as equilibrium exchange of P_i in nonrespiring mitochondria (Ligeti *et al.*, 1985), or as net P_i uptake of respiring mitochondria coupled to the simultaneous accumulation of K^+ (Ligeti and Fonyó, 1984). For the equilibrium exchange measurements mitochondria corresponding to 4.2 mg protein were preincubated in 2 ml of a medium containing 250 mM sucrose, 2 mM KCl, 10 mM Tris-HCl, pH 7.2, 1 mM Tris-P_i 5 mM butylmalonate, and 1 μM rotenone at 25°C. Equisetin when present was added immediately after the mitochondria. After 1 min P_i transport was initiated by the addition of a tracer amount of [^{32}P]P_i

in 10 μl to ensure final specific activity of approximately 1000–1500 cpm/nmol P_i. After 30 sec the P_i transport was stopped by 25 nmol mersalyl/mg protein. After rapid centrifugation the supernatant was discarded, and the tube rinsed with ice-cold 0.25 M sucrose and wiped dry. The pellet was extracted with perchloric acid. An aliquot of the protein-free extract was assayed for radioactivity measured as the Cherenkov effect. Corrections were made by running parallel samples in which mersalyl was added before [^{32}P]P_i. Net P_i uptake was measured similarly except that 60 sec after the addition of the isotope 0.1 μg valinomycin/ml was added and respiration was initiated by 5 mM Tris-ascorbate + 50 μM TMPD. The uptake was terminated after 30 sec by mersalyl.

Measurement of Succinate Uptake

Mitochondria were loaded with L-malate, as described earlier (König *et al.*, 1982). Malate-loaded mitochondria (3 mg protein) were incubated in a medium (1 ml) containing 100 mM KCl, 40 mM K-Hepes, pH 7.2, 1 mM EGTA, 2 μg oligomycin, 2 μg antimycin A, 1 μM rotenone, and equisetin as indicated in the figure at 0°C. The uptake was started by the addition of 1 mM [U-^{14}C]succinate (1000 cpm/nmol) and after 30 sec stopped by adding 10 mM phenylsuccinate to the reaction mixture. After rapid centrifugation, radioactivity was assayed in the same way as described at ATP-uptake. Corrections were made by running parallel samples in which phenylsuccinate was added to the incubation medium 30 sec before the labeled substrate. Other experimental details are given in the legend to the figure.

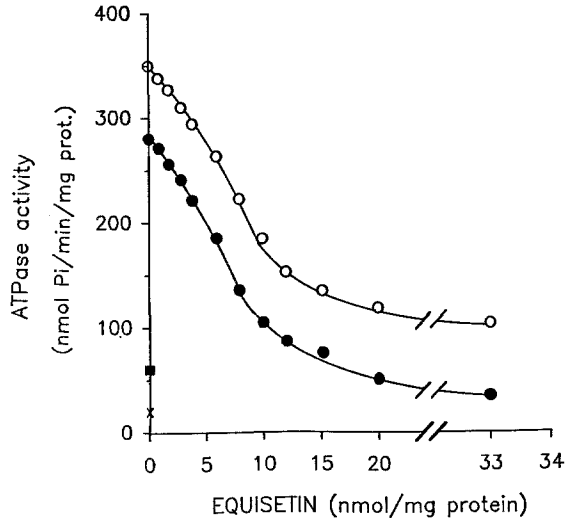


Fig. 1. Effect of equisetin on the DNP-stimulated ATPase activity of mitochondria. Rat liver mitochondria (3 mg protein/ml) were incubated at 37°C with 6 mM ATP in the presence of 100 μM DNP under conditions described in Materials and Methods. After 3 min the reaction was stopped by trichloroacetic acid, centrifuged, and inorganic phosphate (P_i) determined. (●) no $MgCl_2$; (○) +3 mM $MgCl_2$. Control ATPase activities are indicated on the ordinate: (×) no DNP, no $MgCl_2$; (■) no DNP + 3 mM $MgCl_2$.

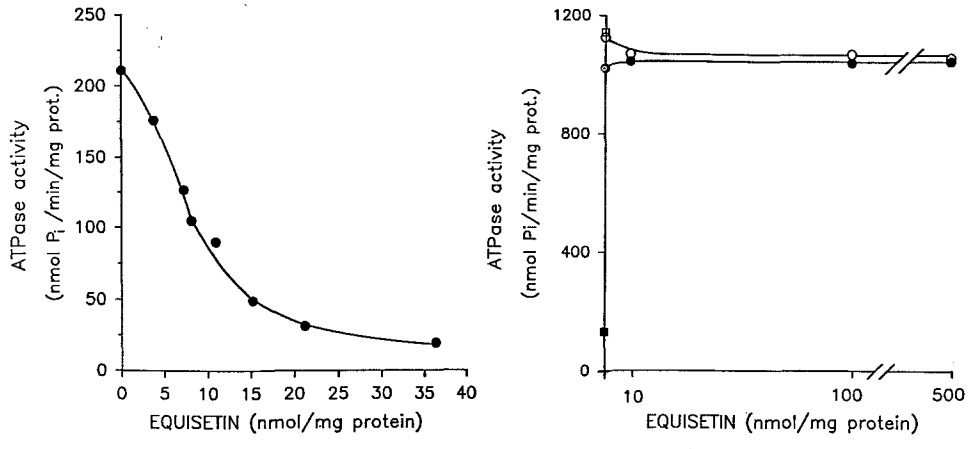


Fig. 2. (A) Effect of equisetin on the DNP-stimulated ATPase activity of mitoplasts. Experimental conditions were exactly the same as described in Fig. 1, except that mitoplasts (1.7 mg protein/ml) were used instead of mitochondria. (B) Effect of equisetin on the ATPase activity of submitochondrial particles. Submitochondrial particles (0.67 mg protein/ml) were incubated at 37°C with 6 mM ATP under conditions described in Materials and Methods. After 3 min the reaction was stopped by trichloroacetic acid, centrifuged, and P_i determined. (●) no further addition; (○) + 100 μM DNP; (■) + 100 μM DNP + 4 μg oligomycin/ml; (□) + 100 μM DNP + 20 μM carboxyatractylate.

Measurement of the Membrane Potential

The mitochondrial membrane potential was followed by a tetraphenylphosphonium (TTP^+) electrode according to Kamo *et al.* (1974). Mitochondria (3 mg) were incubated at 25°C in a medium containing 250 mM sucrose, 10 mM Tris-CL, pH 7.2, 1 μM rotenone, 500 μM ascorbate + 50 μM TMPD, and 1.5 μM TPP^+. Other experimental details are given in the legend to the figure.

Protein Determination

The protein content of mitochondria and mitoplasts was determined according to Schacterie and Pollak (1973).

RESULTS

Effect on ATPase Activity

Equisetin inhibited DNP-stimulated ATPase activity of rat liver mitochondria in a concentration-dependent manner. 50% inhibition was found at about 8 nmol antibiotic/mg protein (Fig. 1). Similar inhibition of the ATPase activity was observed if DNP was replaced by other uncouplers or by valinomycin (not shown). Mg^{2+} did not influence the inhibition of DNP-stimulated ATPase by equisetin (Fig. 1). The extent of the Mg^{2+}-stimulated ATPase activity and the difference between the parallel run of curves indicate that Mg^{2+}-stimulated ATPase activity—probably due to contamination with other cell constituents of the mitochondrial preparation—was not inhibited by equisetin. In separate experiments we found that equisetin had absolutely no effect on the ATPase activity measured in the presence of $MgCl_2$ but in the absence of DNP, and this ATPase activity was oligomycin insensitive (not shown). If mitochondria were preincubated with equisetin for 5 min before starting the reaction with ATP, the inhibition was found to be more powerful, showing a 50% inhibition at about 3 nmol equisetin/mg protein (not shown). The effect of equisetin on the DNP-stimulated ATPase activity of mitoplasts was also measured. As Fig. 2A shows, a dose-dependent inhibition was found. In fact, the dose-dependence curves of mitochondria and mitoplasts were identical. However, in contrast with mitochondria and mitoplasts, submitochondrial particles were not affected at all by equisetin even at high concentrations (Fig. 2B). In conformity with this latter finding, purified F_1-ATPase was also not inhibited by the antibiotic (not shown).

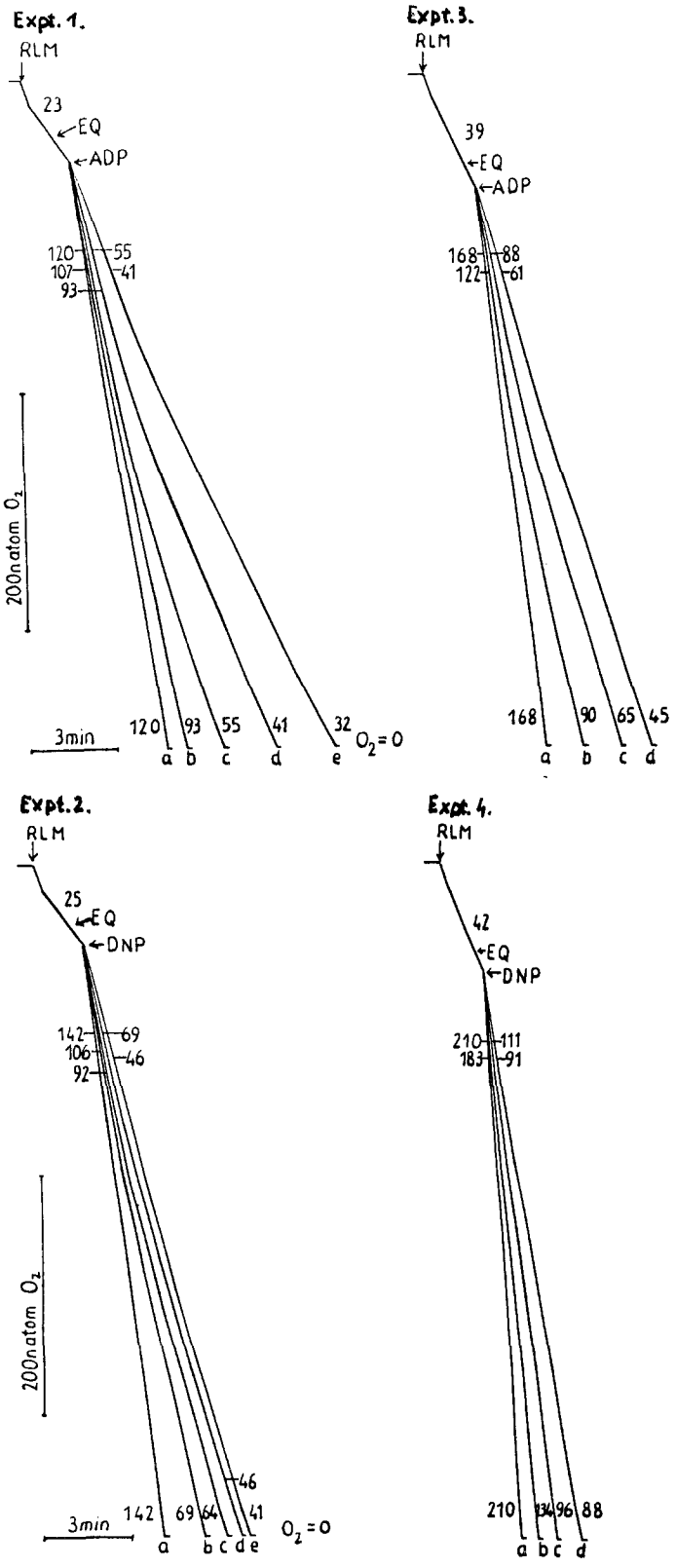
Effect on Respiration

If a substance inhibits the DNP-stimulated ATPase activity of intact mitochondria, one can expect that it inhibits also the ADP-stimulated respiration. As Fig. 3. shows, this is in fact the case. Equisetin inhibited in a concentration-dependent manner the ADP-stimulated oxygen uptake of mitochondria in the presence of glutamate + malate (Fig. 3, Expt. 1), succinate (Fig. 3, Expt. 3), or TMPD + ascorbate (Table I) as respiratory substrates. 50% inhibition of the initial rate of ADP-stimulated respiration was found at about 10 nmol equisetin/mg protein if the substrate was glutamate + malate and about 25 nmol/mg protein when succinate was oxidized. The inhibition increased in time, approximating the state 4 value at higher antibiotic concentrations. However, a similar but somewhat smaller inhibition of the oxygen uptake of mitochondria by the antibiotic could be observed if respiration was stimulated by DNP instead of ADP in the presence of glutamate + malate or succinate (Fig. 3, Expts. 2, 4). On the contrary, no inhibition was found if TMPD + ascorbate were oxidized in the uncoupled state (Table I). The oxidation of succinate or NADH of submitochondrial particles by oxygen was found to be completely insensitive to equisetin (not shown), indicating that the respiratory chain itself is not influenced by the antibiotic.

Effect on Ca^2-Uptake

It is generally accepted that the active Ca^{2+}-uptake

Fig. 3. Effect of equisetin on the respiration of mitochondria under different experimental conditions. Oxygen uptake was measured polargraphically at 37°C in the medium described in Materials and Methods. Expt. 1: Substrates, 5 mM glutamate + 1.7 mM malate. The following additions were made (indicated by the arrows): 1.35 mg mitochondrial protein (RLM), 1.5 mM ADP; (a) 0, (b) 4, (c) 8, (d) 20, and (e) 40 nmol equisetin/mg protein (EQ). Expt. 2: as Expt. 1 except that 100 μM DNP was added instead of ADP. Expt. 3: Substrate 8 mM succinate in the presence of 2 μM rotenone. The following additions were made: 1.2 mg mitochondrial protein (EQ), 1.5 mM ADP; (a) 0, (b) 25, (c) 50, and (d) 100 nmol equisetin/mg protein (EQ). Expt. 4: as Expt. 3 except that 100 μM DNP was added instead of ADP. The numbers represent oxygen uptake expressed in natom/min/mg protein.


Expt. 1.
RLM
23
EQ
ADP
120
107
93
55
41
200natom O2
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a b c d e
Expt. 3.
RLM
39
EQ
ADP
168
122
88
61
168
90
65
45
a b c d
Expt. 2.
RLM
25
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DNP
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106
92
69
46
200natom O2
3min
142
69
64
46
41
O2=0
a b c d e
Expt. 4.
RLM
42
EQ
DNP
210
183
111
91
210
134
96
88
a b c d

Table I. Effect of Equisetin on the Oxidation of TMPD + Ascorbate in State 4, in State 3, and in the Uncoupled State[a]

Added equisetin (nmol/mg protein)	Rate of oxygen uptake (natom/min/mg protein)		
	None	ADP	DNP
0	134	203	234
10	130	157	220
25	132	136	222
50	128	128	222

[a] Oxygen uptake was measured polarographically at 37°C in a medium described in Materials and Methods, containing also 3 mM ascorbate + 0.3 mM TMPD. The reaction was started by the addition of 1.0 mg mitochondrial protein. Thereafter equisetin was added as indicated. State 3 and uncoupled respiration was initiated by the addition of 1.5 mM ADP or 100 μM DNP, respectively. The reactions were linear with time.

by mitochondria requires the existence of a proton electrochemical gradient generated either by respiration or by ATP hydrolysis (Nicholls and Akerman, 1982). Since both mitochondrial respiration and ATPase activity were inhibited by the antibiotic, one would expect that it should also inhibit active Ca^{2+}-uptake by mitochondria driven either by respiration or by ATP hydrolysis. As shown in Fig. 4, this is indeed the case. Equisetin inhibited the Ca^{2+} accumulation by mitochondria in a concentration-dependent manner. Ca^{2+} transport driven by succinate oxidation and by the hydrolysis of ATP were inhibited by 50% at about 18 and 28 nmol equisetin/mg protein concentration, respectively. The inhibitory effect of equisetin on Ca^{2+}-uptake was not influenced by Mg^{2+} and was found to be greater if mitochondria were preincubated for 5 min with the antibiotic (not shown). Equisetin was without effect on the uncoupler-induced swelling of nonrespiring mitochondria in isoosmotic calcium acetate, i.e., on the passive Ca^{2+}-uptake (not shown). These data clearly indicate that equisetin inhibits the Ca^{2+}-accumulation of mitochondria indirectly, i.e., by cutting the energy supply, without influencing the calcium uniporter itself.

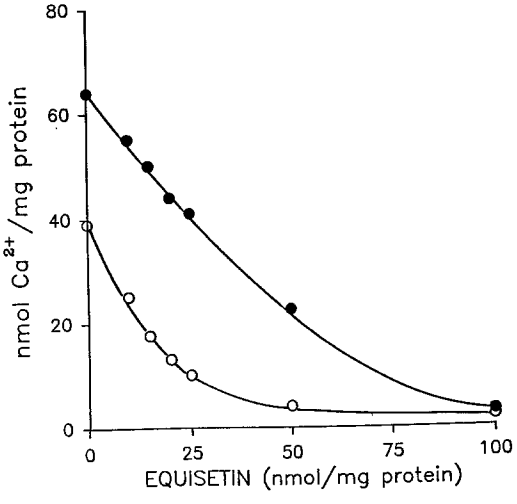

Fig. 4. Effect of equisetin on active calcium uptake by rat liver mitochondria. 200 μg mitochondria protein was incubated at 20°C in a medium detailed in Materials and Methods either in the presence of 0.5 mM ATP (●) or 10 mM Na-succinate (○). The reaction was started by the addition of $CaCl_2$ (50 μM final concentration including $^{45}Ca^{2+}$ tracer). Samples were taken after 1 min incubation. After rapid filtration, calcium uptake was measured as described in Materials and Methods.

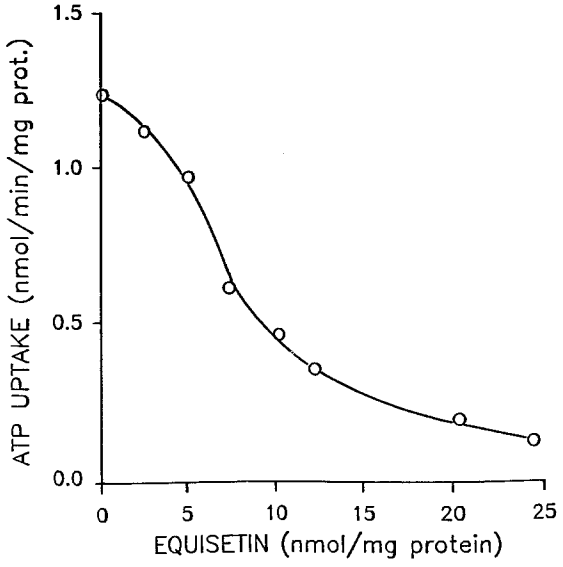

Fig. 5. Effect of equisetin on the ATP uptake by rat liver mitochondria. The reaction mixture contained 100 mM KCl, 1 mM EDTA, 20 mM HEPES-Tris, pH 7.2, 10 μg oligomicyn, 1 μM CCCP, mitochondria (2.5 mg protein/ml), and equisetin at the concentrations indicated. Temperature was 0°C. The uptake was started by adding 20 μM [U-^{14}C] ATP. After 10 sec the reaction was stopped by the addition of 50 μM carboxyatractylate. After rapid centrifugation the pellet was washed twice, then extracted with perchloroacetic acid and assayed for radioactivity. Corrections were made by running parallel samples in which carboxyatractylate was added to the incubation medium 10 sec before the labeled substrate. Other conditions are as described in Materials and Methods.

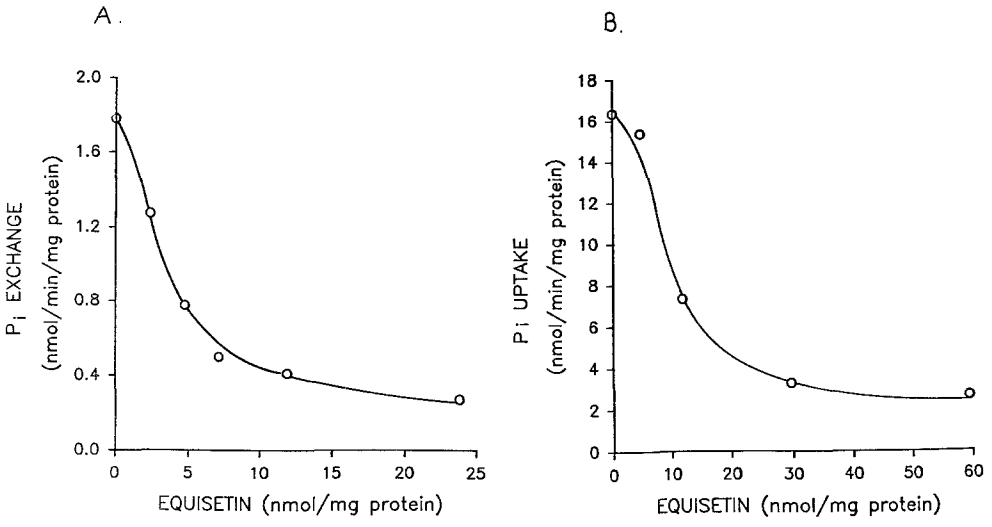

Fig. 6. Effect of equisetin on P_i transport of rat liver mitochondria. (A) Equilibrium exchange of P_i in nonrespiring mitochondria. Different concentrations of equisetin were added to mitochondria (4.2 mg protein) incubated in a medium described in Materials and Methods at 25°C. After 1 min P_i transport was initiated by the addition of $[^{32}P]P_i$. After 1 min the transport was stopped by mersalyl. After rapid centrifugation, P_i transport was measured as described in Materials and Methods. (B) P_i accumulation by respiring mitochondria. Net P_i uptake was measured similarly except that 60 sec after the addition of $[^{32}P]P_i$, 0.1 μg valinomycin/ml was added and respiration was initiated by 5 mM ascorbate + 50 μM TMPD. The uptake was terminated after 30 sec by mersalyl.

Effect on Anion Transport

In order to localize the possible site(s) of equisetin action, its effect on some mitochondrial anion transport processes were studied. As Fig. 5 shows, equisetin inhibited the ATP uptake in a concentration-dependent fashion with a 50% inhibition of about 8 nmol antibiotic/mg protein concentration. In fact, the dose-dependence curves of the ATPase activity of mitochondria, of mitoplasts, and that of ATP uptake by mitochondria are identical (cf. Figs. 1 and 2). As shown in Fig. 6, equisetin inhibited also the mitochondrial P_i carrier in a concentration-dependent fashion. Both P_i exchange and P_i accumulation were inhibited, the former seeming to be slightly more sensitive to equisetin. Fig. 7 shows that succinate uptake of mitochondria was inhibited by equisetin, demonstrating that the dicarboxylate carrier is also sensitive toward this antibiotic.

Effect on the Membrane Potential

To see whether equisetin interfered with the integrity of the energy-transducing membrane, its effect on the membrane potential was studied. Mitochondria respiring on TMPD + ascorbate built up a membrane potential of about 220 mV. This potential value was not decreased by equisetin even at concentrations as high as 150–200 nmol/protein. But after subsequent addition of CCCP the potential difference collapsed (Fig. 8). This experiment clearly demonstrated that equisetin did not uncouple mitochondria. Equisetin was also without effect on the

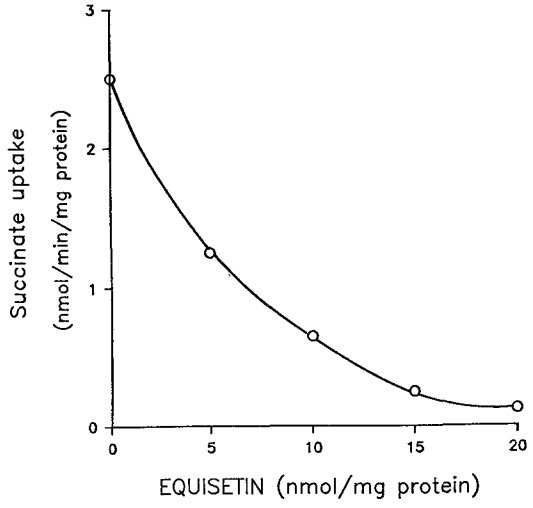

Fig. 7. Effect of equisetin on succinate uptake of rat liver mitochondria. Different concentrations of equisetin were added at 0°C to the reaction medium described in Materials and Methods, containing 3 mg mitochondrial protein. After 1 min the uptake was started by adding 1 mM succinate (including [U-^{14}C]succinate) and stopped after 30 sec by the addition of 10 mM phenylsuccinate. After rapid centrifugation, succinate uptake was measured as described in Materials and Methods.

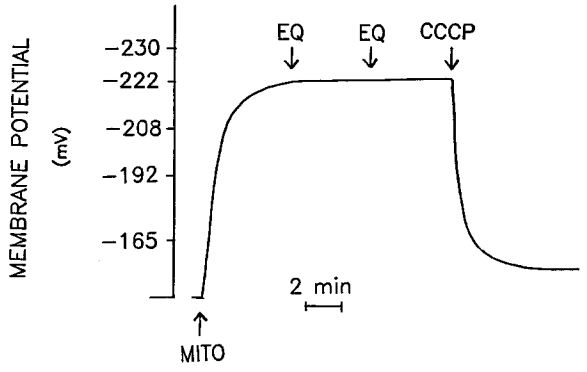

Fig. 8. Effect of equisetin on the membrane potential of rat liver mitochondria. Mitochondrial membrane potential was followed under conditions described in Materials and Methods by a TPP-electrode. At the arrows (EQ) 100–100 nmol equisetin/mg protein, (CCCP), 1 μM CCCP were added, respectively.

membrane potential when sucrose was replaced by KCl (not shown), suggesting that it did not open any pathway through the inner membrane for this monovalent cation to enter the matrix.

Effect on Other Systems

Equisetin up to 300–1000 nmol/mg protein was without effect on the following systems studied: the NADH-cytochrome *c* and NADPH-cytochrome *c* reductase activities of rat liver microsomes; the Na^+, K^+-ATPase activity of rat brain synaptosomes; active Ca^{2+}-uptake by human red cell membrane inside-out vesicles and on its activation by calmodulin (not shown). Dr. M. Colombini demonstrated that equisetin up to 150 nmol/ml had no effect on the VDAC (i.e., mitochondrial porin) conductance in a multichannel membrane or on the voltage dependence (personal communication). However, equisetin inhibited effectively the proton pump activity of bacteriorhodopsin, showing a 50% inhibition at about 6–8 equisetin/bacteriorhodopsin molar ratio König *et al.* (1986). To obtain further information about the nature of inhibition exerted by the antibiotic, the following experiment was made. Mitochondria were incubated with 8 nmol equisetin/mg protein in the standard medium used for ATPase activity determination at 37°C. After 3 min the reaction mixture was diluted with the medium used for the washing of mitochondria during preparation containing also 1% bovine serum albumin and centrifuged. The mitochondria were washed twice with the same medium. Appropriate control was run parallel. Thereafter, the specific activity of the DNP-stimulated ATPase was measured. The values showed that equisetin-treated mitochondria were practically completely inhibited, while the specific activity of the control was even slightly increased. We can thus conclude that the inhibition by equisetin is irreversible.

DISCUSSION

Equisetin powerfully inhibits the uncoupler-stimulated ATPase activity, the respiration stimulated by either ADP or uncoupler in the presence of glutamate + malate or succinate as substrates. However, if TMPD + ascorbate are oxidized, only the ADP-stimulated respiration is inhibited, while the uncoupler stimulated respiration is not affected. Since F_1-ATPase is unaffected and neither the ATPase activity nor the NADH and succinate oxidation of submitochondrial particles are inhibited by equisetin, a direct effect of the antibiotic on F_1-ATPase and F_0F_1-ATPase or on the respiratory chain can be ruled out. On the other hand, the findings that equisetin inhibits the mitochondrial transport of ATP, P_i, and succinate provide a satisfactory explanation for the inhibition by the antibiotic of both the uncoupler-stimulated ATPase activity and respiration stimulated either by ADP or DNP. The finding that equisetin has no effect on VDAC excludes the involvement of the outer membrane. In fact, the following observations provide strong support that it is the inner membrane on which the antibiotic acts: (1) the equisetin dose-dependence curves of ATPase activity of mitochondria and mitoplasts are found to be the same; (2) using TMPD + ascorbate as substrates, the state 3 respiration is inhibited by equisetin, while the uncoupled one remains unchanged. (It should be remembered that ADP has to be translocated through the inner membrane for initiating a state 3 respiration while these substrates react directly with cytochrome *c* on the outer surface of the inner membrane.) Therefore, our results—the inhibition by equisetin of ATP, succinate, and phosphate uptake—clearly indicate that at least three different inner membrane substrate anion carriers, i.e., the ATP/ADP, the P_i, and the dicarboxylate ones, are inhibited by this antibiotic. It remains to be determined whether other inner membrane carriers are also affected by equisetin. However, it should be emphasized that the mitochondrial inner membrane Ca^{2+}-uniporter or the Ca^{2+}-ATPase of red blood cell membrane and the Na^+, K^+ATPase of synaptosomes are found to be unaffected by equisetin.

Furthermore, it is without effect on the electron transport chain of mitochondria and microsomes. Therefore, an unspecific membrane action can be ruled out. In fact, the only "nonmitochondrial" effect of equisetin found so far by us is the inhibition of proton pumping by bacteriorhodopsin. In a recent paper Nyrén and Strid (1989), examining the effect of equisetin on energy-linked reactions in *Rhodospirillum rubrum* chromatophores, have arrived at a conclusion proposing that equisetin acts nonspecifically on membranes and hydrophobic domains of proteins. They also attribute an uncoupling effect to equisetin. We have no clear explanation for the apparent contradiction in our "specific" mitochondrial effects and the nonspecific effects on chromatophores of equisetin found by Nyrén and Strid (1989). One possible interpretation could be based on the phospholipid composition of the chromatophores (see later). The mitochondrial effect of equisetin studied so far seems to be specific, i.e., an inhibition of the inner membrane substrate anion carriers. Regarding the mechanism of the inhibitory effect of equisetin on these carriers, we can only speculate. Recent works have shown that mitochondrial substrate carriers have homologous sequences and are similar in their structure. (Aquila *et al.*, 1987; Krämer and Palmieri, 1989; Palmieri *et al.*, 1990). One possibility would be if equisetin interacted with different carrier proteins at this common site. There are several lines of evidence showing that for the proper functioning of mitochondrial substrate anion carriers, a specific phospholipid environment, namely, cardiolipin, is an absolute requirement. For the inhibition of mitochondrial substrate anion carriers by doxorubicin, its direct interaction with the cardiolipin–carrier protein system is thought to be responsible (Müller *et al.*, 1984; Paradies and Ruggiero, 1988). The other possibility might be that equisetin acts similarly to doxorubicin by disturbing the specific interaction between the carrier proteins and their functionally essential cardiolipin surroundings. Further studies are required to clarify the exact mechanism of inhibition by equisetin of mitochondrial substrate carriers.

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