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INTERACTION OF THE AMINOGLYCOSIDE ANTIBIOTIC DIHYDROSTREPTOMYCIN WITH THE H^+-ATPASE OF MITOCHONDRIA

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Abstract—In this paper a study is presented of the effect of dihydrostreptomycin on the H^+-ATPase of the inner mitochondrial membrane. The antibiotic caused at concentrations of $1–5 \times 10^{-3}$ M a marked enhancement of the hydrolytic activity of the H^+-ATPase complex in intact mitochondria and submitochondrial particles which was accompanied, in the latter, by enhancement of passive transmembrane proton conduction by the complex. The stimulation by dihydrostreptomycin of ATP hydrolysis resulted in a suppression of the sensitivity of this activity to inhibition by oligomycin. On the other hand the dihydrostreptomycin-promoted proton conduction in submitochondrial particles was suppressed by oligomycin. At concentrations above 10^{-2} M dihydrostreptomycin caused inhibition of the activity of both membrane bound and isolated H^+-ATPase. In submitochondrial particles devoided of the catalytic moiety (F_1) of the H^+-ATPase complex, dihydrostreptomycin caused partial inhibition of proton conductivity. It is concluded that the antibiotic decouples the hydrolytic activity of the catalytic moiety (F_1) from transmembrane proton conduction by the membrane sector (F_0) of the ATPase complex. This effect can be followed at higher concentrations of dihydrostreptomycin by inhibition of the catalytic activity of F_1 and, when F_1 is removed from the membrane, by inhibition of transmembrane proton conduction by F_0.

Treatment of various species of animals with aminoglycoside antibiotics produces nephro- and neurotoxicity [1–4]. It has been suggested that the toxicity of these compounds can be determined by two factors: (1) accumulation of the drug within the renal cortex; (2) alteration of structures and functions of intracellular organelles [4], like mitochondria [3].

Rats treated with the aminoglycoside antibiotic Gentamycin show alteration of the ultrastructure of mitochondria, i.e. swelling and irregular shape [3, 4]. Furthermore Gentamycin administered *in vitro* to isolated renal cortical mitochondria induced enhanced permeability to monovalent cations [5, 6].

Aminoglycoside antibiotics are polybasic molecules since of aminogroups in the side-chains and are polycationic at physiological pH. It is conceivable that their activity on biological membranes is due to electrostatic interaction with their anionic components [7]. In line with this, is the finding that dihydrostreptomycin interacts with bilayer membranes made with phosphatidylserine but not with phosphatidylcholine [8].

In this paper we have investigated the effect of the polycationic aminoglycoside dihydrostreptomycin on the H^+-ATPase of the mitochondrial membrane. It is shown that dihydrostreptomycin induces oligomycin-insensitive stimulation of the hydrolytic activity of the ATPase complex in the mitochondrial membrane, which is accompanied by oligomycin-sensitive enhancement of passive transmembrane proton conductivity by the complex. These and other results show that dihydrostreptomycin causes decoupling of the hydrolytic activity in the catalytic moiety (F_1) from proton conduction in the membrane sector (F_0) of the H^+-ATPase. This effect can be followed, at high concentrations of dihydrostreptomycin, by inhibition of the catalytic activity of F_1.

MATERIALS AND METHODS

Dihydrostreptomycin was obtained from Serva (Germany), valinomycin, oligomycin and CCCP (Carbonylcyanide-chlorophenylhydrazone), were obtained from Sigma Chemical Co. (St. Louis, Missouri); phosphoenolpyruvate, pyruvate-kinase, lactate dehydrogenase, β-nicotinamide-adenine-dinucleotide reduced form (NADH) and catalase from Boehringer (Mannheim, F.R.G.). All other chemicals were of high purity grade.

Preparation of beef-heart mitochondria, submitochondrial particles and H^+*-ATPase.* Beef-heart mitochondria were prepared as described by Löw and Vallin [9], "inside out" submitochondrial particles were obtained by exposure of beef-heart mitochondria to ultrasonic energy in the presence of EDTA at pH 8.5 (ESMP) [10]. H^+-ATPase complex was prepared as described by Stigall *et al.* [11].

Determination of ATPase activity. The ATPase activity was determined in the presence of added pyruvate kinase, phosphoenolpyruvate and lactate dehydrogenase, by following spectrophotometrically NADH oxidation at 340 nm [12].

The reaction mixture contained 250 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Tris–HCl, pH 7.4, 0.1 mM NADH, 0.5 μg rotenone, 1 mM phosphoenolpyruvate, 0.1 mM ATP, 5 units lactate

dehydrogenase, 2 units pyruvate kinase and 20–30 μg protein submitochondrial particles in a final volume of 1 ml.

Measurement of proton translocation. Submitochondrial particles, 3 mg protein/ml, were incubated in a reaction mixture containing 250 mM sucrose, 30 mM KCl, 0.2 mg/ml purified catalase and 20 mM succinate as respiratory substrate; final volume 1.5 ml; pH 7.5. Incubation was carried out in a glass vessel, under a constant stream of N_2, thermostated at 25 ± 0.01°. Respiration driven proton translocation was activated by repetitive additions of 1–3% H_2O_2 (5 μl/ml). The pH of the suspension was monitored potentiometrically with a combination electrode (No. 39030, Beckman Instruments International, Geneva, Switzerland), connected to a differential electrometer amplifier (mod. 604, Keithley Instruments) and from this to a strip chart recorder (Leeds & Northrup). The overall response time of the pH recording system used was about 300 msec at 25° [13].

For the kinetic analysis of the anaerobic proton release from submitochondrial particles, the potentiometric traces were converted into proton equivalents by double titration with standard HCl and KOH and treated by a double exponential equation [14].

RESULTS

Figure 1(a) presents a titration curve of the effect of dihydrostreptomycin on the ATPase activity of beef-heart mitochondria. The antibiotic caused a substantial activation of the activity; half-maximal stimulation occurred at ≈ 3×10^{-3} M dihydrostreptomycin. Dihydrostreptomycin produced marked stimulation of the ATPase activity also when added in the presence of the protonophoric uncoupler CCCP.

Figure 1(b) shows a titration of the effect of dihydrostreptomycin on the ATPase activity of vesicles of the inner mitochondrial membrane obtained by exposure of beef-heart mitochondria to ultrasonic energy in the presence of EDTA (ESMP). Concentrations of dihydrostreptomycin in the order of $1–5 \times 10^{-3}$ M caused, also in this case, a significant stimulation of the ATPase activity. This was, however, followed by marked inhibition at higher concentrations of the drug. The inhibition of the ATPase activity by dihydrostreptomycin was of non-competitive nature with a K_i of 38 mM (Fig. 2). It seems, therefore, to occur, at difference of the stimulatory effect, at concentrations too high to be of significance with respect to the pharmacological activity of the drug.

When dihydrostreptomycin was tested on the isolated ATPase complex (Fig. 1c) there occurred a marked inhibition of the activity. Fifty percent inhibition occurred at dihydrostreptomycin concentration close to the K_i observed in submitochondrial particles. Interesting enough the inhibition was not preceded, in this case, by significant stimulation at low concentrations of dihydrostreptomycin.

It is therefore apparent that the stimulatory effect exerted by dihydrostreptomycin on the ATPase activity of the complex in the membrane has to do with disturbance of the coupling of the catalytic activity with transmembrane proton translocation. Hence the extent of the stimulatory effect exerted by

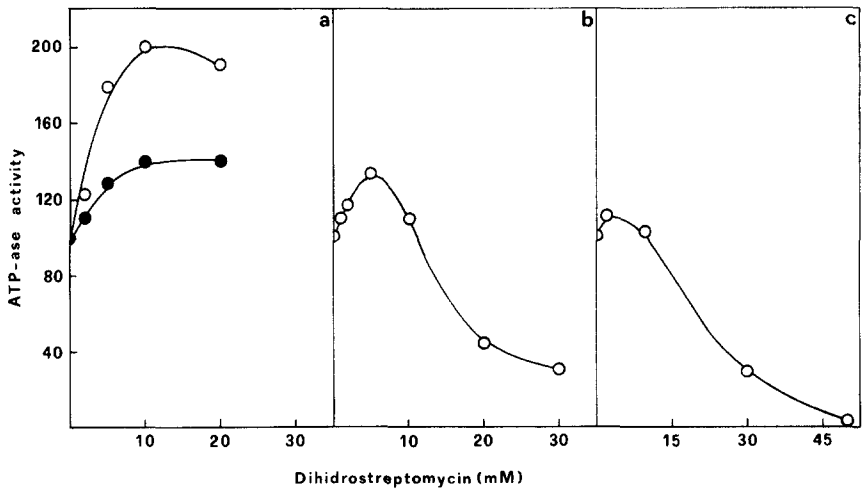

Fig. 1. Titration of the effect of dihydrostreptomycin on ATPase activity of beef-heart mitochondria, submitochondrial particles and purified H^+-ATPase (Complex V). Beef-heart mitochondria (1a) or submitochondrial particles (1b) (50 μg/ml) were incubated in the reaction mixture reported in the Materials and Methods. Mitochondria or submitochondrial particles were incubated 5 min with dihydrostreptomycin, at the concentrations reported, before the reaction was started by ATP addition. The data are expressed as % of the ATPase activity in the absence of dihydrostreptomycin. Control values: (a) 0.58 μmoles ATP hydrolyzed/min/mg protein in the absence (●——●) and 0.68 in the presence of CCCP (○——○). (b) Control value 1.2 μmoles ATP hydrolized/min/mg protein. (c) The ATPase activity of purified Complex V was determined as described in ref. [12]. The enzyme was incubated with dihydrostreptomycin, at the concentrations reported in Figure, 5 min before reaction was started. Control value 4 μmoles ATP hydrolyzed/min/mg protein.

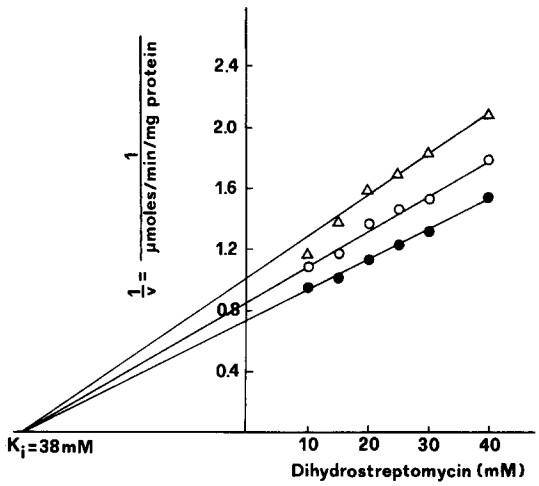

Fig. 2. Dixon plot for dihydrostreptomycin inhibition of ATPase activity in ESMP. For experimental conditions see legend to Fig. 1 (b). Symbols: ●——●, 1 mM ATP; ○——○, 0.5 mM ATP; △——△, 0.25 mM ATP.

the antibiotic varies with the membrane preparation used, being clearly larger in the native coupled enzyme *in situ* in the membrane of intact mitochondria than in partially uncoupled submitochondrial particles and in the isolated F_1–F_0 complex. Preliminary experiments showed, on the other hand, that dihydrostreptomycin exerted, at concentrations in the order of 10^{-2} M, inhibition of the activity of soluble F_1 ATPase prepared as in ref. [19].

The effect of the antibiotic on the membrane bound ATPase was therefore examined in relation to that exerted by the protonophoric uncoupler CCCP and oligomycin. The latter inhibits the H⁺-ATPase by blocking H⁺-conduction by the membrane sector (F_0) of the complex [12, 15].

Figure 3 shows that dihydrostreptomycin stimulated the ATP-ase activity of ESMP also when these were treated with a concentration of oligomycin causing a large inhibition of the enzyme. Dihydrostreptomycin released, in fact, to a large extent the inhibitory effect of oligomycin. This situation is different from that induced by FCCP, where oligomycin suppressed the stimulatory effect exerted on the ATP-ase activity by the uncoupler (Fig. 3B).

Further insight into the mechanism of action of dihydrostreptomycin on the H⁺-ATPase complex was provided by a study of its effect on passive transmembrane proton translocation in ESMP, which takes practically place through the proton conduction pathway in the H⁺-ATPase complex [12, 16].

Figure 4 shows that dihydrostreptomycin, like the uncoupler CCCP, stimulated passive proton conduction in ESMP. This was documented by decrease of the $t_{\frac{1}{2}}$ of the anaerobic release of the protons taken up by the particles during respiratory pulses and by decrease of the extent of proton uptake during the aerobic steady-state.

Kinetic analysis of anaerobic H⁺ release from ESMP, shows a biphasic pattern than can be resolved in two first order processes (Fig. 4B; see also ref. [12, 16]). Whilst the acidic uncoupler CCCP, changed the biphasic pattern in a monophasic first order kinetic, dihydrostreptomycin did not abolish the biphasic pattern but enhanced the kinetic constant of both phases of proton release. This observation indicates that the mechanism by which

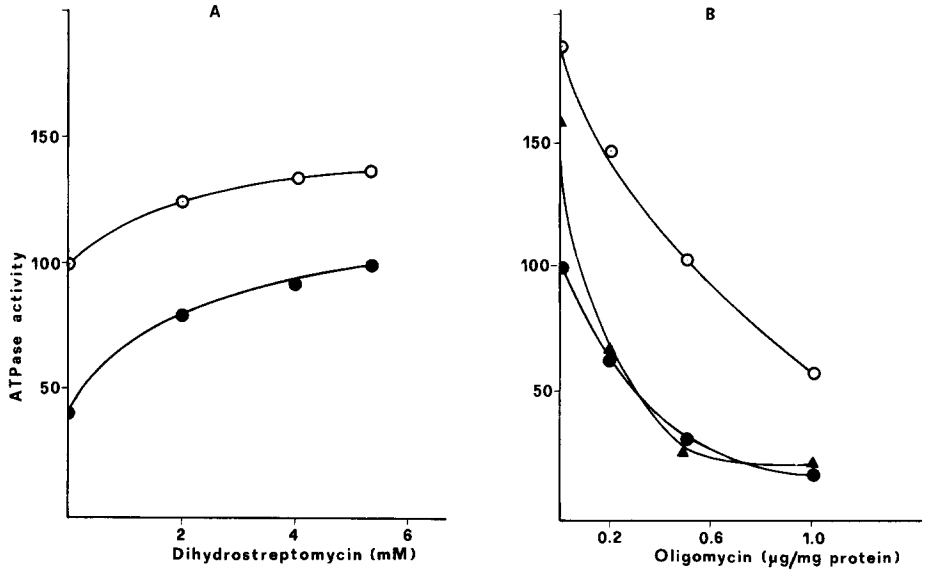

Fig. 3. Titration curves for the stimulatory effect of dihydrostreptomycin (A) and for the inhibitory effect of oligomycin (B) on ATPase activity of ESMP. For experimental conditions and procedure see Materials and Methods and legend to Fig. 1. The data reported in the figure are expressed as % of the ATPase activity of ESMP in the control (1.2 μmoles ATP hydrolized/min/mg protein). Additions: Experiment A: ○——○, none; ●——●, + 0.2 μg/mg protein oligomycin. Experiment B: ●——●, none; ○——○, + 5 mM dihydrostreptomycin; ▲——▲, + 2 μM CCCP.

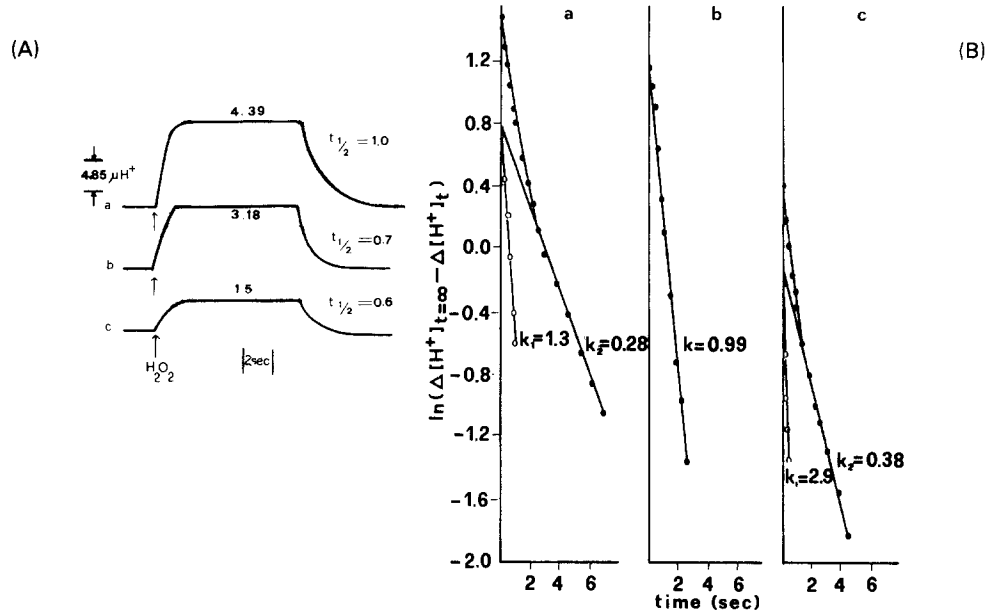

Fig. 4. Effect of dihydrostreptomycin and of the acidic uncoupler CCCP on proton translocation induced by oxygen pulses in ESMP. For experimental conditions and procedure see Materials and Methods. Where indicated the particles were incubated 5 min before the addition of oxygen, with 5 μM CCCP or 6 mM dihydrostreptomycin. Panel A: Cycles of proton translocation. The number on the top of the cycles refer to the extent of aerobic proton uptake expressed in ng ions H^+/mg protein; Panel B: Kinetic analysis of anaerobic proton release. Additions: trace a, none; trace b, + 5 μM CCCP; trace c, + 6 mM dihydrostreptomycin.

dihydrostreptomycin increases proton permeability is different from that of CCCP.

This is further documented by the fact that the stimulatory effect exerted by dihydrostreptomycin on the anaerobic relaxation of the respiratory proton gradient was abolished by the presence of valinomycin, which collapses the transmembrane potential ($\Delta\psi$) set up by respiratory proton uptake (Fig. 5 and Table 1). The stimulatory effect of CCCP was, on the contrary, not abolished by valinomycin (Table 1).

It can be noted that dihydrostreptomycin lowered the extent of aerobic proton uptake by ESMP also in the presence of valinomycin when it had no effect on the anaerobic proton relaxation (Table 1). This may have to do with inhibition by dihydrostreptomycin of valinomycin-mediated K^+ diffusion across the phospholipid bilayer, as observed with positively charged local anaesthetics [17].

The enhanced proton conductivity induced by treatment of ESMP with dihydrostreptomycin was markedly suppressed by oligomycin (Fig. 6). This

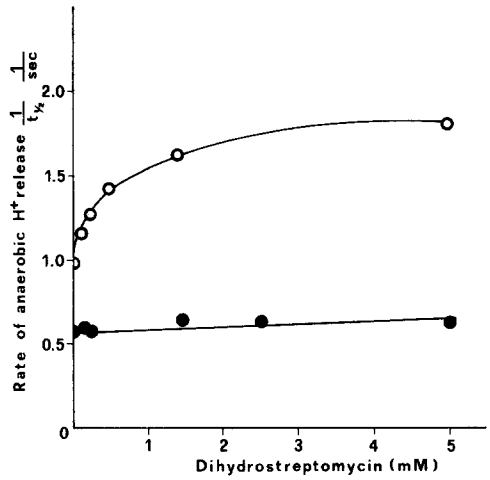

Fig. 5. Titration of the effect of dihydrostreptomycin on anaerobic proton release. Effect of valinomycin ($+K^+$). For experimental conditions see legend to Fig. 4. Additions: ○——○, none; ●——●, + 0.7 μg/mg protein valinomycin.

Table 1. Comparison of the effect of acidic uncoupler and dihydrostreptomycin on proton translocation in ESMP in the absence and in the presence of valinomycin ($+K^+$)

Additions	Control Extent (ngions H^+/mg protein)	Control $t_{\frac{1}{2}}$ (sec)	+Valinomycin Extent (ngions H^+/mg protein)	+Valinomycin $t_{\frac{1}{2}}$ (sec)
—	4.39	1.0	11.2	1.7
CCCP (5 μM)	3.18	0.7	3.45	0.5
Dihydrostreptomycin (5 mM)	1.50	0.6	3.49	1.8

For experimental conditions see legends to Figs. 6 and 7; where indicated 0.7 μg valinomycin/mg protein was present.

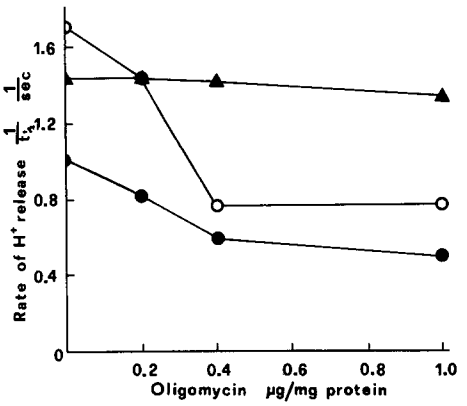

Fig. 6. Titration curves for the inhibitory effect of oligomycin on H^+ conduction in ESMP. For experimental conditions see legend to Fig. 4. Additions: ●——●, none; ○——○, + 5 mM dihydrostreptomycin; ▲——▲, + 5 µM CCCP.

shows that the enhanced proton conductivity induced by the dihydrostreptomycin takes place via the proton conductivity in the H^+-ATPase complex. In fact the stimulatory effect of CCCP, resulting directly from transmembrane proton conductance by the acidic uncoupler, was not depressed by oligomycin (Fig. 6).

Removal of the F_1 moiety from the mitochondrial membrane, effected by treatment of ESMP with urea, results in acceleration of the anaerobic relaxation of the respiratory proton gradient. Dihydrostreptomycin did not cause any stimulation of anaerobic proton relaxation from these particles, it rather produced a significant inhibition of the process (Fig. 7).

DISCUSSION

The results presented reveal a complex and interesting interaction of dihydrostreptomycin with the H^+-ATPase of mitochondria.

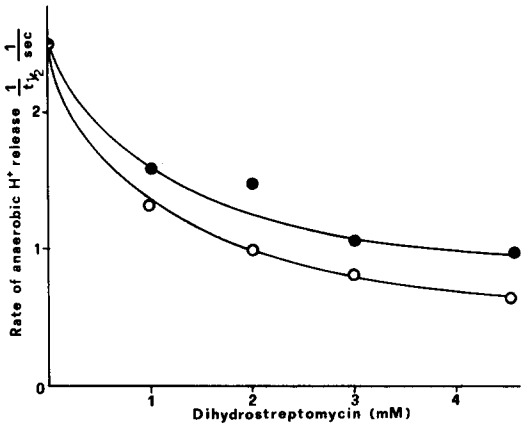

Fig. 7. Effect of dihydrostreptomycin on anaerobic H^+ release from F_1 depleted particles. F_1 depleted particles were prepared as described by Racker and Horstman [19]. For experimental conditions and procedure see under Materials and Methods and legend to Fig. 6. Symbols: ●——●, control; ○——○, + 0.5 µg/mg protein valinomycin.

Hydrolysis, or synthesis, of ATP by the intact H^+-ATPase complex in the mitochondrial membrane is compulsorily linked to transmembrane proton translocation by the enzyme. The coupling capacity seems to involve weak interactions of ionic nature (hydrogen bonds, electrostatic salt-bridges) between the catalytic peripheral moiety (F_1) of the complex and the proton translocating membrane integral sector (F_0) [18].

Dihydrostreptomycin when added at relatively low concentrations (1×10^{-3}, 5×10^{-3} M) to mitochondria or "inside out" submitochondrial particles (ESMP) causes an apparent decoupling of ATP hydrolysis from transmembrane proton conduction in the H^+-ATPase complex. This is documented by: (i) oligomycin-insensitive stimulation of ATP hydrolysis by the membrane bound H^+-ATPase which is not observed in the isolated soluble enzyme; (ii) enhancement of proton-conductivity by the H^+-ATPase complex in F_1–F_0 submitochondrial particles (ESMP) which is suppressed by oligomycin. It has to be noted that the stimulation of the ATPase activity of membrane bound H^+-ATPase by acidic uncoupler, which is due to direct oligomycin insensitive transmembrane proton equilibration by these substances, is suppressed by oligomycin.

The decoupling effect exerted on the H^+-ATPase by dihydrostreptomycin could result from physical displacement of F_1 from the membrane sector (F_0), due to electrostatic disturbance by the polycationic molecule of dihydrostreptomycin of the ionic interactions holding F_1 in its specific, functional association with F_0 in the membrane. Decoupling could, however, result also from more specific interaction of dihydrostreptomycin with aminoacid residues and/or phospholipids specifically involved in the functional interactions of F_1 with F_0. In this respect it is interesting to note that the stimulation of proton conduction exerted by dihydrostreptomycin in ESMP is abolished when the aerobic membrane potential is abolished by valinomycin.

Interaction of dihydrostreptomycin with critical groups in the enzyme could represent the basis for the inhibition of the hydrolysis of ATP observed when concentrations of dihydrostreptomycin higher than 10^{-2} M are presented to the H^+-ATPase in the native membrane (ESMP) or in the isolated soluble state.

Dihydrostreptomycin can apparently interact also with the F_0 moiety of the H^+-ATPase complex, judging from the inhibition of proton conduction caused by the drug in the F_1 depleted submitochondrial particles. The interaction sites in F_0 become, however, accessible to dihydrostreptomycin only after removal of F_1.

Dihydrostreptomycin treatment in the man can result in hematic concentrations in the order of 50 µM [20]. Furthermore it has been found that the antibiotic is actively accumulated by certain tissues (renal cortex) where it concentrates into the mitochondrial fraction [3, 4].

The effects exerted by dihydrostreptomycin on the H^+-ATPase of mitochondria, besides their pharmacological implications, may provide new

approaches to the study of the F_1–F_0 interactions specifically involved in the coupling activity of the H^+-ATPase.

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