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THE EFFECTS OF SOME NUTRITIVE ANTIBIOTICS ON THE RESPIRATION OF RAT LIVER MITOCHONDRIA

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Abstract—(1) Seven antibiotics used as feed additives in animal breeding were investigated for their effects on isolated rat liver mitochondria. Three were found to interfere with mitochondrial energy metabolism. (2) Zinc-bacitracin completely inhibits mitochondrial respiration in the micromolar range, as do other inhibitors known to be highly effective against electron transport system. From studies of this antibiotic on the redox state of cytochromes, as measured by split beam spectra, it is concluded that the site of inhibition is located between cytochrome *b* and c_1 (antimycin A site). The effect is completely reversed by chelating agents, suggesting that Zn^{2+} ions are required for full activity of the cyclic peptide antibiotic. (3) Flavomycin, a polar glycolipid, linearly stimulates oxygen consumption of mitochondria under state 4 conditions in concentrations greater than 100 μmole/gram of protein. Lower concentrations of the antibiotic inhibits respiration of coupled as well as DNP- or FCCP-uncoupled mitochondria by about 70 per cent. While the uncouplinglike effect at high concentrations of the compound can be attributed to nonspecific surface activity which might facilitate proton conductance, the inhibitory activity seen at lower concentrations is assumed to be located near the second phosphorylation site of the respiratory chain. (4) The influence of chlortetracyclin (aureomycin) on mitochondrial activity was found to be dependent on the identity of the substrate. Succinate respiration was more sensitive to chlortetracyclin (CTC) addition by comparison with NAD-linked substrate oxidation, 45 μmole of CTC/gram of protein decreased succinate respiration to half maximal values, whereas glutamate plus malate or β-hydroxybutyrate respiration were inhibited by only 25 per cent. CTC partially inhibits the dehydrogenation of succinate by the succinate dehydrogenase. Uncoupling of oxidative phosphorylation completely abolished CTC-inhibited respiration of NAD-linked substrates, while succinate respiration remained inhibited by 25 per cent. The results of these experiments are discussed in terms of two sites of action for CTC, one located close to the phosphate carrier, while the second interferes with succinate dehydrogenase.

Since many years certain antibiotics which appear to stimulate breeding are used as feed additives. The main scientific interest therefore concerning this group of antibiotics were focused upon their pharmacological, toxicological and microbiological activities. In the biochemical field extensive studies were carried out to elucidate the mechanism of the appearing stimulation of protein synthesis [1, 2]. Even most potent inhibitors of respiratory and phosphorylating system in mitochondria have been found among the antibiotics [3–10] the above mentioned group of compounds were not examined so far for their effects on mitochondrial metabolism. It is the purpose of the present paper therefore to present and discuss results of corresponding investigations.

MATERIALS AND METHODS

Mitochondria. Rat liver mitochondria were isolated as described [11] and suspended in 0.25 M sucrose, 20 mM triethanolamine–HCl buffer pH 7.2, and 2 mM EDTA at 25°, unless otherwise indicated. Mitochondria with acceptor control ratios lower than 4.0 using succinate as substrate were not used for our experiments.

Chemicals. The antibiotics were generous gifts from the following: zinc-bacitracin, H. Lundbeck and Co. A/S, Copenhagen, Denmark; flavomycin, Hoechst AG., Frankfurt, Germany: chlortetracyclin, Cyanamid G.M.B.H., Munich, Germany; oxytetracyclin and oleandomycin, Pfizer G.M.B.H., Karlsruhe, Germany; virginiamycin, Smith Kline Animal Health Products, Brussels, Belgium; tylosin, Centrafarm b.v., Rotterdam, Holland.

Determination of respiratory activity. The oxygen consumption was recorded amperometrically at 25° using a micro Clark-type electrode of own design (Platinium electrode covered with teflon, designed in the laboratory of Prof. Klingenberg).

Determination of the redox states of the cytochromes. The redox states of the cytochromes were estimated from difference spectra at room temperatures using an Aminco DW-2-spectrophotometer, split beam mode. (American Instrument Company, MD, USA).

Determination of the cytochrome c *oxidase* (*EC* 1.9.3.1). Oxygen consumption was measured polarographically at 25° in an assay system containing 50 mM phosphate buffer, pH 7.2, 30 μM cytochrome *c* (horse heart type III, Sigma Biochemical, St. Louis). 5 mM sodium ascorbate, and 0.5 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD).

Succinate-dehydrogenase (*EC* 1.3.99.1) *determination.* The reaction mixture contained 50 mM potas-

sium phosphate buffer. pH 7.2 and 1 mM KCN. About 0.5 mg of mitochondrial protein was incubated for 4 min with this mixture; the reaction was started by the rapid addition of 0.05 mM dichlorophenolindophenol and 6 mM succinate. Absorbance changes were followed at 600 nm. An extinction coefficient of 21 mM^{-1} cm^{-1} for dichlorophenolindophenol was used.

ATPase (EC 3.6.1.3) determination. A coupled enzyme system was used (Pullman *et al.* 1960) containing 50 mM Tris–sulphate pH 9.4, 5 mM phosphoenolpyruvate, 5 mM MgCl, 2 mM ATP, 10 μg pyruvate kinase (Sigma type II) and 0.2–0.5 mg of mitochondrial protein. The reaction was started by the addition of 0.15 mM NADH. The decrease in absorbance was followed at 340 nm; an extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADH was applied.

Protein determination. Mitochondrial protein was determined by a modified biuret method [12].

RESULTS

The influence of the antibiotics on succinate-respiring mitochondria are given in Fig. 1.

Effect on energy-linked mitochondrial respiration. At 100 μmole/gram of protein three antibiotics exerted

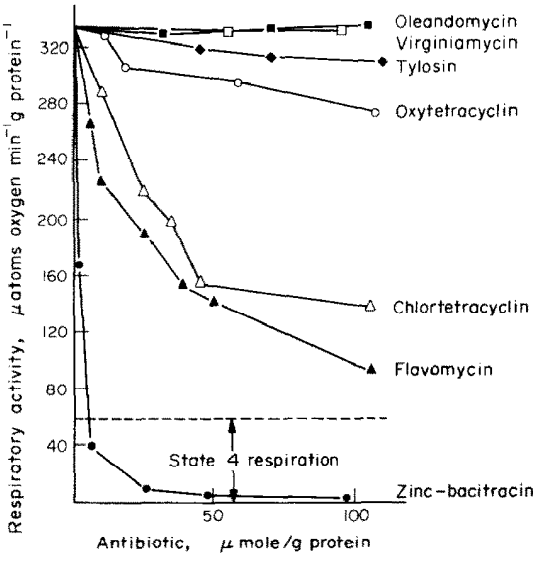

Fig. 1. Effects of seven nutritive antibiotics on coupled state 3 respiration from succinate as substrate. The mitochondria were suspended in 0.25 M sucrose, 2 mM EDTA (except for zinc-bacitracin), and 20 mM triethanolamine buffer pH 7.2. Respiration rates were measured at 25° using a Clark-type electrode. Mitochondrial protein (0.5 mg) was added to 0.5 ml air-saturated reaction mixture containing 0.25 mM sucrose, 2 mM EDTA (not for exps. with zinc-bacitracin), 20 mM triethanolamine pH 7.2, 2 mM potassium phosphate and 12 mM succinate. Shortly after the initiation of state 3 respiration by ADP (220 μM final concentration) different amounts of antibiotics were added and the respiration rate was measured again. The dotted line indicates respiration rates in the absence of ADP (state 4; Chance and Williams, 1955). Results are mean (n = 10) ± S.E.M. is significant for CTC, flavomycin and zinc-bacitracin values, not significant for the oleandomycin, virginiamycin and tylosin group.

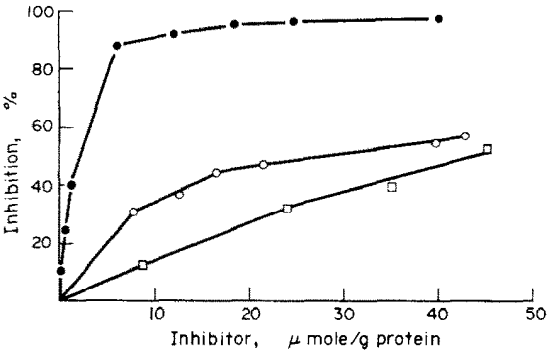

Fig. 2. Comparison of the inhibitor activity of zinc-bacitracin (●); flavomycin (○); and CTC (□) on state 3 respiration. For conditions see Fig. 1.

a marked inhibition of state 3 respiration, one of them inhibiting even the state 4 level. The others affected mitochondrial respiration only slightly or not at all. The inhibitory activities of zinc-bacitracin flavomycin and CTC on mitochondrial oxidation, in ranges of concentration close to the activities of well known mitochondrial enzyme or enzyme-complex inhibitors, are shown in Fig. 2. All three antibiotics inhibited oxygen consumption linearly. The most important observation was the powerful inhibition of the electron transport system by zinc-bacitracin. In the presence of 2 μmole of zinc-bacitracin per gram of protein, oxygen consumption was decreased by nearly 50 per cent. Comparable inhibition by flavomycin and CTC required 15- and 20-fold higher concentrations respectively. Almost complete inhibition was established with zinc-bacitracin at 20 μmole/gram of protein, while the two other antibiotics did not exert an inhibition of more than about 30–45 per cent at comparable concentrations.

Dependence on substrates for respiration. As can be seen from Fig. 3, the identity of the substrate was not critical for zinc-bacitracin and flavomycin, whereas inhibition of mitochondrial respiration by CTC was most effective with succinate. It was of interest, therefore to discover if and to what extent electron transport to the second phosphorylation site from succinate to dichlorophenolindophenol was blocked on addition of CTC. As judged from the results, 28 per cent of the inhibition of succinate oxidation in the presence of 50 μmole of CTC per gram of protein could be attributed to the inhibition of succinate dehydrogenase activity. This was in agreement with expectation since NAD-linked substrate oxidation was shown to be sensitive as well, reaching nearly half the values of total inhibition of succinate respiring mitochondria.

Dependence on oxidative phosphorylation. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and 2,4-dinitrophenol (DNP) did not prevent or abolish the inhibition induced by zinc-bacitracin or flavomycin, indicating that associated energy coupling processes are not involved. This observation is supported by other experiments where the influence of the antibiotics on the hydrolysis of ATP by mitochondrial ATPase was studied . None of these agents inhibited ATPase, as measured by the spectrophotometric

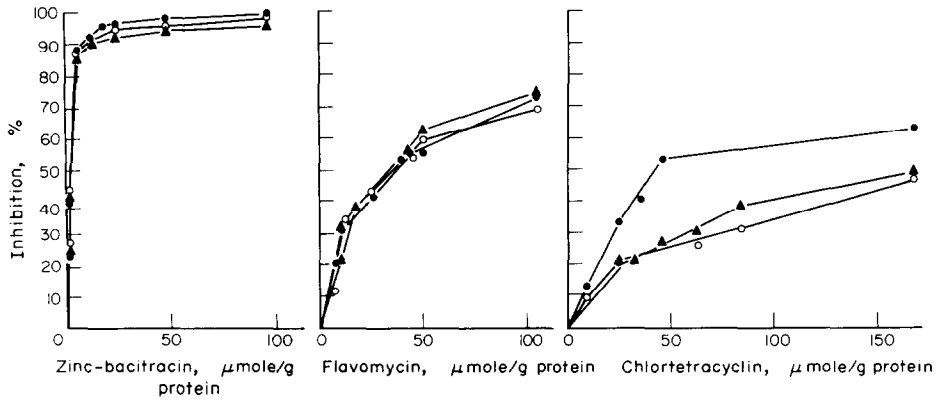

Fig. 3. Influence of substrates for respiration on the inhibitory effects of zinc-bacitracin, flavomycin and CTC. Substrates were added to make a final concentration of 12 mM. Succinate (●); β-hydroxybutyrate (○); glutamate plus malate (▲). Other conditions are those of Fig. 1.

absorbance decrease of NADH in a coupled enzyme system or from the Pi liberated, according to the method of Emmelot and Bos [13, 14]. Uncoupler stimulated respiration, however, was found to be insensitive to CTC. Only when succinate was the substrate for respiration could a remaining inhibition be observed, not higher than 25 per cent. According to these observations the inhibition of oxygen consumption under state 3 conditions could be due to inhibition of the phosphate or adenine nucleotide carriers. Flavomycin-inhibited state 3 respiration was restored to its original level when concentrations where applied slightly above those required for maximal inhibition. In this concentration range state 4 respiration was increased to state 3 level (Fig. 4). From this observation one may conclude that flavomycin acts as a classical uncoupler when present in concentrations sufficient to achieve maximal inhibition.

Influence on segments of the respiratory chain. In order to pinpoint further the sites of action of the three antibiotics, their influence on cytochrome *c* oxidase activity with ascorbate plus TMPD as electron donor was measured. With this assay system, however, no inhibitory activity could be observed. The succinate induced reduction of cytochrome *b* was estimated from the double beam spectra followed at 575–566 nm. When 40 μmole of CTC per gram of protein was added to the mitochondrial suspension the redox state of cytochrome *b* decreased by 23 per cent. As estimated from the split beam spectra a transient oxidation of cytochromes c_1 and aa_3 could be produced with flavomycin. Original redox states could be established again if the suspension was carefully stirred. In the presence of zinc-bacitracin a permanent and concentration-dependent oxidation of the above mentioned cytochromes with a simultaneous high reduction state of cytochrome *b* was observed.

Figure 5 shows that inhibition of succinate respiration by zinc-bacitracin is accompanied by a concomitant oxidation of cytochrome c_1, while the reduction state of cytochrome *b* is unaffected (not shown in this figure). The reduction of cytochrome c_1 starts at 65 per cent and reaches about 5 per cent at full inhibition. The nonlinear curve of dependence of the oxidation of cytochrome c_1 on zinc-bacitracin concentration inversely follows the curve for the respiratory activity. The potent inhibition of mitochondrial respiration by zinc-bacitracin was only observed in the absence of chelating agents. This finding suggested that dissociated Zn^{2+} ions were also responsible for the effect of the antibiotic. Reversibility of the inhibition could therefore be expected by adding chelating agents which form stable Zn^{2+} complexes. Zinc-bacitracin induced inhibition of respiration was therefore tested for complete reversibility by adding increasing concentrations of EDTA. As judged from the type of plot (Fig. 6), complete reversibility of zinc-bacitracin-induced inhibition could be achieved when the EDTA concentration was increased to a point at which the uninhibited oxygen consumption rate v_0

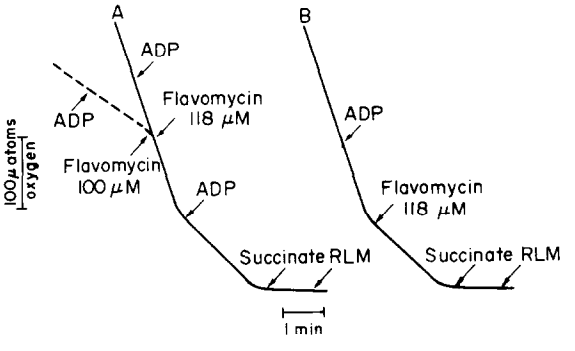

Fig. 4. Effect of high concentrations of flavomycin on state 3 respiration (A) and respiration in the absence of ADP (B). The reaction mixture was similar to that in Fig. 1. With concentrations of flavomycin higher than 100 μM inhibited state 3 respiration (dotted line) turned over to an apparent uncoupling, reaching original level of respiration at 118 μM of flavomycin (A). Additional stimulation with higher concentrations could not be obtained. Oxygen consumption of state 4 respiration increased to state 3 level when 118 μM of flavomycin were added (B). ADP was added to demonstrate respiration is not inhibited by acceptor control (A—dotted line) or uncoupling effect of increasing flavomycin concentration respectively is in the range of state 3 respiration (A; B).

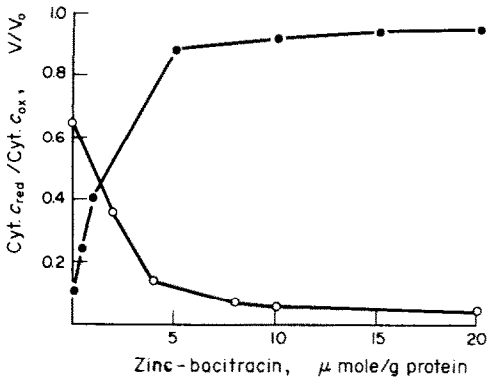

Fig. 5. Zinc-bacitracin titration of the respiratory activity and the redox state of cytochrome c_1. The oxygen consumption was measured as described in Fig. 1. (●) Respiratory activity expressed as rate of oxygen consumption, v, as a fraction of initial rate, v_0; (○) redox state of cytochrome c_1 expressed as cyt c reduced as a fraction of total cyt c. Values for cytochrome c_1 are taken from split beam spectra at 552 nm. Varying amounts of zinc-bacitracin were added 2 min prior to succinate addition (6 mM final conc.). Mitochondrial protein concentration was 1 mg/ml.

over the rate v following the addition of the inhibitor and reversor had a value of 1.

DISCUSSION

It is likely that antibiotics exert their effect by inhibiting enzymes; it may be anticipated that the binding of an especially toxic compound to its susceptible enzyme site might be stoichiometric and strong. It can be assumed therefore, that effective concentrations of specific enzyme-inhibitors will not greatly exceed physiological amounts of their susceptible enzymes. On the other hand, it is not likely that alterations of the activity of organized enzyme systems such as are found in mitochondria by very high concentrations of antibiotics or other compounds can be attributed to specific enzyme effects. The question whether flavomycin exerts both its inhibition and its uncoupling effect at the same site or at separate sites must be discussed therefore in the following way. The chemical structure of this antibiotic consisting of a highly polar sugar and an apolar lipid moiety exhibits the characteristics of a surface-active agent. Considering the relatively high concentrations necessary for apparent uncoupling, flavomycin might act as a detergent under these conditions. The ability of agents to increase proton conductance across lipid membranes or membranes of submitochondrial particles correlates exactly with uncoupling activity [15–18]. On the other hand, facilitation of proton movement across non-respiring mitochondria, as induced by uncouplers, fits quite well with its activity in stimulating uncoupled respiration [19]. FCCP- and DNP-uncoupled respiration, however, can be inhibited by small amounts of flavomycin. From this data one may postulate a different and more specific site for the inhibition and a rather unspecific site for the uncoupling. However the inhibition of the electron flow by flavomycin appears to be incomplete since oxidized cytochromes c_1 and aa_3 became reduced if the suspension was not fully air-saturated. At high concentrations such as need for uncoupling micellar concentration may be reached and the compound removed from its inhibitory site.

CTC is known to be a potent chelating agent that forms stable complexes with such metals as play important roles in many enzyme reactions [20–22]. It might therefore be expected that this antibiotic could influence different enzyme reactions by this mechanism when exposed to complex biological systems like mitochondria. Since succinate dehydrogenase contains iron as an additional prosthetic group, it might be predicted that CTC exerts its inhibitory activity on succinate-respiring mitochondria partly by chelating iron ions of this flavoprotein. However this interpretation seems unlikely since iron is tightly bound to the protein by covalent linkage. Furthermore cytochrome oxidase, which contains iron and copper, was found to be insensitive upon CTC addition. These data support the conclusion that metals acting as additional prosthetic groups of succinate dehydrogenase cannot be the site of action for CTC. Following the concept of Brüggemann *et al.* [23] CTC catalyses the oxidative inactivation of SH-groups, with especially high activity towards flavoproteins. Adapting this basic assumption for the interpretation of the results presented in this context at least two sites of action for CTC on mitochondrial respiration must be discussed. The remaining inhibitory activity of CTC in uncoupled succinate-respiring mitochondria as well as partial inhibition in coupled mitochondria may be due to oxidative inactivation of SH-groups of the succinate dehydrogenase. This is strongly supported by the finding that the additional inhibition of succinate-respiring mitochondria correlates poorly with the inhibition of uncoupler stimulated respiration. Since adeninenucleotide translocation is controlled indirectly by phosphate transport and not sensitive to SH-reagents itself [24], it appears that the major part of inhibition is attributed to the oxidation of the SH-dependent phosphate carrier. Respiration consequently may be inhibited following the addition of CTC by acceptor control of

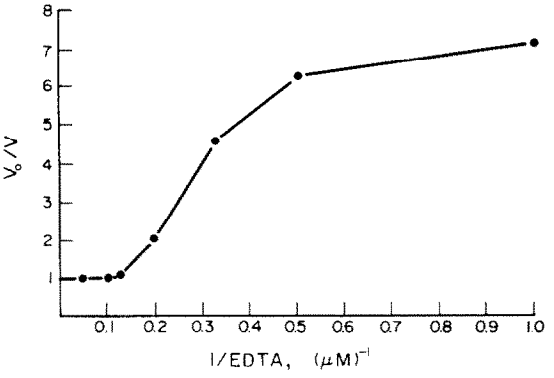

Fig. 6. Reversibility of zinc-bacitracin induced respiratory inhibition by varying concentrations of EDTA. Respiratory activity is expressed as the quotient of the oxygen consumption rate by uninhibited mitochondria (v_0) over the oxygen consumption rate after the addition of zinc-bacitracin (10 μM final concn) and EDTA (v). Final concentration of mitochondrial protein was 1 mg/ml.

the energy conserving system. This is in agreement with observations of other groups who found oxidative phosphorylation and mitochondrial respiration inhibited in the presence of CTC [25–31].

The data with zinc-bacitracin strongly support the hypothesis that this antibiotic is a powerful inhibitor of the mitochondrial electron transport system. Uncoupler-stimulated respiration did not abolish the inbinding site for zinc-bacitracin was assumed to be mechanism by which state 3 respiration is blocked cannot be related to associated energy coupling. The binnding site for zinc-bacitracin was assumed to be the antimycin A site between the cytochromes *b* and c_1, since in the presence of this agent an oxidation of cytochrome c_1 and a reduction of the *b* cytochromes could be observed. The reversibility of the inhibitory activity by EDTA suggested the divalent metal ion is primarily responsible for the results presented here. It was reported earlier that less than 4 μM Zn^{2+} inhibits electron transport in uncoupled but not in coupled mitochondria. Furthermore the Zn^{2+}-binding site was proposed to be located between ubiquinone and the *b* cytochromes [32, 33]. Differently from the free metal ion zinc-bacitracin was shown to act in this concentration range with identical effects on coupled and uncoupled respiration and binds at the antimycin A site. These data indicate that Zn^{2+} ions are required for the activity of bacitracin but do not exert their effects in a direct way. From its capacity to block the electron transport it would appear that Zn^{2+} ions are able to regulate the flow of electrons within the complex.

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