EFFECT OF XANTHOTHRICIN ON THE RESPIRATORY CHAIN

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Received 11 March 1974
Revised version received 26 June 1974

1. Introduction

Xanthothricin (toxoflavin) represents a new class of antibiotics based on 1,6-dimethyl-5,7-dioxo-1,5,6,7-tetrahydropyrimido-(5,4-e)-*as*-triazin [1], it possesses a broad spectrum of antibacterial, anticancer and toxic effects [2,3].

There is only one publication [4] showing that this antibiotic stimulates endogenous respiration of homogenates and cultures of yeast and other microorganisms and that it abolishes the effects of the inhibitors of electron transfer, antimycin A and cyanide. The oxidation of added NADH in the presence of xanthothricin caused the formation of H_2O_2.

From their data the authors concluded that the antibiotic was shunting the respiratory chain, oxidizing NADH with the formation of H_2O_2, the latter causing the antibiotic activity and toxic effect of this compound [4].

In our opinion existing data are insufficient to prove the proposed mechanism. Further H_2O_2 is a normal cell metabolite [5] so its formation cannot explain xanthothricin's biological activity.

In the present paper data on the effects of xanthotricin on the respiratory chain in yeast mitochondria, rat-liver mitochondria and intact Ehrlich ascites tumour cells are reported. These preparations were chosen to cover all the three aspects of xanthothricin's effects.

2. Methods

Rat-liver mitochondria were isolated by Schneider's method [6]. Yeast mitochondria were isolated from *Candida lipolytica* [7]. Ehrlich ascites tumour cells were obtained by the method of Galeotti et al. [8].

Mitochondrial respiratory rate was determined with an LP-7 polarograph provided with a closed Clark-type platinum electrode. The respiratory medium contained, for rat-liver mitochondria, 0.25 M sucrose, 5 mM potassium phosphate, 5 mM $MgSO_4$, 0.1 mM EDTA, pH 7.5; for yeast mitochondria, 0.65 M mannite, 0.05% bovine serum albumin, 0.3 mM EDTA, 10 mM Tris–phosphate, pH 7.0; for ascites cells, 154 mM NaCl, 6.2 mM KCl, 11 mM phosphate buffer, pH 7.5. Sample volume, 2 ml. Room temperature, 22–25°C.

The degree of reduction of pyridine nucleotides was determined by their fluorescence at 460 nm. Incubation was in respiratory medium.

ATPase activity of rat-liver mitochondria was determined by measuring the acidification of the incubation medium with an LPU-01 pH-meter coupled to an automatic KSP-4 potentiometer [9]. The incubation medium contained 0.25 M sucrose, 2 mM potassium phosphate, 50 mM KCl, pH 7.5. Sample volume, 4 ml. Room temperature, 22–25°C. Protein in mitochondria and cells was determined by the biuret method [10].

3. Results

Measurements of respiratory rate of animal and yeast mitochondria in the presence of varying amounts of added xanthothricin showed a maximal stimulation

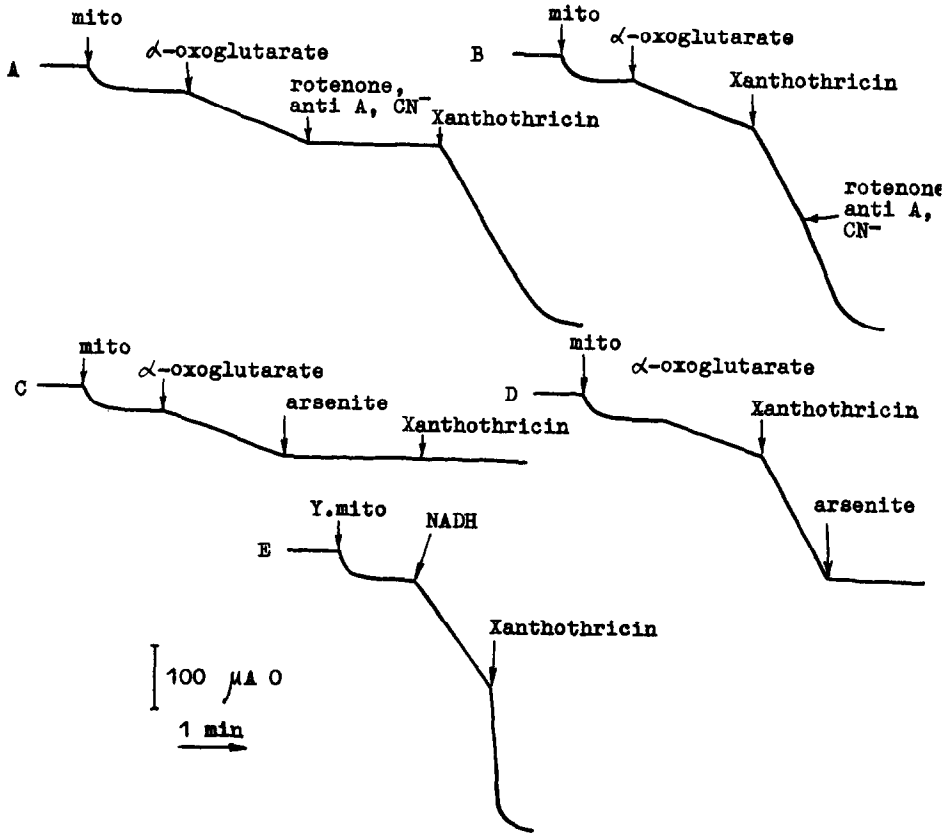

Fig. 1. Effect of xanthothricin on oxidation of NAD-linked substrates and NADH. Additions: rat liver mitochondria 2–3 mg protein/ml; yeast mitochondria (Y.mito) 0.75 mg/ml; 5 mM α-oxoglutarate; 1 mM NADH; 10 μM rotenone; 1 μg/ml antimycin A; 1 mM cyanide; 1 mM arsenite; 10 μM xanthothricin.

at 10 μM antibiotic. Further increase of xanthothricin concentration did not increase the rate of oxygen consumption. Therefore all further measurements were made with 10 μM xanthothricin.

The effect of xanthothricin on oxidation of NAD-linked substrates and NADH is shown in fig. 1, which shows that the antibiotic not only removes the inhibitory effects of rotenone, antimycin A and cyanide but stimulates mitochondrial respiration 2–3 times when oxidizing α-oxoglutarate (curve A). Addition of these inhibitors after xanthothricin does not remove the latter's stimulating effect, even increasing it slightly (curve B). On the contrary, arsenite entirely removes the xanthothricin effect (curve D) and, when added after arsenite, xanthothricin has no effect (curve C). Oxidation of exogenous NADH by yeast mitochondria is also stimulated by xanthothricin (curve E), the extent of stimulation being greater than with α-oxoglutarate. These results allow one to suppose that xanthothricin transfers the reducing equivalents from NADH-dehydrogenase to oxygen, by-passing the respiratory chain.

We then studied the effect of xanthothricin on succinate oxidation (fig. 2). This figure shows that xanthothricin stimulates succinate oxidation but that malonate, antimycin A and cyanide still completeley inhibit respiration (curve B). When xanthothricin is added after these inhibitors it does not stimulate respiration (curve A). It is important to notice that though rotenone does not inhibit succinate oxidation, it prevents the stimulating effect of xanthothricin (curve C) and addition of rotenone after xanthothricin prevents the

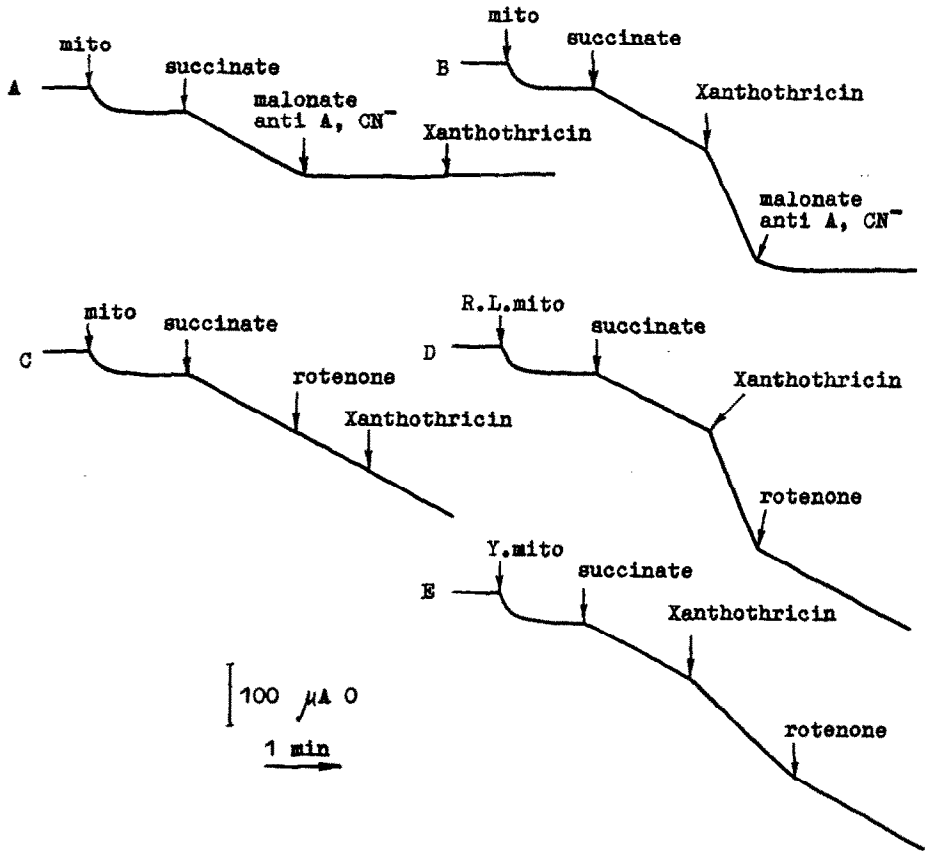

Fig. 2. Effect of xanthothricin on succinate oxidation. Additions: rat liver mitochondria 2–3 mg protein/ml; yeast mitochondria (Y.mito) 1.25 mg protein/ml. 5 mM succinate, 5 mM malonate, 1 μg/ml antimycin A, 1 mM cyanide, 10 μM rotenone, 10 μM xanthothricin.

latter's stimulatory effect (curve D). Succinate oxidation by yeast mitochondria is also stimulated by xanthothricin (curve E) but the respiratory increase is less than with rat-liver mitochondria.

Xanthothricin has no effect on ATPase activity.

The effect of xanthothricin on intact Ehrlich ascites tumour cells is almost the same as on mitochondria, but for maximal activity a concentration of about 300 μM is needed. This may be explained by the additional barrier to penetration of xanthothricin which the cytoplasmic membrane offers.

4. Discussion

The present results show that xanthothricin removes the effect of rotenone in repressing the oxidation of NADH-dehydrogenase but not that of arsenite in inhibiting NAD reduction by α-oxoglutarate (fig. 1, curves A and C). On the other hand, xanthothricin prevents the inhibition of α-oxoglutarate oxidation by rotenone but not by arsenite (curves B and D). Thus xanthothricin by-passes the rotenone-sensitive site in the respiratory chain. As xanthothricin does not directly oxidize NADH and as its redox potential, 0.049 V [11], is near to that of NADH-dehydrogenase, one may suppose that xanthothricin accepts electrons at the level of NADH-dehydrogenase. The insensitivity of respiration to antimycin A and cyanide in the presence of xanthothricin (fig. 1, curves A and B) demonstrates the shunting of the wole respiratory chain from NADH-dehydrogenase to oxygen by this antibiotic.

Fig. 1 also shows that xanthothricin considerably

stimulates the oxidation of exogenous NADH by yeast mitochondria (curve E). The antibiotic increases the rate of oxidation of NAD-linked substrates 2–3 times but the rate of exogenous NADH oxidation is increased 15–20 times. This great difference may probably be explained either by the readier availability of the dehydrogenase oxidizing exogenous NADH, which is on the outer side of the inner mitochondrial membrane [12], or by the greater affinity for xanthothricin of the 'exogenous' rather than the 'endogenous' NADH-dehydrogenase.

Xanthothricin also stimulates mitochondrial respiration during succinate oxidation but, as follows from fig. 2, this is due to reversed electron transfer to NAD and then via xanthothricin to oxygen and not by the oxidation of succinate dehydrogenase by the antibiotic. Xanthothricin does not alter the effects of the inhibitors of succinate oxidation, malonate, antimycin A and cyanide (curves A and B). Rotenone, which does not inhibit succinate oxidation but does inhibit reversed electron flow from succinate, prevents the xanthothricin effect (curve C) and decreases respiration to the former level (curve D). A considerably smaller xanthothricin effect in yeast

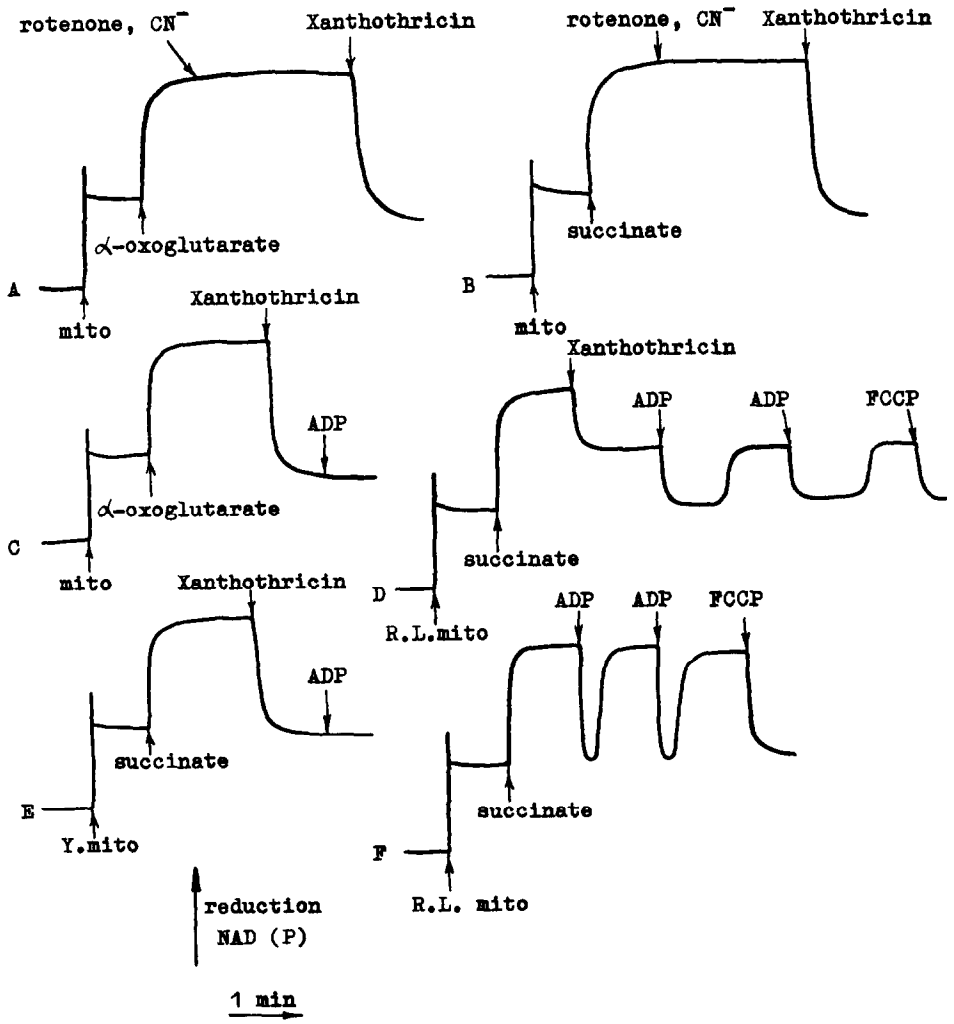

Fig. 3. Effect of xanthothricin on the degree of reduction of pyridine nucleotides in mitochondria. Additions: rat-liver mitochondria (R.L.mito) 2.4 mg protein/ml; yeast mitochondria (Y.mito) 2.6 mg protein/ml; 5 mM α-oxoglutarate, 5 mM succinate; 10 μM rotenone; 1 mM cyanide; 200 μM ADP; 1 μM FCCP; 10 μM xanthothricin.

mitochondria oxidizing succinate (curve E), may probably be explained by the lower intensity of reverse electron transfer in yeast than in rat-liver mitochondria.

The polarographic data are confirmed by the data obtained by fluorometry of pyridine nucleotides (fig. 3). Curves A and B show that the presence of rotenone or cyanide does not prevent the oxidation by xanthothricin of pyridine nucleotides reduced by an NAD-linked substrate or by succinate. Phosphorylation during oxidation of NAD-linked substrates in the presence of xanthothricin is absent (curve C)

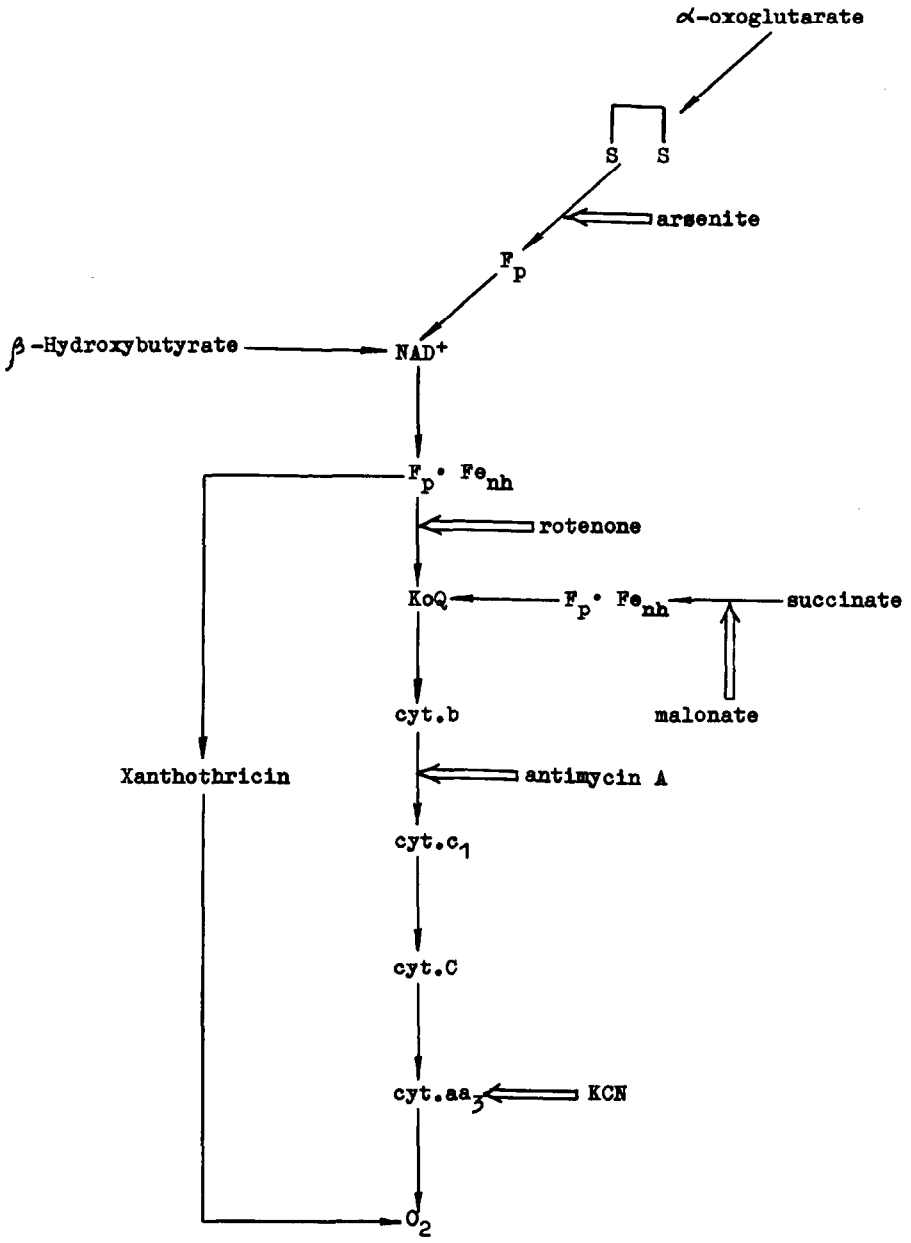

Fig. 4. Scheme showing the effect of xanthothricin on the electron transport chain of mitochondria.

as xanthothricin shunts all three coupling sites. During succinate oxidation in the presence of xanthothricin phosphorylation does take place though its efficiency is decreased (curves D and F). This may probably be explained by supposing that the macroergic compounds formed are used to support reversed electron transfer. During succinate oxidation by yeast mitochondria, xanthothricin completely oxidizes pyridine nucleotides (curve E), which is probably connected with the low rate of reversed electron transfer.

Preservation of oxidative phosphorylation during succinate oxidation in the presence of xanthothricin and an absence of an effect of this antibiotic on ATPase activity suggests that xanthothricin has no effect on the energy transfer chain or on the coupling sites.

From the data presented one may conclude that xanthothricin is a specific carrier of reducing equivalents from NADH-dehydrogenase to oxygen (fig. 4). Xanthothricin prevents phosphorylation during oxidation of NAD-linked substrates and reduces its efficiency during succinate oxidation. Being unregulated by the respiratory chain, NADH oxidation by xanthothricin leads to a decrease of the reduced pyridine nucleotide pool and of the pool of macroergic compounds both of which are necessary for normal cell metabolism particularly for protein synthesis. This probably explains its antibiotic activity, toxicity and anticancer effect.

References

[1] Davis, Jr., G. D., Robins, R. K. and Cheng, C. C. (1962) J. Am. Chem. Soc. 84, 1724–1729.
[2] Machlowitz, R. A., Fischer, W. P., McKay, B. S., Tytell, A. A. and Charney, J. (1954) Antibiotics & Chemotherapy 4, 259.
[3] Van Damme, P. A., Johannes, A. G., Cox, H. C. and Berends, W. (1960) Rec. Trav. Chim. 79, 255–267.
[4] Latuasan, H. E., Berends, W. (1961) Biochim. Biophys. Acta 52, 502–508.
[5] Loschen, G., Azzi, A., Flohé, L. (1973) FEBS Letters 33, 84–88.
[6] Schneider, W. C. (1948) J. Biol. Chem. 176, 259.
[7] Akimenko, V. K., Golovchenko, N. P., Medentsev, A. G., Mikrobiologiya (in press).
[8] Galeotti, T., Azzi, A., Chance, B. (1969) Arch. Biochem. Biophys. 131, 306.
[9] Chance, B., Nischimura, M. (1967) Methods in Enzymol. 10, 641.
[10] Gornall, A. G., Bardawill, C. J., David, M. M. (1949) J. Biol. Chem. 177, 751.
[11] Stern, K. G. (1935) Biochem. J. 29, 500–508.
[12] Von Jagow, G., Klingenberg, M. (1970) Eur. J. Biochem. 12, 583–592.