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INHIBITION OF CREATININE PHOSPHOKINASE AS A POSSIBLE MECHANISM FOR CREATINURIA PRODUCED BY TWO TOXIC ANTIBIOTICS, MUCONOMYCIN A AND B

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Abstract—Muconomycin A (M-A) and muconomycin B (M-B), two nonnitrogenous antibiotics derived from culture broths of *Myrothecium verrucaria*, possess unique structural features and high mammalian toxicity. The LD_{50} in mice (i.p.) was found to be 0·50 to 0·75 mg/kg for both antibiotics. M-A induced a severe creatinuria in rats, and this effect was attenuated by pre-administration of vitamin E. Both antibiotics were found to inhibit creatine phosphokinase (CPK) *in vitro*; I_{50} values were $29{\cdot}8 \times 10^{-4}$ M for M-A and $7{\cdot}0 \times 10^{-4}$ M for M-B. CPK was then assayed, at various concentrations of substrate, with M-A. A Hunter-Downs plot of the data showed that M-A was a noncompetitive inhibitor of CPK; the K_I was $3{\cdot}5 \times 10^{-4}$ M. It is suggested that the high toxicity of these antibiotics, as well as their ability to cause creatinuria, may be related to their inhibitory effect on CPK.

MUCONOMYCIN A (M-A)† and Muconomycin B (M-B), two antibiotics derived from culture broths of *Myrothecium verrucaria*, possess unique structural features and exhibit a wide variety of biological activity. In this report are presented data showing that M-A induces creatinuria in rats and that both antibiotics inhibit the enzyme creatine phosphokinase (CPK) *in vitro*.

The toxicity of M-A and M-B is very high. In our laboratory, we found that when these compounds are administered i.p. to mice, the LD_{50} for both is in the range of 0·50 to 0·75 mg/kg. Rusch and Stahelin[1] administered M-A i.v. to rats and reported a similar LD_{50}, 0·8 mg/kg. They also found that M-A was 20 times more potent than methotrexate against P-815 mastocytoma cultures, and they concluded that M-A was one of the most active of known cytostatic agents.

M-A and M-B are macrocyclic triesters with an exocyclic methylene epoxide group and do not contain nitrogen (Fig. 1). However, M-A and M-B possess structural features in common with antimycin, and were therefore considered as possible antagonists of vitamin E.[2] Such antagonists would be expected to heighten the degree of creatinuria seen in rats deprived of vitamin E;[3] our data show that M-A does induce creatinuria and that the degree of creatinuria is inversely proportional to the amount of vitamin given to the animals. Two possible explanations for this creatinuria-inducing effect are: selective inhibition of oxidative phosphorylation,[4] and decreased

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† A Swiss group, led by Charles Tamm, has published the structural elucidation of M-A, but designates the compound as Verrucarin A (J. GUTSWILLER and C. TAMM, *Helv. chim. Acta* **46**, 1786 (1963).

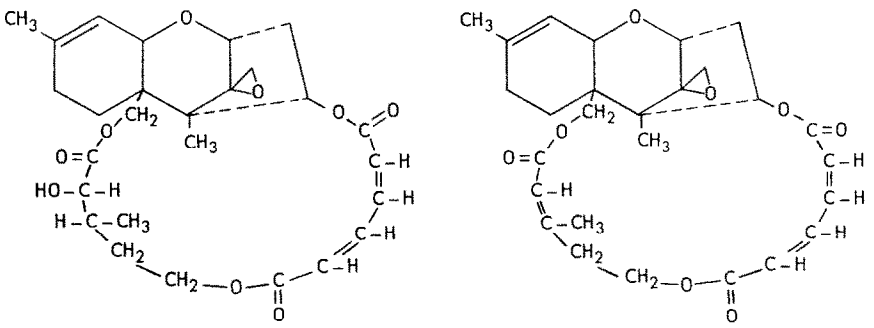

FIG. 1. Structural formulas for muconomycin A and muconomycin B.

activity of creatine phosphokinase.[5] Rusch and Stahelin[1] studied the effects of M-A on energy metabolism and reported that the antibiotic had no significant effect on aerobic or anaerobic glycolysis in cells or tissues suspended in Krebs–Ringer solution. Since oxidation thus seemed not to be affected by M-A, it became of interest to us to examine the possibility that M-A and M-B might inhibit creatine phosphokinase. Our data show that CPK is inhibited *in vitro* by M-A and by M-B.

MATERIALS AND METHODS

Weanling rats of the Sprague–Dawley strain (Charles River) were fed a vitamin E-deficient diet (General Biochemicals, Chagrin Falls, Ohio). After they had been maintained on this diet for 4 months, the rats were divided into 5 groups of 3 rats each. Once each day for 10 days, after a method of Hove,[3] rats in groups 1, 2 and 3 received 10, 25 and 100 mg/kg, respectively, of vitamin E (*dl*-α-tocopherol, Calbiochem) administered orally in olive oil. Group 4 was given olive oil only. The fifth group was given no vitamin E; this group was starved during the same period that the other groups received 0·25 mg/kg of muconomycin A* in propylene glycol via the peritoneal route.

Before treatment with M-A was begun, normal creatine excretion patterns were established by daily analysis of 24-hr urine samples pooled from each group. Urinary creatine was assayed by the method of Folin[6] and reported as mg/100 g body wt/24 hr. The mean creatine output for the 10-day vitamin E treatment period was determined and divided into the creatine output observed for each day of the antibiotic treatment period. This quotient appears in Fig. 2 as the "creatinuria ratio." As some treated animals did not eat well, the starved group was included in order to determine whether decreased food consumption might affect urinary creatine levels.

Creatine phosphokinase (ATP: creatine phosphotransferase) was assayed by the method of Tanzer and Gilvarg.[7] Crystalline CPK was purchased from Calbiochem as were all other biochemicals used in this procedure.

In order to determine the type of inhibition effected by the antibiotics, CPK was assayed at various substrate concentrations in the presence and in the absence of M-A and of M-B. The antibiotics were routinely preincubated with enzyme alone for 10 min,

* Antibiotic samples of high purity were kindly supplied by Dr. B. M. Vittimberga, Department of Chemistry, University of Rhode Island, Kingston, R.I.

since preincubation with enzyme and substrate did not change the type of inhibition obtained. The relative potencies of M-A and M-B were determined by calculating the I_{50} (antibiotic concentration required for 50 per cent inhibition of reaction rate) for each compound.

In the assay method employed in this work, two enzymes are coupled to CPK. It was necessary, therefore, to demonstrate that only the CPK reaction was susceptible to the action of the antibiotics. We found that the coupled enzymes, pyruvate kinase and lactic dehydrogenase, were not affected by M-A or by M-B.

RESULTS AND DISCUSSION

Data in Fig. 2 illustrate the ability of vitamin E to reverse the creatinuria induced by muconomycin A. Rats which had received high doses of the vitamin (25 or 100 mg/kg)

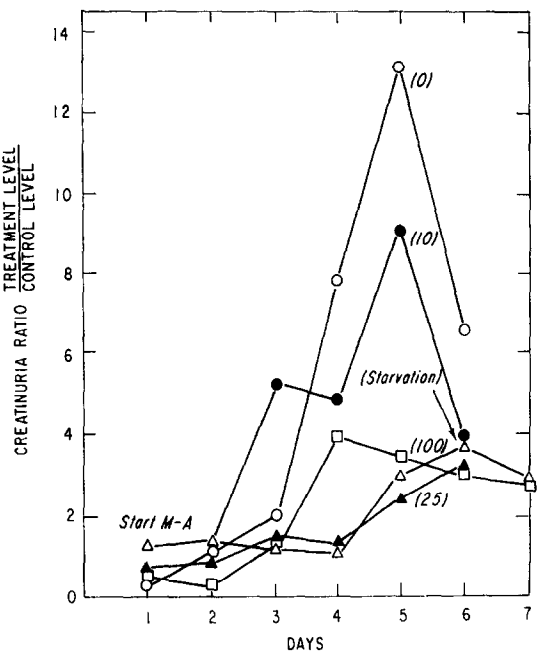

FIG. 2. Effect of muconomycin A (M-A) on creatinuria of rats previously treated with vitamin E. Intraperitoneal doses of 0·25 mg/kg of M-A were given daily for 7 days to 4 groups of 3 rats each, previously treated daily for 10 days with the following doses (mg/kg) of vitamin E: O——O, 0; ●——●, 10; ▲——▲, 25; □——□, 100. The fifth group of rats (△——△) received vitamin E and no M-A, but was starved during the 7-day M-A treatment period. In the "creatinuria ratio", the "control level" = mean daily urinary creatine output for each group during the 10-day vitamin E treatment period and "treatment level" = daily urinary creatine output for each group during the 7-day M-A treatment period.

displayed a low level of creatinuria during treatment with M-A. On the other hand, rats which had received only 10 mg/kg of the vitamin or none at all displayed a high level of creatinuria. This is especially obvious after 5 days of treatment with the antibiotic. Starvation did produce a slight increase in creatinuria, but this was insignificant as compared with the creatinuria induced by M-A.

The loss of body stores of creatine is serious, since creatine as the phosphate is stored in muscle and provides a reserve of "high energy phosphate," regenerating ATP as required. Anything interfering with the synthesis of creatine phosphate (CP) might therefore be expected to cause creatinuria. Creatine phosphokinase, the enzyme which synthesizes CP, is inhibited *in vitro* by muconomycin A (Fig. 3).

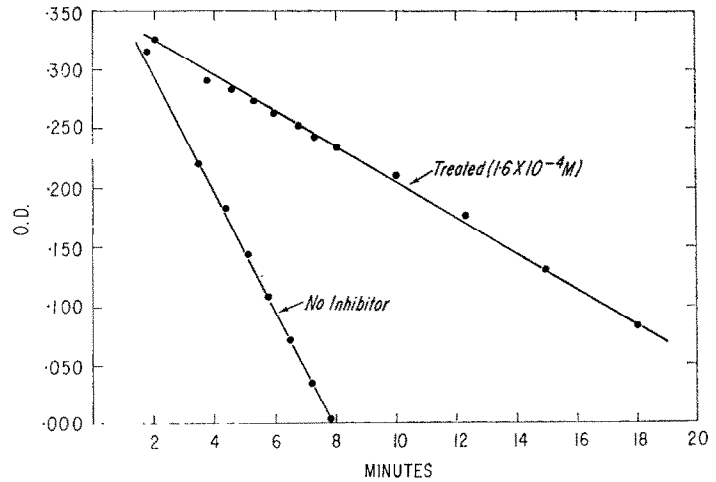

FIG. 3. Effect of muconomycin A on creatine phosphokinase activity. The reaction mixture contained: glycine buffer (pH 9), 150 μmole; NADH, 0·32 μmole; ATP, 1·0 μmole; phosphoenolpyruvate, 2·0 μmole; $MgCl_2$, 1·0 μmole; lactic dehydrogenase, 0·1 mg; pyruvate kinase, 0·1 mg; creatine, 110 μmoles; creatine phosphokinase, 1·68 μg in a total volume of 3·0 ml.

The method of Hunter and Downs[8] was used to determine the K_i (inhibitor constant) for M-A (Fig. 4). The K_i was $3{\cdot}5 \times 10^{-4}$ M and since the slope of the line was zero,

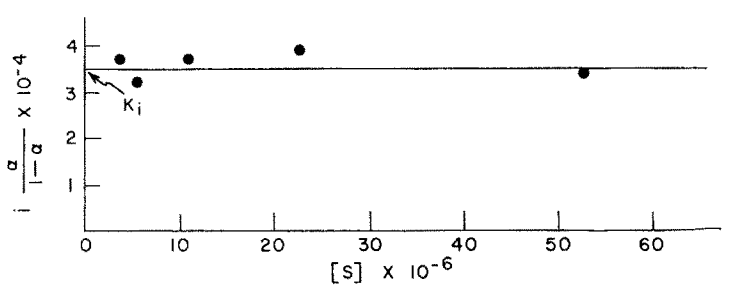

FIG. 4. Inhibition of creatine phosphokinase by muconomycin A. Graphical determination of the inhibitor constant (K_i) was by the method of Hunter and Downs.[8]

the inhibition was noncompetitive.[8] In Fig. 5, the graphic method for the determination of the I_{50} for M-A and for M-B indicates that M-B is about 4 times more potent an inhibitor of CPK than is M-A.

This 4-fold difference in potency suggests that the minor structural difference between M-A and M-B is important. There is an additional ethylenic bond in M-B, and it has been postulated that this bond in similar esters leads to reactivity with —SH groups.[9, 10] Both antibiotics contain other ethylenic bonds adjacent to carbonyl

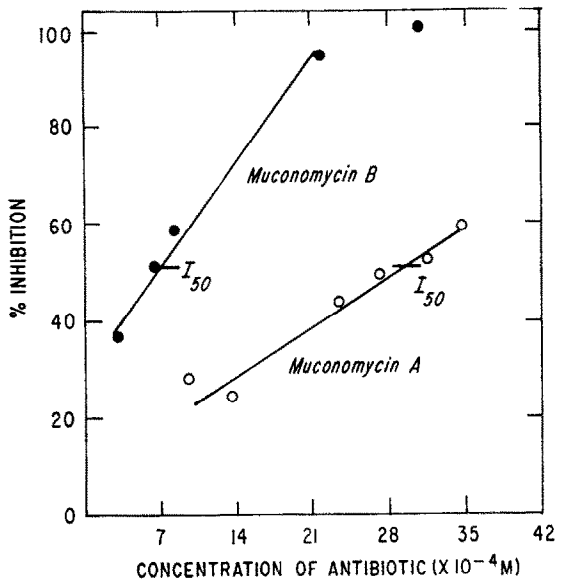

FIG. 5. Calculation of I_{50} for muconomycin A and muconomycin B on creatine phosphokinase. The reaction mixture was the same as indicated in Fig. 3, except that the concentration of the antibiotics was changed as shown.

groups as well as exocyclic methylene epoxide groups; any of these groups may react with thiols. Since at least one —SH group has been reported to be present in the active center of CPK,[11] it is possible that these antibiotics may be thiol-alkylating reagents. The fact that the muconomycins have several possible thiol-reactive sites may possibly explain in part why these compounds appear to be more potent than other —SH reagents.

Our study shows, in summary, that M-A and M-B are highly toxic, that M-A induces severe creatinuria and that both M-A and M-B inhibit creatine phosphokinase *in vitro*. It is suggested that these effects may be due to a reaction with a common underlying factor.

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REFERENCES

1. M. E. RUSCH and H. STAHELIN, *Arzneimittel-Forsch.* **15**, 893 (1965).
2. K. O. DONALDSON, A. NASON and R. H. GARRETT, *J. biol. Chem.* **233**, 566 (1958).
3. E. L. HOVE, *Ann. N.Y. Acad. Sci.* **52**, 202 (1949).
4. H. WEIL-MALHERBE, *A. Rev. Biochem.* **17**, 1 (1948).
5. C. NICHOL, P. L. MCGEER and J. R. MILLER, *Can. J. Biochem. Physiol.* **40**, 443 (1962).
6. O. FOLIN, *J. biol. Chem.* **97**, 469 (1914).
7. M. L. TANZER and C. GILVARG, *J. biol. Chem.* **234**, 3201 (1959).
8. A. HUNTER and C. E. DOWNS, *J. biol. Chem.* **157**, 427 (1945).
9. A. M. BACQ, *Experientia* **2**, 349 (1946).
10. J. F. MACKWORTH, *Biochem. J.* **42**, 82 (1948).
11. A. H. EANOR and H. ROSENBERG, *Biochem. J.* **57**, 203 (1954).