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SUPEROXIDE DISMUTASE FROM *TRICHURIS OVIS*, — INHIBITION BY BENZIMIDAZOLES AND PYRIMIDINE DERIVATIVES

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Three superoxide dismutase isoenzymes of different cellular location were detected in an homogenate of Trichuris ovis. *Each of these molecular forms was purified by differential centrifugation and precipitation with ammonium sulphate, followed by chromatography on DEAE-cellulose and Sephadex G-75 columns. The activity levels of the two molecular forms detected in the mitochondrial (one cyanide sensitive Cu-Zn-SOD and the other cyanide insensitive Mn-SOD) were higher than that of the superoxide dismutase detected in the cytoplasmic fraction (cyanide sensitive Cu-Zn-SOD). All molecular forms present evident differences to the SODs contained in the host liver. Molecular mass and some of the physical and chemical properties of the enzyme was determined for all three molecular forms.*

An inhibitory effect on the SOD of the parasite an the host was detected with a series of compounds, some of which markedly inhibited parasite ensyme but not host enzyme.

Key words: *Trichuris ovis* – superoxide dismutase – characterization – inhibition

Superoxide dismutase (E.C. 1.15.1.1.) (SOD) is a widely distributed enzyme in animal tissues. The principal physiological role of the enzyme SOD is to protect cells from the superoxide radical, a potential agent of oxygen poisoning (McCord et al., 1971). It catalyses the cleavage of the radical according to the following reaction:

$$2\ O_2 + 2\ H^+ \longrightarrow H_2O_2 + O_2$$

The superoxide radical can acts equally as a reducing or and oxidizing agents, and is capable of modifying a large number of mollecules of biological importance. The enzyme SOD also eliminates the cytotoxic superoxide radicals (Klebanoff & Clark, 1978).

The superoxide dismutase has been found in a variety of forms. The copper-zinc enzyme is found in the cytosol and in the mitochondrial intermembrane space of eukaryotic cells and is sensitive to high concentration of cyanide (McCord & Fridovich, 1969). The mitochondrial matrix also contains cyanide-insensitive manganese enzyme similar to that form in prokaryotes (Beauchamp & Fridovich, 1971).

SOD activity has been reported only in a few helminth tissues (Paul & Barret, 1980; Rhoads, 1983; Leid & Suquet, 1986; Sanchez-Moreno et al., 1987, 1988). The mayority of these authors documentd the existence of the enzyme only; little is known about its molecular and physical properties. The studies published to date suggests that the metalloenzyme SOD may play important roles either as a component of leukocytes or as a defense mechanism against them and a detoxifier or superoxide anion radicals, the metabolites of oxygen compsuntion. Meshnick et al. (1986), pointed out the posible use of SOD as a target for the action of antiparasitic drugs in view of its important role in the removal of radicals. Its also been observed that benzimidazoles, described as powerful, broad-spectrum anthelmintics, interference with the synthesis of energetic compounds and with helminth glucose metabolism in general. However, the mode of action of these compounds is still unclear, and they may be acts upon other enzymatic systems.

Pyrimidine derivatives appear to be potential chemoterapeutic agents as many of them inhibit or modify the activities of metallo-enzymes, and some also show antitumoral activity (Salas-Peregrin et al., 19850.

This study forms part of a program developed in our laboratory to characterize SOD in several

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helminth species. Its implicit significance in parasite survival could well make SOD an appropriatte target for chemotherapeutic agenst. The present paper assesses the effects of several benzimidazoles and recently synthesized pyrimidine derivatives on purifeid SOD from Trichuris ovis.

MATERIALS AND METHODS

Biological sample – Adult specimens of *T. ovis* and livers from goats (*Capra hircus*) were obtained from slaughterhouse of Granada (Spain) and transported to the laboratory in NaCl (0.9% w/v) at 37 °C. On arrival the samples were prepared for extraction and purification of SOD.

Isolation and purification of SOD – The samples were chopped into small pieces inmediately before being homogenized in a Potter-Elvehjem homogenizer using 0.25 M sucrose, 20 mM Tris-HCl (pH 7.4) and 1 mM EDTA at a tissue/solution ratio of 1:6 (w/v) at 4 °C. The homogenate was centrifuged for 10 min at 1500xg, and the supernatant was collected and stored at 4 °C. The precipitate was resuspended in the homogenizing buffer and centrifuged again at 1500 x g for 10 min, and the resulting supernatant was added to that obtained previously. The precipitate was resuspended, homogenized and recentrifuged, the third supernatant being added to the other and the precipitate discarded.

The combined supernatant were centrifuged at 10,000 x g for 20 min and the resulting supernatants and precipitate were considered as the cytoplasmic and mitochondrial fractions, respectively.

The cytoplasmic fraction was precipitated with ammonium suphate at a final concentration of 60%, followed inmediately by centrifugation at 5000 x g for 20 min. The resulting supernatant fraction was re-precipitated with ammonium sulphate at a final concentration for 90% and centrifuged as before. Practically all the enzymatic activity was found in the precipitate, which was dialysed with continuous stirring for 6 h at 4 °C against distilled water. The dialysed fraction was placed on a 1x30 cm DE-22 DEAE-cellulose column prebuffered with 0.01 M K2HPO, pH 8.5 and 0.1 mM EDTA, pH 8.5. The elution process was in two phases: First, a continuous gradient was established between a basic solution of 0.05 M KH2PO4, pH 8.5 (150 ml) and a solution gradient of 0.05 M KH2PO4 which was simultaneously eluted with 0.1 M NaCl, pH 4.4 (250 ml). The second phase was eluted with a solution of 0.1 m KH2PO4, pH 4.4 (300 ml) at an elution rate of 0.4 ml per min. Fraction of 5 ml were collected.

Those fraction which showed enzyme activity were concentrated by precipitation with 90% ammonium sulphate, followed by dialysis against a 0.05 M phosphate buffer at pH 7.8. The diffusate was run on a 1x100 cm Sephaex G-75 column with 0.05 M phosphate buffer at pH 7.8. Elution was performed with a potassium phosphate buffer, pH 7.8 (0.05 M K2HPO4 and 0.1 mM EDTA) at a flow rate of 0.4 ml/min. Fractions of 10 ml were collected.

The mitochondrial fraction was resuspended in homogenization buffer with 10% digitonin 1:6 (mass/vol) and sonicated (in an sonifer mode B-12 sonicator) for six periods of 30 sec at 1.5 mA before being subjected to the fractionation process on the DE-22 DEAE-cellulose column from which active fractioins of 5 ml were obtained. The fractions were separately collected and concentrated in preparation for molecular filtration on G-75 Sephadex columns: the elution conditions for both the cytoplasmic and mitochondrial fraction wre the same.

The active fractions obtained after Sephadex filtration were concentrated with 90% ammonium sulphate and dialysed against distilled water.

Enzyme activity measurements – SOD assays were performed with the xanthine-xanthine oxidase superoxide radical generating system coupled with p-nitrotetrazolium blue (NBT), according to the method of McCord & Fridovich (1969). All measurements were made with a Beckman moel DBG specgtrophotomoter at 560 nm and 25 °C. One unit of SOD activity was defined as that amount of enzyme which inhibited the reduction of NBT by 50%. Protein concentrations were determined by the method of Lowry (Lowry et al., 1951). All biochemicals were parchesed from Sigma Chemical Co.

Physicochemical characterization

Molecular weights – The molecular weights of the enzyme were determined by gel filtration on a Sephadex G-75 column (1x100 cm), equilibrated with 0.05 M phosphate buffer (pH 7.8) containing 0.1 M KCl, using standard protein markers. The eluted fractions were monitored photometrically at 280 nm.

Electrophoresis – Crude homogenates were obtained from single individual specimens crushed

in 20 µl of distilled water and absorbed on to 4 x 5 mm chromatography paper strips (whatman 3), which were inserted is starch gel trays. Horizontal elecrtrophoresis was carried out at 7-8 V/cm for 5 h at 5 °C. The staining techniques were, with some modifications, those described by Selander et al. (1971).

Metal analysis – The enzyme (4 mg) was heated in 1 ml of a 60% HNCO3 plus 60% HC103 mixture (1:1, v/v) at 100 °C for 1 h. Metals were determined by atomic absorption spectrophotometry with an instrumentation Lab. model 751.

pH stability – Pre-prepared buffers (citrate-phosphate; phosphate-potassium; and carbonate-bicarbonate) with a pH range from 3.0 to 11.0 were used to determine enzyme activities at different pH values.

Inhibition of SOD – For *in vitro* assays, three benzimidazoles (BZ) (mebendazole-MBZ-, parbendazole-PBZ- and thiabendazole-TBZ) and six pyrimidine derivatives (Fig. 1) were used. All chemicals were tested at three different concentration (10^{-7}, 5 x 10^{-6} and 10^{-6} M). The pyrimidine derivatives were recently synthesized in the Departamento de Quimica Inorganica de la Facultad de Ciencias, Universidad de Granada (Spain). Percentage inhibition of SOD by these derivatives was determined in a manner similar to that described for enzyme activity. The measurements were taken after incubation at 37 °C for 30 min (longer incubation times failed to raise percentage inhibition). The preparations were dissolved in a stock solution of ethanol to a concentration of 3.3% (v/v/). All experiments were repeated with 5 separate enzyme preparations and one control as well as an ethanol blank were run for each experiment.

RESULTS AND DISCUSSION

Isolation and purification of SOD – Whole homogenates of *T. ovis* (Table I) showed considerable SOD activity, which was similar to the levels documented by other authors for the nematode *Trichinella spiralis* (Rhoad, 1983), and significantly higher than that detected in the cestodes *Hymenolepis diminuta, Moniezia expansa, M. benedeni, Dipylidium caninum and Taenia hydatigena* (Paul & Barret, 1980; Sanchez-Moreno et al., 1989).

. In whole extracts of *T. ovis*, two molecular forms of SOD were detected (Table I). One of these was identified as Cu-Zn SOD based on its sensitivity to cyanide and hidrogen peroxide, whereas the other was identified as manganese-containing SOD. Three SOD isoenzymes were detected during purification, one in the cytosol fraction and another in the mitochondrial fraction. Both were found to be cyanide/peroxide-sensitive (Cu-Zn-SOD I and II). A third isoenzyme, found in the mitochondrial fraction, was cyanide/peroxide insensitive (Mn-SOD). All three forms of the enzyme were obtained in highly purified form (130-270 U/mg protein).

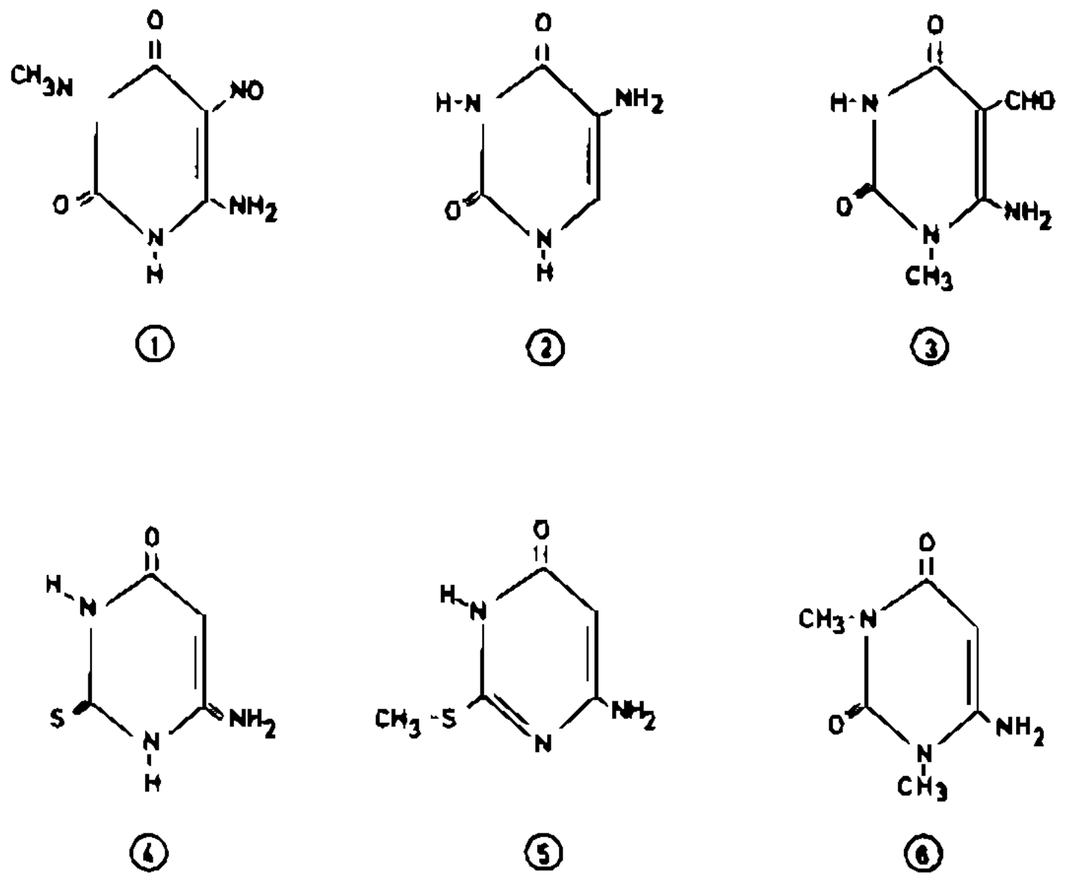

Fig. 1: 4 amino-1 methyl-5 mitroso-uracil (MNU). 2: 5 amino-uracil (5-AU). 3: 4 amino-5 phormil 3-methyl-uracil (5-FMU). 4: 4 amino-2 thiouracil (TU). 5: 4 amino-2 methyl--thio-uracil (MTU). 6: 4 amino-1,3 dimethyl-uracil (DTU).

The three isoenzymes showed different levels of activity. The activity of both mitochondrial forms was significantly greater than that found in the cytosol fraction. All values were within the range of SOD activity detected in mammals (Henry et al., 1980).

Very little information is available regarding multiple SOD ensymes of different locations and activities in helminth parasites. As in our previousk study in *Ascaris suum* (Sanchez-Moreno et al., 1987), three isoenzymes were detected in the present study of *T. ovis*. Although no other studies have reported the existence of these three SOD isoenzymes in parasites, similar results were obtained by authors working with other eukaryotic and prokaryotic organimss (Henry et al., 1980; Anderson, 1982; Blum & Fridovich, 1983).

The high levels of SOD activity found in *T. ovis*, as well as the low or absent catalase activity reported in some nematode species (Roahd,

TABLE I

Isolation and purification of SOD from *Trichuris ovis*

Procedure	Protein total(mg)	Activity Total (U)	Specific activity (U/mg) ± SD	Purific.
Homogenate	267.40	2193.92	8.20 ± 0.63	—
Cu-Zn-SOD	"	138.22	5.17 ± 1.00	—
Mn-SOD	"	86.17	3.02 ± 0.85	—
Cystosolic fraction				
(NH2SO4	50.00	3970.34	79.41 ± 6.03	15.4
DAEE-cellulose	1.92	391.78	204.05 ± 12.17	39.5
Sephadex G-75	.03	20.41	680.37 ± 21.01	131.6
MItochondrial fraction				
(NH4)2SO4	8.03	439.52	54.73 ± 8.75	5.2
Cu-Zn-SOD	"	296.47	36.92 ± 7.33	7.1
Mn-SOD	"	172.56	21.49 ± 2.14	6.7
DEAE-cellulose:				
Cu-Zn-SOD	.19	41.46	218.20 ± 13.16	42.2
Mn-SOD	.16	21.09	131.81 ± 11.07	40.9
Spehadex G-75				
Cu-Zn-SOD	.04	35.86	896.54 ± 51.00	173.4
Mn-SOD	.02	17.29	864.48 ± 27.35	268.5

1983; Sanchez-Moreno et al., 1987) have been shown to be the principal mechanism in the removal of superoxide radicals in mammals. This suggests, however, that the action of SOD in nematodes is the main, and perhaps the only, system used by these organisms against the toxic efects of these radicals.

Characterization of SOD isoenzymes – The three molecular forms of SOD from *T. ovis* were characterized on the basis of molecular weight, electrophoretic properties, storage, pH and heat stability, and metal content. Sephadex G-75 (Fig. 2) gave approximate molecular weights of 32,000, 36,000 and 64,000 for Cu-Zn-SOD I, Cu-Zn-SOD II and Mn-SOD respectively. Similar values have been obtained for other helminth parasites (Sanchez-Moreno et al., 1988).

The electrophoretic properties of the enzyme were compared in whole homnogenates and purified extracts of *T. ovis* and of the host (*C. hircus*). Figure 3 shows the electrophoretic patterns obtained for whole homogenates and for each purification stage. Treatment withl mM cyanide (Geller & Winge, 1983) led to the disappearance of both Cu-Zn-SOD activities, while Mn SOD activity persisted. Analyses of liver homogenates from the host showed notable differences between the three molecular forms of the host and parasite SODs in terms of electrophoretic mobility and staining intensity.

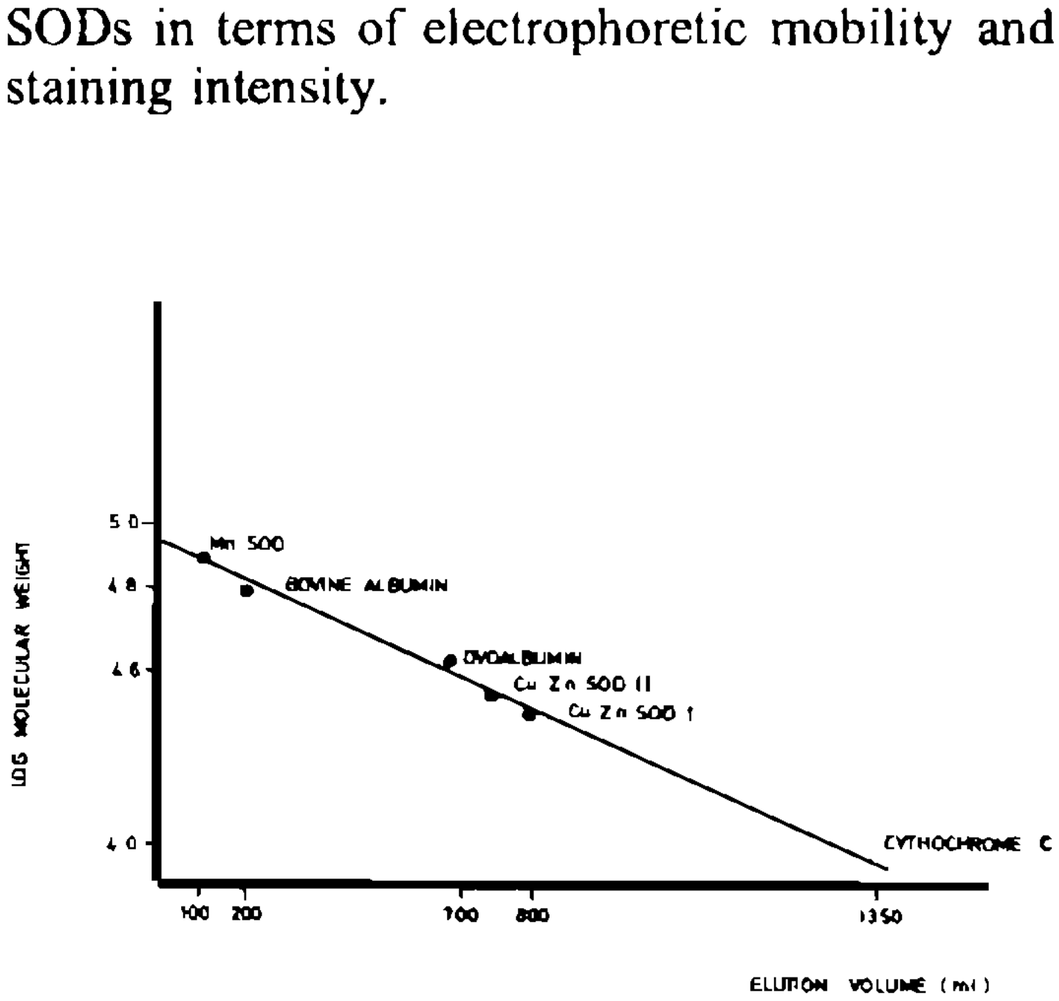

Fig. 2: determination of molecular weights of SODs, for filtration molecular in Sephadex G-75 eluding with aphosphate buffer 50 mM, pH 7.8, using the master protein at a concentration of 5 mg.ml-1.

Our findings also confirmed that the enzyme is stable for at least 3 months when stored at –70 °C. At 4 °C the enzyme lost its catalytic activity within approximately 7 days.

When enzyme solution (23 µg/ml) was incubated for 2 h in various buffers (0.1 M) at a pH

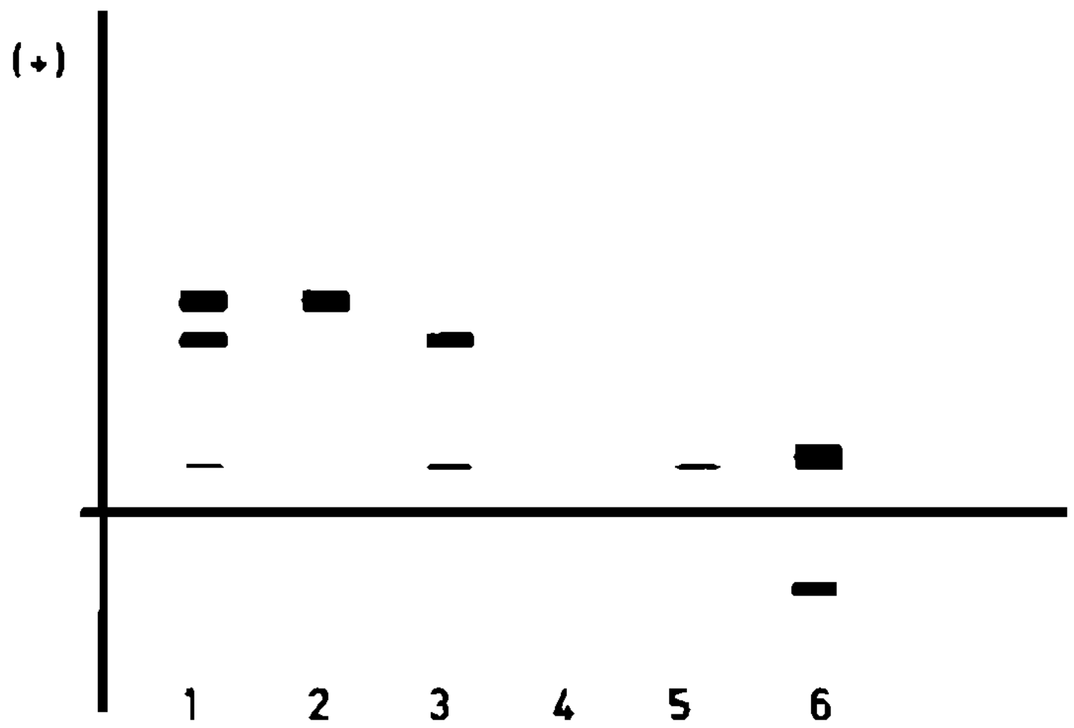

Fig. 3: SOD isoenzymes from Trichyris ovis and Capra hircus by electrophoresis starch gels of different fractions of the purification. 1: homogenate T. ovis (20 µg protein). 2: cytoplasmic fraction (30 µg protein). 3: mitochondrial fraction (16 µg protein). 4: cytoplasmic fraction with cyanide. 5: mitochondrial fraction with cyanide. 6: homogenate C. hircus (20 µg protein>Significant differences between control and results obtained with anthelminthic treatment using paired t-test: a $p < 0.05$; b $p < 0.0025$.).

values ranging from 3.0 to 11.0, the *Trichuris* SODs were stable over a range of 9.5 to 11.0.

Treatment of the enzyme solution at different temperatures for 60 min showed optimal enzyme activity at 37 °C, although activity at 50 °C was still considerable (75%). No activity was recorded at 100 °C.

Atomic absorption spectrometry indicated that *Trichuris* SOD contained 2.1 g Cu and 1.07 g Zn per mole of enzyme.

Inhibition of SOD activity – The results of the inhibition tests for SOD using 3 benzimidazoles and 6 recently synthesized pyrimidine derivatives are shown in Table II. Only the two highest doses of TBZ benzimidazole significantly inhibited the parasite enzymne. PBZ was slightly inhibiting at the highest dose (10 µM), while MBZ was inefective at all doses assayed against all three parasite isoenzymes. None of these three anti-helminthics produced and inhibitory effect on the host enzyme.

Benzimidazoles have traditionally been used as antihelminthics, due to their ability to inhibit energetic metabolism of fumarate reductase and succinate dehydrogenase in the parasite. Some authors have suggested that the action of these compounds may not be limited to one enzyme system. More specifically, it has been theorized that TBZ may inhibit enzyme systems — including SOD — indrectly related with electron transport (Malkin & Camacho, 1972); Bhem & Bryant, 1979; Sharman, 1987). Our findings clearly support these theories, particularly in light of the marked degree of inhibition of SOD by TBZ in *T. ovis* as well as in *M. expansa* (Sanchez-Moreno et al., 1989).

Somewhat unexpectedly, the inhibition observed with three of the pyrimidine compounds tested 91.5% 5-FMA, 85.3-MTU and 63.8 DMU - was much stronger than that produced by TBZ. Both 5-FMU and MTU, at the lowest concentration tested, yielded inhibitionn indices of approximately 50%, 5-FMU leading to complete inhibi-

TABLE II

Percentage of inhibition of SOD activity from *Trichuris ovis* by benzimidazoles and some pyrimidine derivatives

	% Inhibition			
	SOD from *T. ovis*			SOD from *C. hircus*
	Concentration (M)			
Inhib	10^{-7}	5×10^{-6}	10^{-6}	10^{-6}
MBZ	–	–	13.5	–
PBZ	14.7	28.6	33.1[a]	–
TBZ	20.5	41.9[a]	63.2[b]	3.3
MNU	6.0	16.0	28.4	–
5-FMU	62.4[b]	82.4[b]	91.5[b]	9.0
5-AU	–	–	5.6	–
DMU	16.1	39.7[a]	53.8[b]	27.5
MTU	42.2[b]	52.3[b]	85.3[b]	33.0
TU	–	–	–	–

Significant differences between control and results obtained with anthelminthic treatment using paired t-test: *a* $p < 0.05$; *b* $p < 0.0025$.

tion at 10 µM. The pyrimidine compounds, like the benzimidazoles, did not significantly inhibit the host enzyme. Salas-Peregrin et al. (1985) found that these derivatives are able to modify the activity of some metalloenzymes. SOD is a metalloenzyme whose catalytic function depends mainly on the metallic ion it carries. Fee (1977) reporteed diminished levels of enzyme activity, or complete loss of activity, after the removal or substitution of a metallic ion. This suggests that the inhibition of SOD produced by 5-FMU, MTU and DMU may be conditioned by a modification of its prosthetic group, which is linked to the metallic ion. Further studies of these compounds will be needed to explain the fact that despite the structural similarity of the pyrimidine compounds we tested, only three were able to inhibit SOD of *T. ovis*.

Our findings, toghether with the fact that these compounds are relatively inexpensive, readily synthesized and nontoxic to mammals (Salas-Peregrin et al., 1985), point to the huge potential of pyrimidine derivatives in antihelminthic treatment. This potential is further supported by the possibility that pyrimidine derivatives, like benzimidazoles, may inhibit other enzyme systems in addition to the SODs.

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