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ANTIOXIDANT ENZYMES IN *ACANTHOCHEILONEMA VITEAE* AND EFFECT OF ANTIFILARIAL AGENTS*

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Abstract—Adult worms of *Acanthocheilonema viteae* were found to be susceptible to the reactive oxygen intermediates (ROI) generated by the xanthine–xanthine oxidase (X–XO) system. The damage caused by this system was completely abolished by superoxide dismutase (SOD) and catalase but not by mannitol. The results, therefore, suggest that superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) alone or in combination might be toxic to the filariid. *A. viteae* exhibited the presence of an active enzyme system to protect itself against the oxidants. SOD and catalase were present in high levels of activities and appeared to constitute the major defence system. The role of glutathione peroxidase (GPx), on the other hand, seemed less important due to the weak activities of glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH). *A. viteae* also released SOD, catalase and GPx in the ambient medium, which appear useful in protecting the filariid against ROI generated by the host in the immediate surroundings of the parasite. Antifilarial agents, diethylcarbamazine (DEC) and 2,2′-dicarbomethoxylamino-5,5′-dibenzimidazolyl ketone (82/437) appreciably inhibited catalase and GPx of *A. viteae*. Inhibition of these enzymes appears to render the parasite prone to H_2O_2 toxicity leading to death. No adverse effect on antioxidant enzymes of liver, lungs and subcutaneous tissue of *Mastomys natalensis* recorded as a result of exposure to 82/437 suggests a non-toxic nature to the compound.

Several intermediates of oxygen metabolism, O_2^- and H_2O_2 in particular, are cytotoxic and kill a variety of infectious agents [1–4]. These intermediates are known to exert deleterious effects against helminth parasites also, including some filariids [5–9]. The ability of a parasite to survive in the host has, therefore, been related with its capacity to deal with the toxicity of such species of oxygen. This capacity, to a large extent, is judged by the level of antioxidant enzymes possessed by the parasite. For instance, *Nematospiroides dubius* contains 2–3 times as much superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) as does *Nippostrongylus brasiliensis* and hence, the former nematode can thrive in the host for several months whilst the latter is expelled within 10–12 days only [10]. Likewise, in case of *Trichinella spiralis*, greater resistance of muscle stage larvae and adults compared to new born larvae has been attributed to the higher levels of antioxidant enzymes in the former stages of the parasite [6].

Acanthocheilonema viteae (earlier known as *Dipetalonema viteae*) which is being used in this laboratory as a model for various studies including screening of antifilarial compounds, survives for a considerably long period in its laboratory host, *Mastomys natalensis*. Although antibody mediated adherence of effector cells is known to damage larval as well as adult stages of the parasite [11–13], no information on the filariid concerning sensitivity to reactive oxygen species or endowment with antioxidant enzymes is yet available. Investigations on these aspects of *A. viteae* adults were, therefore, carried out, and the results are presented in this communication. Attempts have also been made to assess the susceptibility of antioxidant enzymes of the filariid and the host tissues to diethylcarbamazine (DEC) and 82/437 (a candidate macrofilaricidal agent developed at the Institute, Fig. 1) [14, 15] for exploring the possibility of employing these enzymes as target for filarial chemotherapy.

MATERIALS AND METHODS

M. natalensis bearing 75–90-day-old infection of *A. viteae* were killed after deep anaesthesia with ether. Liver, lungs, subcutaneous tissue as well as adult worms were immediately collected and washed three times with normal saline.

Effect of artificially generated oxidants. For evaluating the effect of reactive oxygen intermediates (ROI) *in vitro*, the xanthine–xanthine oxidase (X–XO) system was employed as the source of oxidants and the damage was assessed by monitoring the leakage of preabsorbed [^{14}C]adenine [16]. In brief, 30–40 adult worms of *A. viteae* were incubated with 5.0 mL RPMI-1640 medium containing [8-^{14}C]adenine (1 μCi/mL) at 37°. After 2 hr, the worms were removed and washed three times with Hank's balanced salt solution (HBSS) to make them free of the adhering radioactivity. The worms were subsequently divided into seven groups and transferred to 10-mL beakers containing 2.0 mL HBSS and other constituents described in Fig. 2. SOD, catalase and mannitol were used as antioxidants. After 4 hr, the worms were removed from the medium, washed twice with HBSS and transferred to the scintillation vials. The parasites were solubilized in Triton X-100 and determined for radiocarbon content. The incubation medium (0.4 mL) was

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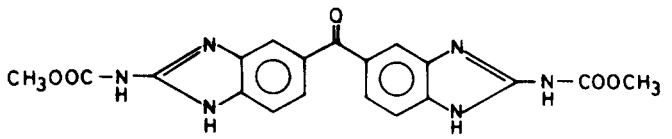

Fig. 1. Compound 82/437 i.e. 2,2'-dicarbomethoxylamino-5,5'-dibenzimidazolyl ketone.

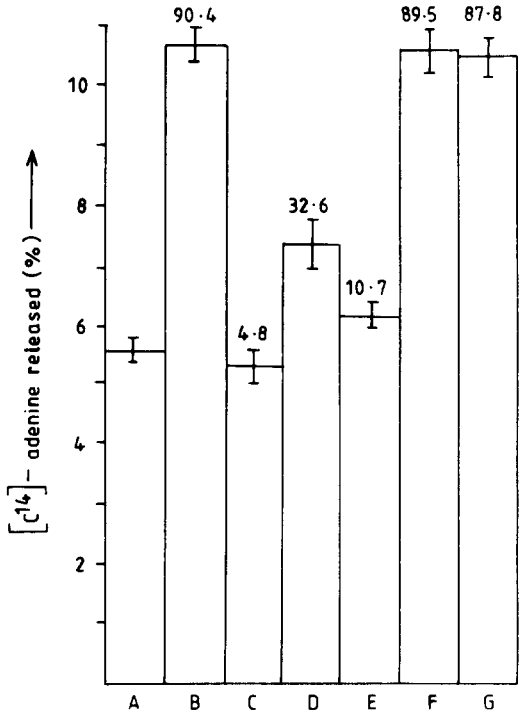

Fig. 2. Leakage of preincorporated [^{14}C]adenine by *A. viteae* adults during 2 hr incubation in HBSS plus other constituents. (A) Control (no other constituents); (B) xanthine (2 μM); xanthine oxidase (40 mUnits/mL); (C) X–XO + SOD (50 units/mL); (D) X–XO + catalase (2000 units/mL); (E) X–XO + catalase (6000 units/mL); (F) X–XO + mannitol (10 mM); (G) X–XO + mannitol (25 mM). Numbers above the bars denote per cent change with respect to control (A).

also counted simultaneously using a LKB 1209 Rackbeta liquid Scintillation counter. The per cent leakage was calculated as follows:

$$\text{Leakage} = \frac{\text{Total dpm released in the medium}}{\text{Total dpm in the medium} + \text{Total dpm in the worms}} \times 100.$$

Preparation of homogenate. A 10% (w/v) homogenate of the parasite and tissues was prepared in isotonic KCl using a Potter–Elvehjem tissue grinder fitted with a Teflon pestle. The homogenate was centrifuged at 9000 *g* for 30 min and the supernate was respun at 105,000 *g* for 60 min. The cytosolic fraction thus obtained was assayed for antioxidant enzymes. All the above operations were carried out at 4°.

Release of antioxidant enzymes. Adult (100–200 mg) *A. viteae* were incubated at 37° in 5.0 mL HBSS containing glucose (50 mM). After 3 hr, the worms were taken out and the medium was assayed for SOD, catalase and GPx.

Enzyme assays. Xanthine oxidase (XO) was assayed by the method of Horecker and Heppel [17]. The reaction mixture in 1.5 mL contained (in μmoles): phosphate buffer (pH 8.0), 150; cytochrome *c*, 0.032; xanthine, 1; catalase, 2 units; and a suitable aliquot of the extracts. Reduction of cytochrome *c* was followed at 550 nm.

The activity of SOD was determined by the method of Misra and Fridovich [18]. The reaction mixture in a final volume of 1.5 mL contained (in μmoles): carbonate buffer (pH 10.2), 500; epinephrine, 50; and a suitable aliquot of the extracts. One enzyme unit is defined as the amount of protein that inhibits the auto-oxidation of epinephrine by 50% under specified conditions.

Catalase activity was measured by determining the decomposition of H_2O_2 at 230 nm [19]. The assay mixture in 1.5 mL contained (in μmoles): phosphate buffer (pH 7.0), 150; H_2O_2, 0.5; and a suitable aliquot of the enzyme source.

GPx was assayed at 340 nm [20]. The assay mixture in 1.5 mL contained (in μmoles): phosphate buffer (pH 7.0), 250; NADPH, 0.12; GSH, 3.25; EDTA, 0.1; sodium azide, 0.1; glutathione reductase, 0.2 unit; H_2O_2, 0.5; and a suitable amount of the enzyme source.

Glutathione reductase (GR) was assayed by following the oxidation of NADPH at 340 nm [21]. The reaction mixture in 1.5 mL contained (in μmoles): phosphate buffer (pH 7.4), 50; NADPH, 0.12; oxidized glutathione, 3.3; bovine serum albumin (BSA), 1.5 mg; and a suitable aliquot of the extracts.

The assay of glucose-6-phosphate dehydrogenase (G6PDH) was carried at 340 nm following the reduction of NADP [22]. The reaction mixture in addition to enzyme in 1.5 mL contained (in μmoles): phosphate buffer (pH 7.4), 150; glucose-6-phosphate (G6P), 3; $MgCl_2$, 75; and NADP, 0.12.

Protein content was measured colorimetrically using BSA as a standard [23].

Reagents. G6P, H_2O_2, NADP, xanthine and reduced and oxidized forms of glutathione were purchased from SISCO Research Laboratories, Bombay, India. BSA, NADPH, cytochrome *c* (oxidized), catalase, GR, XO and SOD were obtained

Table 1. ROI metabolizing enzymes of *A. viteae* adults

Enzymes	Specific activity (units/mg protein)
Xanthine oxidase*	2.16 ± 0.13
Superoxide dismutase†	6.30 ± 0.51
Catalase*	1.32 ± 0.13
Glutathione peroxidase‡	31.0 ± 2.0
Glutathione reductase‡	7.1 ± 0.4
Glucose-6-phosphate dehydrogenase‡	5.3 ± 0.5

* μmol/min.
† One enzyme unit is that amount of protein which inhibits the autoxidation of epinephrine by 50%.
‡ nmol/min.

Table 2. Release of superoxide dismutase, catalase and glutathione peroxidase *in vitro* by *A. viteae*

Enzymes	Release (units/hr/g worm)
Superoxide dismutase	3.5
Catalase	1.7
Glutathione peroxidase	32.0

Units are described in Table 1.

from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Epinephrine and mannitol were procured from Romali, Bombay, India.

RESULTS

Effect of X–XO generated ROI on A. viteae *adults*

A. viteae adults were found to release preabsorbed [^{14}C]adenine into the medium (Fig. 2). The leakage of the radionucleotide was, however, markedly enhanced by exposure of the worms to the X–XO system. SOD completely abolished this increase and brought the leakage back to the rate shown by the control set. Catalase exhibited a concentration dependent effect and 6000 units of the enzyme provided nearly complete protection of the parasite. Mannitol, on the contrary, proved ineffective in this respect.

Endowment of antioxidant enzymes

A. viteae exhibited the presence of all the major antioxidant enzymes namely, SOD, catalase, GPx, GR and G6PDH (Table 1). The former two enzymes were detected in substantially higher levels of activities compared to the latter three enzymes. SOD showed maximum activity, while G6PDH registered minimum. XO, the enzyme which leads to the generation of O_2^-, was also found in significant amounts.

Release of enzymes

The three major defence enzymes namely, SOD, catalase and GPx were found to be released by the filariid into the ambient medium (Table 2). The rates of their secretion showed a parallel relation with their relative concentrations in the parasite, i.e. SOD > catalase > GPx.

Effect of antifilarials

DEC and 82/437 inhibited both catalase and GPx of *A. viteae* in a concentration dependent manner (Fig. 3). Nonetheless, a comparatively much higher concentration of the former drug was required to produce an effect equivalent to that caused by the latter filaricide. For instance, 82/437 at 10 μM concentration inhibited 38 and 30% activities of catalase and GPx (I_{50}: 20.3 and 30.7 μM), respectively, while DEC produced a similar effect at 100 μM concentration and yielded 175 and 261 μM respectively as I_{50} values. SOD, on the contrary, exhibited stimulation. However, the effect was statistically insignificant at all of the concentrations of the drugs employed.

SOD and catalase of subcutaneous tissue of *M. natalensis* showed no significant deviation in their activities as a result of exposure to DEC or 82/437 (Fig. 4). GPx was, nonetheless, markedly enhanced. The stimulation was concentration dependent and became statistically significant at higher concentrations of the drugs.

82/437 also activated SOD and GPx of liver and lungs of *M. natalensis* in a concentration dependent manner (Fig. 5). Catalase of the two organs, however, exhibited different effects; the liver enzyme showed stimulation while the lung enzyme displayed inhibition. In the case of liver, enhancement of all the three enzymes, which was insignificant at 5 μM concentration of 82/437, became highly significant at 25 μM concentration. On the contrary, amongst the lung's enzymes, only GPx showed a significant change.

DISCUSSION

Enhancement in the leakage of preabsorbed [^{14}C]adenine by *A. viteae* and *Brugia malayi* has been regarded as an index of damage to the cuticular surface [16]. Hence, the increased leakage of the nucleotide observed by exposing *A. viteae* to the X–XO system suggests that oxygen derived intermediates are injurious to the parasite (Fig. 2). X–XO is known to generate O_2^-, H_2O_2 and ·OH [7]. Complete protection by SOD and catalase

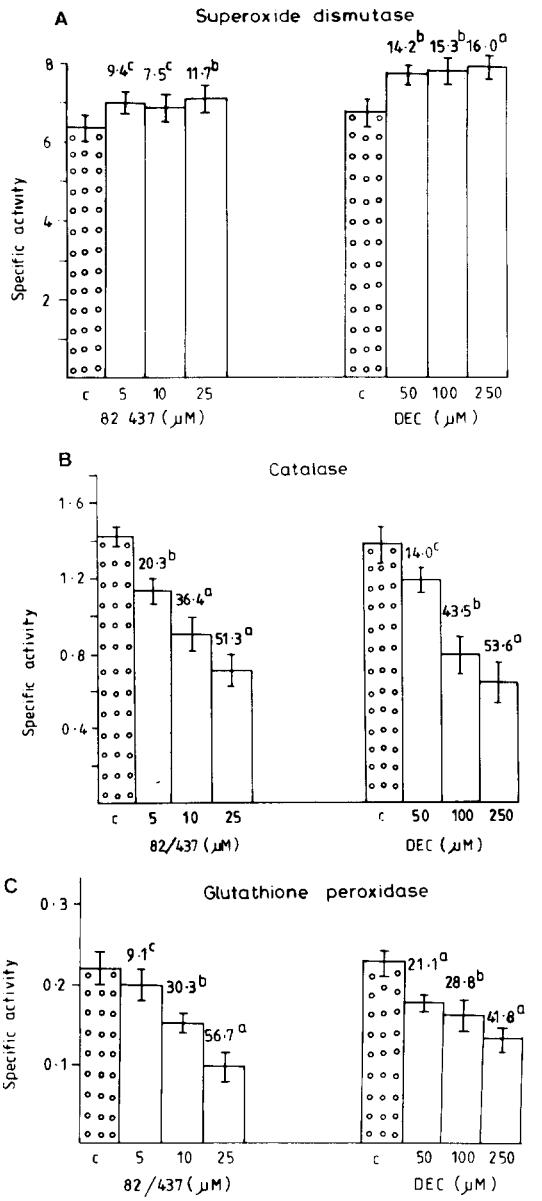

Fig. 3. Effect of 82/437 and DEC on (A) SOD, (B) catalase and (C) GPx of *A. viteae* adults. Numbers and letters above the bars denote per cent change and their statistical significance, respectively. [a] $P < 0.005$ (highly significant); [b] $P < 0.05$ (significant); [c] $P > 0.05$ (insignificant).

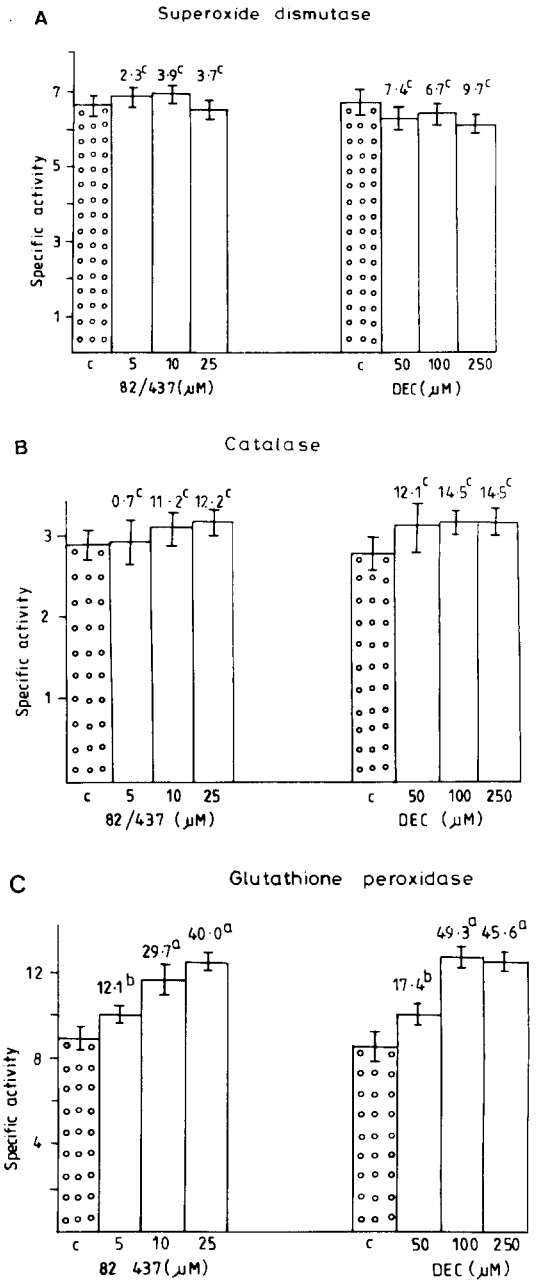

Fig. 4. Effect of 82/437 and DEC on (A) SOD, (B) catalase and (C) GPx of subcutaneous tissue of *M. natalensis*. Other details are similar to those described for Fig. 3.

accompanied with the ineffectiveness of mannitol indicates that O_2^- and H_2O_2 but not ·OH are deleterious to *A. viteae*.

It is pertinent to mention that the host derived macrophages and granulocytes, which employ respiratory burst as one of the effector mechanisms, are known to damage the filariid for nearly a decade [11–13]. Interestingly, liver and spleen of the host (*M. natalensis*) have recently been shown to possess altered activities of ROI metabolizing enzymes in response to *A. viteae* infection. These changes have been related with the host's attempt to reject the infection by producing oxygen intermediates in greater amounts [24].

In view of the above discussed toxicity of ROI, the ability of *A. viteae* to survive in the host may be attributed to the presence of antioxidant enzymes (Table 1). Highly active SOD may efficiently scavenge O_2^- and convert it into H_2O_2 which is also cytotoxic. To detoxify peroxide, the filariid possesses both catalase and GPx. The latter enzyme can not act independently, as it has to depend on GR for an optimum recycling of oxidized glutathione (GSSG)

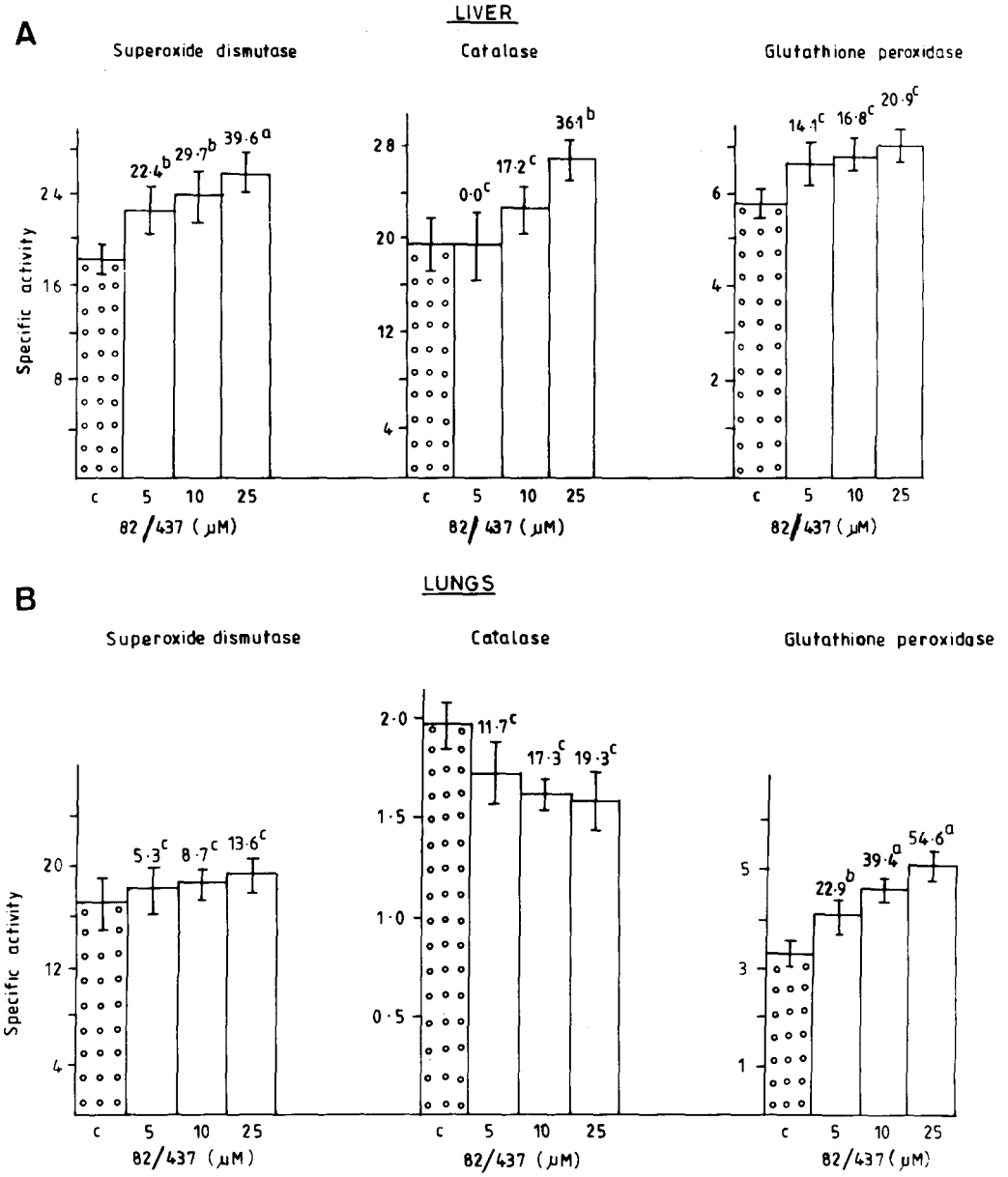

Fig. 5. Effect of 82/437 on SOD, catalase and GPx of (A) liver and (B) lungs of *M. natalensis*. For other details see Fig. 3.

into reduced glutathione (GSH). GSH *per se* requires NADPH, which is generated by the reduction of NADP by G6PDH. Interestingly, the levels of GR and G6PDH are substantially lower in *A. viteae* and this may limit the optimal functioning of GPx which itself is present with much lower activity than catalase. It would, therefore, appear that in *A. viteae* catalase acts as a major defence enzyme against H_2O_2 while GPx plays only a minor role. A perusal on the relative rate of release of antioxidant enzymes by the filariid also leads to a similar conclusion (Table 2). The released enzymes appear important for protecting the parasite in its immediate surroundings against oxygen species generated by the host. *Onchocerca cervicalis* and *Dirofilaria immitis*, the other two filarial parasites, unlike *A. viteae*, possess negligible amounts of catalase [7]. In these parasites, GPx seems to constitute the major defence against H_2O_2.

Marked inhibition of *A. viteae* catalase and GPx by DEC and 82/437 would significantly hamper the parasite's ability to detoxify H_2O_2 (Fig. 3). Since, H_2O_2 is deleterious to the filariid, the non effect of the above antifilarials appears to conclude in death of the parasite. 82/437 is a promising macrofilaricide and kills adult worms of *Litomosoides carinii*, *A. viteae* and *B. malayi* when administered orally or intraperitoneally into the host [15]. The compound also enhances the leakage of preincorporated [^{14}C]adenine by *A. viteae* and thereby damages the

parasite *in vitro* [25]. 82/437, therefore, appears to exert its macrofilaricidal effect by making the parasite prone to peroxide toxicity by inactivating scavenging enzymes, catalase and GPx at very low concentrations (I_{50}: 20.3 and 30.7 μM, respectively). 82/437 also appreciably depresses uptake of glucose and transport of methyl glucose by *A. viteae* but at a comparatively much higher concentration [$I_{50} > 110$–$160\ \mu$M] (Srivastava VML, unpublished data). It may also be argued that the latter described effects are the secondary reflections of the cuticular damage resulting from H_2O_2 toxicity following marked inhibition of catalase. Likewise, the inability of DEC (a microfilaricidal agent) to kill adult worms *in vitro* as well as *in vivo* may be attributed to its greater I_{50} value; 175 and 261 μM for catalase and GPx, respectively. Interestingly, neither DEC nor 82/437 exert any adverse effect on the antioxidant enzymes of the liver, lungs and subcutaneous tissue of *M. natalensis* (Figs 4 and 5). The data, therefore, suggest that these antifilarials do not disturb normal metabolism of oxygen species in the host tissues. Stimulated GPx would be helpful for quicker disposal of H_2O_2 to avoid the possibility of any damage by the oxidant. Enhanced or undisturbed levels of these enzymes in the liver and lungs of *M. natalensis* further strengthen the non-toxic effect of 82/437 towards the host tissues (Fig. 5). This interpretation is substantiated by the chronic toxicity study of compound 82/437 in rodents, where the total safety of the animals was observed up to a maximum dose of 500 mg/kg, which is five times greater than the therapeutic dose (Chatterjee RK, unpublished data).

It may, therefore, be concluded that *A. viteae* thrives *in situ* in the host by counteracting obnoxious O_2 metabolites of host origin with the help of its antioxidant enzymes. 82/437 inhibits catalase and hampers this evading mechanism and thereby kills the parasite. Since the compound does not inhibit the host counterpart, it may be non-toxic to the host. It is pertinent to mention here that according to Saz [26] *A. viteae (D. viteae)* and *Brugia pahangi* resemble human filarial parasites in their lack of a major requirement of O_2. This conclusion has been based on the observations made on the carbohydrate metabolism [27] and the effect of cyanine dyes [28, 29]. Furthermore, the World Health Organization has also recommended the use of the above filariids for screening compounds for antifilarial activity [30]. The results of the present study, therefore, warrant comprehensive investigations on catalase which appears to have a potential of being utilized as a target for filarial chemotherapy.

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