pressure at 60°C and treated with activated charcoal. To the cool and clear filtrate was added distilled ethanol up to 70% and kept in cold for about 2 h to allow the white precipitate to settle. It was removed by filtration. The aqueous alcohol phase was concentrated again under reduced pressure at 45°C. Crystalline white precipitate separated after allowing it to stand in cold for several hours. White precipitate was collected by filtration, dissolved in sufficient amount of 1N HCl, and subjected to Dowex-50 ion exchange column chromatography. Eluants used were 80% ethyl alcohol, and 0.4 N, 0.8 N, 2 N and 4 N of NH_4OH solution in the same order. Of these fractions, solid separated from ethyl alcohol fraction was recrystallised from acetone. The crystalline solid thus obtained was identical to an authentic sample of oxalic acid in its physical and chemical properties namely, solubility, chromatographic mobility, crystalline structure, melting point (102°C-monohydrate) and equivalent weight (63).

Fifty mg of this solid and the other fractions from Dowex-50 column were tested for aflatoxin-inhibiting activity by adding each into the medium[4] inoculated with toxigenic *A. flavus*, and incubated for seven days at 28°C. The culture broths were extracted with methanol and chloroform for aflatoxins. Aflatoxins were estimated by visual TLC method[5]. Results indicated that the ethyl alcohol fraction exhibited maximum antagonistic potential curtailing aflatoxin biosynthesis to the extent of 85%.

After identifying the inhibitor as oxalic acid, an authentic sample (BDH India, GR grade) of oxalic acid was tested for its inhibitory activity by supplementing various levels (50 to 1000 ppm) to the culture medium and incubating with *A. flavus* for production of aflatoxin under conditions previously mentioned. Extraction and estimation procedure for aflatoxin was the same as described earlier. Figure 1 indicates that there was no lowering of pH at the end of seven days as a result of addition of oxalic acid, which implies that decrease in production of aflatoxin by oxalic acid is not by lowering the pH. The figure also reveals that

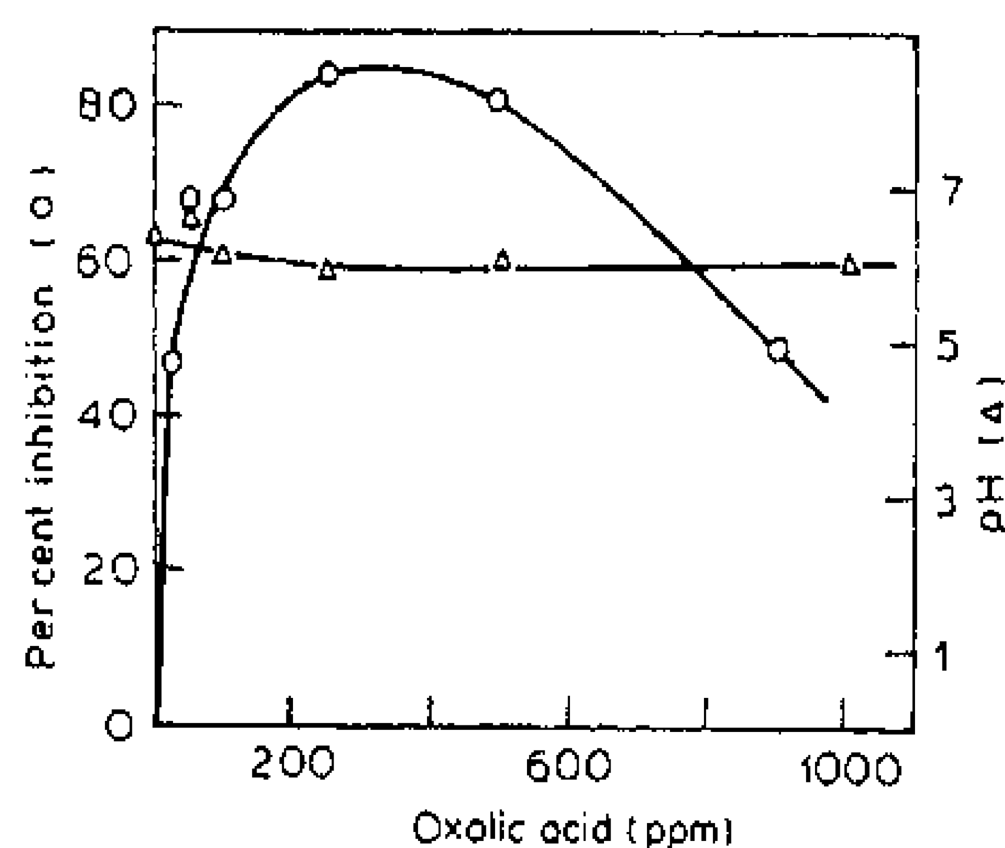

Figure 1. Inhibition of aflatoxin production by *A. flavus* in presence of oxalic acid in Czapek-Dox liquid medium in seven days at 28°C.

maximum inhibition occurred at 250 ppm level of oxalic acid. The mycelial mats collected (as described earlier) on preweighed folded filter paper circles were dried at 90°C to a constant weight. The weights recorded were very close (0.9 g) to each other indicating that the decrease in aflatoxin production is not due the inhibition of the growth of aflatoxin-producing organism.

The study unravels that oxalic acid is one of the major inhibitory factors produced by *A. niger* to antagonize biosynthesis of aflatoxin by *A. flavus* in liquid synthetic medium. The inhibition is not due to the lowering of pH. Although production of oxalic acid was reported in 1956 itself by Hayashi *et al.*[6], it has only now been recognized as a factor inhibiting aflatoxin synthesis.

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Difluoromethylornithine sensitivity of ornithine decarboxylase from *Acanthamoeba culbertsoni* and *Giardia lamblia*

P. Kishore, N. Gupta*, A. K. Agarwal* and O. P. Shukla

Divisions of Biochemistry and *Microbiology, Central Drug Research Institute, Lucknow 226 001, India

Acanthamoeba culbertsoni **showed remarkable resistance to α-difluoromethylornithine (DFMO), while multiplication of *Giardia lamblia* is inhibited by this compound. Ornithine decarboxylase (ODC) activity has been detected in both these parasites. DFMO inhibits ODC of *A. culbertsoni* and *G. lamblia* to a similar extent in the cell-free extracts as well as whole cells. The refractoriness of ODC to DFMO or the impermeability of cells to the inhibitor do not seem to be involved in DFMO resistance of *A. culbertsoni*.**

DL-α-Difluoromethylornithine (DFMO, eflornithine) is a

specific enzyme-activated irreversible (suicide) inhibitor of ornithine decarboxylase (ODC), a key enzyme of polyamine biosynthesis in most prokaryotes and eukaryotes[1]. Inhibition of this enzyme has important pharmacological and chemotherapeutic implications for cancer and protozoal diseases[2,3]. DFMO has been reported to inhibit growth of *Trypanosoma brucei*, *Eimeria tenella*, *Plasmodium* sp., *Pneumocystis* carinii[3,4] and *Giardia lamblia*[5] while certain other protozoa, viz. *T. cruzi*, *Entamoeba histolytica*, *Trichomonas vaginalis*, *Acanthamoeba* and *Leishmania*[5-9] have been reported to be resistant. North *et al.*[10] found that ODC of *T. vaginalis* was strongly inhibited by DFMO and this resistance may be associated with uptake of polyamines from the growth medium. The use of defined and semi-defined media has demonstrated the sensitivity of *T. vaginalis*, *P. falciparum*, *L. donovani* and *A. castellanii* to DFMO[11-14]. *Acanthamoeba culbertsoni* has displayed remarkable resistance to DFMO[7] but the mechanism of this resistance is not known; failure of the drug uptake and refractoriness of the enzyme to the inhibitor could be involved. The action of DFMO on the ODC of *A. culbertsoni* was therefore compared with that on the ODC of *G. lamblia*, a sensitive organism, and results are reported in this communication.

A. culbertsoni (strain A-1 of Culbertson: ATCC No. 30171) obtained from American type culture collection, Rockville, USA, was employed for the study. The amoebae were grown axenically in peptone medium containing per litre (l^{-1}): peptone 20 g; NaCl 5 g; thiamine 10 mg; and cyanocobalamin 5 μg as described earlier[15]. DFMO (gift from Merrell Dow Co. Cincinnati, Ohio) was dissolved in water, sterilized separately by filtration through 0.22 μm membrane filter (Whatman), and added aseptically to the medium in the desired concentration.

Axenic strain of *G. lamblia* was regularly maintained in TYI-S-33 medium of Diamond *et al.*[16] of composition (g/100 ml): trypticase (BBL) 2.0; yeast extract (Oxoid) 1.0; NaCl 0.2; K_2HPO_4 0.1; K_2HPO_4 0.06; cysteine hydrochloride 0.1; L-ascorbic acid 0.02; ferric ammonium citrate 0.0022; bovine serum (heat inactivated) 10 ml; vitamin mixture 2.5 ml, pH 6.8. The medium was sterilized by filtration through a Seitz filter assembly, pre-autoclaved at 1.06 kg/cm^2 for 15 min *G. lamblia* culture was grown in screw capped glass tubes (16 × 125 mm), containing 9 ml of TYI-S-33 medium and giardial trophozoites as inoculum. The tubes were incubated at 37°C for 3–4 days to yield cell density of 3–4 × 10^6 cells/ml.

The reaction mixture for the assay of ODC contained in the final volume of 250 μl: potassium phosphate buffer, pH 8.0, 50 mM; DTE 0.2 mM; EDTA 2 mM; pyridoxal phosphate 40 μM and ornithine-1-^{14}C 250 nCi. Whole cell samples and homogenates, of *A. culbertsoni* and *G. lamblia*, made by hand homogenization in an all glass homogenizer (50 up and down strokes were found to yield complete cell breakage with no loss of activity) were employed as the source of enzyme. The reaction mixture was incubated with shaking at 37°C for 1 h in tubes sealed with rubber stoppers. The reaction was stopped by injection of 0.5 ml of 50% TCA and CO_2 absorbed on filter papers soaked with 50 μl KOH (40%). The filter paper strips containing absorbed CO_2 were transferred to scintillation fluid (PPO 0.4%, POPOP 0.01% in 500 ml toluene and 500 ml methoxy ethanol) and radioactivity counted in LKB liquid scintillation counter.

The results presented in Table 1 demonstrate ornithine decarboxylase activity in *A. culbertsoni* as well as *G. lamblia*. The enzyme activity was much lower in *A. culbertsoni* compared to that in *Giardia*. In both the organisms similar level of enzyme activity was detectable in the whole cells and cell homogenates indicating that the permeability of the substrate was not a limiting factor. Table 1 also indicates that ODC from *G. lamblia* was inhibited very strongly by DFMO yielding about 90–95% inhibition at 1 mM concentration of the inhibitor. An essentially similar level of inhibition was obtained using unbroken *Giardia* cells

Table 1. Effect of α-DFMO on the activity of ornithine decarboxylase of *Acanthamoeba culbertsoni* and *Giardia lamblia*.

	DFMO (mM)	*Acanthamoeba culbertsoni* Enzyme activity (nmol CO_2 h mg protein)	% Activity	*Giardia lamblia* Enzyme activity (nmol CO_2 h mg protein)	% Activity
Cells					
Control	—	0.186±0.022	100	2.17±0.12	100
DFMO	0.1	0.180±0.16	96	ND	ND
DFMO	1.0	0.137±0.005	74	0.28±0.09	12.9
DFMO	10.0	0.089±0.010	48	0.031±0.01	1.43
Cell homogenate					
Control	—	0.182±0.055	100	2.47±0.14	100
DFMO	0.1	0.142±0.015	62	ND	ND
DFMO	1.0	0.058±0.016	17	0.14±0.01	5.67
DFMO	10.0	0.027±0.001	12	0.017±0.001	0.69

ND, Not done.

or cell homogenates as the source of enzyme; the cells would thus seem to be freely permeable to DFMO. ODC of *A. culbertsoni* was also strongly inhibited in the cell homogenate by DFMO yielding about 70 and 85% inhibition at 1 mM and 10 mM concentrations, respectively. The enzyme of *A. culbertsoni* is, therefore, fully sensitive to the action of this inhibitor. DFMO, however, yielded somewhat lower inhibition when whole cells were used as the source of enzyme yielding about 25% and 50% inhibition at 1 mM and 10 mM concentrations. These results suggest somewhat lower permeability of DFMO in the trophozoites of *A. culbertsoni*. However, it cannot fully explain the resistance of the organism to DFMO as essentially no growth inhibition was obtained when cells were cultivated in peptone medium containing 20 mM DFMO[7] which ensures continuous presence of the inhibitor with the cells. The actual mechanism of DFMO uptake by organisms and its relationship to DFMO resistance are not yet properly understood[17,18]. Our results presented in this communication would exclude impermeability of DFMO as well as insensitivity of amoebic ODC to this inhibitor as possible causes of the apparent DFMO resistance of *A. culbertsoni*. Diaminopropane has been detected as the principal polyamine of this amoeba (unpublished observations) that may substitute for putrescine and could be involved in the DFMO resistance of *A. culbertsoni*. Further studies on the effect of DFMO and related inhibitors are needed to understand their action on polyamine metabolism of amoebae and *Giardia* and their chemotherapeutic potential.

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