Abuse and was purified to 99 per cent by thin-layer chromatography (t.l.c.) using a hexane–acetone (4:1) system. The incubation conditions were based on those previously reported [3], and the products were extracted and isolated in the same manner as those from a similar study in the mouse [4]. Briefly, this involved exhaustive solvent extraction with ethyl acetate of the incubation media, separation into radioactive zones by t.l.c. and isolation and identification of the metabolites by gas–liquid chromatography–mass spectrometry (g.l.c.–m.s.). The results are summarized in Table 1.

More than 70 per cent of the recovered radioactivity consisted of unchanged Δ^1-THC. This is in marked contrast to the extensive conversions previously observed by us in the rat and mouse [4]. The known metabolites, 7-hydroxy-Δ^1-THC and 6α,7-hydroxy-Δ^1-THC, were found in t.l.c. zones 3 and 4 respectively; their chromatographic mobilities and GLC–MS properties were identical to those of authentic standards.*

Zone 2 was further separated by g.l.c. into two monooxygenated metabolites of Δ^1-THC. Comparison with standards showed that the more mobile peak was 1,2α-epoxyhexahydrocannabinol [5, 6] and the other was 6α-hydroxy-Δ^1-THC.† The epoxide has been previously reported in only one species, the squirrel monkey [5], while the other product has been found in the rat and mouse [4] and in human plasma [7]. Both of these metabolites showed activity in the rhesus monkey [6, 8] and, in addition, 6α-hydroxy-Δ^1-THC showed some activity in the mouse [7].

Our finding that the epoxide is not a unique metabolite of the squirrel monkey suggests that this metabolic route may also exist in man. It seems unlikely that a reactive species such as an epoxide would be a final product, raising the question of further transformation products being formed *in vivo*. Since epoxides are believed to react with macromolecules such as proteins [9], one can speculate on the occurrence of such a process here. This could give rise to immunologically reactive substances, as has been postulated by Nahas *et al.* [10], and may also account for the unextractable products which have been observed in various experiments *in vitro*.‡ In fact, Willinsky *et al.* [11] have also reported non-extractable binding of metabolites to tissue constituents possibly involving epoxides.

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* Obtained from the National Institute on Drug Abuse.

† A sample of the epoxide was prepared using the procedure in reference 6; authentic 6α-hydroxy-Δ^1-THC was obtained from the National Institute on Drug Abuse.

‡ Unpublished results from this laboratory.

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Effect of penicillamine on some metals and metalloproteins in the rat

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In an attempt to gain a better understanding of the mechanism of copper exchange between plasma proteins and cellular compartments of rat tissues, we have altered the mobilization and the excretion of this metal by administration of penicillamine, the chelating agent commonly used in Wilson's disease. In humans, D-penicillamine (D-PAM) is well tolerated even in cases of prolonged treatment. The substance is not cytotoxic although, some side effects, ascribed to allergic, idiosyncratic or immunological mechanisms, have been reported [1].

In this study we have determined the extent to which copper, zinc and iron are removed by treatment with D-PAM. In addition, we have checked if the activity of cytoplasmic superoxide dismutase (SOD) and serum ceruloplasmin are affected by depletion of copper and zinc.

Male Wistar rats weighing about 300 g were kept in metabolic cages made of plexiglas and fed dried pellets of standard composition. Each rat was injected intramuscularly with 15 mg/100 g body wt of D-PAM at intervals of 12 hr for 20 days. Control animals were kept under the same environmental conditions. Twenty animals were used in each group.

Copper, zinc and iron in urine and in homogenized tissues were estimated by atomic absorption (Perkin Elmer model 300) after oxidation with nitric and perchloric acids.

SOD activity in the supernatant obtained from homogenates after centrifugation at 105,000 *g* for 4 hr was determined according to Beauchamp and Fridovich [2]. The oxidase activity of ceruloplasmin was estimated using *p*-phenylendiamine according to Ravin [3], and D-PAM in the blood was estimated by the method of Pal [4]. Water was deionized on Amberlite resins and checked

Table 1. Copper, zinc and iron content in normal and treated rats (μg/g fresh tissue).

Tissue		Control	Treated	P
Copper	liver	4·76 ± 0·81	3·56 ± 0·19	<0·01
	spleen	2·68 ± 0·45	2·26 ± 0·25	<0·05
	kidneys	9·40 ± 1·33	4·67 ± 0·87	<0·001
	heart	6·65 ± 0·84	4·40 ± 0·55	<0·02
	lungs	2·18 ± 0·45	1·58 ± 0·20	<0·02
	brain	4·40 ± 0·30	3·45 ± 0·37	<0·02
	plasma	1·76 ± 0·17	0·93 ± 0·19	<0·001
	red cells	1·95 ± 0·22	0·55 ± 0·08	<0·001
Zinc	liver	35·75 ± 2·40	2·86 ± 2·60	<0·01
	spleen	33·72 ± 8·04	22·91 ± 5·75	<0·02
	kidneys	31·94 ± 4·51	21·28 ± 2·32	<0·001
	heart	29·68 ± 2·31	15·68 ± 1·80	<0·001
	lungs	22·23 ± 3·93	14·02 ± 0·96	<0·01
	brain	23·71 ± 3·67	12·45 ± 0·58	<0·01
	plasma	5·36 ± 0·23	2·22 ± 0·25	<0·001
	red cells	6·16 ± 0·13	5·48 ± 0·94	<0·05
Iron	liver	115·63 ± 20·62	74·28 ± 6·14	<0·01
	spleen	684·00 ± 109·77	378·00 ± 86·74	<0·02
	kidneys	89·00 ± 6·94	46·13 ± 8·48	<0·001
	heart	67·30 ± 7·31	40·40 ± 3·55	<0·01
	lungs	100·87 ± 17·50	54·50 ± 20·93	>0·05
	brain	26·88 ± 3·86	15·48 ± 2·30	<0·05
	red cells	429·00 ± 8·48	248·31 ± 32·29	<0·02

Table 2. Activity of superoxide dismutase (enzymatic units/g fresh tissue) in normal and D-PAM treated rats.

Tissue	Control	Treated	P
Liver	19,138 ± 2304	15,298 ± 2990	<0·05
Spleen	7597 ± 1731	5950 ± 1365	>0·05
Kidneys	12,636 ± 2439	8665 ± 1296	<0·02
Heart	5335 ± 831	4356 ± 459	<0·05
Lungs	3158 ± 278	2719 ± 204	<0·05
Brain	2737 ± 207	2209 ± 360	<0·05
Red cells	4105 ± 1113	2719 ± 406	<0·02

for the presence of metals by atomic absorption analysis; D-PAM chloride was purchased from Signa.

The urinary excretion of copper and zinc increased the second day after treatment with D-PAM and remained constant thereafter. Even after the administration of D-PAM for 20 days, the daily urinary excretion of copper was increased 400–500 per cent (from 10 to 47 μg). Zinc excretion increased from 15 to 106 μg a day, an increase of nearly 700 per cent. In contrast, iron excretion increased only 30 per cent.

Table 1 shows that the concentrations of copper, zinc and iron decreased in all organs examined. The differences are significant ($P < 0·02$ or $P < 0·01$) except in the spleen for copper ($P < 0·05$), in the red cells for zinc ($P < 0·05$) and in the lungs and the brain for iron ($P < 0·05$). The reduction is highly significant ($P < 0·001$) for all the elements in the kidneys and the plasma.

The copper content of the organs was followed also during a 120-day treatment with D-PAM; the animals were killed, two at a time, at fixed intervals. There was a sharp decrease in the copper content of the kidney, heart and lung after the first week. In the other tissues, a more gradual decrease was observed.

Table 2 shows that the drop in SOD activity which accompanies D-PAM treatment is significant ($P < 0·02$) in the red blood cells and the kidneys. The SOD activity in the other tissues dropped by 5–17 per cent; therefore, there is no apparent correlation between the activity of the enzyme and the copper and zinc levels of the organs.

D-PAM has no effect *in vitro* on SOD activity, measured using the purified enzyme from horse liver [5]. D-PAM has a marked effect on the serum ceruloplasmin level, as determined by its oxidase activity on *p*-phenylendiamine.

After 20 days of treatment with D-PAM, total serum ceruloplasmin concentration decreased from 0·49 mg/ml to 0·16 mg/ml (68·5 per cent). There was a concomitant decrease in the total serum copper (Table 1). The complete absence of D-PAM in the serum of killed animals indicates that the treatment actually affects the ceruloplasmin content of the blood.

The results presented here indicate that the D-PAM treatment significantly decreases the metal content of most rat tissues. The reduction in copper, zinc and iron concentrations *in vitro* has secondary effects, as shown by decreases in SOD activity and ceruloplasmin concentration.

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Action of imidazole and metronidazole on the differentiation of *Hartmannella culbertsoni**

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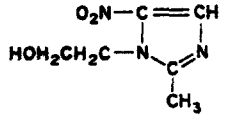
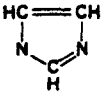
Metronidazole (I) (1-β-hydroxyethyl-2-methyl-5-nitroimidazole) is a potent amoebicidal agent [1–3] and is structurally related to imidazole (II) which is known to possess antilipolytic activity [4–6], to stimulate glycolysis in rat diaphragm [7] and to activate cyclic 3',5'-adenosine monophosphate phosphodiesterase [8–13].



(I)



(II)

Taurine and biogenic amines induce the differentiation of *Hartmannella culbertsoni* into viable cysts [14, 15]. Besides the extensive degradation of lipids and glycogen [16, 17] that occurs in the amoebae committed to encyst under the triggering action of taurine or epinephrine, a membrane localized adenylate cyclase is also activated [14, 15, 18, 19]. It was of interest, therefore, to examine the effect of imidazole and metronidazole on the differentiation of this free living amoeba.

MATERIALS AND METHODS

Hartmannella culbertsoni [20], from the collection of Dr. B. N. Singh of this Institute, was grown axenically in a medium containing 2% (w/v) peptone (British Drug Houses, India), 0·5% (w/v) NaCl and 10 mg thiamine HCl/l. and 5 μg canocobalamin/l. (D. C. Kaushal and O. P. Shukla, unpublished observations from this laboratory). One-litre Erlenmeyer flasks containing 250 ml of the above medium (pH 6·8) were autoclaved at 15 lb/in.^{2} for 20 min and inoculated with 50 ml of 4-day-old culture (10^8 cells). The flasks were shaken in a rotary shaker (Emenvee Engineering Co., Poona, India) at 300 rev/min and maintained at 34 ± 2°. During 4 days growth 2 × 10^6 cells/ml was obtained. The cells were harvested by centrifugation at 800 *g* for 10 min and washed twice by dispersal in sterile 150 mM NaCl followed by centrifugation at 800 *g* for 10 min and finally suspended in the required media. All the operations were carried out aseptically.

The cells suspended in 150 mM NaCl were homogenized with a Potter Elvehjem type of tissue grinder using a Teflon pestle by eight up and down strokes and a rotor speed of 2000 rev/min. The homogenizer and contents were chilled during homogenization. Microscopic examination showed that all the cells were ruptured by this procedure.

Imidazole or metronidazole were dissolved in 150 mM NaCl to the required concentration and the pH was adjusted to 7·0 and the solutions seitz filtered. Freshly harvested amoebae suspended in 150 mM NaCl were exposed to a sterile solution of the drug under test for the required period. The cells were then recovered by centrifugation, washed by dispersal in sterile 150 mM NaCl followed by centrifugation and finally inoculated into axenic medium. Growth was followed by counting with the aid of a haemocytometer.

Freshly harvested cells (3 × 10^6) were suspended in 10 ml medium containing 80 mM NaCl, 20 mM taurine, 15 mM $MgCl_2$ and imidazole or metronidazole in desired concentrations. The cells were shaken at 37 ± 1° for 6 hr in a metabolic shaker (80 horizontal strokes/min, 1·5 cm amplitude). The cells were recovered by centrifugation and washed twice in 150 mM NaCl. The cells were then spread over plain non-nutrient agar plates and incubated at 27 ± 1°. Samples were examined microscopically for morphological changes. Counts of trophozoites and cysts were made in triplicate samples in a haemocytometer.

Cyclic AMP synthesis was followed by prelabelling ATP *in situ* with adenine-8-^{14}C and measuring the amount of cyclic AMP-8-^{14}C formed in the presence of theophylline [14]. The identity of cyclic AMP was established by chromatography and chemical and enzymic degradation using an authentic sample of cyclic AMP.

Cyclic AMP phosphodiesterase (EC 3.1.4.1) was assayed using the method of Butcher and Sutherland [8].

Table 1. Effect of imidazole and metronidazole on growth of *H. culbertsoni*

Concentration of drug (mM)	Imidazole Time of exposure (hr) 6	72	Metronidazole Time of exposure (hr) 6	72
0	20·0	20·5	21·0	17·0
1	—	—	21·5	11·5
2	—	—	23·0	9·7
5	—	—	22·0	0
10	—	—	20·0	0
20	19·9	17·0	20·5	0
40	20·2	0	18·0	0
100	19·7	0	17·5	0

2 × 10^5 freshly harvested cells were incubated with 0–100 mM imidazole or metronidazole in 150 mM NaCl for 6 and 72 hr at 34 ± 2°, on a rotary shaker (300 rev/min). At intervals cells were harvested, washed free from drug and inoculated into growth medium in small test tubes. Haemocytometric counts were made each day. The results show cells × 10^5/ml on the fourth day of growth.

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