D-2 receptors whilst Class II binding sites which are poorly recognised by domperidone ($IC_{50} = 10\ \mu M$) should be termed D-3 receptors. Labelling of D-1 receptors is not thought to be involved in [^{3}H]-apomorphine binding studies at nanomolar concentrations, because the recognition sites of the dopamine-sensitive adenylate cyclase are stimulated by micromolar concentrations of dopaminergic agonists [6]. Nevertheless, the proposal that the two classes of binding site labelled by [^{3}H]-apomorphine represent distinct dopamine receptors must be regarded with caution. Since (−)NCA can selectively inhibit binding of [^{3}H]-apomorphine to Class I binding sites, the use of (−)NCA as a selective irreversible antagonist at D-2 receptors may provide an important biochemical and pharmacological tool to help clarify the present confusion regarding multiple subtypes of dopamine receptor.

In summary, the novel aporphine (−)NCA, appears selectively to inhibit binding of [^{3}H]-apomorphine to domperidone-sensitive binding sites (Class I) which probably represent D-2 receptors, in rat striatal tissue.

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Effects of oxygen tension and reducing agents on sensitivity of *Giardia lamblia* to metronidazole *in vitro*

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Metronidazole [Flagyl; 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] is one of the most important drugs used in the treatment of giardiasis [1]. Nonetheless, there are few *in vitro* studies of the metronidazole sensitivity of the causative agent, the flagellated protozoan *Giardia lamblia* [2–4], and the mechanism of killing of *Giardia* by this drug is not known. Metronidazole is highly specific for anaerobic organisms—both prokaryotic and eukaryotic. Trichomonads (e.g. *Trichomonas foetus*, a parasite of cattle, and the human pathogen *Trichomonas vaginalis*), as well as susceptible bacteria, reduce metronidazole to a toxic form via certain low redox potential reactions which are either absent or unimportant in aerobic organisms [5, 6]. Reduction of metronidazole decreases the intracellular concentration of the unchanged drug, creating a concentration gradient which drives its uptake [7]. The cellular target of the reduced metronidazole has not been positively identified.

The uptake of radioactive metronidazole by *T. foetus*, *T. vaginalis* and *Entamoeba invadens* (a pathogen of carnivorous reptiles) was strongly inhibited under aerobic conditions [6]. Oxygen competes with metronidazole for the electrons necessary for reduction. Killing of *T. foetus* was related to the amount of drug taken up; higher drug concentrations were required for killing under aerobic conditions [6].

It is likely that a similar reduction-driven uptake and activation mechanism is responsible for the sensitivity of *G. lamblia* to metronidazole. If so, killing of the parasite should be decreased under aerobic conditions. This has not been tested previously. Test conditions must be selected to avoid killing of *G. lamblia* by prolonged exposure to atmospheric concentrations of O_2 [8]. We have shown recently that this parasite tolerates exposure to O_2 for up to 8 hr (without growth) [8]. Thus, it is possible to examine the sensitivity of *G. lamblia* to metronidazole during shorter exposure to aerobic conditions. In this report, metronidazole sensitivity was measured with a routinely isolated strain [9] as well as a strain of *G. lamblia* recently isolated from a patient who had repeatedly been treated unsuccessfully with metronidazole [10].

Methods

Giardia lamblia Portland-1 strain [9] (PO; American Type Culture Collection No. 30888) was obtained from Drs. G. Visvesvara [11] and G. Healy (Center for Disease Control, Atlanta, GA). The WB strain (ATCC No. 30957) was isolated in this laboratory from duodenal fluid of a 27-year-old male with symptomatic giardiasis of 2.5-years duration despite four courses of metronidazole and three courses of quinacrine therapy [10].

Trophozoites were grown axenically (with subculture twice weekly) to log phase in filter-sterilized TYI-S-33 medium [12] modified by the addition of bovine bile (bacteriological, 500 μg/ml; Sigma Chemical Co., St. Louis, MO), and doubling the L-cysteine concentration (D. B. Keister, manuscript in preparation). Parasites were enumerated with a Coulter Counter (Coulter Electronics, Hialeah, FL).

Metronidazole sensitivity. Metronidazole was a gift of G. D. Searle & Co. (San Juan, Puerto Rico). Two methods of determining the metronidazole sensitivity of *G. lamblia*

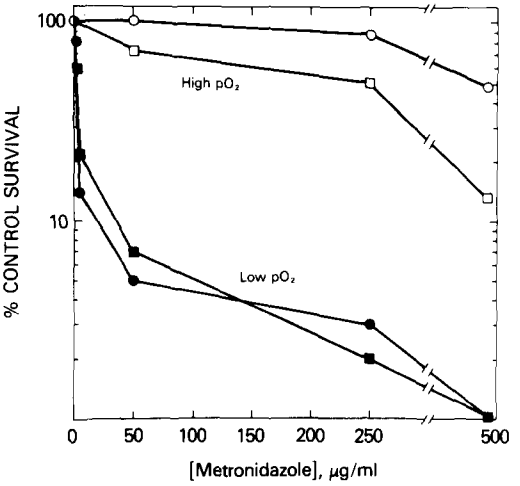

Fig. 1. Effects of oxygen tension and reducing agents upon killing of *G. lamblia* (Portland-1 strain) by metronidazole. Trophozoites were exposed to drug for 5 hr in liquid medium under high pO_2 or low pO_2 conditions with or without fresh cysteine and ascorbic acid (3 mM) as described in Methods. Then survival was determined by colony assay without drug or fresh reducing agents. Survival of controls without metronidazole was not affected by the presence or absence of cysteine and ascorbic acid. Key: (○, ●) without fresh reducing agents; and (□, ■) with fresh reducing agents. Open symbols: high pO_2; and filled symbols: low pO_2. Survival in controls lacking metronidazole was 30,350 colonies/ml.

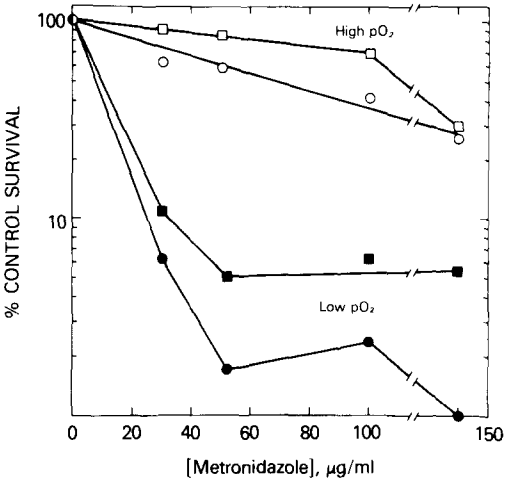

Fig. 2. Sensitivity of Portland-1 and WB strains of *G. lamblia* to metronidazole under high pO_2 and low pO_2 conditions (as described for Fig. 1). Key: (□, ■) Portland-1; and (○, ●) WB. Open symbols: high pO_2; and filled symbols: low pO_2. Survival in controls was 40,800 colonies/ml for strain PO and 48,200 for WB.

trophozoites were utilized. In early experiments, differences in freshness of the media gave rise to variability [4] as the cysteine in the medium was oxidized during the first few days after preparation [13]. Therefore, the medium was aged at 4° for 7–14 days prior to experiments, and little variability was observed.

High versus low pO_2. Trophozoites were suspended (50,000/ml) in liquid medium containing metronidazole (2–500 μg/ml) and, where indicated, freshly prepared reducing agents (3 mM) in addition to that in the medium. The final volume was 4 ml in a 4.7-ml capacity screw-capped glass vial. After mixing, 0.5 ml was transferred to a second vial. The oxygen tension (pO_2) under the latter condition was ~140 mm Hg or approximately atmospheric pO_2 (high pO_2). In contrast, the pO_2 at the bottom of the remaining 3.5 ml of medium where most of the trophozoites attached was similar to that in our standard growth conditions (~30 mm Hg, low pO_2) [8]. The vials were incubated upright at 35.5 ± 0.5° for 5 hr. The cultures were then chilled and mixed, and duplicate 10-μl samples were withdrawn for colony assay of surviving organisms in drug-free semi-solid medium [4]. This sample volume was small enough that the amount of drug transferred did not inhibit colony formation.

Direct colony assay (low pO_2). Inhibition of clonal growth of *G. lamblia* was assayed in various concentrations of metronidazole and, where indicated, freshly prepared L-cysteine or reduced or oxidized glutathione (Sigma, 3 mM, final concentration) in semi-solid agarose medium as described [4].

Results

Giardia lamblia was strikingly less sensitive to metronidazole under high pO_2 compared with low pO_2 conditions in liquid medium (Fig. 1). The LC_{50} (calculated from semi-logarithmic plots) was 500 μg/ml under aerobic conditions, almost 200-fold greater than the LC_{50} under low pO_2 conditions (2.8 μg/ml). Parasite killing was due to the drug and not to O_2 as survival in controls lacking drug was similar under high and low pO_2 conditions.

Table 1. Effects of glutathione and cysteine upon sensitivity to metronidazole*

Metronidazole (μg/ml)	Survival (% control)			
	No addition	+ Glutathione		+ Cysteine
		Oxidized	Reduced	
1.00	78	86	100	100
1.25	28	17	78	93
1.5	6	4	53	71
1.8	2	0	38	50
2.5	0	0	6	9

* Measured by the direct colony assay (low pO_2). Metronidazole at the concentrations indicated and cysteine or reduced or oxidized glutathione (3 mM) were mixed with the melted agarose medium. After addition of trophozoites (0.1 ml of a suspension containing 3500 cells/ml), the medium was mixed and solidified. Survival in controls lacking metronidazole was 1252 (±84) colonies/ml and was not affected by the presence of cysteine or glutathione.

Metronidazole sensitivity of the WB strain of *G. lamblia* was compared with that of the Portland-1 strain under both high and low pO_2 conditions (Fig. 2). This was necessary because strains of *T. vaginalis* which were resistant to metronidazole *in vivo* were only resistant *in vitro* under aerobic conditions [14]. The WB strain appeared to be slightly more sensitive to metronidazole than the PO strain. Both strains showed greatly decreased susceptibility to metronidazole under aerobic conditions (Fig. 2).

We have shown previously (using a different medium, TP-S-1) that, when the sensitivity of *G. lamblia* to metronidazole was assayed by the colony method, fresh cysteine and ascorbic acid in the semi-solid medium partially protected the parasites [4]. However, it might be expected that reducing agents would potentiate the effects of metronidazole. Therefore, the effects of fresh cysteine and ascorbic acid upon metronidazole sensitivity were compared in liquid medium under low and high pO_2 (Fig. 1) and in semi-solid medium (Table 1).

The magnitude of the effects of cysteine and ascorbic acid was much less than that of oxygen and depended upon the assay. In the high pO_2 assay, cysteine and ascorbic acid slightly, but consistently, potentiated the effect of metronidazole, decreasing the LC_{50} from 500 μg/ml to 250 μg/ml (Fig. 1). Under low pO_2 conditions (in liquid medium), reducing agents had no significant effects (LC_{50} 2.8 vs 2.5 μg/ml, Fig. 1).

In contrast, the parasites were partially protected from metronidazole by fresh cysteine in the agarose direct colony assay (Table 1), in agreement with earlier results [4]. Similar protection was observed with reduced, but not with oxidized, glutathione (Table 1).

Discussion

We have investigated the effects of two variables on the metronidazole sensitivity of *G. lamblia*. First, increasing the oxygen tension strikingly increased (> 100-fold) the resistance of the parasites to metronidazole (Fig. 1). Second, the addition of the reducing agents, cysteine and ascorbic acid or glutathione, had lesser effects depending upon the assay (Table 1 and Fig. 1).

The first observation is concordant with the decreased uptake of drug by *E. invadens*, *T. vaginalis* and *T. foetus* and the decreased killing of the latter under aerobic conditions [6]. Our results suggest that *G. lamblia*, like these parasites, reduces metronidazole to convert it to a toxic form and cannot do so under aerobic conditions. It was necessary to use a short assay time (5 hr) as *G. lamblia* was killed by prolonged (> 8 hr) exposure to atmospheric pO_2, even in the presence of reducing agents [8].

Under high pO_2, fresh cysteine and ascorbic acid slightly, but consistently, decreased the resistance of *G. lamblia* to metronidazole (Fig. 1). Possibly these reducing agents enabled the parasites to reduce some of the drug. In contrast, in the direct colony assay, fresh cysteine or reduced glutathione were protective (Table 1). Cysteine is oxidized rapidly in liquid medium even under low pO_2 conditions [13]. The oxidation would be retarded in the agarose medium. This medium provides a low pO_2 environment, with slow diffusion of O_2 in which the parasites may possess their maximal capacity to reduce metronidazole without the fresh reducing agents. Moreover, the protective effect of fresh cysteine (and reduced glutathione) in the colony assay may be non-specific since partial protection of *G. lamblia* from quinacrine, emetine and chloramphenicol, as well as metronidazole, by L-cysteine was also observed [4].

Several cases of persistent giardiasis despite one or more courses of appropriate drug therapy have been described [10, 14, 15]. It is important to know whether the parasites are resistant to metronidazole. The strain (WB) we isolated from one such patient was not resistant when tested by the direct colony assay. In the case of *T. vaginalis* however, it was necessary to assay under aerobic conditions in order to demonstrate the resistance of a strain [16]. In this communication, we demonstrated that the WB strain, like the Portland-1 strain of *G. lamblia*, was sensitive to metronidazole under low pO_2 conditions and resistant under aerobic conditions (Fig. 1).

Resistance to drug therapy may be due to qualities of both host and parasite. Oxygen tension and reducing agents may affect the sensitivity of *G. lamblia* to metronidazole *in vivo*. Oxygen in the upper small intestine (where *G. lamblia* trophozoites live) is derived primarily from swallowed air [17]. Also, bile has been shown (in the rat) to contain 2–4 mM reduced glutathione [18]. Both oxygen tension and reducing agent concentration could vary among individuals. Both would be affected by the intestinal flora. Thus, *G. lamblia* may be protected from killing by metronidazole in a host with increased small intestine pO_2 or reducing agents.

In summary, the effects of oxygen tension and reducing agents upon the sensitivity of *Giardia lamblia* to metronidazole *in vitro* were determined using two different assays. First, the parasites were exposed to drug under high or low oxygen tension in liquid medium with or without fresh cysteine and ascorbic acid. Survival was determined by colony assay. Second, the effect of thiol reducing agents was measured directly by colony assay in semi-solid media (low pO_2 conditions). Trophozoites were strikingly (> 100-fold) less sensitive to metronidazole under the aerobic condition of the first assay. In contrast, the reducing agents had much lesser effects (2- to 5-fold) depending upon the assay. Finally, *G. lamblia* isolated from a patient treated with metronidazole unsuccessfully four times showed similar metronidazole sensitivity to a standard strain under both aerobic and reduced O_2 tension conditions.

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Deuterium isotope effect on the enzymatic oxidation of dopamine and serotonin

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Monoamine oxidase (EC 1.4.3.4, MAO) inactivates various amines. The reaction involves the cleavage of an α-hydrogen from the side-chain carbon atom. It has been observed that substitution of this α-hydrogen with deuterium in *p*-tyramine results in a profound reduction in the rate of oxidation [1, 2]. This deuterium isotope effect was found to be related to hydroxyl substitution on the phenyl ring with respect to *p*-tyramine, *m*-tyramine and β-phenylethylamine [1]. We have now investigated further the deuterium isotope effect in the oxidative deamination of the putative neurotransmitters, dopamine and serotonin (5-HT).

Rat liver mitochondrial MAO was used in this study. The mitochondrial fractions were obtained by differential centrifugation, and the mitochondrial membrane fragments were prepared by lysing the mitochondria in chilled distilled water and then centrifuging at 105,000 *g* for 30 min as previously described [1]. The membrane preparations were then washed by suspension in chilled distilled water and centrifuged. MAO activity was measured using a high performance liquid chromatographic (HPLC) method based on the estimation of the disappearance of substrates [3]. The enzyme was incubated in 1×10^{-4}M non-deuterated or $\alpha\alpha$-d_2, $\beta\beta$-d_2, and $\alpha\alpha\beta\beta$-d_4 deuterated dopamine or serotonin in 0.05 M phosphate buffer (pH 7.5). The reactions were stopped by addition of 1% perchloroacetic acid containing isoproterenol or *N*-methyldopamine as internal standard for the HPLC system. The system consisted of a Waters (Melfort, MA) model 6000 solvent delivery pump, a Waters model UK injector, an Altex (Arlington Heights, IL) Ultrasphere C_{18} ion pairing column, a Bioanalytical System LC-2A electrochemical detector (West Lafayette, IN) with carbon paste electrode, and a Fisher Recordall series recorder. A base solvent of 0.1 N HNO_3 titrated to pH 2.7 with concentrated NaOH and containing 20 mg/l EDTA was modified by adding 2% methanol for dopamine analysis and 10% methanol for serotonin analysis. A rate of 1 ml/min was maintained for dopamine and 0.9 ml for serotonin. The internal standards were *N*-methyldopamine for dopamine measurements and isoproterenol for serotonin analysis. Calibration curves were prepared daily prior to the analysis. An electrode potential of 0.72 V with respect to Ag/AgCl reference electrode was used. A 1–5 μl injection and a sensitivity range of 10–20 nano-amps/V gave a good signal for the measurement of peak heights.

Dopamine-$\alpha\alpha$-d_2 was prepared by reduction of 3,4-dimethoxyphenylacetonitrile with lithium aluminum deuteride and, then, demethylation in glacial acetic acid and hydrogen bromide. Dopamine-$\beta\beta$-d_2 was prepared by refluxing 3,4-dimethoxyphenylacetonitrile in ethanol-OD, -D_2O, -NaOD and KCN, followed by reduction with lithium aluminium hydride and demethylation. The deuterated dopamine analogues were then isolated and purified by recrystallization of the hydrochloride from ethanol-ether. 5-Hydroxytryptamine-$\alpha\alpha$-d_2 was prepared from 5-benzyloxyindole-3-acetonitrile. The nitrile ester was reduced with lithium aluminium deuteride and hydrogenated over palladium on charcoal to remove the benzyl

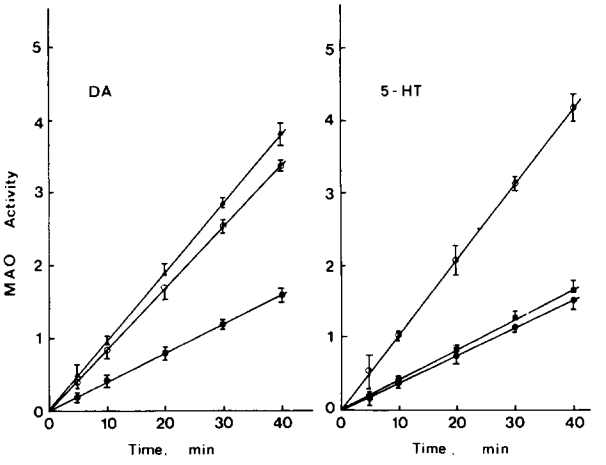

Fig. 1. Initial velocities of the deamination of different deuterated analogues of dopamine and serotonin. Enzyme activity [nmoles · (mg protein)$^{-1}$ · min^{-1}] was measured using the HPLC method with $\alpha\alpha$-d_2 (●——●), $\beta\beta$-d_2 (▲——▲), $\alpha\alpha\beta\beta$-d_4 (■——■), and nondeuterated (○——○) amines as substrate. The substrate concentrations used were 1×10^{-4} M. Each value is the means ± S.E.M. of triplicate determinations.