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Reductive Metabolism of Niridazole by Adult *Schistosoma mansoni*

Correlation with Covalent Drug Binding to Parasite Macromolecules

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SUMMARY

Niridazole, an antischistosomal nitrothiazole derivative, is metabolized by adult *Schistosoma mansoni* to one or more reactive intermediates, as evidenced by extensive covalent binding of [^{14}C]niridazole to parasite macromolecules. When worm pairs were incubated for 16 hr in culture medium containing 70 μM [^{14}C]niridazole, 26–34% of the total parasite-associated radioactivity was irreversibly bound to trichloroacetic acid-precipitable material. Drug binding was both time- and [^{14}C]niridazole concentration-dependent. Of the bound drug fraction, 85–90% was associated with parasite proteins, 3–5% with RNA and 4–7% with DNA. When schistosomes were recovered from infected mice, treated with periodic doses of [^{14}C]niridazole, over 40% of the total parasite-associated radioactivity was bound to macromolecules. Niridazole caused up to a 40% decrease in the concentration of total nonprotein thiols in intact schistosomes incubated with the drug over an 8-hr period. Under strictly anaerobic conditions, cell-free schistosome preparations catalyzed a reduced pyridine nucleotide-dependent reduction of niridazole's essential nitro group, as evidenced by disappearance of absorption at 400 nm. Net nitroreduction did not occur under aerobic conditions, although the drug did stimulate oxidation of the pyridine nucleotide cofactor. Covalent binding of [^{14}C]niridazole also took place in this cell-free system, with requirements identical with those needed for enzymatic nitroreduction. Covalent drug binding, but not nitroreduction, was inhibited up to 80–85% by 2 mM L-cysteine, *N*-acetyl-L-cysteine, or glutathione; *S*-carboxymethyl-L-cysteine, which has no free sulfhydryl group, was not inhibitory. [^{14}C]4′-Methylniridazole, a nonschistosomicidal analogue of niridazole, was taken up by intact schistosomes *in vitro*, but was not metabolized and did not bind covalently to parasite macromolecules. Furthermore, 4′-methylniridazole did not affect the concentration of nonprotein thiols in intact parasites and did not serve as a substrate for schistosomal nitroreductase *in vitro*. These results indicate a positive correlation between proximal metabolic activation of niridazole within these facultative anaerobic organisms and its antiparasitic activity.

INTRODUCTION

Niridazole, an antischistosomal nitrothiazole derivative (Fig. 1), is effective against three major schistosome species that infect humans (1), protozoa [e.g., *Entamoeba histolytica* (2)], and a number of anaerobic and facultative anaerobic bacteria (3, 4). Early studies of niridazole disposition in schistosomes revealed that the drug is taken up and converted extensively to unknown metabolites (5), but the pathways of drug metabolism and the relationship of drug metabolism to toxic efficacy in this parasite have not been elucidated.

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Earlier we reported that TCI[1] is a product of niridazole metabolism in adult *Schistosoma mansoni*.[2] This immunoactive metabolite results from cleavage of the nitrothiazole ring (Fig. 1) (6). The findings that TCI can be formed from niridazole either by chemical reduction with zinc dust[3] or by microflora in mammalian intestine (7) suggested that niridazole is reductively metabolized by schistosomes. In agreement with this hypothesis, no 360-

[1] The abbreviations used are: TCI, 1-thiocarbamoyl-2-imidazolidinone; Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; HBSS, Hanks' balanced salt solution; HPLC, high-pressure liquid chromatography; NPSH, nonprotein thiols; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

[2] B. A. Catto, J. W. Tracy, and L. T. Webster, Jr., in preparation.

[3] S. B. Wilson, E. H. Fairchild, and J. W. Tracy, unpublished observations.

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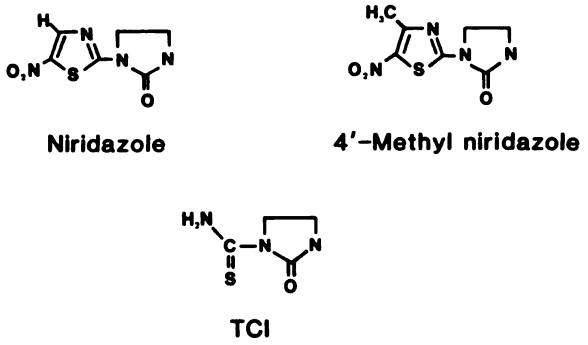

FIG. 1. *Structures of niridazole, 4′-methylniridazole, and TCI*

nm-absorbing metabolites characteristic of oxidative hepatic niridazole metabolism (8) were detected in adult *S. mansoni* incubated with the drug.[2]

We now report that the proximal step of niridazole metabolism by *S. mansoni* involves enzymatic reduction of the drug's essential nitro group. Such metabolism results in formation of one or more chemically reactive drug species, as evidenced by extensive covalent binding of [^{14}C]niridazole to parasite macromolecules. In contrast, macromolecular drug binding was not found when the niridazole analogue, [^{14}C]4′-methylniridazole (Fig. 1), was tested under identical conditions. This derivative can be chemically reduced to TCI,[3] but it has no demonstrable antischistosomal activity (9). Like niridazole, the analogue was taken up by schistosomes, but (unlike niridazole) it was recovered unchanged from these organisms. Niridazole also decreased NPSH levels in schistosomes, whereas the inactive analogue did not. These results indicate a positive correlation between proximal metabolic activation of niridazole and its antiparasitic activity.

EXPERIMENTAL PROCEDURES

Materials

Chemicals. Niridazole was generously donated by Ciba-Geigy Corporation (Summit, N.J.). [^{14}C]Niridazole, 1-(5-nitro-2-[2-^{14}C]thiazolyl)-2-imidazolidinone (5.2 mCi/mmole; radiochemical purity ≥98%) was custom-synthesized (10) by Amersham Corporation (Arlington Heights, Ill.). [^{14}C]Thiourea (58 mCi/mmole), NCS tissue solubilizer, and OCS and ACS-II scintillation cocktails were also purchased from Amersham. RPMI medium 1640 with 25 mM Hepes buffer (pH 7.3), HBSS, L-glutamine, and a penicillin-streptomycin mixture were purchased from M. A. Bioproducts (Walkersville, Md.). Protease (Type XIV), DNase-I (Type I), RNase A (Type III-A), catalase (C-40), glucose oxidase (Type X), glucose 6-phosphate dehydrogenase (Type XXIII), D-glucose, D-glucose 6-phosphate (monosodium salt), cysteine, *N*-acetyl-L-cysteine, *S*-carboxymethyl-L-cysteine, GSH, and DTNB were purchased from Sigma Chemical Company (St. Louis, Mo.). NADH and NADPH were obtained from P-L Biochemicals, Inc. (Milwaukee, Wisc.). Thiourea (99+%) and 1-bromo-2,2-dimethoxypropane (99%) were products of Aldrich Chemical Company (Milwaukee, Wisc.). Bovine serum albumin (monomer standard) was obtained from Miles Laboratories, Inc. (Elkhart, Ind.). 2-Chloroethyl isocyanate (Fluka Chemical Corporation, Hauppauge, N.Y.) was redistilled under vacuum before use. Solvents (HPLC grade) were purchased from Fisher Scientific Company (Pittsburgh, Pa.). Water was purified by passage through a Milli-Q system (Millipore Corporation, Bedford, Mass.).

Source of adult Schistosoma mansoni. Female mice (CF_1 strain, 18–20 g, Charles River Breeding Laboratories, N. Wilmington, Mass.) were each injected subcutaneously with 200 *S. mansoni* cercariae (Puerto Rican strain). After 45–60 days, adult schistosomes were recovered by portal perfusion (11). The parasites were washed five times with 50 ml of HBSS before use.

Methods

Chromatography. The HPLC system consisted of a Model 6000A pump, Model 660 solvent programmer, a Model U6K injector (Waters Associates, Milford, Mass.), a Model 153 UV detector equipped with either a 254-nm or 365-nm filter (Beckman Instruments, Inc., Berkeley, Calif.), and a Supergrator 3 computing integrator (Columbia Scientific Industries Corporation, Austin, Tex.). Reverse-phase HPLC was done on a 5-μm Beckman Ultrasphere-ODS column (0.46 × 25 cm). Conditions for the analysis of niridazole, its oxidative metabolites, and TCI have been reported (6, 8). Conditions for analysis of 4′-methylniridazole were as follows: 20% acetonitrile in water; flow rate, 1.5 ml/min (elution volume, 24.3 ml). 4′-Methylniridazole was also analyzed on a 10-μm silica column (μPorasil, 0.4 × 30 cm, Waters Associates) with 30% (v/v) acetone in toluene as the mobile phase (elution volume, 18.5 ml). Radioactivity in chromatographic peaks was determined by counting 0.4-min fractions (in 5 ml of ACS-II) collected from the detector outlet.

Measurement of radioactivity. Aqueous samples (100 μl) were mixed with 0.8 ml of NCS. After 4 hr, 15 ml of OCS were added, and the samples were counted in a Model LS-7500 liquid scintillation counter (Beckman Instruments, Inc., Irvine, Calif.) equipped with an automatic quench correction program. The counting efficiency (unquenched standard) was 96.6 ± 0.2%; background radioactivity was 15 ± 3 cpm.

Protein determination. Protein concentrations were measured by the method of Lowry *et al.* as modified by Peterson (12). Crystalline bovine serum albumin was used as the standard.

Synthesis of 4′-methylniridazole. 2-Amino-4-methyl-5-nitrothiazole, prepared from thiourea and 1-bromo-2,2-dimethoxypropane (13) was allowed to react with 2-chloroethyl isocyanate (10) to give 4′-methylniridazole. This product was recrystallized twice from *N,N*-dimethylformamide/absolute ethanol (98:2) to give small yellow crystals (m.p. 248–250°, decomp.). HPLC analysis at either 254 nm or 365 nm showed a single component. The UV spectrum of 4′-methylniridazole showed absorption maxima at 242 nm and 375 nm (λ_{max}; E_M = 10,100) and a minimum at 290 nm. The electron impact mass spectrum of 4′-methylniridazole gave a molecular ion at *m/z* 228.0358 (calculated for $C_7H_8N_4O_3S$, *m/z* 228.0371) and a fragmentation pattern consistent with the assigned structure. Chemical ionization mass spectral analysis (with isobutane) gave a quasimolecular ion (*M* + 1) at *m/z* 229. [^{14}C]4′-Methylniridazole, 1-(4-methyl-5-nitro-2-[2-^{14}C]thiazolyl)-2-imidazolidinone (5.8 mCi/mmole; radiochemical purity ≥98%), was synthesized, starting from [^{14}C]thiourea (13).

Incubation of schistosomes. Groups of 10 worm pairs were incubated at 37° (5% CO_2/95% air) in six-well plastic dishes (Costar, Cambridge, Mass.) containing 5 ml of RPMI medium 1640 supplemented with 56 mM D-glucose, 2 mM L-glutamine, penicillin (50 units/ml), streptomycin (50 μg/ml), and either [^{14}C]niridazole (5.2 mCi/mmole) or [^{14}C]4′-methylniridazole (5.8 mCi/mmole). For large-scale preparations, 100 worm pairs were incubated in 75 cm^2 plastic flasks (Corning Glass Works, Corning, N.Y.) containing 50 ml of medium. After 0–24 hr, parasites were collected on 25-mm nylon filters (50-μm mesh, Filtron Medical, Inc., Santa Barbara, Calif.) and washed with drug-free HBSS. To obtain separated male and female schistosomes, worm pairs were chilled on ice for 30 min and gently mixed for 15 sec.

Disposition of [^{14}C]niridazole in schistosomes incubated in vitro. Worm pairs, incubated for 16 hr in the presence of 70 μM [^{14}C]niridazole, were homogenized at 0° with a Potter-Elvehjem tissue grinder in 0.05 M potassium phosphate buffer (pH 7.5) (Fig. 2). Total parasite-associated radioactivity was quantitated by counting triplicate samples of homogenate. Homogenates were extracted five times with 2 volumes of water-saturated ethyl acetate, and the organic solvent was evaporated under vacuum. The residue was analyzed by HPLC for niridazole and its metabolites. The aqueous phase was treated at 0° with an equal volume of 20% trichloroacetic acid. After 30 min, the precipitate was collected by centrifugation (10 min at 5000 × *g*) and was washed with three successive 30-ml portions each of ice-cold 10% trichloroacetic

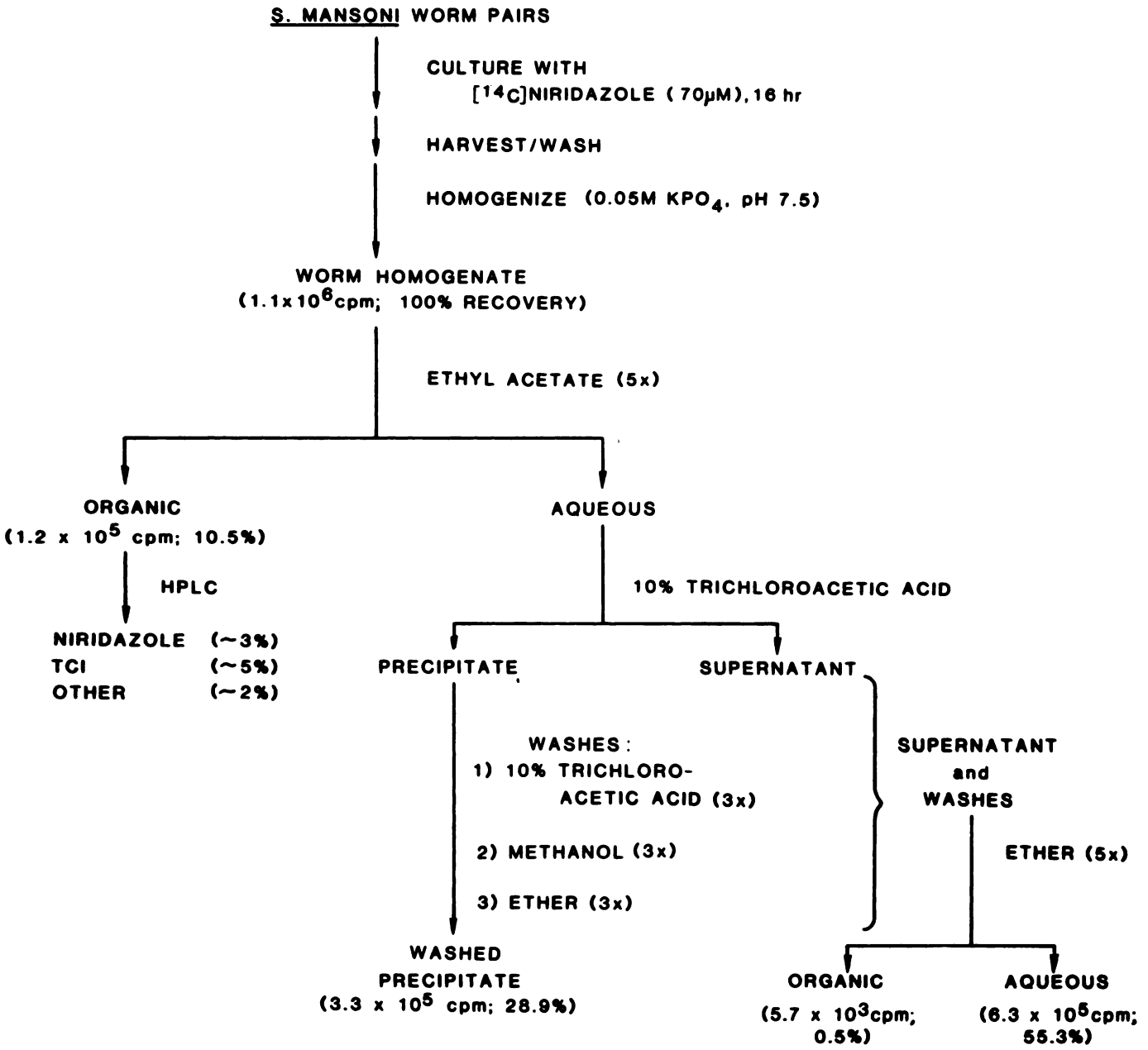

FIG. 2. *Experimental protocol for determining the disposition of [14C]niridazole in Schistosoma mansoni incubated in vitro*
The numbers in parentheses refer to the measured radioactivity (CPM) and the percentage of total parasite-associated radioactivity (%) in individual fractions from one representative experiment. Details are given under Experimental Procedures. The over-all recovery was 95.3%.

acid, methanol, and diethyl ether. Triplicate samples of the washed precipitate, resuspended in 0.05 M potassium phosphate buffer (pH 7.5) were used to quantitate protein and bound drug. Wash fluids were combined with the original acid supernatants and evaporated to dryness. Residues were dissolved in water (50 ml) and extracted with diethyl ether. Radioactivity remaining in the aqueous phase was taken as a measure of water-soluble drug metabolites.

Quantitation of total parasite-associated drug and drug binding to parasite macromolecules. Triplicate samples of worm homogenates were counted to estimate total parasite-associated drug radioactivity. Results are expressed as nanomoles of drug per milligram of protein. Macromolecular drug binding was routinely determined by precipitating triplicate samples (50–250 μl; ≤1.0 mg of protein) with ice-cold trichloroacetic acid (10%, final concentration). After 30 min at 0°, the precipitates were collected on 25-mm Whatman GF/A filters. Before counting, each filter was washed three times with 5 ml of ice-cold 10% trichloroacetic acid, four times with 5 ml of methanol, and four times with 5 ml of diethyl ether. Results are expressed as specific drug binding (nanomoles per milligram of protein). For some experiments, male and female schistosomes were homogenized separately at 0° in 20 mM Tris-HCl buffer (pH 7.5) containing 0.3 M sucrose. Subcellular fractions, i.e., nuclei, mitochondria, microsomes, and cytoplasm, were isolated by differential centrifugation (14). Triplicate samples of each fraction were analyzed for total radiolabeled drug, specific drug binding, and protein concentration.

Drug binding to parasite DNA, RNA, and protein. Worm pairs, incubated *in vitro* for 8 hr with 90 μM [14C]niridazole, were homogenized at 0° in 0.05 M potassium phosphate buffer (pH 7.5) containing 0.3 M NaCl. Macromolecules were precipitated at 0° by adding six volumes of ice-cold 95% ethanol. The precipitate was collected by centrifugation at 0° for 15 min at 5000 × *g*; washed exhaustively with 95% ethanol, methanol, and diethyl ether; and resuspended in 3 ml of sterile 0.3 M NaCl. Specific drug binding and protein concentration were each determined in triplicate. Other triplicate samples were each mixed with

an equal volume of the following sterile solutions: (a) 0.2 M sodium acetate buffer (pH 5.0) containing 10 mM $MgSO_4$ and 60 units of DNase-I; (b) 0.2 M sodium acetate buffer (pH 5.0) containing 15 units of RNase A, previously heated to 100° for 10 min to inactivate any contaminating DNase; and (c) 0.2 M potassium phosphate buffer (pH 7.5) containing protease (100 μg/ml). Enzymes were omitted from control tubes. After 2 hr at at 37°, the samples were treated with trichloroacetic acid and processed as usual. The decrease in amount of trichloroacetic acid-precipitable radioactivity after specific enzyme treatment was taken as a measure of drug binding to that class of macromolecules. A more direct measure of macromolecular drug binding was obtained by separating parasite DNA, RNA, and protein on cesium chloride gradients (15). Fractions corresponding to schistosome RNA, DNA, and protein were precipitated with ethanol as before, and the washed precipitates were resuspended in 0.3 M NaCl for counting. Samples of the pooled fractions were also subjected to specific enzyme digestion as outlined above; in each case, at least 96% of the bound radioactivity could be solubilized.

Dialysis of schistosome protein fractions containing bound drug against guanidine hydrochloride. A washed ethanol precipitate, prepared as described above from 100 worm pairs incubated for 10 hr with 90 μM [^{14}C]niridazole, was resuspended in 5 ml of 0.1 M sodium acetate buffer (pH 5.0) containing 10 mM $MgSO_4$, 60 units of DNase-I, and 15 units of RNase A. After 2 hr at 37°, the undigested material was precipitated with ethanol. The precipitate was dissolved in 10 ml of 6 M guanidine hydrochloride containing 1 mM dithiothreitol, and was dialyzed at 25° against 250 ml of the denaturant. The dialyzing medium was changed four times during the next 24 hr. Specific drug binding and protein concentration were compared before and after dialysis.

Drug binding to schistosomes from infected mice treated with [^{14}C] niridazole. Groups of five mice, infected 56 days earlier, were given six i.p. doses of [^{14}C]niridazole suspended in water (10 mg/kg/dose; 2.6 mCi/mmole) at 8-hr intervals. Twelve hours after the last dose, the schistosomes were recovered by portal venous perfusion (11) and were homogenized in 0.05 M potassium phosphate buffer (pH 7.5). Quadruplicate samples of the homogenate were used to determine total parasite-associated drug radioactivity, specific drug binding, and protein concentration.

Schistosome NPSH. Groups of 10 worm pairs were homogenized with a microscale tissue grinder (K-885470, Kontes, Vineland, N.J.) in 200 μl of ice-cold 5% sulfosalicylic acid. The pestle was washed with 50 μl of acid solution. After centrifugation at 4° (10 min at 12,800 × *g*), 150 μl of the supernatant were withdrawn and added to 50 μl of 2.0 M Tris base (final pH 8.0 ± 0.1 at 25°). Next, 500 μl of 1 mM DTNB in 0.02 M Tris-HCl (pH 7.3) were added, and the absorbance at 412 nm (E_M = 13,600) was recorded after 10 min (16).

Cell-free schistosome preparations. Worm pairs or separated male and female parasites were homogenized at 0° with a Potter-Elvehjem tissue grinder in 0.1 M potassium phosphate buffer (pH 7.5). The homogenate was centrifuged at 0° for 15 min at 2000 × *g*. The supernatant was decanted through a two layers of cheesecloth to yield a crude cell-free preparation, which was kept at 0° and used within 24 hr.

Nitroreductase assay. The spectrophotometric assay for schistosomal nitroreductase activity (17) was carried out in Thunberg-type quartz cuvettes (Type 26, Precision Cells, Inc., Hicksville, N.Y.) under anaerobic conditions in the presence of an oxygen-scavenging enzymatic system (18). Routinely, the cuvette contained 0.15 mmole of potassium phosphate buffer (pH 7.5), 300 nmoles of niridazole or 4′-methylniridazole, 0.5 mg of schistosome protein, 6 units of glucose 6-phosphate dehydrogenase, 0.06 unit of glucose oxidase, and 1100 units of catalase in a volume of 1.3 ml. The side arm contained 1.5 μmoles of NADH or NADPH and 15 μmoles of D-glucose 6-phosphate in a volume of 0.15 ml. The cuvette was purged for 2 min with N_2 (99.9995%; Matheson, Dayton, Ohio) that had been bubbled through Feiser's solution (18), evacuated to about 20 mm Hg, and purged again. Fifty microliters of 0.3 M β-D-glucose solution were added to the cuvette, and the purging cycle was repeated. After equilibration at 37° for 5 min, the absorbance at 400 nm was recorded for 3 min (Model 2400 spectrophotometer; Gilford Instruments, Inc., Oberlin, Ohio). The reaction was initiated by mixing the contents of the cuvette and side arm, and the decrease in absorbance was recorded. A control mixture lacking NAD(P)H and D-glucose 6-phosphate was used as a reference. One unit of nitroreductase activity was defined as the amount of enzyme which catalyzed the disappearance of one nanomole of substrate per hour, where E_M (niridazole) = 10,400 (17) and E_M (4′-methylniridazole) = 5,700. Specific activity was expressed as units per milligram of protein. For experiments under aerobic conditions, niridazole-dependent oxidation of NADPH by cell-free preparations was monitored at 340 nm (17).

Drug binding in cell-free schistosome preparations. Experimental conditions for binding of [^{14}C]niridazole and [^{14}C]4′-methylniridazole to macromolecules in cell-free preparations were identical with those used for the nitroreductase assay, except that reactions were carried out in Thunberg tubes (A. H. Thomas, Inc., Philadelphia, Pa.). After 0–60 min, the tubes were opened and triplicate samples were withdrawn for determination of total drug radioactivity and specific drug binding. In some experiments, ethyl acetate extracts of the reaction mixtures were analyzed for drug and drug metabolites by HPLC.

RESULTS

Drug binding to macromolecules after in vitro incubation of schistosomes with [^{14}C]niridazole. Schistosoma mansoni worm pairs, which had been incubated overnight in the presence of 70 μM [^{14}C]niridazole, were treated according to the scheme shown in Fig. 2. Only 11% of the total parasite-associated radioactivity was extracted from the homogenate with ethyl acetate. As noted previously,[2] the two major radiolabeled components of such organic extracts were found by HPLC to be unchanged niridazole and its metabolite, TCI; no 360 nm-absorbing, oxidative metabolites (8) were detected. When the aqueous phase was treated with trichloroacetic acid, over 40% of the total radioactivity was recovered in the resulting precipitate. Even after extensive washing with 10% trichloroacetic acid, methanol, and ether, nearly 30% of the total radioactivity remained associated with the insoluble fraction. In five separate experiments, the fraction of total radioactivity recovered in the washed precipitate varied from 26% to 34%.[4] The balance of the radioactivity (50–60%) was present in water-soluble drug products which were not identified.

Both the net accumulation of radiolabeled drug and its binding to parasite macromolecules were monitored over a 24-hr period in worm pairs maintained *in vitro* (Fig. 3A). The biphasic time course for net accumulation of total radioactivity was reminiscent of that reported by Faigle and Keberle (5). However, drug binding to macromolecules displayed a more complex time course. An initial lag phase of 1–2 hr was followed by a 10- to 12-hr period during which binding increased linearly. The rate of binding then declined until, by 24 hr, little additional drug binding was observed. After 16 hr, many parasites became unpaired and nonmotile, whereas parasites maintained in drug-free medium remained paired and motile. Both net accumulation of total radioactivity and drug binding to macromolecules after 6 hr were [^{14}C]niridazole concentration-dependent over a range of 5–180 μM (Fig. 3B). To focus on early events of schistosomal niridazole metabolism, incubation times were subsequently limited to 8–10 hr. Under these conditions, virtually all parasites remained paired and motile.

[4] By comparison, less than 0.02% of the total radioactivity was precipitated with trichloroacetic acid when an equivalent amount of [^{14}C]niridazole was added to homogenates of control parasites.

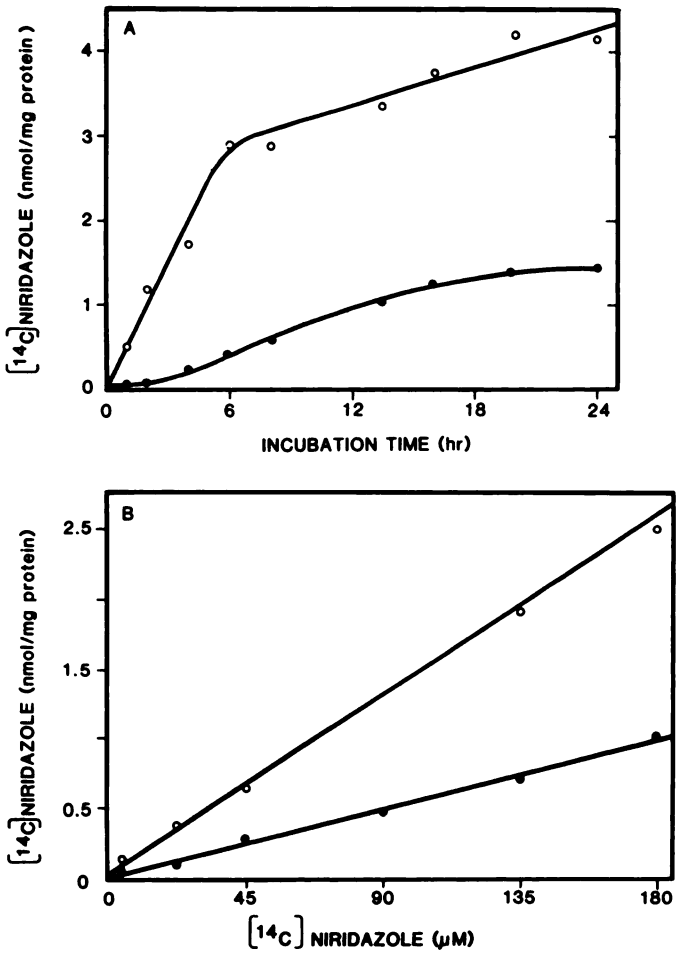

FIG. 3. *Net drug accumulation and specific drug binding to macromolecules in adult Schistosoma mansoni incubated with [^{14}C]niridazole*

Both the net accumulation of drug (○) and specific drug binding (●) were quantitated in homogenates of groups of 10 adult *S. mansoni* worm pairs as described under Experimental Procedures. Each point represents the average value of two experiments. A. Time course: [^{14}C] niridazole concentration, 70 μM. B. [^{14}C]Niridazole concentration dependence measured after 6 hr of incubation.

Net accumulation of radioactivity and drug binding to macromolecules were also compared in male and female schistosomes, separated after incubation with [^{14}C]niridazole. The data in Table 1 demonstrate that females bound twice as much [^{14}C]niridazole per milligram of protein as did males. Because drug binding might occur preferentially in a particular subcellular fraction, binding was quantitated in fractions of separated male and female parasites (14). Such experiments revealed that bound radioactivity was randomly distributed among nuclear, mitochondrial, microsomal, and cytoplasmic fraction. Specific drug binding ranged from 0.56 to 0.69 nmole/mg of protein in female worm fractions and from 0.29 to 0.38 nmole/mg protein in males.

Parasite binding of [^{14}C]niridazole showed marked specificity for the class of macromolecules involved. The data in Table 2 illustrate that digestion with nonspecific protease solubilized about 88% of the bound radioactivity, whereas treatment with DNase or RNase solubilized only

TABLE 1

Drug binding to macromolecules of female and male adult Schistosoma mansoni incubated in vitro with [^{14}C]niridazole

S. mansoni worm pairs were maintained for 8 hr at 37° in supplemented RPMI medium 1640 containing 90 μM [^{14}C]niridazole. Female and male parasites were separated, washed with drug-free HBSS, and homogenized in 0.1 M potassium phosphate buffer (pH 7.5). Drug binding to trichloroacetic acid-precipitable material was quantitated as described under Experimental Procedures. Values reported for each experiment are the means ± standard deviation of three determinations.

Experiment no.	Specific drug binding		Ratio[a]
	Females	Males	
	nmoles/mg protein		
1	0.62 ± 0.02	0.34 ± 0.01	1.8
2	0.67 ± 0.03	0.35 ± 0.01	1.9
3	0.80 ± 0.03	0.38 ± 0.02	2.1
4	0.65 ± 0.02	0.34 ± 0.01	1.9

[a] Ratio of drug binding in females to drug binding in males.

about 5% and 3%, respectively. Sequential treatment of the precipitate with DNase, RNase, and protease converted virtually all of the bound radioactivity into trichloroacetic acid-soluble forms. In four experiments, 86%–92% of the total trichloroacetic acid-precipitable radioactivity present was bound to protein; no more than 10%–12% was bound to nucleic acids. When parasite DNA, RNA, and proteins were separated on cesium chloride gradients and the specific drug binding to each fraction was quantitated, 85%–90% of the radioactivity was associated with protein, 3%–5% with RNA and 4%–7% with DNA.

Attempts to remove bound drug from parasite macromolecules by extraction with a number of organic solvents or by competition with unlabeled niridazole were unsuccessful (data not shown). To exclude further the possibility of noncovalent interactions between [^{14}C]niridazole products and parasite proteins, a washed protein precipitate was exhaustively dialyzed against 6 M guanidine hydrochloride containing 1 mM dithiothreitol. A comparison of specific drug binding before and after dialysis revealed that bound radioactivity was not removed by this treatment (Table 3). The accumulated

TABLE 2

Binding of [^{14}C]niridazole to schistosome DNA, RNA, and protein fractions as estimated by specific enzyme digestion

Samples of a washed ethanol precipitate, prepared from a homogenate of worm pairs incubated for 10 hr at 37° with 90 μM [^{14}C]niridazole, were incubated for 2 hr at 37° with the indicated enzymes. Undigested macromolecules were precipitated with trichloroacetic acid to quantitate residual bound radioactivity. Further details are given under Experimental Procedures. Each value is the mean ± standard deviation of three determinations performed on a typical ethanol precipitate.

Treatment	Specific drug binding	% Solubilized
	nmoles/mg protein	
None	1.04 ± 0.03	0
DNase-I	0.99 ± 0.02	4.8
RNase A	1.01 ± 0.03	2.9
Protease	0.13 ± 0.01	87.5
DNase, RNase, protease[a]	0.03 ± 0.02	97.1

[a] Sequential 2-hr digestions.

TABLE 3

Dissociation of bound [^{14}C]niridazole from a schistosome protein fraction by dialysis against guanidine hydrochloride

A washed ethanol precipitate, prepared from an homogenate of *Schistosoma mansoni* worm pairs incubated for 8 hr at 37° with 90 μM [^{14}C]niridazole, was treated with a mixture of DNase-I and RNase. Undigested material was precipitated with ethanol, and the precipitate was dissolved in a solution of 6 M guanidine hydrochloride containing 1 mM dithiothreitol. The protein solution was dialyzed for 24 hr at 25° against the denaturant with four changes of dialyzing medium. Specific drug binding was determined before and after dialysis. Further details are given under Experimental Procedures. Values reported for each experiment are the means ± standard deviation of three determinations.

Experiment no.	Niridazole bound		% Recovery
	Before dialysis	After dialysis	
	nmoles/mg protein		
1	0.72 ± 0.02	0.71 ± 0.03	98.6
2	0.92 ± 0.03	0.90 ± 0.02	97.8
3	0.80 ± 0.02	0.79 ± 0.04	98.8

evidence is consistent with the idea that drug is bound covalently to parasite proteins. These results also indicate that the simple trichloroacetic acid-precipitation/washing procedure used provided an accurate estimate of covalent drug binding.

Covalent drug binding in parasites recovered from [^{14}C]niridazole-treated mice. To determine whether covalent binding to parasite macromolecules occurs *in vivo*, groups of *S. mansoni*-infected mice were treated with periodic subcurative doses of [^{14}C]niridazole. Twelve hours after the last dose, the parasites were isolated, and the extent of covalent drug binding was determined (Table 4). In two experiments, the fractions of total parasite-associated radioactivity bound to macromolecules were 43% and 47%.

Reductive metabolism and covalent binding of niridazole in cell-free schistosome preparations. The finding that TCI is a metabolite of niridazole in schistosomes indicated that niridazole might be reductively metabolized by the parasite.[2] Therefore, the ability of crude cell-free schistosome preparations to catalyze disappearance of the nitrothiazole chromophore was tested. When a complete reaction mixture with NADPH as the cofactor was incubated under anaerobic conditions, the absorbance at 400 nm decreased linearly with time (Fig. 4). In contrast, when either the cofactor or the parasite preparation was omitted, no change in absorbance occurred. Likewise, no net reduction of niridazole was observed under aerobic conditions. Although reduction of niridazole in air could not be detected spectrophotometrically, the drug did stimulate oxidation of NADPH by cell-free preparations. In the absence of drug, the rate of NADPH oxidation was 7.5 ± 0.8 nmoles/hr/mg of protein, whereas in the presence of 200 μM niridazole, it increased to 48.3 ± 5.6 nmoles/hr/mg of protein. The cofactor was not oxidized in the absence of schistosome protein. No 360-nm-absorbing oxidative metabolites of niridazole were detected under these conditions.

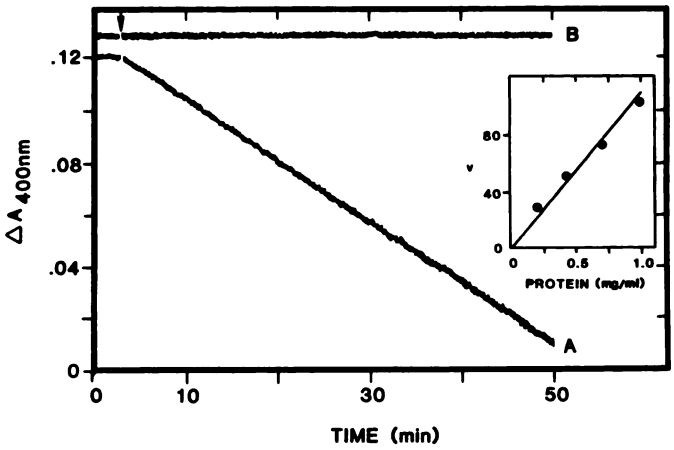

FIG. 4. *Spectrophotometric assay of niridazole nitroreductase activity in cell-free preparations of Schistosoma mansoni worm pairs*

A typical recorder tracing is shown of the disappearance of the nitrothiazole chromophore at 400 nm as a function of time.

A. The reaction was carried out at 37° in a Thunberg-type quartz cuvette under anaerobic conditions. The complete reaction mixture contained, in a total volume of 1.5 ml, 15 mmoles of potassium phosphate buffer (pH 7.5); 300 nmoles of niridazole; 1.5 μmoles of NADPH, 0.5 mg of schistosome protein; a reduced pyridine nucleotide-regenerating system consisting of 6 units of glucose 6-phosphate dehydrogenase and 15 μmoles of D-glucose 6-phosphate; and an oxygen-scavenging enzymatic system consisting of 0.06 unit of glucose oxidase, 1100 units of catalase, and 15 μmoles of β-D-glucose. The reaction was initiated (*arrow*) by adding NADPH and D-glucose 6-phosphate from the sidearm of the cuvette.

B. Reaction mixture as in A, but lacking NADPH and D-glucose 6-phosphate. Identical tracings were obtained when schistosome protein was omitted or when the reaction was carried out in air.

Inset. Protein concentration dependence of apparent initial reaction rate. v = nanomoles of niridazole reduced per hour.

The initial rate of niridazole reduction under anaerobic conditions was proportional to the protein concentration over a range of 0.2–1.0 mg/ml (Fig. 4, *inset*). A comparison of cell-free preparations from male and female parasites revealed that niridazole nitroreductase activity was comparable in both sexes (Table 5). Furthermore, NADH could also serve as a cofactor for the reaction, although NADPH was preferred. The ratio of NADPH-dependent to NADH-dependent activity was about 1.5.

When cell-free schistosome preparations were incubated in the presence of [^{14}C]niridazole under the conditions of the nitroreductase assay, radiolabeled drug bound covalently to trichloroacetic acid-precipitable material. The data in Table 6 show that the requirements

TABLE 4

Covalent drug binding to macromolecules of adult Schistosoma mansoni recovered from [^{14}C]niridazole-treated mice

Twelve hours after the sixth intraperitoneal dose of [^{14}C]niridazole (10 mg/kg/8 hr; 2.6 mCi/mmole), schistosomes were recovered by portal perfusion from groups of five mice. Both total radioactivity and specific drug binding in parasite homogenates were quantitated as described under Experimental Procedures. Approximately 0.05% of the total administered drug dose was recovered in the parasites. Values reported for each experiment are the means ± standard deviation of four determinations.

Experiment no.	[^{14}C]Niridazole		% Bound
	Total	Bound	
	nmoles/mg protein		
1	1.31 ± 0.04	0.56 ± 0.03	42.7
2	1.43 ± 0.05	0.67 ± 0.02	46.8

TABLE 5

Comparison of NADPH and NADH as cofactors for niridazole nitroreductase activity in cell-free preparations of worm pairs and separated female and male parasites

Nitroreductase activity was determined as described in the legend to Fig. 4 with 200 μM niridazole as the substrate. Values reported are the means ± standard deviations of four separate determinations.

Preparation	Nitroreductase activity		Ratio[a]
	NADPH	NADH	
	nmoles/hr/mg protein		
Worm pairs	36.2 ± 1.1	24.8 ± 1.2	1.5
Males	35.6 ± 0.9	25.4 ± 0.8	1.4
Females	37.4 ± 1.3	24.5 ± 0.6	1.5

[a] Ratio of NADPH-dependent to NADH-dependent activities.

for macromolecular drug binding in this cell-free system were identical with those for demonstrating niridazole nitroreductase activity. In experiments not illustrated, the rate of drug binding was found to be dependent on both time and protein concentration. Analysis of ethyl acetate extracts of the complete reaction by HPLC confirmed that TCI was a product of the enzymatic reduction of niridazole.

Effect of niridazole on parasite NPSH. Formation of electrophilic drug species within cells is often associated with a depletion of intracellular NPSH (19). To determine whether niridazole affects parasite NPSH, groups of worm pairs were incubated in the presence or absence of drug, and the relative concentration of NPSH was determined as a function of time (Fig. 5). In the absence of drug, NPSH concentrations remained constant over an 8-hr period (2.1 ± 0.06 nmoles/worm pair; mean ± standard deviation; $n = 8$). In contrast, incubation of schistosomes in the presence of 90 μM niridazole resulted in a 40% decrease in NPSH concentrations.

Because NPSH, and particularly GSH, are thought to be involved in the detoxification of chemically reactive drug species (19), the ability of NPSH to inhibit covalent binding of such species to parasite macromolecules was examined in the cell-free system (Fig. 6). At a concentration of 2 mM, cysteine, *N*-acetyl-L-cysteine, and GSH inhibited covalent binding by 80–85%. In contrast, *S*-carboxymethyl-L-cysteine, which has no free sulfhydryl group, was not inhibitory. None of these compounds

TABLE 6

Covalent binding of [^{14}C]niridazole to parasite macromolecules in vitro-catalyzed by cell-free Schistosoma mansoni preparations

The complete reaction mixture, with either 1 mM NADPH or 1 mM NADH as cofactor, is described in the legend to Fig. 4. Incubations were carried out for 60 min at 37° in Thunberg tubes under anaerobic conditions, except as otherwise noted. Specific drug binding was quantitated as described under Experimental Procedures. Values reported are the means ± standard deviation of five separate experiments.

Incubation mixture	Niridazole bound
	nmoles/mg protein
Complete (NADPH)	1.24 ± 0.09
Complete (NADH)	0.88 ± 0.05
−NAD(P)H	≤0.02
Heat-denatured worm preparation[a]	≤0.02
+Air	≤0.02

[a] 10 min at 100°.

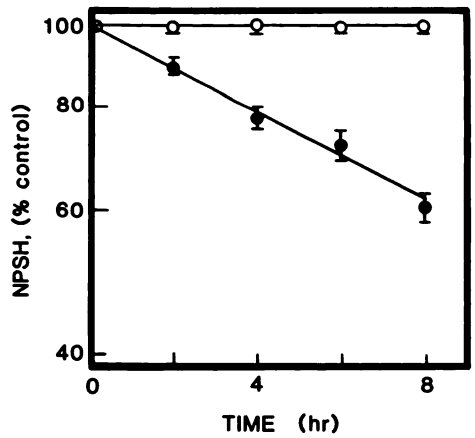

FIG. 5. *Effect of niridazole on total NPSH levels in Schistosoma mansoni maintained in vitro*

Groups of 10 worm pairs were incubated in supplemented RPMI medium 1640 in the absence (○) and presence (●) of 90 μM niridazole. At various times, NPSH concentrations were determined by titration with DTNB as described under Experimental Procedures. Results are expressed relative to the amount of NPSH in freshly perfused schistosomes at zero time (2.1 ± 0.06 nmoles/worm pair).

inhibited the anaerobic enzymatic reduction of niridazole by more than 5% (data not shown).

Studies with [^{14}C]4′-methylniridazole. To determine whether proximal reductive metabolism of niridazole and subsequent covalent drug binding to schistosome macromolecules was correlated with niridazole's antischistosomal activity, parallel studies were carried out with the pharmacologically inactive niridazole analog, 4′-methylniridazole (9). Groups of worm pairs were incubated in the presence of either [^{14}C]niridazole or [^{14}C]4′-methylniridazole (Table 7). Both drugs were taken up by the parasites over an 8-hr period. Whereas niridazole was extensively metabolized (95–97%) and was bound covalently (25%), virtually all of the radioactivity absorbed by

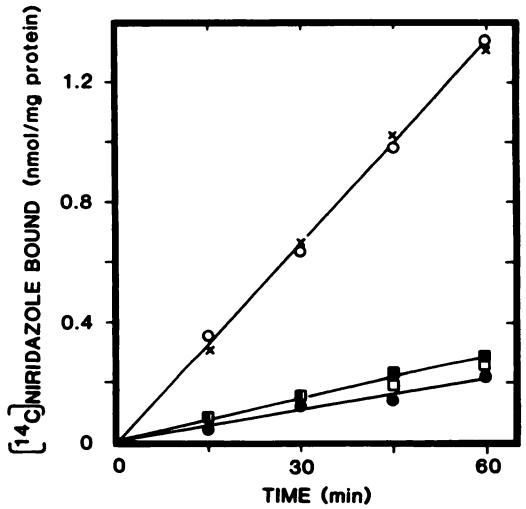

FIG. 6. *Inhibition by NPSH of covalent binding of [^{14}C]niridazole to macromolecules in cell-free schistosome preparations*

The complete reaction mixture is described in the legend to Fig. 4. Specific drug binding was quantitated as described under Experimental Procedures. ○, no inhibitor; ●, 2 mM GSH; □, 2 mM *N*-acetyl-L-cysteine; ■, 2 mM L-cysteine; (×) 2 mM *S*-carboxymethyl-L-cysteine.

TABLE 7

Uptake and covalent binding of [^{14}C]niridazole and [^{14}C]4′-methylniridazole by adult Schistosoma mansoni incubated in vitro

Groups of 10 worm pairs were maintained at 37° in 5 ml of supplemented RPMI medium 1640 containing 90 μM [^{14}C]niridazole or 90 μM 4′-methylniridazole. At the indicated times, parasites were washed with drug-free HBSS and homogenized. Both total radioactivity and specific drug binding were quantitated in triplicate. The fraction of total radioactivity present as unchanged drug was determinated by HPLC analysis, after extraction into ethyl acetate. Further details are given under Experimental Procedures. The values reported are the means ± standard deviation of three separate experiments.

Incubation time	[^{14}C]Niridazole		[^{14}C]4′-Methylniridazole	
	4 hr	8 hr	4 hr	8 hr
Total drug (nmoles/mg protein)	2.06 ± 0.14	3.17 ± 0.22	2.44 ± 0.18	4.31 ± 0.27
% Total drug as unchanged drug	5 ± 2	3 ± 1	94 ± 4	95 ± 3
Bound drug (nmoles/mg protein)	0.39 ± 0.04	0.80 ± 0.05	≤0.01	≤0.01

parasites incubated with [^{14}C]4′-methylniridazole was recovered as the unchanged parent compound.

Unlike niridazole, the inactive methyl analogue did not serve as a substrate for schistosomal nitroreductase in cell-free preparations (≤0.6 nmole/hr/mg of protein, with 1 mM NADPH). Furthermore, incubation of [^{14}C]4′-methylniridazole with cell-free parasite preparations under conditions optimal for nitroreduction did not result in covalent drug binding to trichloroacetic acid-precipitable material. Finally, at concentrations of up to 200 μM, 4′-methylniridazole did not affect the concentration of NPSH in schistosomes incubated *in vitro* (data not shown).

DISCUSSION

The results of this study support the hypothesis that metabolic activation of niridazole by adult *S. mansoni* involves enzymatic reduction of the essential nitro group and that such reduction is an obligatory step in the expression of niridazole's antiparasitic action. As evidenced by extensive covalent drug binding to parasite macromolecules, niridazole is metabolized to one or more reactive intermediates. A comparison of results obtained with niridazole and its non-schistosomicidal 4′-methyl analogue (9) reveals a positive correlation between reductive metabolic activation and toxic drug efficacy. Although both compounds can be reduced chemically to TCI[3] and both are taken up by schistosomes, only niridazole was extensively metabolized, served as a substrate for schistosomal nitroreductase, depleted parasite NPSH, and became covalently bound to parasite macromolecules in a manner inhibitable by physiological thiol compounds.

In contrast to results obtained with intact schistosomes, net reduction and covalent binding of [^{14}C]niridazole in cell-free preparations were demonstrable only under strictly anaerobic conditions. Although nitroreduction of niridazole in air could not be detected spectrophotometrically, the drug did stimulate oxidation of NADPH, indicating that it was undergoing metabolism. These results are consistent with formation of a nitro anion radical as the first intermediate of the reaction (20). The apparent inhibition of nitroreduction in air has been attributed to spontaneous reaction of such drug radicals with molecular oxygen to form superoxide and regenerate the parent nitro compound (21). The lack of covalent drug binding in the cell-free system in air can be explained in two ways. First, the rate at which the putative niridazole anion radical reacts with oxygen may be sufficiently rapid that it is reoxidized (22) before it can interact with parasite macromolecules. Alternatively, the nitro radical may not be the drug form involved in covalent binding. Under anaerobic conditions, further reduction of the radical might give rise to other electrophilic species, such as the nitroso and/or hydroxylamino derivatives, which could bind covalently to tissue nucleophiles (23, 24). The present experiments do not distinguish between these possibilities.

The fact that covalent binding of [^{14}C]niridazole does proceed in intact schistosomes incubated under aerobic conditions suggests that the intracellular concentration of molecular oxygen available for reoxidation of drug radicals is very low. This is consistent with the view that adult *S. mansoni* are facultative anaerobes. Although these parasites have a few mitochondria and may consume small amounts of oxygen (25, 26), the major source of metabolic energy seems to be derived from glycolysis. When incubated in air, the parasites utilize glucose at a rate equivalent to 15–26% of their body dry weight per hour, of which 80–90% is metabolized to lactic acid (26). It has yet to be determined whether lactate fermentation also predominates in parasites within the mammalian host.

Niridazole is reductively metabolized by other facultative anaerobic organisms too. We have reported that, in mammals, niridazole is converted to TCI by the action of intestinal microflora (7). Recently, Blumer *et al.* (27) demonstrated that both the antibiotic and mutagenic activities of niridazole toward histidine auxotrophic strains of *Salmonella typhimurium* are related to the specific activity of bacterial nitroreductases. Mutants of these strains, selected for their ability to grow in the presence of niridazole, were found to have only low levels of nitroreductase activity. These nitroreductase-deficient mutants were much less sensitive to drug-mediated mutagenesis and killing than were the parent strains (27). Thus, niridazole toxicity in this bacterial system can be correlated with reductive metabolism.

The metabolic fate of niridazole in mammals is strikingly different from that in schistosomes and bacteria. Although Feller *et al.* (17) demonstrated formation of a hydroxylamino derivative of niridazole by rat liver microsomes under strictly anaerobic conditions, they were unable to detect product formation in air. Even if one assumes that niridazole is reduced to a nitro anion radical, there is yet no evidence to support a physiological role for reductive metabolism of this drug in mammals. On the contrary, evidence accumulated in this laboratory

indicates that hydroxylation of the 2-imidazolidinone ring, catalyzed by an inducible, microsomal mixed-function oxidase system, is the major pathway of hepatic metabolism of niridazole (8). Furthermore, our recent results demonstrate that covalent binding of [^{14}C]niridazole and [^{14}C]4′-methylniridazole in mouse liver microsome preparations proceeds under aerobic conditions, in the presence of NADPH.[5] This binding can be completely inhibited by carbon monoxide.[5] Therefore, whereas niridazole toxicity in the parasite can be correlated with reductive metabolism, toxic side effects in the host (28) may be related to oxidative metabolism (8).

The present study may shed new light on the mode of action of niridazole in schistosomes, even though a variety of nitro compounds may produce similar effects in other organisms (29). Bueding and Fisher (30) suggested that niridazole acts by depleting glycogen from the musculature of male parasite. They attributed this effect, which was first evident 18–24 hr after treatment of infected mice, to inhibition of glycogen phosphorylase inactivation, resulting in an increased rate of glycogenolysis (30). However, glycogen depletion probably is a late event relative to both reductive metabolism and covalent drug binding and may be unrelated to the drug's primary mode of action. Considering that niridazole is a relatively slow-acting metabolic poison, it may be impossible to identify a single molecular mechanism for its toxic efficacy. However, our finding that reductive metabolism leads to the formation of electrophilic drug species which bind covalently to parasite macromolecules, particularly proteins, offers one explanation. Inhibition of covalent binding in cell-free preparations by low molecular weight thiols (Fig. 6), together with a preliminary identification of cysteine as the principal target amino acid residue,[5] suggest that binding preferentially involves free sulfhydryl groups. This in turn might cause inhibition or inactivation of critical parasite enzymes whose activities depend on essential sulfhydryl groups. Proof of this putative mechanism of drug toxicity will require isolation and structural characterization of appropriate drug-amino acid adducts and a demonstration that candidate enzymes are indeed selectively inhibited by niridazole.

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REFERENCES

1. Katz, N. Chemotherapy of *Schistosoma mansoni*. *Adv. Pharmacol. Chemother.* **14**:1–70 (1977).
2. Sankale, M., P. Satge, M. Lariviere, M. Moulanier, A. Bourgeade, C. Debroise, J. P. Ancelle, and A. M. Sow. Efficacy of niridazole in amoebic dysentery. *Ann. N. Y. Acad. Sci.* **160**:755–763 (1969).
3. Collins, F. M. Effect of oral niridazole treatment on some bacterial infections in mice. *Antimicrob. Agents Chemother.* **7**:453–456 (1975).
4. Hof, H., V. Sticht-Groh, and K. Muller. Comparative in vitro activities of niridazole and metronidazole against anaerobic and microaerophilic bacteria. *Antimicrob. Agents Chemother.* **22**:332–333 (1982).
5. Faigle, J. W., and H. Keberle. Metabolism of niridazole in various species including man. *Ann. N. Y. Acad. Sci.* **160**:544–557 (1969).
6. Tracy, J. W., E. H. Fairchild, S. V. Lucas, and L. T. Webster, Jr. Isolation, characterization and synthesis of an immunoregulatory metabolite of niridazole: 1-thiocarbamoyl-2-imidazolidinone. *Mol. Pharmacol.* **18**:313–319 (1980).
7. Tracy, J. W., and L. T. Webster, Jr. The formation of 1-thiocarbamoyl-2-imidazolidinone from niridazole in mouse intestine. *J. Pharmacol. Exp. Ther.* **217**:363–368 (1981).
8. Blumer, J. L., R. F. Novak, S. V. Lucas, J. M. Simpson, and L. T. Webster, Jr. Aerobic metabolism of niridazole by rat liver microsomes. *Mol. Pharmacol.* **16**:1019–1030 (1979).
9. Schmidt, P., K. Eichenberger, A. O. Ilvespan, and M. Wilhelm. Nitro heterocycles with antiparasitic effects. *Ann. N. Y. Acad. Sci.* **160**:530–535 (1969).
10. Faigle, J. W., and H. Keberle. Synthesis of ^{14}C-labeled 2-aminothiazole, 2-amino-5-nitrothiazole, 2-chloro-5-nitrothiazole, and 1-(5-nitro-2-thiazolyl)-2-imidazolidinone (active substance of Ambilhar). *J. Labelled Cmpd.* **5**:173–180 (1969).
11. Duval, R. H., and W. B. De Witt. An improved perfusion technique for recovering adult schistosomes from laboratory animals. *Am. J. Trop. Med. Hyg.* **16**:483–486 (1967).
12. Peterson, G. L. A simplification of the protein assay of Lowry *et al.* which is more generally applicable. *Anal. Biochem.* **83**:346–356 (1977).
13. Wilhelm, M., F. H. Marquardt, K. Meier, and P. Schmidt. Synthese und abbau von 1-(5-nitro-2-thiazolyl)-2-imidazolidinone und derivaten. *Helv. Chim. Acta* **49**:2443–2452 (1966).
14. Smithers, R. S. R., D. B. Roodyn, and R. J. M. Wilson. Biochemical and morphological characteristics of subcellular fractions of male *Schistosoma mansoni*. *Exp. Parasitol.* **16**:195–206 (1965).
15. Shaw, J. L., J. Blanco, and G. C. Mueller. A simple procedure for isolation of DNA, RNA and protein fractions from cultured animal cells. *Anal. Biochem.* **65**:125–131 (1975).
16. Harris, J. W. Non-protein sulfhydryl content of Erlich ascites tumor cells exposed to hyperbaric oxygen or X-irradiation. *Exp. Cell Res.* **50**:293–305 (1968).
17. Feller, D. R., M. Morita, and J. R. Gillette. Enzymatic reduction of niridazole by rat liver microsomes. *Biochem. Pharmacol.* **20**:203–215 (1971).
18. Blumer, J. L., and J. J. Mieyal. Genetic expression of microsomal electron transport in mice: different patterns of dependence of constitutive cytochrome P-450 reductase activity on pyridine nucleotide concentrations. *J. Biol. Chem.* **253**:1159–1166 (1978).
19. Nelson, S. D., M. R. Boyd, and J. R. Mitchell. Role of metabolic activation in chemical-induced tissue injury, in *Drug Metabolism Concepts* (D. M. Jerina, ed.). American Chemical Society, Washington, D. C., 155–185 (1977).
20. Mason, R. P., and J. L. Holtzman. The mechanism of microsomal and mitochondrial nitroreductase: electron spin resonance evidence for nitroaromatic free radical intermediates. *Biochemistry* **14**:1626–1632 (1975).
21. Mason, R. P., and J. L. Holtzman. The role of catalytic superoxide formation in the O_2 inhibition of nitroreductase. *Biochem. Biophys. Res. Commun.* **67**:1267–1274 (1975).
22. Wardman, P., and E. D. Clarke. Oxygen inhibition of nitroreductase: electron transfer from nitro radical-anions to oxygen. *Biochem. Biophys. Res. Commun.* **69**:942–949 (1976).
23. Eyer, P. Reactions of nitrosobenzene with reduced glutathione. *Chem.-Biol. Interact.* **24**:227–239 (1979).
24. Weisburger, J. H., and E. K. Weisburger. Biochemical formation and pharmacological, toxicological and pathological properties of hydroxylamines and hydroxamic acids. *Pharmacol. Rev.* **25**:1–66 (1973).
25. Coles, G. C. Oxidative phosphorylation in adult *Schistosoma mansoni*. *Nature (Lond.)* **240**:488–489 (1972).
26. Bueding, E. Carbohydrate metabolism of *Schistosoma mansoni*. *J. Gen. Physiol.* **33**:475–495 (1950).
27. Blumer, J. L., A. Friedman, L. W. Meyer, E. H. Fairchild, L. T. Webster, Jr., and W. T. Speck. Relative importance of bacterial and mammalian nitroreductases for niridazole mutagenesis. *Cancer Res.* **40**:4599–4605 (1980).
28. Blumer, J. L., J. M. Simpson, S. V. Lucas, and L. T. Webster, Jr. Toxicogenetics of niridazole in inbred mice. *J. Pharmacol. Exp. Ther.* **212**:509–513 (1980).
29. Mason, R. P. Free radical intermediates in the metabolism of toxic chemicals, in *Free Radicals in Biology*, (W. A. Pryor, ed.) Vol. 5. Academic Press, New York, 161–212 (1982).
30. Bueding, E., and J. Fisher. Biochemical effects of niridazole on *Schistosoma mansoni*. *Mol. Pharmacol.* **6**:532–539 (1970).

[5] J. W. Tracy, unpublished observations.

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