THESIS

This is to certify that the

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DYNAMICS OF GLUTATHIONE REGULATION IN SCHISTOSOMA MANSONI: CORRELATIONS WITH THE ACUTE EFFECTS OF OLTIPRAZ

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Dan Douglas Morrison

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DYNAMICS OF GLUTATHIONE REGULATION IN *SCHISTOSOMA MANSONI*: CORRELATIONS WITH THE ACUTE EFFECTS OF OLTIPRAZ

By

Dan Douglas Morrison

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ABSTRACT

DYNAMICS OF GLUTATHIONE REGULATION IN SCHISTOSOMA MANSONI:
CORRELATIONS WITH THE ACUTE EFFECTS OF OLTIPRAZ

By

Dan Douglas Morrison

Glutathione is present in adult Schistosoma mansoni (0.336 ± 0.012 nmoles/mg protein) at significantly lower levels than uninfected host liver tissues (1.051 ± 0.013 nmoles/mg protein) or host kidney tissue (0.627 ± 0.013 nmoles/mg protein). Host hepatic glutathione levels decline significantly during the course of an infection by the parasite, while renal cortical glutathione levels are unaffected. Of the enzymes regulating glutathione utilization, glutathione reductase in the male parasite exhibits a specific activity of 10.3 ± 4.2 mU/mg protein, 15% that of uninfected liver. The apparent glutathione S-transferase activity depended on substrate tested, 26 ± 7 µmole conjugate formed/min/mg protein with p- nitrobenzyl chloride as substrate (13% of hepatic values) and 526 ± 18 µmole conjugate formed/min/mg protein with 1-chloro-2,4-dinitrobenzene as substrate (43% of hepatic values). Male schistosomes exhibited negligible glutathione peroxidase activity (5 ± 9 nmoles NADPH oxidized/min/mg protein), while the uninfected host exhibited values of 258 ± 19 nmoles NADPH oxidized/min/mg protein for this enzyme. During the course of infection, the specific activity of hepatic glutathione S-transferase

was depressed to 26% of uninfected control values (p- nitrobenzyl chloride as substrate). Similarly, hepatic glutathione peroxidase activity was reduced to 37% of control values. Hepatic glutathione reductase was not significantly affected during the infection. Also, neither of the renal enzymes, glutathione peroxidase or glutathione reductase, were affected. Oltipraz, an antischistosomal compound, effected a significant depletion of parasite and host glutathione levels within 1 h of exposure *in vivo* and *in vitro* at a dosage of 250 mg/kg and 10 μM, respectively. Host tissue glutathione levels returned to, or above, control levels by 6 h after oltipraz administration, while parasite glutathione levels remained significantly depressed. Uptake of [^{35}S]cysteine or [^{35}S]cystine by schistosomes was inhibited by oltipraz. However, the drug did not alter the relative distribution of label once incorporated into the parasite, indicating that the enzymes of glutathione synthesis were not directly inhibited. Oltipraz significantly depressed surface electrical activity and the tegument potential of male schistosomes. However, both the biochemical and physiological effects of oltipraz (10 μM) were prevented by coincubating the parasite in 1 mM cysteine, methionine or glutathione. The amelioration of oltipraz-induced effects was not dependent on free thiol groups, as neither dithiothreitol nor 2-mercaptoethanol attenuated the schistosomicidal effect of oltipraz. The inactive analog of oltipraz (RP 36 642, 100 μM) did not produce effects mimicking those seen with oltipraz.

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LIST OF ABBREVIATIONS

AT-125 = L-(αS,5S)-α-Amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid

BCNU = 1,3-bis [2-chloroethyl]-1-nitrosourea

BSO = L-buthionine-S-R-sulfoximine

DTT = Dithiothreitol

GPx = Glutathione peroxidase (EC 1.11.1.9)

GRed = Glutathione reductase (EC 1.6.4.2)

GSH = Glutathione, reduced form (γ-L-glutamyl-L-cysteinylglycine)

GTp = Glutathione transpeptidase (EC 2.3.2.2)

GTr = Glutathione S-transferase (EC 2.4.1.18)

HPLC = High pressure liquid chromatography

HS/RPMI = 1:1 mixture of horse serum (heat-inactivated) and RPMI 1640, to which penicillin (100 U/ml), streptomycin (100 µg/ml) and Fungizone (0.5 µg/ml) were added

NAD(P)H = Nicotinamide-adenine dinucleotide (phosphate)

S.E. = Standard error of the mean

TCA = Trichloroacetic acid

INTRODUCTION

A. The parasite, Schistosoma mansoni:

Schistosoma mansoni is a digenetic parasite, which debilitates millions of people in tropical regions throughout the world. The regions of endemic schistosomiasis are restricted to tropical climates because of the presence of the snail vector in which the schistosome develops during one stage of its two-host life cycle. S. mansoni adults, residing in the mesentaric venules lining the intestines of the human, oviposit approximately 300 eggs/day/worm pair [99]. Up to 70% of these are dislodged from the venule into the blood stream and become entrapped within the hepatic sinusoids of the liver or the microfenestrations of the spleen, resulting in the hepatosplenic pathology which characterizes long-term, heavy worm burden infections [130,131,132]. The overt disease symptoms, hepatomegaly, splenomegaly, portal hypertension and esophageal varices, can be prevented, even in mice heavily infected with schistosomes by inhibition of oviposition. Also, the disease state shows signs of amelioration, by gradual host repair of damaged tissues, if egg production is halted after the onset of hepatosplenic disease [129].

An increase in the prevalence and intensity of schistosomiasis is occurring in the tropics because agricultural irrigation is rapidly expanding in countries where the disease is endemic, providing a larger habitat for the snail host. To interrupt transmission of the disease requires effective sanitary waste disposal, large-scale snail control, or mass chemotherapy. However, field-trials have indicated that the best results are obtained with chemotherapy [78], and chemotherapy will likely remain the only viable approach in the control of the disease for the extended future. However, due to the proclivity of the human population to become reinfected, mass chemotherapy is an expensive temporary solution.

B. Chemotherapy:

1. General: Several compounds are available which exhibit antischistosomal activity (Table 1, from [16]), however, the use of the antimonials, niridazole and hycanthone is limited due to host toxicity. Metrifonate is not effective in eliminating mansoni schistosomiasis, which limits its use. Research is warranted on the other antischistosomal compounds to attempt to determine their modes of action in efforts to design more efficacious drugs or cheaper substitutes that still retain schistosomicidal activity. Further research is also warranted to forestall or circumvent possible problems of resistance that may develop to drugs currently available and in use, or the possible discovery of untoward effects of the newer drugs on the host. Ideally, the antiparasitic would be effective in a single oral dose, of low cost, and effective against all stages of the parasite

Table 1: Drugs Used in the Treatment of Human Schistosomiasis

Antischistosomal compound (date)[a]	Antischistosomal Spectrum of Action[b]	Comments
Antimonials (1918)	*S. m.*, *S. j.*, *S. h.*	Limited use due to toxicity
Metrifonate (1962)	*S. h.*	Minor side effects
Niridazole (1965)	*S. m.*, *S. h.*	Convulsant, carcinogenic
Hycanthone (1967)	*S. m.*, *S. h.*	Hepatic necrosis, carcinogenic
Oxamniquine (1973)	*S. m.*	Minor side effects
Amoscanate (1978)	*S. j.* *S. m.* (?), *S. h.* (?)	Hepatotoxicity
Praziquantel (1979)	*S. m.*, *S. j.*, *S. h.*	
Oltipraz (1979)	*S. m.*, *S. h.*	

[a] Date of first reported use in humans with schistosomiasis.
[b] *Schistosoma mansoni* (S. m.), *Schistosoma haematobium* (S. h.) and *Schistosoma japonicum* (S. j.).

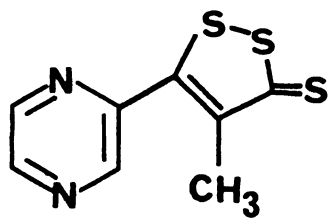
[67].

2. Oltipraz structure and structure-activity relationships: Oltipraz (35 972 R.P.; 4-methyl-5-[2-pyrizinyl]-1,2-dithiol-3-thione; Figure 1) was synthesized and patented in the mid-1970's by Rhone Poulenc Sante, Paris [9]. Congeners of this drug exhibit amebicidal [9] and fungicidal [12] activity, while some other compounds with the 1,2-dithiole-3-thione ring structure exhibit diuretic activity [38]. The structure-activity relationship for oltipraz is rather stringent [25]. The presence of the thione and an adjacent aromatic ring containing two nitrogens are structural properties required for antischistosomal activity [23]. Further, while oltipraz is extensively metabolized [19], none of the metabolites which have been identified possess any antischistosomal activity [77].

3. Oltipraz spectrum of action: In man, oltipraz is effective in erradicating S.mansoni, S.haematobium and S.intercalatum infections [49,108], but the drug is ineffective against S. japonicum [24]. Oltipraz is less active against schistosomula than against mature adult schistosomes [89].

4. Oltipraz mechanism of action: Oltipraz is a slow-acting drug, two or more months may pass before the worm burden is totally erradicated with a curative dose. In mice given very high doses of the drug, evidence of worm lethality is observed after 48 h, with all schistosomes being killed by 14 days post-treatment [89]. The mechanism of action which leads eventually to the schistosomacidal

Figure 1: Chemical structure of oltipraz.



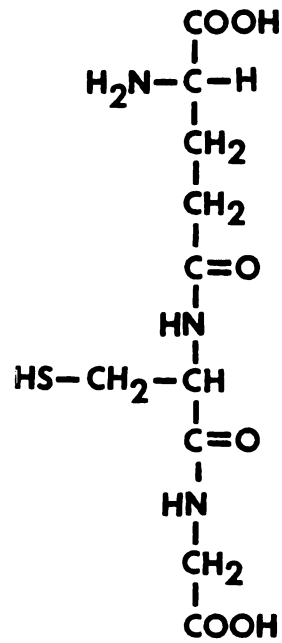
OLTIPRAZ

outcome remains unknown. There are no published reports on acute effects of this drug, nor have reports been published on in vitro studies to determine the direct action of this compound on the parasite which are independent of the variables of the host response to drug action. With oltipraz, and various structural analogs of oltipraz, a positive correlation exists between antischistosomal activity and the depression of glutathione levels in the parasite [25]. Although not studied acutely, these investigators also observed a concomittant elevation in the glutathione levels of various host tissues post-treatment, suggesting that a biochemical difference exists between host and parasite in the regulation of this important intracellular thiol compound.

C. Glutathione and enzymatic regulation of glutathione:

1. Glutathione: Glutathione (γ-L-glutamyl-L-cysteinylglycine; GSH) is a tripeptide of molecular weight 307.33, shown in Figure 2. The pKa of the sulfhydryl is 9.2, therefore GSH carries a net negative charge at physiological pH (as does cysteine, with a pKa of the sulfhydryl at 8.18). GSH oxidizes spontaneously at physiological pH to form the disulfide, oxidized GSH. Alternatively, GSH can react with cysteine or protein sulfhydryls to form mixed disulfides. The role of GSH in the overall regulation of enzyme function is an area of much current study. "Essential" protein sulfhydryl groups are ubiquitous, whether in a catalytic role or in determining protein conformation, as is indicated by the inactivation of a large number of these enzymes by oxidants such as diamide, N-ethylmaleimide or 5,5′-dithiobis

Figure 2: Chemical structure of glutathione



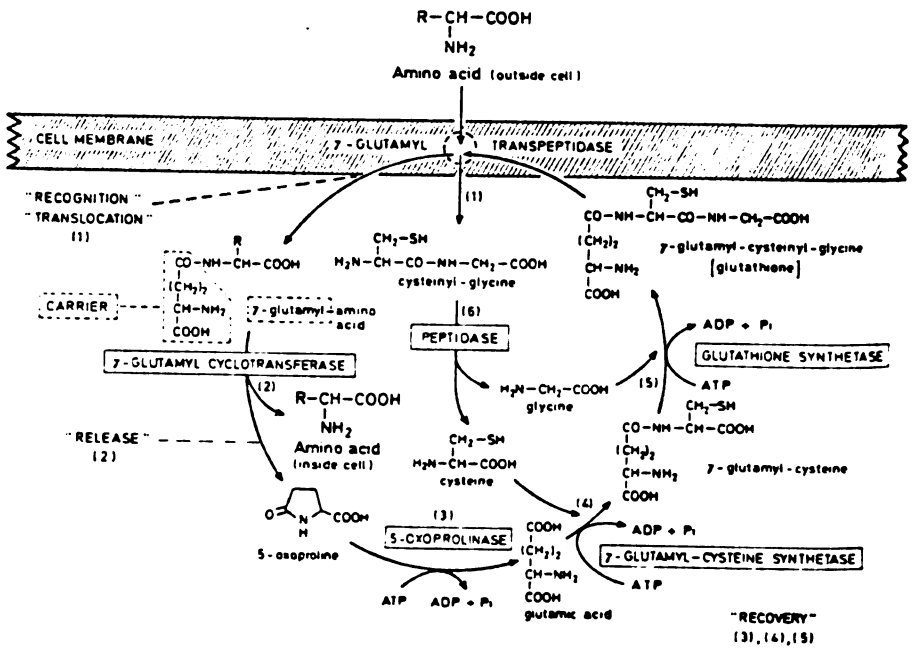
GLUTATHIONE

γ-L-glutamyl-L-cysteinylglycine

(2-nitrobenzoic acid). For this reason, reducing agents, such as 2-mercaptoethanol or dithiothreitol are commonly used to stabilize enzymatic function. In light of the effects of thiol/disulfide exchange on enzyme activity, this covalent modification may play an important role in the biological control of various metabolic pathways. Further, the thiol/disulfide status of GSH may modulate enzyme activity [50], and recently, GSH disulfide has been shown to inactivate, destabilize, and enhance proteolytic susceptibility of fructose-1,6-bis-phosphate aldolase [104]. The importance of GSH is underscored by its ubiquitous occurrence in every organism examined. GSH is a cofactor, coenzyme or substrate for an encyclopedic number of enzymatic reactions. On the molecular level, the most conspicuous aspect of cellular GSH depletion is its relationship to cell damage. That GSH provides an important cellular defense against oxidant injury has been well documented [16,68,133]. During a state of GSH deficiency, reactive metabolites, formed in situ, bind to macromolecules of biologic importance and cellular toxicity results [50]. The formation of oxygen-dependent free radicals and peroxides result in the peroxidation of membrane polyunsaturated fatty acids.

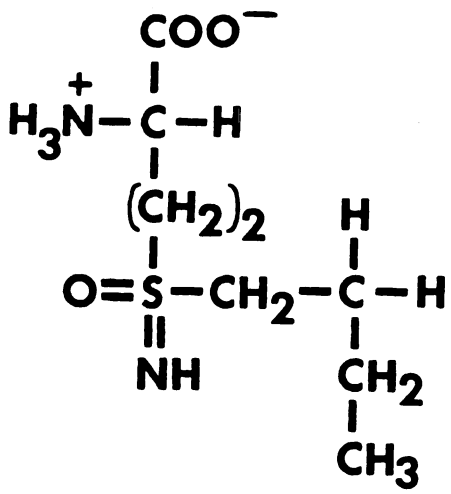
2. Enzymatic synthesis: The synthesis of GSH occurs in a series of 6 reactions of the γ-glutamyl cycle from precursor amino acids (Figure 3, from [92]). The enzymes of this cycle have been reviewed [94,96]. The availability of the precusor amino acid, cysteine, is the rate-limiting factor in GSH synthesis. The physiologic concentration of cysteine is about an order of magnitude lower than the Km of γ-glutamylcysteine synthetase for cysteine [114]. According to the

Figure 3: γ-glutamyl cycle


R–CH–COOH
NH2
Amino acid (outside cell)
CELL MEMBRANE
γ-GLUTAMYL TRANSPEPTIDASE
"RECOGNITION"
"TRANSLOCATION"
(1)
CARRIER
γ-glutamyl-amino acid
γ-GLUTAMYL CYCLOTRANSFERASE
(2)
R–CH–COOH
NH2
Amino acid (inside cell)
"RELEASE"
(2)
5-oxoproline
(3)
5-OXOPROLINASE
ATP
ADP + Pi
cysteinyl-glycine
(6)
PEPTIDASE
glycine
cysteine
(4)
ADP + Pi
γ-GLUTAMYL-CYSTEINE SYNTHETASE
ATP
glutamic acid
γ-glutamyl-cysteinyl-glycine
[glutathione]
(5)
ADP + Pi
GLUTATHIONE SYNTHETASE
ATP
γ-glutamyl-cysteine
"RECOVERY"
(3), (6), (5)

report of Beatty and Read [14], cysteine or cystine supported GSH synthesis in isolated rat hepatocytes better than methionine. However, the determination of GSH synthesis by these authors, which was by the rate of accumulation of cysteine-glutathione mixed disulfide in the incubation media, probably resulted in erroneous estimates of GSH synthesis, due to inhibition of GSH efflux from isolated rat hepatocytes by methionine [7]. Also, methionine, a precursor of cysteine via the cystathionine pathway, has been shown to be a good precursor for rapid GSH synthesis in hepatocytes [13,113]. The kidney differs from the liver, in that cysteine, as cystine, is readily taken up by tubular epithelium, supporting the requirements for the rapid turnover of glutathione in kidney [98,106], whereas methionine is poorly supportive of rapid GSH synthesis, probably because of a deficiency of cystathionase [98]. GSH regulates its own synthesis by non-allosteric competitive inhibition of the γ-glutamate binding site of γ-glutamylcysteine synthetase [39,114]. The γ-glutamyl bond in the tripeptide renders GSH resistant to degradation by α-peptidases, however, this bond is susceptible to hydrolytic and transpeptidation reactions leading to products recoverable by the cell. GSH synthesis can be inhibited by buthionine sulfoximine (Figure 4), a potent and specific inhibitor of γ-glutamylcysteine synthetase [54,55,56,58]. L-2-imidazolidone-4-carboxylate inhibits the enzyme 5-oxoprolinase [58]. Potent, irreversible inhibition of γ-glutamyl transpeptidase activity can be attained with AT-125 [61]. The structure-activity relationships of substrate analogs on the inhibition of the γ-glutamyl cycle enzymes have been studied [57].

Figure 4: Chemical structure of buthionine sulfoximine.



BUTHIONINE SULFOXIMINE

3. Glutathione utilization: Intracellular GSH is converted to oxidized GSH by selenium-containing glutathione peroxidase (GPx), which also catalyzes the reduction of H_2O_2 and other peroxides. GPx is specific for its hydrogen donor, GSH, and nonspecific for the hydroperoxide [95]. Therefore, GPx catalytically detoxicates a range of substrates from H_2O_2 to organic hydroperoxides. Although GPx shares the substrate H_2O_2 with catalase, it alone can react effectively with organic hydroperoxides as well. Selenium-independent GPx activity describes the peroxidase activity of glutathione transferase [88]. In male weanling rats maintained on a selenium-deficient diet, GPX activity fell to undetectable levels. When these animals were also deprived of vitamin E and sulfur-containing amino acids, a fatal hepatic necrosis developed [63,116].

The reduction of oxidized GSH, with concomitant oxidation of NADPH, is catalyzed by the flavoprotein glutathione reductase (GRed) in an essentially irreversible reaction, accounting for the very high GSH:oxidized GSH ratios found in cells [95]. The importance of this enzyme is most apparent in cells which no longer synthesize macroomolecules, such as the mature erythrocyte where maintainance of adequate levels of reduced GSH is essential for the deformability and flexibility of these cells [71]. In rat hepatocytes, the protective role of GRed against adriamycin-mediated toxicity was demonstrated by the inactivation of this enzyme with BCNU [8]. In these experiments, the rapid recycling of oxidized GSH to GSH by GRed ameliorated the lipid peroxidation seen when GRed was inactivated. Glutathione S-transferase performs the catalytic function of conjugating

potentially harmful electrophilic compounds of hydrophobic character with the nucleophilic GSH to produce a compound less toxic and more water soluble than the parent compound. By reason of their high affinity and concentration, they may also act as scavengers for alkylating agents. In a suicide-manner, the covalent bond formation between the transferase and the highly reactive electrophile detoxicates the compound, at the expense of further enzyme function [73].

RATIONALE

The overall research objective for studying biochemical differences between the host and parasite is the possibility that this approach could lead to the rational development of new families of drugs which exhibit antiparasitic activity at levels of drug which are not toxic to the host. The rationale for investigating the mechanism of action of oltipraz is to determine the biochemical differences between these organisms which result in schistosomicidal activity with only minor side effects noted in the host. This may, in turn, lead to the development of better, safer and less expensive new chemotherapeutics.

MATERIALS AND METHODS

A. Preparation of host and parasite tissue: Adult Schistosoma mansoni, 45-55 days post-infection were dissected from the hepatic and mesentaric veins of Swiss Webster female mice by the method of Fetterer et al. [45]. Where male parasites were used, worms were dissected into media containing 0.05% sodium pentobarbital, mechanically separated and the males placed into fresh media and maintained at 37°C until assay.

For in vivo experiments, mice were dosed by gavage at either 125 mg/kg or 250 mg/kg oltipraz in peanut oil or peanut oil vehicle and sacrificed at various times post dosing.

For anaerobic experiments, 40 male parasites were incubated 18 hours at 37 °C in 25 ml HS/RPMI. Prior to incubation and immediately after addition of worms to the media, nitrogen gas was passed through an oxygen trap (0.2 M pyrogallol in 0.2 N NaOH), then bubbled through the media to replace dissolved oxygen.

For egg-laying experiments, fifteen pairs of animals were transferred at random to 50 ml of media in 250 ml Erlenmeyer flasks. The medium was a 1:1 mixture of RPMI 1640 and heat inactivated horse serum, to which had been added 100 U/ml penicillin and 100 ug/ml streptomycin (media components from Gibco Laboratories, Grand Island, NY). Mercaptoethanol was added to a final concentration of 50 μM. Drug or vehicle were added to the media in a volume not exceeding 100

µl. Flasks were then incubated for 72 h at 37°C, with continuous mechanical agitation.

B. Glutathione assay: At the end of the incubation period, worms were filtered, weighed and homogenized (1:10, w/v) in 6% trichloroacetic acid (TCA). Host tissues were perfused with ice-cold 1.15% KCl to clear blood from the organ and then homogenized 1:20 (w:v) in 6% TCA. Aliquots were removed for protein determination by the method of Albro [2]. Following 1000 x g centrifugation, GSH was determined by a modification of the method of Ellman [44], where to 100 µl aliquots of supernatant were added 800 µl of 0.3 M Na_2HPO_4, pH 8.2 and 100 µl 0.04% 1-fluoro-2,4 dinitrophenol. Absorbance was recorded at 412 nm for freshly prepared standards and samples.

C. Manipulation of schistosome GSH levels in vitro: Schistosome GSH levels were altered by treatment of male parasites with diamide [88]. To determine the extent of lipid peroxidation, malondialdehyde, which is formed as a breakdown product of polyunsaturated fatty acids was measured by the thiobarbituric acid assay of Buege and Aust [29].

Parasite GSH levels were measured after incubation in varying concentrations of BCNU (1,3-bis [2-chloroethyl]-1-nitrosourea) for 3 h or BSO (L-buthionine-S-R-sulfoximine) for 6 h. The dose-dependency of oltipraz in depleting parasite GSH levels was established for 1 h, and the antagonism of oltipraz depression of schistosomal GSH by various compounds was evaluated at 18, 36 and 72 h.

D. High pressure liquid chromatography (HPLC): Male schistosomes were transferred to wells (multi-well tissue culture plates, Flow Laboratories, Inc., McLean, VA) containing 2.5 ml RPMI 1640, 3 x 10^5 dpm/well [^{35}S]cysteine or [^{35}S]cystine or [^{14}C]glucose (Amersham Corp., Arlington Heights, IL) and other exogenously added components as indicated. Plates were maintained at 37 C in a dark chamber, for the time periods specified. Parasites were weighed and homogenized 1:10 (w:v) in 3.5% perchloric acid. The homogenate was centrifuged 12000 x g, 10 min. One-half ml aliquots of acid-soluble supernatant were derivatized and the derivatives analyzed by HPLC as described by Reed et al. [114]. Eluant was monitored at 352 nm and collected in 0.5 ml fractions. Five ml ACS (Amersham Corp., Arlington Heights, IL) was added to each fraction and fractions were assayed for radioactivity by liquid scintillation (Beckman Instruments, Inc., Fullerton, CA).

E. Enzyme assays: The enzyme activity of γ-glutamyl transpeptidase (γ-GTP) was determined according to a modification of the method of Orlowski and Meister [108]. Tissue homogenates (1:20, w:v) were prepared in 0.1 M K_2HPO_4, 1 mM GSH, pH 8.0. A 20 µl aliquot of the supernatant fraction (25,000 x g, 20 min) was added to 480 µl substrate buffer consisting of 5.55 mM L-γ-glutamyl-p-nitroanilide, 11.1 mM $MgCl_2$ and 110 mM Tris-HCl. Enzymic activity was monitored at pH's from 6.0 to 9.5 in increments of 0.5 pH unit. The reaction was stopped with 1 ml 1.5 N acetic acid and the p-nitroaniline product formed was measured at 410 nm against a reference solution. Enzyme activity is expressed as µmoles p-nitroaniline formed/min/ mg protein.

Glutathione S-transferase (GTr) activity was examined in tissue cytosol fractions, prepared as above, in the presence of 1mM GSH, with 1,2-dichloro-4-nitrobenzene or p-nitrobenzyl chloride as substrate. The initial velocities of glutathione conjugate formation were measured spectrophotometrically at ambient temperature according to the procedure of Habig et al. [64]. Specific activity is expressed as nmoles product/min/mg protein, after correction for spontaneous nonenzymatic conjugation.

Glutathione reductase (GRed) activity was assayed by the method of Beutler [17]. Either 50 µl of 2 mM NADPH (or NADH) was used as substrate and the rate of change in optical density at 340 nm was recorded. The oxidation of 1 µmole NADPH (or NADH) per minute was used as a unit of GRed activity.

Glutathione peroxidase (GPx) was measured by both direct and indirect assay methods. Both assays measure selenium- and nonselenium-dependent GPx. The direct assay was performed by the method of Mills [100] as modified by Hafeman et al. [65]. Parasite tissues were prepared as described for the Y-GTP assay (rather than 4 volumes 0.15 M KCl used by Hafeman et al). The indirect assay for the measurement of GPx was by the method of Beutler [18]. The rate of formation of oxidized GSH was measured by the change in absorbance upon the oxidation of NADPH to $NADP^+$.

F. Mechanical, surface electrical and tegumental potential measurements: Recordings of longitudinal muscle tension and muscle activity were made from male schistosomes as previously described [45]. Surface electrical activity was recorded by the use of suction electrodes as described by Semeyn et al., [122]. Recordings of tegumental potentials were obtained by using glass microelectrodes as described by Thompson et al. [129].

CHAPTER ONE: HOST-PARASITE DIFFERENCES IN THE REGULATION OF GLUTATHIONE

Introduction:

Schistosome glutathione (GSH) levels declined after *in vivo* treatment with the antischistosomal drug, oltipraz, while the GSH content of various host tissues was observed to increase [26], suggesting the possibility that there may be chemotherapeutically vulnerable sites in the parasite relating to biochemical differences in GSH metabolism. The relationship of GSH levels to the maintenance of schistosome viability has not been examined, although GSH has been detected in relatively high concentrations in almost all cells of living organisms, including the schistosomes. Presence of the enzymes of the γ-glutamyl cycle which regulate GSH synthesis have been reported only for the cestode *Moniezia benedeni* [90]. Because of its reactive sulfhydryl group, GSH is involved in a large variety of chemical reactions. The GSH S-transferase family of enzymes utilize GSH to protect proteins and membranes against reactive electrophilic species by the formation of GSH adducts. GSH serves to protect cells against peroxides formed during oxidative metabolic processes in a GSH peroxidase catalyzed reaction [4]. Further, in addition to its role as cofactor for numerous reactions of cellular metabolism [48,81], GSH regulates the redox status of protein thiol groups, thus modulating various metabolic processes and membrane events [51,106], underscoring the importance of this tripeptide to both host and parasite. The enzymatic regulation of GSH by *S. mansoni* was investigated in this report.

Results:

Diamide effected a dose-dependent depletion of schistosome intracellular GSH (Figure 5). The production of malondialdehyde, however, was not observed after 1 h incubations of parasites in diamide at concentrations as high as 3 mM, even though the parasites were moribund at this concentration.

Male S. mansoni possess low levels of the enzyme γ-glutamyl transpeptidase. The parasite enzyme activity (68 µmoles product formed/min/mg protein) is of the same order as uninfected mouse liver tissue (59 µmoles product formed/min/mg protein). Uninfected mouse kidney cortex preparations exhibited greater activity, 331 µmoles product formed/min/mg protein. The pH optimum for parasite enzyme activity, pH 8.5, is the same as that observed for the host tissues.

Male parasites possess GSH S-transferase activity, with an optimum for catalytic activity at pH 8.0. The activity of the enzyme occurs with a slightly higher Km than host liver, but the reduced affinity for substrate is not due to inhibitory factors in the parasite cytosol, as the cytosolic fraction did not inhibit GTr kinetics (purified GTr from equine liver, Sigma), although specific activity did decline over time, suggesting the presence of proteases in the parasite supernatant. The GTr activities of male and female parasites and of uninfected mouse liver are shown in Table 2.

Figure 5: Dose-dependent depletion of schistosome glutathione by diamide. Twenty adult male schistosomes were incubated in 2.5 ml RPMI 1640 containing various concentrations of diamide, as indicated. Incubations were performed at 37°C, in a dark chamber for 1 h. Values represent two or three separate experiments with triplicate groups of 20 animals per group. Vertical lines are $\pm$ 1 S. E., Asterisk = $p < 0.05$.

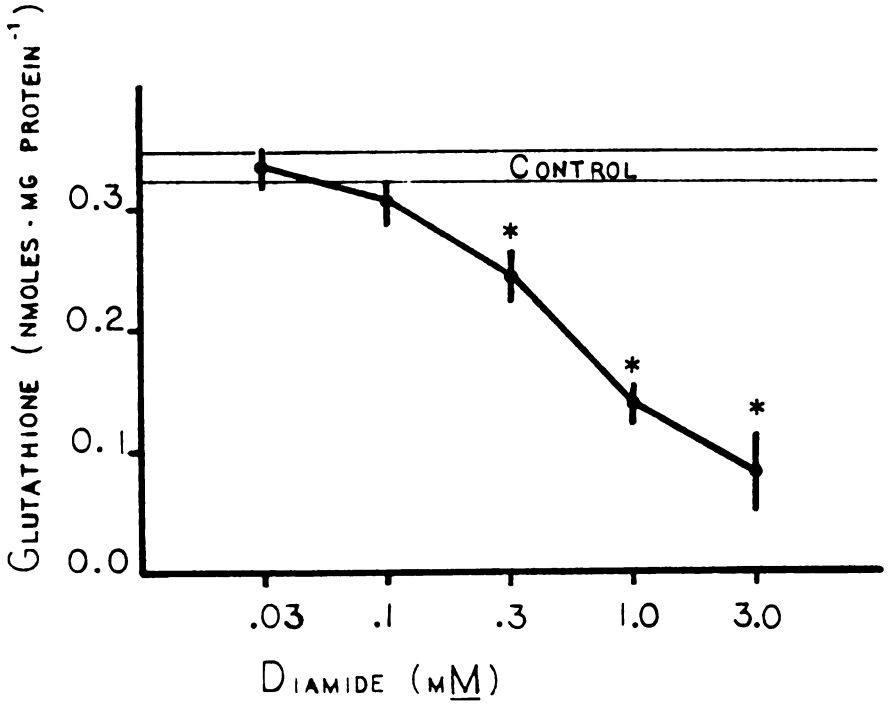

GLUTATHIONE (NMOLES · MG PROTEIN^{-1})
CONTROL
0.3
0.2
0.1
0.0
*
*
*
.03
.1
.3
1.0
3.0
DIAMIDE (mM)

Table 2: Glutathione S-transferase activities of Schistosoma mansoni and uninfected mouse liver tissue.

	Substrate	
	p-Nitrobenzyl Chloride	1-Chloro-2,4-Dinitrobenzene
Liver	199±26	1210±200
S. mansoni male	26± 7	526±169
S. mansoni female	9± 4 *	172±141 *

Glutathione S-transferase activity is expressed as nmoles product/min/mg protein, after correction for spontaneous nonenzymatic conjugation.

Male S. mansoni possess 10.3 ± 0.2 mU/mg protein GRed activity, which is 15.1% of mouse liver enzyme activity with NADPH as cofactor. With NADH, GRed activity was reduced 90% in both parasite and mouse liver tissue. The presence of inhibitory factors in the schistosome homogenate was considered unlikely, as boiled worm homogenate did not inhibit the activity of purified yeast GRed (obtained from Sigma).

Male schistosome GPx activity was not significantly above blank values with either the direct or indirect assay.

Discussion:

In this study, reduction of parasite GSH by diamide treatment in vitro to 30% of control did not yield a measurable increase in malondialdehyde equivalents. GSH depletion to a critical level, about 20% of control, was required before an increase in lipid peroxidation was observed in rat hepatocytes [136]. As the parasites were dead or dying at diamide concentrations effecting 60-70% GSH depletion, it would appear that diamide toxicity may not be dependent on membrane damage due to lipid fragmentation occurring as a result of peroxide attack. The toxicity observed for diamide may be attributed to nonspecific sulfhydryl oxidation [67]. Further, membrane proteins begin to form intrachain disulfides in cells in which GSH has been depleted by 60-70% [89], resulting in altered membrane properties [112,124].

The values obtained for γ-glutamyl transpeptidase activity from parasite supernatants may be misleading, since no ultrastructural studies on the localization of the enzyme were performed and the specific activity reported is for soluble protein from the entire animal. In general, higher enzyme activity is located on the external surface of cells which exhibit prolific secretory or absorptive functions. Therefore the enzyme may be localized to the tegument of the schistosome, which comprises only 1-2% of the total worm protein (unpublished results). As tissues are essentially impermeable to exogenous GSH without the transpeptidase [66], these results suggest that schistosomes may be capable of absorbing GSH from the host blood stream, possibly coupled with amino acid uptake by the classical γ-glutamyl cycle [97,99].

Schistosomes are capable of performing phase 2 conjugation reactions, suggesting that GTr activity may play a role in detoxication of chemotherapeutic agents. Female parasite activity at 30-40% of male GTr values is without explanation, however, other sex-related differences in the activities of schistosomal enzymes have been observed [37]. Further, the large amount of soluble protein released from the female digestive system during homogenization may have resulted in lower specific activities than are actually present intracellularly.

That schistosomal GRed activity was only 15% as active as mammalian liver tissue could indicate that the parasite is more at risk to oxidative damage when GSH levels are depleted. However, in light of the fact that S. mansoni are functional anaerobes [27], the low level of GRed activity is not surprising. The level of GRed would still be the primary determinant of the availability of reduced glutathione because GRed is considered to be the rate limiting factor in the redox cycling of GSH [104,125].

The results with the two pyridine nucleotide substrates on host and schistosomal GRed activity suggest that the enzyme is NADPH specific and that there may be one or more transhydrogenases present in the crude homogenate capable of transferring the reducing equivalent from NADH to $NADP^+$. The importance of functional mitochondria to the maintenance of control levels of reduced GSH in rat tissue was shown by Jocelyn [78], where although the reduction of GSH by diamide was mediated by GSH oxidation, GSH levels were maintained due to regeneration by NADH driven transhydrogenase and $NADP^+$-specific GRed. The functional status of schistosome mitochondria has not been clearly elucidated. The results obtained in the present study showing no lipid peroxidation in the presence of diamide suggest that the activities of parasite GRed and transhydrogenase reactions are adequate to the task of rapidly recycling GSH from oxidized GSH in the face of an oxidant stress.

That the lack of significant GPx activity in the male schistosome is compatible with parasite viability indicates that under physiologic conditions either the parasites are not exposed to peroxide stress or have evolved other mechanisms to minimize or repair peroxide-induced toxicity. Circulating concentrations of lipid peroxides in the blood stream are negligible, likely due to the high GPx activity reported for host red cells [17]. Endogenous peroxide formation is probably minimal in schistosomes as reports on parasite metabolism indicate a reliance on reductive or conjugative reactions for drug metabolism [41,42,43,103]. Also, schistosomes do not possess a functional cytochrome mixed function oxidase system [10], further minimizing the level of toxic insult to the parasite due to GPx deficiency. Furthermore, there is a nonenzymic decomposition of peroxide by GSH, proportional to GSH levels [100], which may provide adequate protection for the parasite from low levels of intracellularly generated peroxides.

A chemotherapeutic possibility, the inhibition of γ-glutamyl transpeptidase would prevent the normal extracellular breakdown of GSH and inhibit translocation of γ-glutamyl amino acids intracellularly. In mice treated with AT 125, an irreversible γ-glutamyl transpeptidase inhibitor, and in two human patients exhibiting congenital γ-GTP deficiency, glutathionemia and glutathionuria developed, with marked enhancement in the renal excretion of γ-glutamylcysteine, cysteine and cystine also being observed [60,63]. The toxicity of irreversible inhibitors of γ-GTP to the schistosome have yet to be studied. Therefore, while enzymatic differences between host and parasite were

observed in this report, the possibility for the rational design of a selective chemotherapeutic compound based on these differences is marginal.

CHAPTER TWO: ACUTE EFFECTS OF OLTIPRAZ AND ITS ANTAGONISM *IN VITRO*

Introduction:

Bueding *et al.* [26] observed a depression of parasite glutathione (GSH) content as one of the earliest biochemical changes after administration of oltipraz *in vivo*. As the reduction in parasite GSH was accompanied by an increase in host tissue levels of GSH, the possibility for the rational design of a drug selectively toxic to the parasite based on interference with parasite glutathione metabolism or synthesis suggests itself. To further define the relationship between the antischistosomal activity of oltipraz and parasite GSH levels, the acute effects of oltipraz on various physiological parameters of *S. mansoni in vivo* and *in vitro* are presented.

Results:

In vivo, oltipraz (250 mg/kg), effected a significant reduction in male parasite, host liver and host renal cortex GSH levels by 1 h, with maximum depression at 1 h for host tissues and 3 h for parasite tissue (Figure 6). GSH levels of the host rebound to or above control levels at 6 h. However, parasite GSH levels remained significantly depressed at that time. *In vitro*, oltipraz (10 μM) significantly depressed schistosome GSH levels by 1 h, (Figure 7) an effect not observed when parasites were coincubated in GSH (1 mM), cysteine (1 mM) or methionine (1 mM). Neither DTT nor 2-mercaptoethanol (100 μM or 1 mM) blocked the oltipraz-induced depression of schistosome GSH *in vitro* (Figure 8).

Figure 6: Oltipraz effect on glutathione content in infected mouse hepatic and renal cortical tissue and in male schistosomes *in vivo*. Infected mice were dosed with 250 mg/kg oltipraz in peanut oil by gavage. Mice were allowed food and water *ad lib*. Worms from 6 mice were pooled and GSH was assayed from groups of approx. 40 males [●]. Tissue slices of host liver [■] or kidney cortex slices [◆] were pooled and 5 groups of approx. 100 mg (wet weight) tissue were assayed for GSH; Asterisk = $p<0.05$ from time 0 levels.

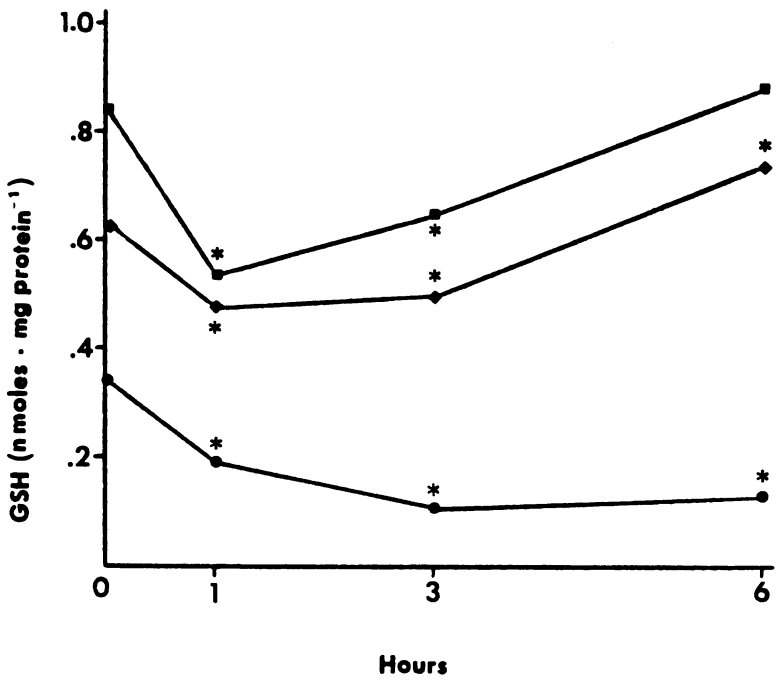

1.0
.8
.6
.4
.2
GSH (n moles · mg protein⁻¹)
0
1
3
6
Hours

Figure 7: Dose-dependent depletion of schistosomal glutathione by oltipraz. Twenty adult male schistosomes were incubated in 2.5 ml RPMI containing various concentrations of oltipraz, as indicated. Incubations were performed at 37°C, in a dark chamber, for 1 h. Values represent two or three separate experiments with triplicate groups of 20 animals per group. Vertical lines are $\pm$ 1 S.E., Asterisk = $p < 0.05$.

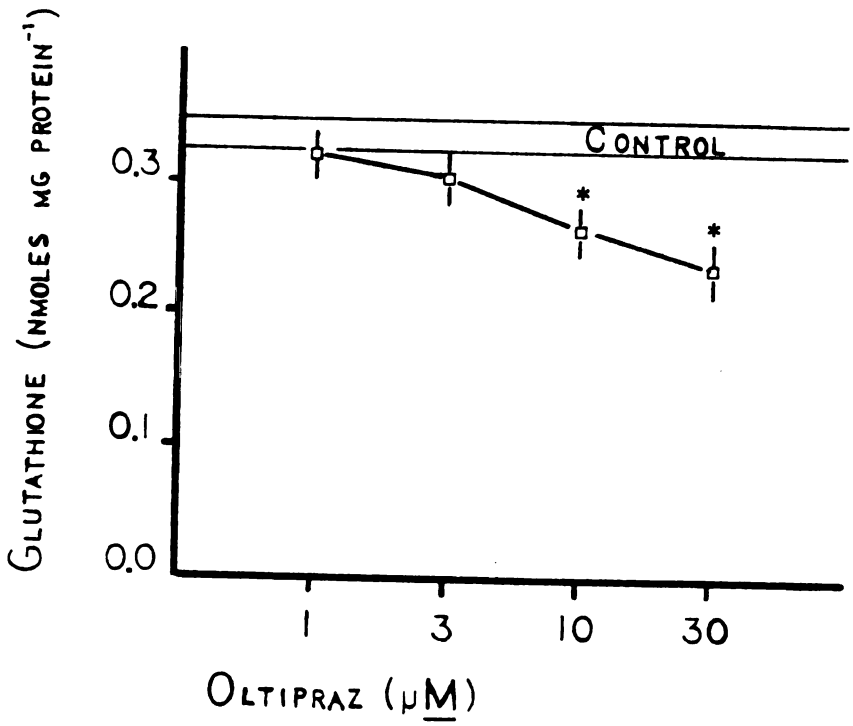
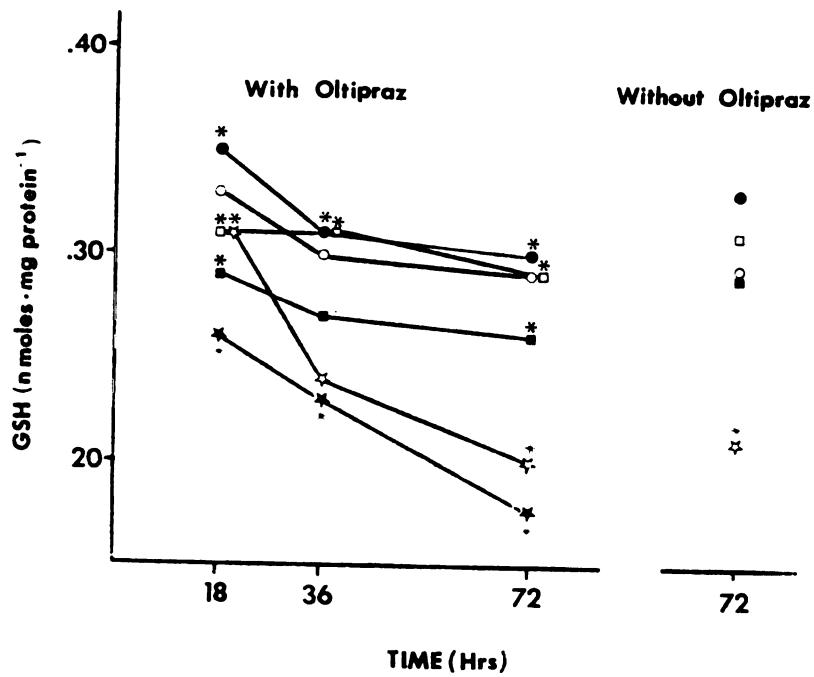

GLUTATHIONE (NMOLES MG PROTEIN^{-1})
0.3
0.2
0.1
0.0
CONTROL
*
*
1
3
10
30
OLTIPRAZ (μM)

Figure 8: Effect of oltipraz and various compounds on schistosome glutathione levels *in vitro*. Paired schistosomes were incubated in 50 ml HS/RPMI at 37°C, in a dark chamber with continuous mechanical agitation. [*] = $p < 0.05$ from control value at the same time point (without oltipraz control). [*] = $p < 0.05$ from oltipraz value at the same time point. + oltipraz is schistosomes incubated in 1 μM oltipraz. [●] is glutathione, reduced form; [■] is cysteine (1 mM); [□] is methionine (1 mM); [☆] is dithiothreitol (100 μM) and [★] is oltipraz (1 μM). Values represent means of triplicate samples of males which were separated from female parasites at the end of the experiment.


.40
.30
20
GSH (n moles · mg protein⁻¹)
With Oltipraz
Without Oltipraz
18
36
72
72
TIME (Hrs)

Surprisingly, oltipraz (1 μM) lowered parasite GSH levels only in an aerobic environment. 10 μM oltipraz produced a more pronounced effect, but again, only under aerobic conditions (Figure 9).

By one h, the uptake of [^{35}S]cysteine or [^{35}S]cystine by male schistosomes was significantly inhibited (41% and 36% of control, respectively) during *in vitro* incubations in the presence of 5 μM oltipraz (Figure 10). [^{35}S]GSH formation occurred in the presence of 5 μM oltipraz, and although less label was incorporated, the relative distribution of either labelled precursor was minimally affected. There were no significant differences between the cysteine and cystine controls.

[^{14}C]glucose uptake was significantly reduced by 5 μM oltipraz only at 60 min (Figure 11). Neither the oxy derivative of oltipraz (100 μM) nor cysteine (1 mM) had any effect on labelled glucose uptake.

The amplitude and frequency of endogenous electrial transients in male *S. mansoni* were profoundly depressed after *in vivo* exposure to oltipraz, even at the low dose of 125 mg/kg. The onset of significant depression occurred by 6 h, with the high amplitude (>40 μV) potentials exhibiting a higher level of sensitivity (Figure 12). Schistosomes exposed to 3.5 μM oltipraz *in vitro* exhibited a similar level of depression to the drug, with high amplitude electrical activity being significantly depressed after incubations as brief as 1 h. The profound depression of surface electrical activity observed after 24 h incubations in 10 μM oltipraz was not observed when the parasites were

Figure 9: Oltipraz effect on schistosomal glutathione levels *in vitro*: Aerobic vs anaerobic environment. Paired schistosomes were incubated as described in Figure 8 in either an aerobic or anaerobic environment, with or without oltipraz, as indicated. For anaerobic experiments, N_2 first bubbled through an oxygen trap occupied the gas phase in the flasks. Values represent the means of triplicate samples of males separated at the end of the experiment. [*] = $p < 0.05$ from control value at the time period specified, [*] = $p < 0.05$ anaerobic value significantly different from corresponding aerobic value. Vertical lines are 1 S.E.

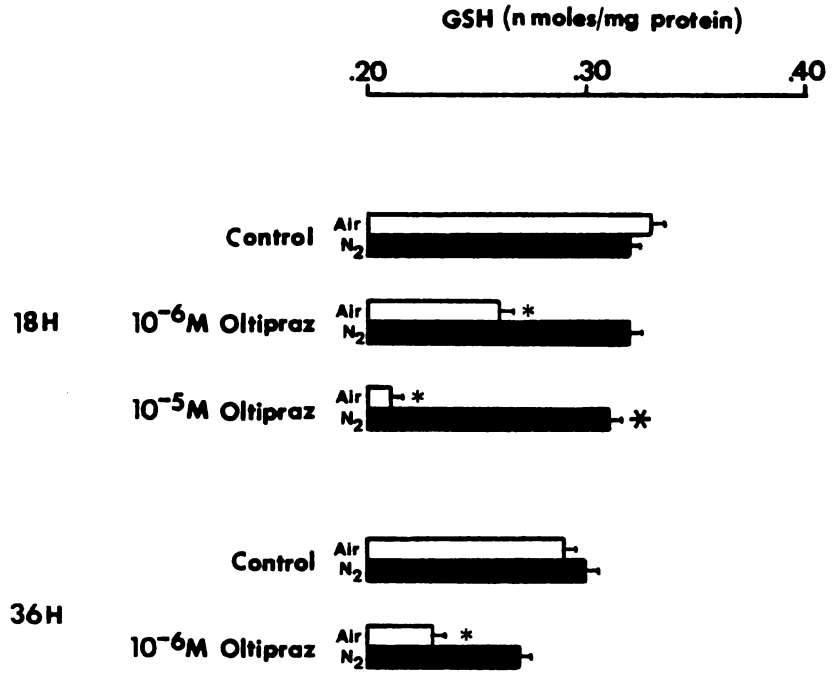

GSH (n moles/mg protein)
.20
.30
.40
Control
Air
N2
18H
10-6M Oltipraz
10-5M Oltipraz
36H
*

Figure 10: [^{35}S]Cysteine uptake and incorporation into schistosomes *in vitro*. Male schistosomes were incubated in 2.5 ml RPMI 1640 containing 3 x 10^5 dpm [^{35}S]cysteine per well at 37°C, in the dark, in the presence of 5 μM oltipraz (solid symbols) or vehicle only (empty symbols). [o or ●] represent total uptake (disintegrations/min/male) of [^{35}S]cysteine, solid line; [□ or ■] represent the relative percentage of total ^{35}S as [^{35}S]GSH and oxidized [^{35}S]GSH, broken line. 10 males per well, values represent means of 4 wells per data point; Asterisk = statistically different ($p<0.05$) from control uptake. Relative distribution of label, once assimilated by the parasite, into [^{35}S]GSH and oxidized [^{35}S]GSH was not statistically different from control.

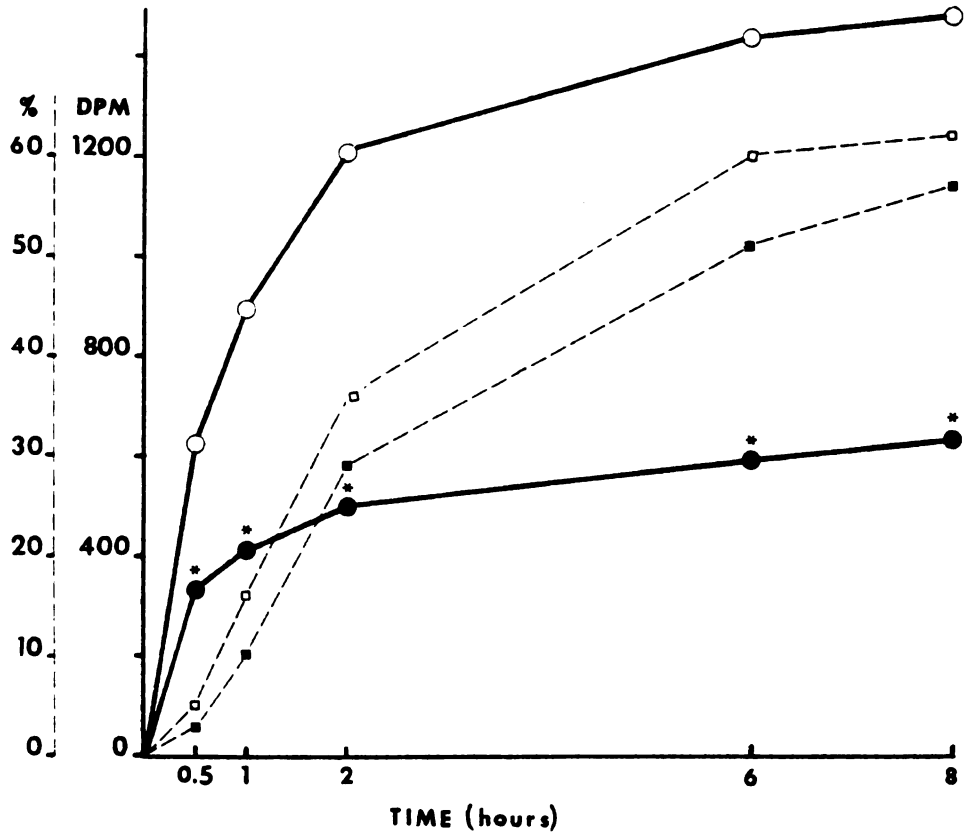

%
DPM
60
1200
50
40
800
30
20
400
10
0
0
0.5
1
2
6
8
TIME (hours)

Figure 11: [^{14}C]Glucose uptake into schistosomes *in vitro*. Male schistosomes were incubated as in Figure 10, except wells contained 3 x 10^5 dpm [^{14}C]glucose per well. [o] represents untreated males, [■] represents oltipraz-treated animals (5 μM), [□] represents parasites treated with the oxy analog of oltipraz (50 μM) and [●] represents male schistosomes coincubated in 5 μM oltipraz and 1 mM cysteine. Values represent means of 4 wells per data point; Asterisk = $p<0.05$.

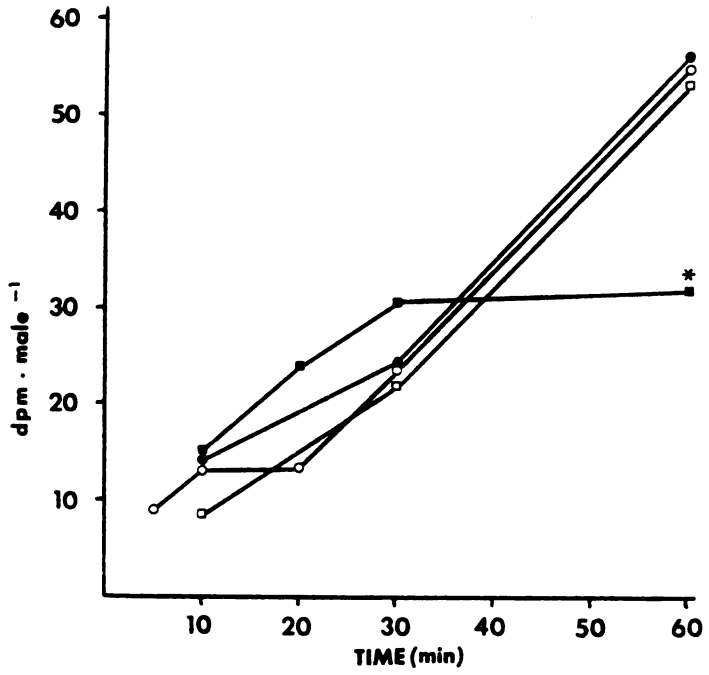
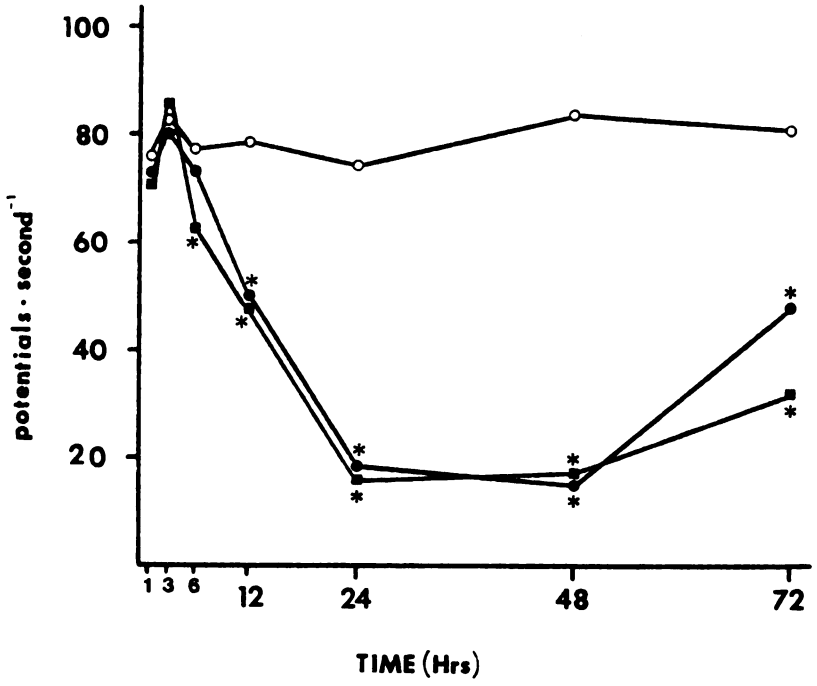

60
50
40
30
20
10
dpm · male⁻¹
10
20
30
40
50
60
TIME (min)
*

Figure 12: Surface electrical activity of male S. mansoni in vivo: Oltipraz effect. Mice (55 days p.i.) were dosed by gavage with 125 mg/kg oltipraz [●], 250 mg/kg oltipraz [■] or peanut oil vehicle [o] and sacrificed at the times indicated. Values presented are the means of 6 to 8 animals analyzed per time point per test dose; vertical lines are 1 S.E. Asterisk = $p<0.05$.


100
80
60
40
20
potentials · second⁻¹
1 3 6
12
24
48
72
TIME (Hrs)

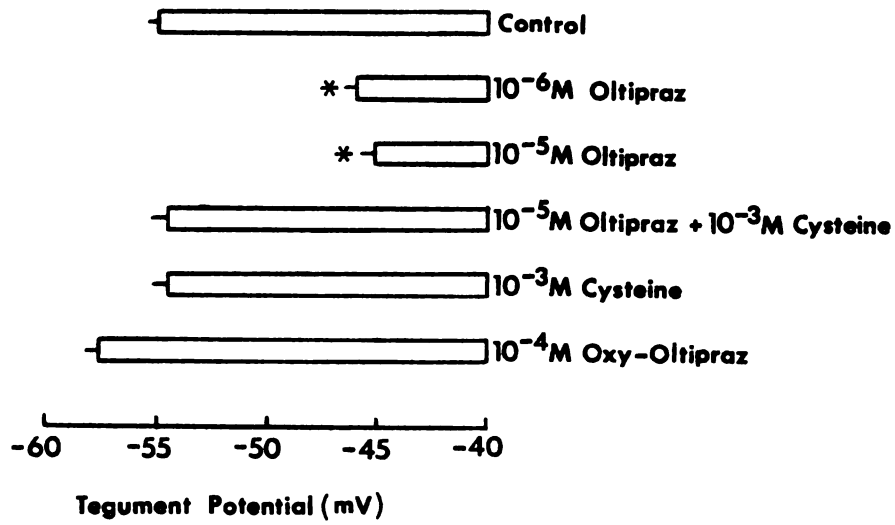
coincubated in 1 mM cysteine or 1 mM glutathione, but not by 1 mM or 100 µM 2-mercaptoethanol. The oxy analog of oltipraz (100 µM) did not alter parasite surface electrical activity from control levels.

Tegument potentials recorded from male schistosomes were significantly depolarized by 1 µM oltipraz following 18 h *in vitro* incubation. This effect was prevented by the addition of 1 mM cysteine to the incubation medium, whereas cysteine by itself did not alter the tegument potential, nor did the oxy analog of oltipraz (Figure 13).

Discussion:

Bueding *et al*. [26] observed that *in vivo* treatment of *S. mansoni* with a 250 mg/kg dose of oltipraz resulted in a depression of parasite GSH levels to roughly 40% of control over the first week after treatment. In the present study, depletion of GSH was observed by 1 h in both parasite and host tissues at a dosage of 250 mg/kg *per os*. The fact that GSH levels in parasite tissue were not rapidly restored to control levels, as was seen in host tissues, indicates that there may be important differences in the biochemical regulation of GSH between the parasite and host. The present results are not in conflict with those of Bueding *et al* which showed depressed parasite GSH and elevated host tissue GSH after oltipraz treatment *in vivo*, as their experiments did not include measurement of the acute response of host tissue or parasite GSH levels to oltipraz. A similar depletion of parasite GSH levels in the presence of oltipraz was observed *in vitro*, allowing the acute actions of oltipraz on the parasite to be studied independent of

Figure 13: Tegument potential of male schistosomes *in vitro*: Effect of Oltipraz and antagonism of effect by cysteine. Male schistosomes were incubated in HS/RPMI containing Oltipraz or oltipraz plus cysteine as indicated, or in the presence of 100 μM oxy-oltipraz, for 12 h at 37 °C, in the dark. Values presented are the means of 5 animals per tegumental potential average, vertical lines are 1 S.E.


Control
* 10⁻⁶M Oltipraz
* 10⁻⁵M Oltipraz
10⁻⁵M Oltipraz + 10⁻³M Cysteine
10⁻³M Cysteine
10⁻⁴M Oxy-Oltipraz
-60
-55
-50
-45
-40
Tegument Potential (mV)

the variables of host reaction to the drug.

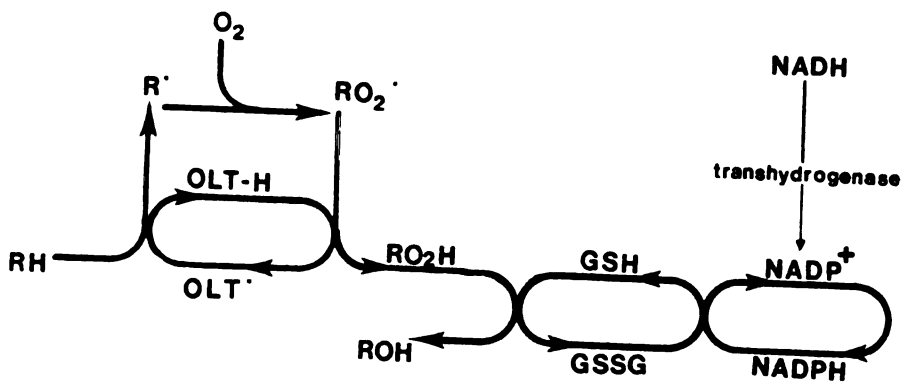
Bueding et al. speculated that the chemotherapeutic activity of oltipraz may be due to competition of the drug with γ-glutamyl cysteine, a precursor of GSH synthesis. By this mechanism, the non-stoichiometric depletion of GSH could occur at a rate dependent on the degree of inhibition of synthesis and the turnover rate for parasitic intracellular GSH. The results of the present study suggest that there may be an alternative explanation for the effect of oltipraz on GSH levels. First, the inhibition of cysteine uptake by 5 μM oltipraz corroborates results by Seed (personal communication) that uptake of both cysteine and glutathione, but not other amino acids, is retarded by oltipraz. Secondly, male schistosomes were able to incorporate [^{35}S]cysteine label into the glutathione pool, as GSH and oxidized GSH, in the presence of 10 μM oltipraz, at a relative percent not significantly different from control. Therefore, under the conditions of acute exposure, it is unlikely that GSH synthesis is being directly inhibited by oltipraz.

That the oxy derivative of oltipraz, which has negligible antischistosomal effect, did not affect the uptake of [^{35}S]cysteine into the parasites, even at 50 μM, suggests that the thione group of the drug molecule is a necessary participant in the oltipraz-induced effects on transport mechanisms.

In the present study, GSH (1 mM), cysteine (1 mM) and methionine (1 mM), but not 1 mM or 100 μM DTT or 2-mercaptoethanol, were able to block the *in vitro* effect of oltipraz on GSH levels occurring during incubation. The antagonism of oltipraz effects by GSH or GSH precursors may be mediated through a process of mass action on the replenishment of GSH stores. DTT and 2-mercaptoethanol, unlike the other compounds tested, are incapable of participating in the production of GSH, which may explain their inability to antagonize the effects of oltipraz. Alternatively, the inactivity of the oxy derivative of oltipraz and the rather stringent structural requirements for the maintenance of antischistosomal activity of a series of oltipraz congeners [26] suggest that oltipraz may influence a membrane receptor, possibly functioning as a dipeptidase or transport carrier molecule for amino acids.

The observation that oltipraz lowered parasite GSH levels only when the parasites were incubated in an aerobic environment indicates that it is probably an oxidative reaction that the GSH is participating in. Figure 14 illustrates a hypothetical scheme which could explain the oxygen-dependency of oltipraz effect on schistosome GSH levels. If oltipraz cycles a free radical, then organic components of the membrane could react with oltipraz to form an organic radical. In an oxygen-dependent reaction, an oxide radical is created which donates the radical back to oltipraz with the abstraction of a hydrogen, forming an organic peroxide. While schistosomes possess negligible GPx activity, GSH detoxication of lipid peroxides occurs nonenzymatically [100]. Similar radical cycling has been suggested for nitrofurans

Figure 14: Hypothetical reaction sequence to explain oxygen-dependency of oltipraz-induced depletion of schistosomal GSH *in vitro*. R represents an organic (presumably a lipid) component of the membrane, OLT refers to oltipraz. GSH and GSSG represents glutathione reduced and oxidized, respectively.



HYPOTHETICAL REACTION SEQUENCE RELATING

OLTIPRAZ TO GLUTATHIONE OXIDATION

[19], adriamycin and daunomycin [52] and paraquat [80].

Oltipraz-induced decrease in glucose uptake may be an indirect consequence of the drug's inhibitory effect on parasite motor activity. This is supported by the observation that high amplitude electrical potentials, which appear to represent summed electrical activity originating from schistosome muscle tissue [122], are reduced in frequency by the drug. Thiol interference by oltipraz could also result in the inhibition of glucose utilization because of the wide range of enzymes that require a thiol group as cofactor for their catalytic activities [52].

The fact that tegumental morphology is not affected by *in vitro* treatment of the parasites with 10 μM oltipraz for 12 h [23] indicates that structural damage to the tegument is not a contributory factor in the induction of acute oltipraz effects on membrane transport. While the structural integrity of the outer tegumental membrane was maintained, microelectrode recordings showing depolarization of the tegument suggest that a significant redistribution of ions across the tegumental membrane had occurred. The effect(s) of a drug-induced ionic imbalance on the Na-driven carrier mediated cotransport of glucose [131] may have contributed to the decrease in glucose uptake observed. In a similar manner, perturbations of ionic equilibria across the tegument may contribute to the inhibition by oltipraz of the uptake of GSH precursors that was observed. Consistent with the effects on other parameters measured, tegument potentials of worms incubated in 1 mM cysteine or 10 μM oltipraz plus 1 mM cysteine were

not significantly different from control values. These results indicate that cysteine is able to prevent oltipraz-induced ionic imbalances *in vitro* without directly affecting ionic fluxes across the membrane. The fact that the oxy derivative of oltipraz did not affect the tegumental membrane potential further suggests that a disruption of membrane conductance may contribute to the antischistosomal efficacy of oltipraz.

Oltipraz is a very lipophilic compound which is rapidly accumulated by the membranes of the parasite, suggesting that there is a hydrophobic nature to the site of drug action. Intercalation of drug into the tegument and muscle membranes may partially explain the effects of oltipraz on tegument potential and surface electrical activity, in a manner reflecting the correlation between the solubility of drugs in membranes and their ability to fluidize and disorder membrane structure [110,121].

From a therapeutic perspective, the report by Ali *et al.* [3] which showed that coadministration of cysteine and oltipraz to monkeys resulted in an enhanced bioavailability of the drug, suggests that the *in vivo* synergistic action of cysteine on oltipraz schistosomicidal effects may be due to this enhancement of oltipraz bioavailability. As the synergistic interaction of cysteine with oltipraz may be indirect and removed from the site of drug action, the *in vitro* evidence presented which show cysteine antagonistic to oltipraz action does not refute the *in vivo* observations. Instead, these findings suggest that although cysteine, GSH or methionine ameliorate oltipraz action acutely

when applied directly to the parasite, in the long-term *in vivo* situation, the enhancement of oltipraz bioavailability is more relevant clinically in the treatment of the disease.

CHAPTER THREE: RELATIONSHIP OF SCHISTOSOME GLUTATHIONE LEVELS TO VARIOUS PHYSIOLOGIC PARAMETERS OF THE PARASITE

Introduction:

Schistosome GSH levels were manipulated by various compounds in order to determine dose-dependency of acute effect and to determine if these effects were also antagonized by cysteine, methionine and/or glutathione in like fashion to the antagonism of oltipraz-induced GSH depression in the parasites.

Results: BCNU, an alkylating agent which inactivates GRed [49], effected a dose-dependent depletion of parasite GSH levels after 3 h incubation (Figure 15). Microelectrode recordings of schistosome tegumental potential revealed that ionic conductance is also altered by BCNU in a dose-dependent manner, an effect which is partially reversed by coincubation of the parasites in 1 mM cysteine (Figure 16). Tegumental potentials for 1 mM cysteine-treated control, for 1 mM cysteine plus 3 mM BCNU-treated parasites, and for 3 mM BCNU-treated animals were statistically different from each of the other groups ($P < 0.01$).

Figure 15: Dose-dependent depletion of schistosomal glutathione by BCNU. Twenty adult male schistosomes were incubated in 2.5 ml RPMI containing various concentrations of BCNU, as indicated. Incubations were performed at 37°C, in a dark chamber, for 3 h. Values represent two or three separate experiments with triplicate groups of 20 animals per group. Vertical lines are $\pm$ 1 S.E., Asterisk = $p < 0.05$.

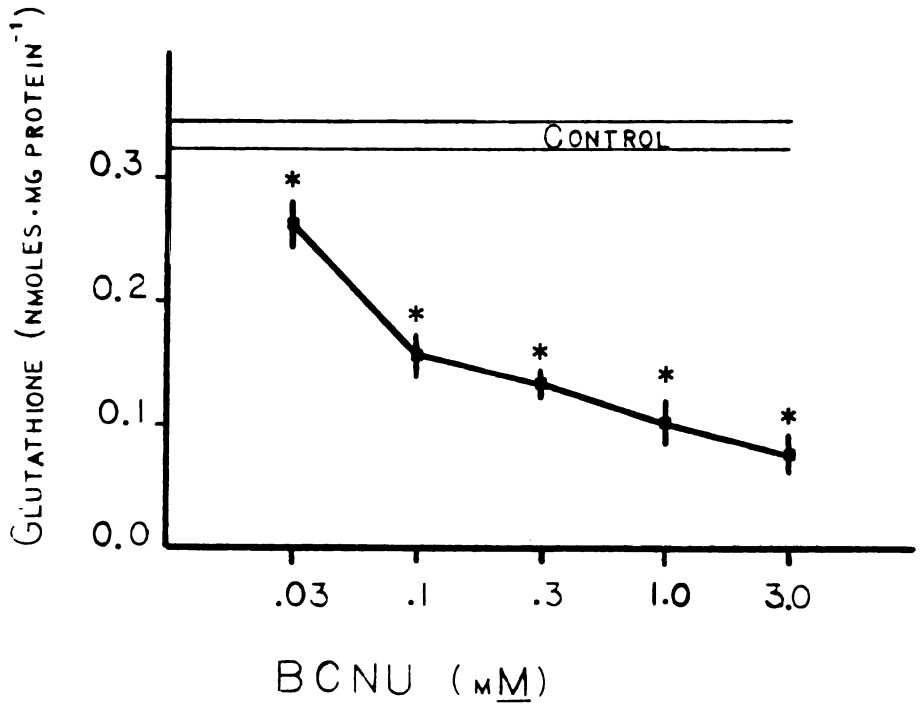
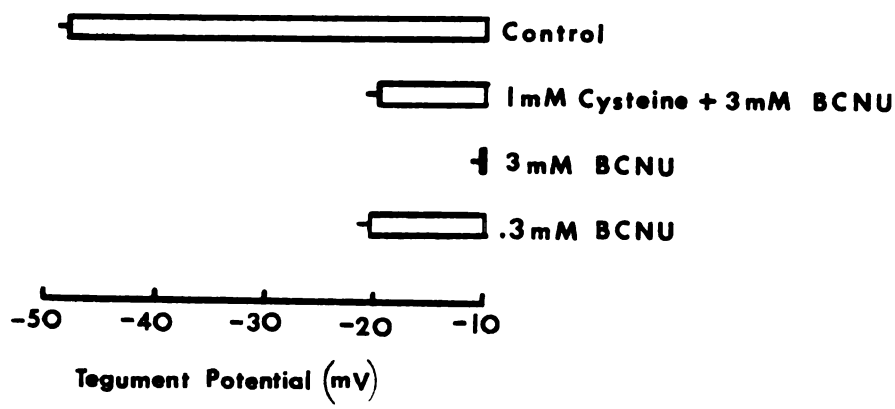

GLUTATHIONE (NMOLES·MG PROTEIN^{-1})
CONTROL
0.3
0.2
0.1
0.0
.03
.1
.3
1.0
3.0
BCNU (mM)

Figure 16: Tegument potential of male schistosomes *in vitro*: Effect of BCNU and antagonism of effect by cysteine. Male schistosomes were incubated in HS/RPMI containing BCNU or BCNU plus cysteine as indicated, for 12 h at 37 °C, in the dark. Values presented are the means of 5 animals per tegumental potential average, vertical lines are 1 S.E.


Control
1mM Cysteine + 3mM BCNU
3mM BCNU
.3mM BCNU
-50
-40
-30
-20
-10
Tegument Potential (mV)

BSO, an inhibitor of γ-glutamyl cysteine synthetase [60], produced a dose-dependent depletion of GSH in male S. mansoni. The decline in GSH levels (Figure 17) was less dramatic than results obtained with diamide or BCNU and required a longer incubation period. No acute differences from control were observed in longitudinal muscle tension development over the first 30 min of exposure to 1 mM BSO. BSO at 100 μM for 18 h produced a significant depression of high ampliture (> 40 μV) electrical potentials recorded from the surface of male parasites (93 ± 47 potentials BSO-treated, 253 ± 81 potentials control). Coincubation in the presence of 1 mM cysteine inhibited BSO-induced depression of electrical activity (262 ± 92 potentials). Surprisingly, BSO-treatment at 1 mM resulted in egg counts not different from controls over the 72 h period, although GSH levels for the paired worms were depressed 30-40% from control levels at 72 h. Oltipraz effects on schistosome fecundity in vitro are shown in Figure 18. The antagonism of oltipraz effects by various compounds in the same assay is shown in Figure 19.

Discussion:

BCNU irreversibly inactivates GRed. This inhibition is selective as BCNU does not inhibit either the enzymes of glucose catabolism or GPx activity [49]. The prevention by cysteine of GRed inactivation by BCNU [49] may explain the antagonism of BCNU-induced depression of schistosomal tegumental potentials observed in this study, alternatively, as cysteine feeds into GSH synthesis, these data are also supportive of the hypothetical reaction sequence shown in Figure

Figure 17: Dose-dependent depletion of schistosomal glutathione by BSO. Twenty adult male schistosomes were incubated in 2.5 ml RPMI containing various concentrations of BSO, as indicated. Incubations were performed at 37°C, in a dark chamber, for 6 h. Values represent two or three separate experiments with triplicate groups of 20 animals per group. Vertical lines are $\pm$ 1 S.E., Asterisk = $p < 0.05$.

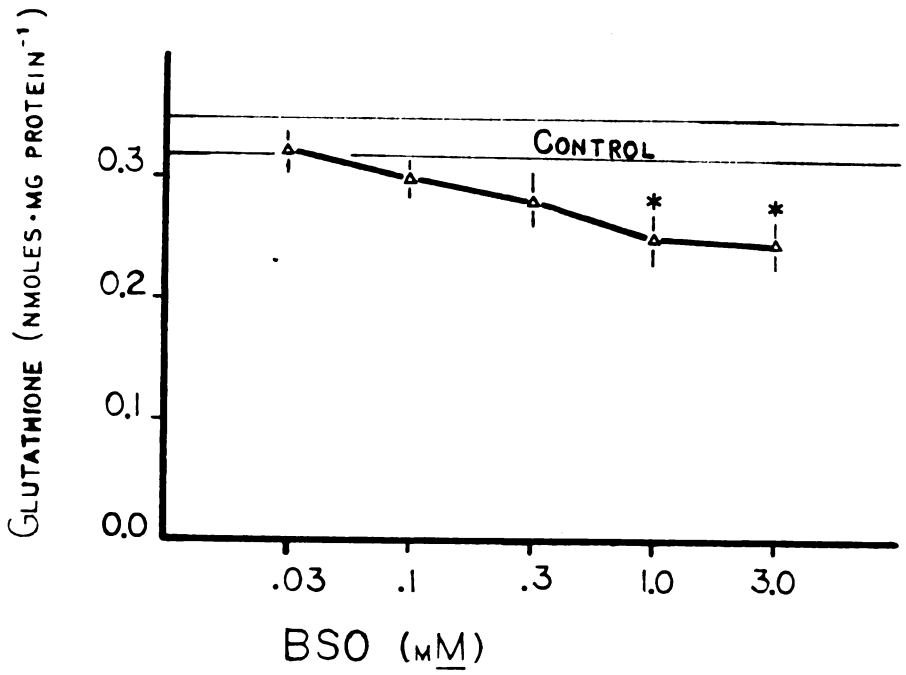

GLUTATHIONE (NMOLES·MG PROTEIN⁻¹)
0.3
0.2
0.1
0.0
CONTROL
*
*
.03
.1
.3
1.0
3.0
BSO (mM)

Figure 18: Oltipraz inhibition of schistosome fecundity *in vitro*. Paired schistosomes (15 pair per 50 ml HS/RPMI in 250 ml Erlenmeyer flasks) were incubated 72 h at 37°C in the presence or absence of oltipraz at the concentrations indicated. Asterisk = $p < 0.05$ from control. Values presented are the means of 5 to 7 flasks run at each concentration; vertical lines are 1 S.E.

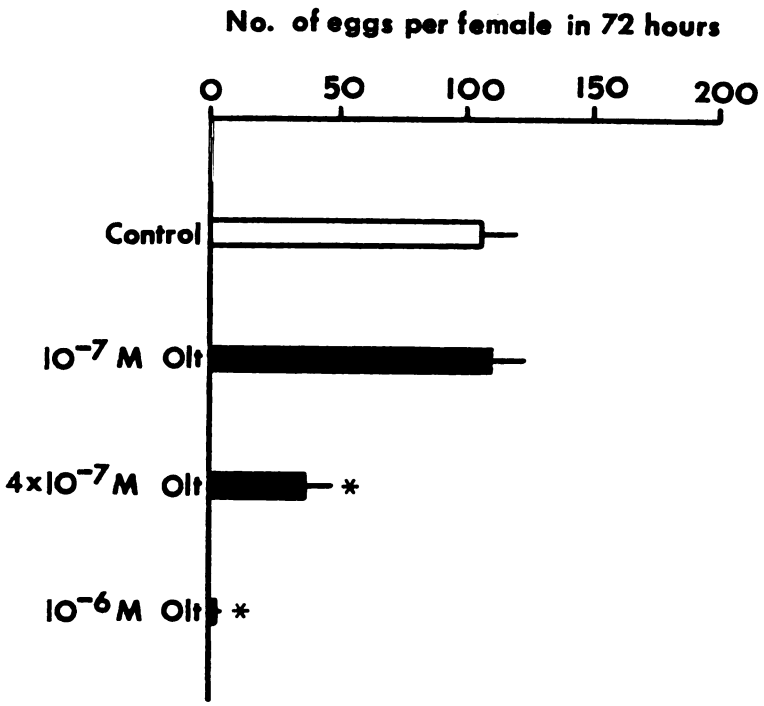
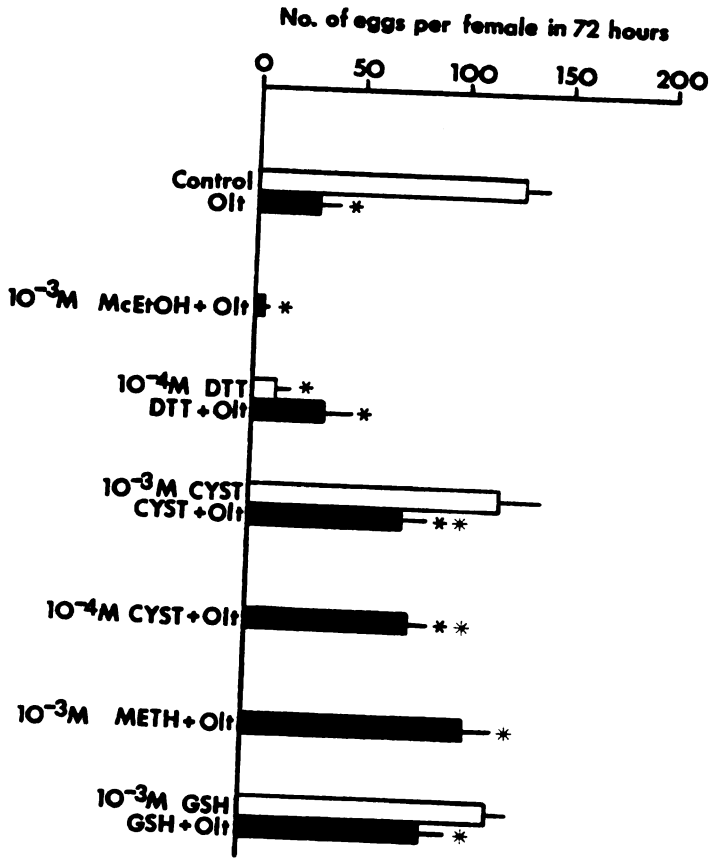

No. of eggs per female in 72 hours
0
50
100
150
200
Control
10⁻⁷ M Olt
4x10⁻⁷M Olt
*
10⁻⁶M Olt
*

Figure 19: Effect of various reagents on oltipraz inhibition of schistosome fecundity *in vitro*. Paired schistosomes were incubated as described in Figure 18. +OLT indicates coincubation of parasites in 0.4 μM oltipraz, McEtOH is 2- mercaptoethanol, DTT is dithiothreitol (Cleland's reagent), CYST is cysteine, METH is methionine and GSH is glutathione, reduced form. [*] = $p < 0.05$ from control, [*] = $p < 0.05$ from oltipraz only, i.e. statistically significant prevention of oltipraz effect. Values presented are the means of 5 to 7 flasks run at each concentration; vertical lines are 1 S.E.


No. of eggs per female in 72 hours
0
50
100
150
200
Control
Olt
10⁻³M McEtOH + Olt
10⁻⁴M DTT
DTT + Olt
10⁻³M CYST
CYST + Olt
10⁻⁴M CYST + Olt
10⁻³M METH + Olt
10⁻³M GSH
GSH + Olt

14.

Despite the toxic effects of BCNU on the schistosome, there is little chemotherapeutic potential for this particular compound. BCNU is an anticancer agent which exhibits mammalian toxicity due to alkylation of biological macromolecules. Thus, while BCNU may selectively jeopardize the schistosome because of its absence of GPx activity, the carcinogenic potential of this drug would limit its usefulness. As BCNU appears to be able to inactivate GRed without metabolic activation, its mechanism of action may not be related to alkylation, hence there may be potential for a BCNU-like compound if the enzymic inhibition properties can be dissociated from the alkylation potential inherent in BCNU.

BSO requires intracellular activation to BSO-phosphate before inhibition of γ-glutamylcysteine synthetase occurs. If the plateauing of GSH depletion at 6 h due to BSO concentrations greater that 1 mM indicates near total inhibition of the enzyme, the slow rate of GSH decline suggests that the turnover rate *in vitro* is on the order of 12.5 h. Turnover rates in the literature vary from estimations of Ø.5 h for rat kidney to 65-96 h for mammalian erythrocytes. Tissue culture and tumor cells exhibit biological half-life for GSH in the range of 6-8 h [87].

Methionine, cysteine and GSH were observed to provide significant ameliorative action on egg laying inhibition due to oltipraz. Both methionine and cystein e may be used for the intracellular enzymatic synthesis of GSH, which may aid in the maintenance of GSH levels, whereas DTT and 2-mercaptoethanol do not contribute to the synthesis of GSH. The poor correlation between GSH levels and fecundity was unexpected, however it may be an artifact of the technique employed, as worms from several flasks were pooled in order to assay for GSH. Alternatively, in vitro culture dramatically affects oviposition in effecting a lower output of eggs, especially during the first 24 h period, when the worm pairs are "in shock" while they adjust to the new media and nonphysiologic conditions [unpublished observations]. As many factors may be affecting the reproductive system which do not influence GSH levels, the amelioration of oltipraz effects on schistosome reproduction may be merely a qualitative finding.

CHAPTER FOUR: EFFECTS OF SCHISTOSOMAL INFECTION ON HOST REGULATION OF GLUTATHIONE LEVELS

Introduction:

The cause of the major disease syndromes of schistosomiasis is the retention of a large number of eggs in host tissue [134]. Most of the eggs freed into the circulation are sieved out by the liver where host granulomatous inflammatory response to the eggs results in a presinusoidal blockage of portal blood flow, as well as hepatic necrosis and subsequent fibrosis.

Monoamine oxidase activity, a sensitive indicator of liver fibrosis [72], is significantly depressed in mice by the 6th week following infection with S. mansoni [1]. There is also a marked depression of microsomal drug-metabolizing enzyme activities of the host liver. A close correlation exists between the onset and degree of enzymic depression with the severity and age of infection [32,33]. In the metabolism of xenobiotics, the tripeptide glutathione (GSH) plays an important role in chemical detoxication processes [52]. Experimental GSH depletion exacerbates toxicity due to protein alkylation or lipid peroxidation caused by chemically reactive, electrophilic chemicals or metabolites [4,77]. Glutathione peroxidase (GPx) is an integral part of the cellular antioxidative system [36], reducing peroxides in a reaction with GSH, which becomes oxidized in the process. Glutathione reductase (GRed) reverts oxidized GSH to its reduced state at the expense of NADPH. The glutathione S-transferase

(GTr) family of enzymes removes potentially cytotoxic alkylating agents through conjugation with GSH [21,84]. GTr also have GPx activity [113] which suggests a secondary role in detoxication of the by-products of oxygen utilization.

As the toxicity of antiparasitic drugs to the host may limit the therapeutic value of these compounds, changes in the ability of the host to detoxify xenobiotics due to the presence of parasitic infections are of considerable importance.

Results:

GSH levels in host liver decline during the course of the infection, while kidney cortex levels remain constant (Figure 20). Hepatic GSH is statistically depressed from uninfected liver values by day 50.

Hepatic enzyme activities are summarized in Table 3. The GRed activity of infected mouse liver was not significantly different from uninfected controls at 50-60 days, however GPx enzymic activity was depressed to 37% of uninfected levels. Hepatic GTr from infected mice exhibited 26% of uninfected liver activity. Neither renal GPx nor GRed enzymic activities were altered due to schistosomal infection. Kidney cortex was not examined for GTr activity.

Figure 20: Glutathione levels in host hepatic and renal cortical tissues during the course of Schistosoma mansoni infection. Means of triplicate samples of organs where the host harbored 15-30 pairs of parasites, n=5 animals per date, [●] represents hepatic GSH, [■] represents renal cortical GSH. Asterisk = statistically different ($p<0.05$) from uninfected control values at the time period specified, vertical lines are 1 S.E.

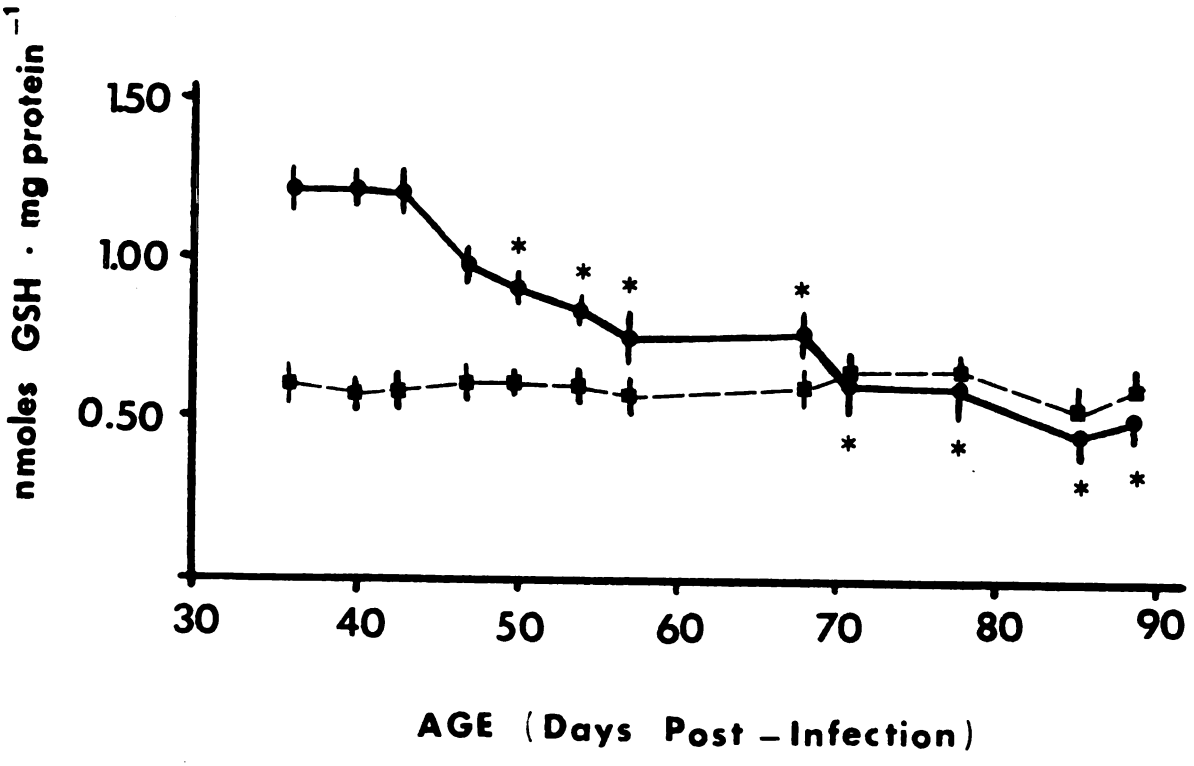

nmoles GSH · mg protein⁻¹
1.50
1.00
0.50
30
40
50
60
70
80
90
AGE (Days Post – Infection)

Table 3: Mouse hepatic glutathione-related enzyme activity: Effect of Schistosoma mansoni infection.

	Hepatic enzyme activity		
	GPx [1]	GRed [2]	GTr [3]
Uninfected liver	440±43	66±7	199±26
Infected liver	163±39 *	62±11 ns	52±22 *

[1] - Glutathione peroxidase activity, with t-butyl hydroperoxide as substrate, is expressed as nmoles NADPH oxidized/min/mg protein.

[2] - A unit of glutathione reductase activity represents the oxidation of 1 µmole NADPH/min/mg protein, results are expressed as mU.

[3] - Glutathione S-transferase activity is expressed as nmoles product per min/mg protein, after correction for spontaneous nonenzymatic conjugation, p-nitrobenzyl chloride was the substrate.

* - Statistically different ($p<0.05$) from uninfected liver value, ns= not significant as determined by Student's t-test.

Discussion:

The relative contribution of GSH depletion or the depression of hepatic enzyme activity to the hepatopathology of schistosomiasis could depend, in part, on the redundancy of the glutathione system. A functional reserve capacity for GPx [127] and GRed [49] has been shown in mice. Further, as the GTr are a family of enzymes with overlapping substrate specificities, and constitute 5-10% of the liver cytosol protein [25], a functional reserve capacity for mouse liver GTr is also likely. In addition, γ-glutamylcysteine synthetase activity may increase as GSH levels decline [76]. However, the rate of synthesis of GSH in the infected animals was inadequate to maintain GSH at control levels, despite possible redundancy in the system. These results suggest that animals already compromised by a schistosomal infection may have enhanced susceptibility for hepatic toxicity from xenobiotic compounds which impose either an oxidative stress or require conjugation for detoxication.

SUMMARY AND CONCLUSIONS

The present study indicates that the prolonged antischistosomal activity of oltipraz may be due, in part, to the acute effects observed *in vivo* and *in vitro*. Disruption of tegumental function in the transport of glutathione or glutathione precursors into the schistosome effects a depletion of intracellular GSH stores. As was observed for other sulfhydryl inhibitors [73], the ionic gradient across the tegumental membrane is disrupted by oltipraz. These effects of the drug on the tegument and the subsequent depletion of schistosomal GSH lead to the depression of basic physiological parameters (frequency and amplitude of surface electrical potentials and inhibition of reproductive processes) and the alteration of biochemical processes, including indirect effects on glucose utilization. The present study also indicates that GSH and GSH precursors inhibit the acute actions of oltipraz *in vitro*. A likely hypothesis for the synergistic action of cysteine on the schistosomicidal activity of oltipraz *in vivo* is related to cysteine's enhancement of the bioavailability of the drug [3]. That DTT and 2-mercaptoethanol do not antagonize GSH effects *in vitro* is probably related, in part, to the fact that these compounds cannot serve as precursors to *de novo* GSH synthesis.

Concerning the enzymes of glutathione regulation, one of the major findings of this study is the elucidation of biochemical differences between the host and the parasite in the level of enzymic activity present in GPx, GRed, GTr and γ-GTP. These differences may be exploitable chemotherapeutically. The fact that the parasite has different enzymic activities in regard to GSH utilization is not surprising because of the difference in the parasites metabolic handling of xenobiotics compared to the host. Further, the parasites do not require oxidative reactions to provide energy, and therefore, do not apparently require similar levels of GSH in order to protect against oxidant injury.

In light of the hepato-pathology of the disease, changes in the biochemical regulation of GSH stores were not unexpected. These changes may indicate that the host is more at risk as the liver is the major organ for detoxication of most xenobiotics.

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