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REDUCTION IN THE RNA CONTENT OF *SCHISTOSOMA MANSONI*: A POTENTIAL MECHANISM FOR THE SCHISTOSOMICIDAL ACTION OF RO 15-5458

By

Feleke Eshete

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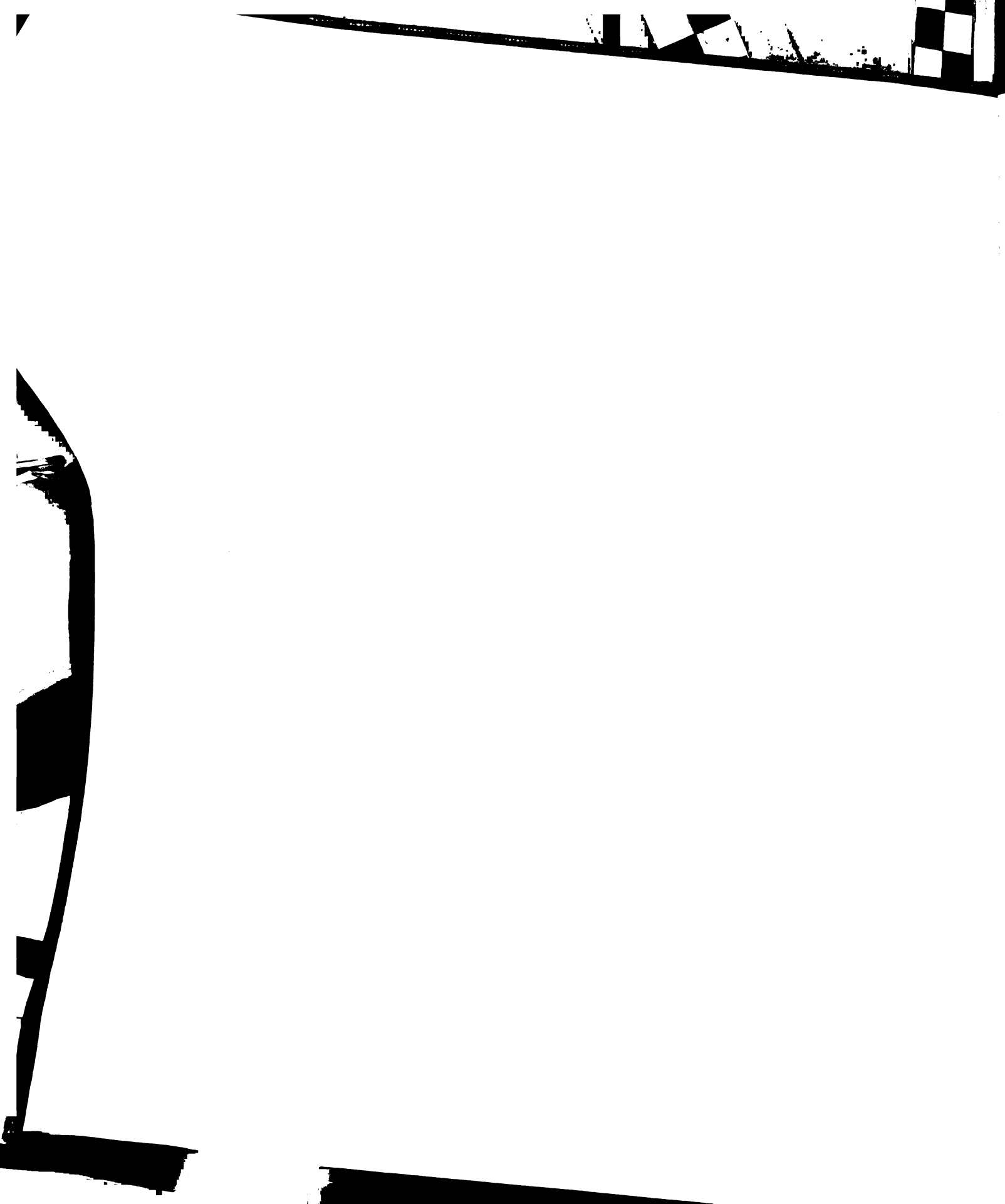

ABSTRACT

Reduction in the RNA Content of Schistosoma mansoni: A Potential Mechanism for the Schistosomicidal Action of Ro 15-5458

by

Feleke Eshete

Ro 15-5458 [10-2-(diethylamino)ethyl-9-acridanone(2-thiazolin-2-yl)hydrazone] has recently been shown by the Hoffmann-LaRoche & Co., Ltd. to be effective in rodents and baboons against the three principal species of schistosomes that cause the human disease, schistosomiasis. Apart from the chemotherapeutic evaluations of Ro 15-5458 against schistosome infections, no attempt has been made to pharmacologically characterize its actions and examine the effects of the compound on the parasites. The main objective of this thesis is to examine the effects of Ro 15-5458 on adult Schistosoma mansoni after exposing parasites to the drug in vivo, with the intent of understanding the mechanism of its schistosomicidal action. In addition, the in vitro effects of the drug on the parasite, as well as its absorption kinetics, have been studied.

In vitro, no antischistosomal effect could be demonstrated when pairs of parasites isolated from mice were incubated for 72 h in a medium containing a 10 μM solution of the drug. However, the drug increased both the contractile activity and longitudinal tension of the parasite musculature. This effect of Ro 15-5458 was reversed by cholinomimetic

agents and does not appear to be associated with the *in vivo* schistosomicidal action of the drug.

Administration of a single dose (15 mg/kg) of Ro 15-5458 to infected mice resulted in the death of all parasites. The drug exhibited a slow onset of activity *in vivo*; with the same dose, a significant dislodgment of worms from the mesenteric to hepatic portal veins (hepatic shift) was observed after a lapse of 4-5 days.

Examination of parasites retrieved from mice dosed with Ro 15-5458 (15 mg/kg) for drug-induced changes before the hepatic shift indicated no alterations in the surface membrane integrity, energy metabolism or erythrocyte consumption up to 3 days postdosing. However, this treatment resulted in a significantly reduced egg output, weight, protein content, and the incorporation of leucine into acid-insoluble fractions of parasites. These defects were preceded by a reduction in the total RNA content of the parasites. Quantitative hybridization of parasite RNA immobilized on nylon membranes with specific probes revealed that treatment reduced the amount of actin and superoxide dismutase mRNA and both the 18S and 28S rRNA content of the parasites. Administration of the same dose of Ro 15-5458 did not exert similar actions on treated but uninfected mouse liver RNA, suggesting selectivity in its effects.

These results suggest that the schistosomicidal effect of Ro 15-5458 and/or its metabolic products is to inhibit the expression of parasite genes. It is postulated that the reduction in the RNA content of the parasite initiates a series of events which alters the host-parasite relationship and gradually results in parasite death.

To my parents, without whose support, encouragement and sacrifice, this would not have been possible.

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CHAPTER I

GENERAL INTRODUCTION

It is estimated that more than 200 million people suffer from schistosomiasis (bilharziasis) and about three times this number are threatened with the infection (Iarotski & Davis, 1981; WHO, 1985). The disease is endemic in about 76 countries. It is most widespread in tropical and subtropical areas of Africa, South America, the Caribbean Islands, the Eastern Mediterranean and the Arabian Peninsula and South-East Asia. It is essentially an infection of rural and agricultural areas, where there exists poverty, ignorance, poor housing and substandard hygienic practices. Schistosomiasis ranks second only to malaria in terms of socioeconomic and public health importance.

Schistosomiasis is caused by digenetic trematodes of the genus Schistosoma, among which S. mansoni, S. haematobium, and S. japonicum are the principal causative agents of human disease. Humans are exposed to the infective form of the parasite in water during occupational or recreational activities; agricultural work in irrigation schemes constitutes one of the schistosomiasis risks (Hunter, 1982; Kloos, 1985; WHO ,1985). In most endemic areas, children 6-15 years of age have been identified as the most highly exposed and heavily infected age group and also contribute most to water contamination (Dalton & Pole, 1978; Kloos et al., 1983). Once the invasive form of the parasite finds its way into the ultimate host, the resulting larvae migrate until the parasites grow

and sexually mature. The male and female worms copulate and, depending on the species, inhabit the mesenteric and intra-hepatic veins or the vesical plexus, where they live for many years producing numerous eggs. Different species of schistosomes differ in geographical distribution, snail-host infectivity, response to drugs, pathogenicity and immunogenicity. The pathology of schistosome infection involves many organs and is mainly due to a vigorous host response against the parasite eggs.

A. Schistosoma mansoni

1. The parasite

The blood fluke S. mansoni is a sexually dimorphic plathyhelminth. The adult worms (male 10-15 mm long and female 15-20 mm long) often live in copula, the female residing within the gynecophoral canal of the male (Figure 1). Parasites attach to the inner wall of the veins of the host through the ventral sucker, a muscular organ capable of sustained contraction.

The digestive system of the parasite is well developed. The oral sucker of both male and female S. mansoni parasites leads into the cecum, through a non-muscular esophagus. The cecal wall consists of a syncytial lining epithelium (gastrodermis). Morphological evidence suggests that the gastrodermis is both secretory and absorptive in function. The gastrodermis cells secrete digestive enzymes, such as hemoglobinase, which break down hemoglobin from the host erythrocytes into a pigment which imparts a brown appearance to the parasites.

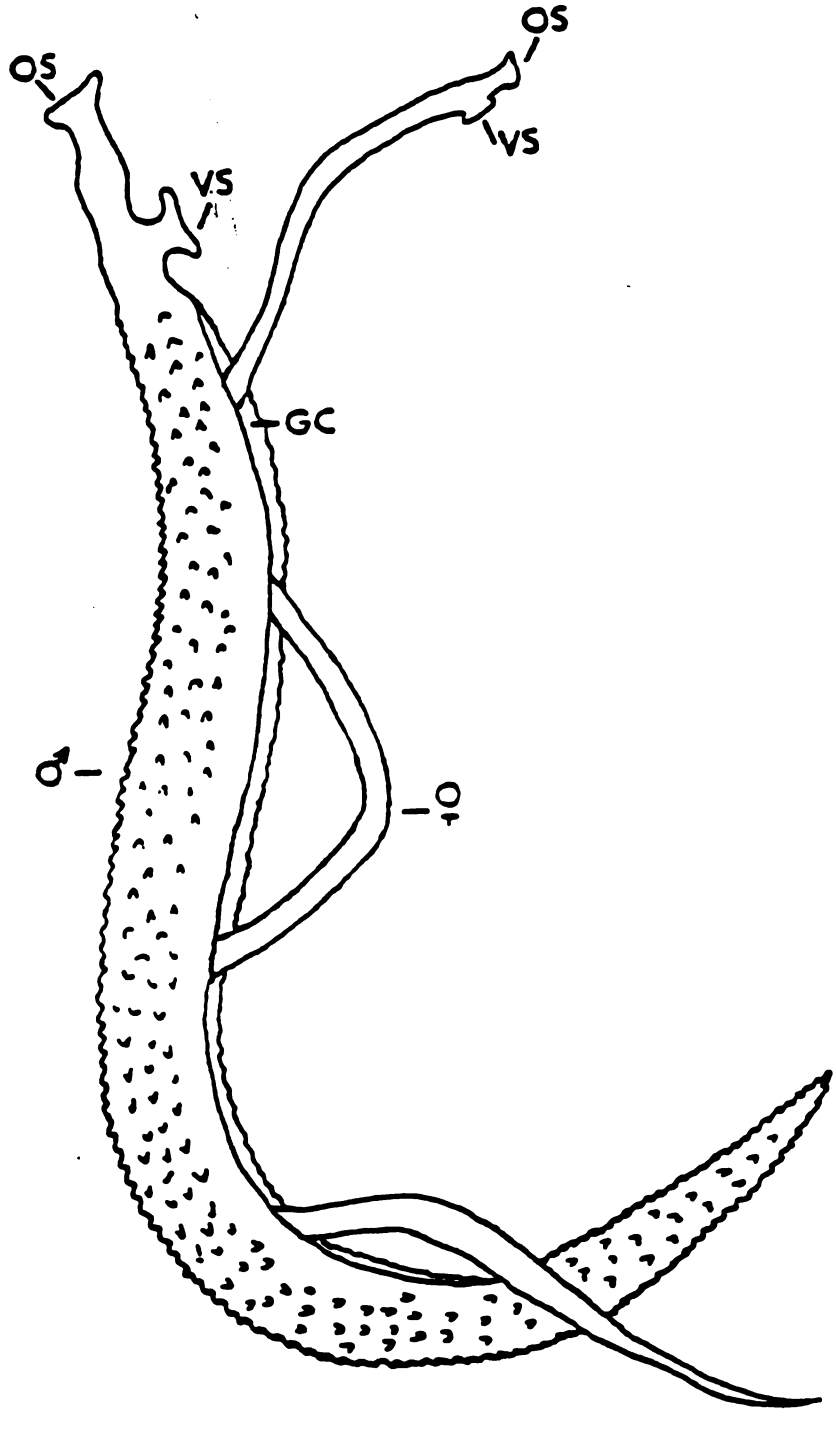


Figure 1. Drawing illustrating the external characteristics of male and female S. mansoni. OS, oral sucker; VS, ventral sucker; GC, gynecophoral canal.

The external surface (tegument) of adult schistosomes is a syncytial interface between the parasite and the host environment. It is an unusual double bilayer structure (McLaren & Hockley, 1977), consisting of an inner bilayer or plasma membrane overlaid by a secreted outer bilayer of lipid rich material (Wilson & Barnes, 1974). The tegument develops within 3 h of host penetration by the cercaria and is thought to be an adaptation to the host environment, enabling the parasite to evade the immune response of the host (Hockley & McLaren, 1973; McLaren, 1984; McLaren et al., 1975).

The surface membrane of the parasite is in a state of rapid turnover with a continuous synthesis and degradation of membrane proteins (Kusel & Mackenzie, 1975; Tavares et al., 1980; Dean and Podesta, 1984) and rapid turnover of membrane lipids (Meyer et al., 1970, Vial et al., 1985). The rate at which the membrane turns over ($t_{1/2}$) has been reported to be in the range of 5 to 45 h by different investigators (Wilson & Barnes, 1977; Ruppel & McLaren, 1986; Saunders et al., 1987). Surface topography of the tegument reveals features consistent with an absorptive function (Hockley, 1973). During short-term in vitro incubations, the tegument appears to be the primary site involved in absorption of low molecular weight solutes such as glucose (Rogers & Bueding, 1975; Uglem & Read, 1975; Mercer & Chappell, 1986a), amino acids (Chappell, 1974; Asch & Read, 1975a,b; Isserof et al., 1976), pyrimidine and purine bases (Levy & Read, 1975a,b; Mercer & Chappell, 1986b) and cholesterol (Haseeb et al., 1985).

Work by Fetterer, Pax & Bennett (1980a) has demonstrated that a well-defined tegumental potential of about -60 mV exists at the dorsal

surface of the male parasite that can be altered by changing physical or chemical qualities of the parasite environment in vitro. These investigators also predicted that a Na^+-K^+-ATPase pump may be operating and that it maintains an ion gradient across the parasite tegument (Fetterer et al., 1980b).

The worms feed on host erythrocytes, utilizing at least part of the hemoglobin molecule (Zussman et al., 1970); the female parasites ingest about 13 times more erythrocytes at a rate 9 times faster than males (Lawrence, 1973). This large requirement for blood cells by the female parasites is necessary to supply precursors for proteins and perhaps nucleic acids in egg production, which is estimated to be as high as 1100 eggs/female/day (Damian & Chapman, 1983). Although the specific roles of the gut and the tegument in the nutrition of these parasites is not understood due to technical limitations, parasites may also absorb solutes from the host body through the oral route and/or the tegument.

2. Life cycle

The life cycle of the parasite involves three biochemically and morphologically distinct forms of the parasite, snail and a vertebrate host (Figure 2). Some of the eggs deposited by the females into the bloodstream of the vertebrate host pass through the venule walls, cross the intestinal mucosa, reach the lumen, and are evacuated with the fecal material. When these eggs come in contact with fresh water, a freely swimming form (miracidium) is released. This form penetrates an appropriate snail host, asexually multiplies in this host and is transformed into a new infectious form, the cercariae. The male and female cercariae shed by the snail penetrate the skin of man or another vertebrate host. In the skin they change into schistosomula and travel in the lymphatic

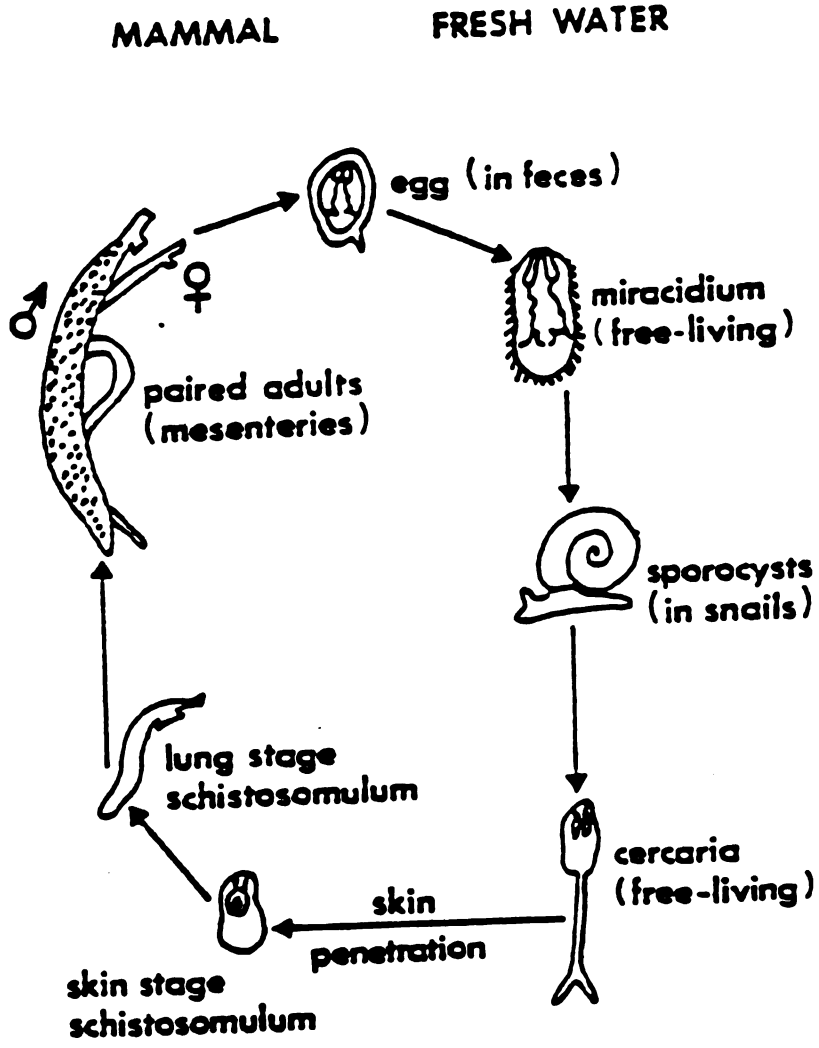


Figure 2. Life cycle of S. mansoni.

system to the right heart and lungs, from which they migrate to the portal circulation. They grow and sexually mature in the liver and descend to the mesenteric veins where they live for as long as 30 years actively producing eggs (Faust et al, 1934).

3. The musculature

The musculature of S. mansoni is located immediately beneath the inner membrane of the tegument and consists primarily of an outer circular and inner longitudinal muscle layer. In the male schistosome, in which both sets of muscles are well developed compared to the female, the longitudinal muscle is important in locomotor activity, while the circular muscle is essential for the formation of the gynecophoral canal and thus maintenance of male-female pairing. The contractile elements of the myofibrils consist of thick myofilaments (18-40 nm diameter), each surrounded by an irregular array of 8-14 thin filaments (5 nm diameter) (Silk & Spence, 1969). The ratio of thin to thick filaments and absence of transverse tubules and microtubules makes the muscles of the worm resemble vertebrate smooth muscles. The sarcoplasmic reticulum is poorly developed or absent, but rough elements can be found scattered; mitochondria appear in sac-like distentions of the sarcoplasm along myofibril bundles. The nuclei are located deeper than the muscle fiber bundles and are connected to them through cytoplasmic processes. Lipid globules as well as α- and β-glycogen particles are distributed throughout the peripheral cytoplasm of muscle cells (Silk & Spence, 1969).

The spontaneous motor activity of the parasite can directly be recorded using suction pipets in circuit with a force transducer (Fetterer et al., 1977). The technique has also been employed in the

measurement of the effects of various pharmacological agents and inorganic ions on the neuromuscular system of the parasites. High K^+ (60 mM) induces contractions of the muscle, by depolarization of the muscle membrane thereby allowing the entry of Ca^{++}; osmolarity greater than 300 mOsm, pH less than 6.8, and the concentration of inorganic ions below or above that found in Hanks' balanced salt solution reduced the contraction rate of the male schistosome muscle (Fetterer et al., 1978; 1980b).

The motor activity of these parasites appears to be modulated and coordinated by the nervous system through the action of neurotransmitters. There is evidence suggesting that 5-hydroxytryptamine (5-HT) functions as an excitatory neurotransmitter, causing an increase in motor activity (Barker et al., 1966; Tomosky et al., 1974; Fetterer et al., 1977; Willcockson & Hillman, 1984). Acetylcholine is believed to function as an inhibitory neurotransmitter, causing flaccid paralysis (Hillman & Senft, 1973; Barker et al.,1966; Fetterer et al., 1977). Catecholamines have also been implicated as putative neurotransmitters (Tomosky, Bennett & Bueding, 1974). A report by Pax et al. (1984) suggests the possibility that the neurotransmitters described above affect the longitudinal and circular muscles of the parasite differentially. Only longitudinal muscle appears to possess cholinergic receptors, while only circular muscle appears to possess dopaminergic receptors. 5-HT receptors appear to be associated with both longitudinal and circular muscle function. Carbachol and the cholinesterase inhibitors physostigmine and metrifonate block electrically-induced contractions of the longitudinal muscle (Pax et al., 1981). The muscarinic receptor antagonist atropine and the antischistosomal agents hycanthone and oxamniquine induce a marked increase in

contractile activity and were observed to reverse the paralytic effect of carbachol on parasite musculature (Hillman & Senft, 1975; Pica-Mattoccia & Cioli, 1986).

4. Intermediary Metabolism

More important data concerning intermediary metabolism has been collected for S. mansoni than any other schistosome species because of its widespread geographical distribution and the simplicity of propagating the parasite in the laboratory. Nevertheless, knowledge on the metabolism of the parasite is far from complete; more research is needed, especially on the anabolic pathways of the parasite. In addition, most of the information on the metabolism of these parasites is derived from experiments carried out in vitro. Whether this information relates to the situation in vivo has remained controversial, because several reports (Floyd & Nollen, 1977; Shaw & Erasmus, 1977; Fried, 1978; Basch & Humbert, 1981) indicate that systems employed for the maintenance of adult schistosomes in vitro are inadequate.

While lengthy survival periods in vitro have been reported for adult S. mansoni (Robinson, 1956; Lancastre & Golvan, 1973), deterioration of worms during culture has been observed. Ultrastructural studies on the reproductive system reveal that the testes (Floyd & Nollen, 1977), and the vitelline gland and ovary (Floyd & Nollen, 1977; Shaw & Erasmus, 1977) of S. mansoni degenerate commencing 5 to 6 days after isolation from the host. Egg production invariably ceases within 2-3 weeks in culture (Michaels & Prata, 1968; Newport & Weller, 1982) and reduction in parasite size, glycogen and protein content has also been observed (Zussman et al., 1970; Basch & Humbert, 1981; Mercer & Chappell, 1985a).

a. Carbohydrate metabolism

Adult S. mansoni obtain the major portion of their energy from glucose. Glucose is taken up primarily through the tegument by mediated transport and simple diffusion (Rogers & Bueding, 1975; Uglem & Read, 1975; Mercer & Chappell, 1986a). The transfer of glucose from male to female parasites has also been observed (Cornford & Huot, 1981). Other hexoses such as galactose (Isseroff, Bonta & Levy, 1972), fructose and mannose (Bruce et al., 1974) are also taken up through the tegument.

The pioneering work of Bueding (1950) suggested that in vitro, the adult parasites consume an amount of glucose equivalent to 20-30% of their dry weight in an hour, converting 80% of the glucose to lactic acid. Glycolysis was then identified to be the main pathway for energy extraction in these parasites. In a more recent study, the same group concluded that glucose utilization, lactic acid production and ATP levels were the same under aerobic and anaerobic conditions and all the ingested glucose and endogenous glycogen are quantitatively metabolized to lactic acid (Bueding & Fisher, 1982). The original work by Bueding (1950), on the other hand, demonstrated the consumption of oxygen by schistosomes. The O_2 uptake was increased by inclusion of glucose in the medium and a difference in the respiratory quotients was observed in the presence and absence of glucose in the medium, suggesting the ability of these parasites to oxidize glucose to water and CO_2. However, in a complete state of inhibition of parasite respiration with cyanine dyes, the rate of glycolysis was not affected and no adverse effects on the parasites were observed. Thus, it was concluded that S. mansoni are homolactic fermenters and derive their ATP solely through this pathway.

The role of O_2 in these parasites is not well understood yet, although the parasites live in an aerobic environment. Recent studies suggest that schistosomes, although relaying heavily on glycolysis for ATP synthesis, generally have wider metabolic capabilities with regard to oxidative pathways. Although glycolysis appears to be the major pathway of glucose metabolism, recent studies indicate that no stochiometric relationship exists between the amount of glucose utilized and lactic acid excreted (Rahman, Mettrick & Podesta, 1985a; McManus, 1986). For instance during 6 h aerobic incubations (McManus, 1986) paired and female S. mansoni produced glucose:lactate ratios of approximately 1:1, while for male parasites the ratio was 1:2, suggesting that the females were channelling a substantial amount of glucose into the synthesis of mucopolysaccharides, glycoproteins and glycolipids (McManus, 1986; Rahman, Mettrick & Podesta, 1985b).

In addition, Van Oordt et al. (1985) incubated parasites in simple salt solution with [6-^{14}C]-glucose and determined CO_2 produced. Subsequent calculations indicated that at least one third of the energy production of adult schistosomes occurs by aerobic processes. However, in addition to using a physiologically stressful medium, they assumed in their calculations that the tricarboxylic acid (TCA) cycle and coupled electron transport chain operate in schistosomes as efficiently as in mammalian tissues, which may not be the case. Ambiguities prevail in the literature about the existence of a functional TCA cycle, since enzymes and intermediates have not been completely demonstrated (Coles, 1972; Smith & Brown, 1977; McManus, 1986). S. mansoni produce CO_2 from glucose, fructose and mannose (Bruce et al., 1974; Van Oordt et al., 1985).

However, the pathway that produces the CO_2 has been assumed to be the TCA cycle but has not been identified.

The existence of an electron transport system and individual cytochromes (b, c_1, c, a/a_3) have been reported (McManus, 1986), which indicates the potential to carry out oxidative phosphorylation. However, there is no study that explains how glycolysis and the electron transport system are coupled in schistosomes.

Another possible source of energy for S.mansoni is endogenous glycogen. Glycogen constitutes about 15% of the dry weight of adult male schistosomes, while the equivalent figure is about 5% for the females (Bueding & Koletsky, 1950; Lennox & Schiller, 1972). The function of the glycogen reserve in these parasites is not well understood. Nonetheless, it may serve as a transient source of energy (Mercer & Chappell, 1985a; Tielens & Van den Bergh, 1987). In addition, schistosomes convert glycogen into lactic acid when parasites are incubated in glucose free medium (Bueding, 1950; Bueding & Fisher, 1982). Rapid synthesis and degradation of glycogen has also been shown in vivo after administration of radiolabeled glucose to infected hamsters (Tielens et al., 1989a). Parasites, maintained in vitro, can synthesize glycogen from other precursors, such as fructose and mannose (Tielens et al., 1989b). The relevance of these studies to glycogen metabolism in vivo is not clear since parasites were incubated in sugar concentrations higher than they would encounter in the host. In vitro studies on the synthesis and degradation of glycogen suggest that, at glucose concentrations near the physiological levels of the human host, parasites rapidly deplete their

glycogen reserve despite increased uptake and incorporation of glucose into glycogen (Bueding, 1950; Mercer & Chappell, 1985a, 1986a).

b. Lipid metabolism

Studies on the lipid composition of adult S. mansoni reveal that schistosomes contain significant amounts of phospholipids, triglycerides, and cholesterol as free sterol, as well as small amounts of cholesterol esters and free fatty acids (Smith & Brooks, 1969; Meyer et al., 1970). Essentially all the phospholipids in these parasites are in the form of glycerophospholipids. Phosphatidyl choline (39%) is the major phospholipid, along with smaller amounts of phosphatidyl ethanolamine (3%). The synthesis of both phospholipids is thought to start with choline (Young & Podesta, 1985). In addition traces of phosphatidyl serine, phosphatidyl inositol and cardiolipin have been detected in these parasites (Meyer et al., 1970).

Schistosomes are host-dependent for sterols and long chain fatty acids, although possessing the capacity to elongate exogenously supplied fatty acids (Smith et al., 1970; Meyer et al., 1970). They incorporate [1-^{14}C]acetate, uniformly labeled [^{14}C]glucose and [1-^{14}C]oleate into their triglycerides and phospholipids. Acetate is incorporated into preformed fatty acids while most of the glucose ends up in the composition of the glycerol backbone of triglycerides and phospholipids.

c. Amino acid and protein metabolism

Little is known about the amino acid and protein metabolism of schistosomes. The small amount of data accumulated in the literature is restricted mainly to uptake and/or incorporation studies (Chappell, 1974; Asch & Read, 1975a,b; Isserof et al., 1976; Chappell &

Walker, 1982; Mercer & Chappell, 1985b) and the amino acid composition of total proteins (Robinson, 1961; Senft et al., 1972; Chappell & Walker, 1982). These studies indicate that, in vitro, amino acids enter through the tegument by diffusion and mediated transport. The uptake of amino acids varied depending on the sex and pairing state of the parasites. Tyrosine is selectively taken up by the female vitelline cells (Erasmus, 1975). Leucine uptake rates were higher for separated parasites compared to pairs (Mercer & Chappell, 1985b).

Limited work has been reported regarding the interconversion and the synthesis of schistosome amino acids using carbon skeletons derived from glucose. A small amount of glucose is converted to amino acids (ala,asp, glu), and only 5 amino acids (ala, arg, asp, gly, ser) were converted to other amino acids. The interconversion is thought to have little significance to schistosome protein synthesis, except for glutamine (from alanine) and proline (from arginine), which were incorporated into proteins (Chappell & Walker, 1982). In addition, histidine has been shown to be metabolized via decarboxylation, deamination and transamination reactions to products including histamine and glutamate (Saber & Wu, 1985). Transamination (Garson & Williams, 1957; Chappell & Walker, 1982) and decarboxylation (Bruce et al., 1972; Foster et al., 1989) reactions have also been examined, but only limited attempts to elucidate pathways of amino acid catabolism and understanding the fate of the products have been made.

Biosynthetic processes in the adult parasite must proceed at a high rate, since schistosomes produce enormous amounts of eggs and are known to rapidly renew their surface membrane. Thus, an

active protein synthetic mechanism must operate in these parasites. However, the mechanism of protein synthesis and the characteristics of enzymes involved in the process remain completely unstudied in schistosomes. Messenger RNA has been isolated and translated in vitro in a rabbit reticulocyte system to products that react with the host immune sera (Tenniswood & Simpson, 1982; Taylor et al., 1983; Knight et al., 1984). A parasite-derived, cell-free protein synthesizing system has also been reported (Lukacs et al., 1980).

d. Nucleotide metabolism and structure of nucleic acids

Nucleic acids are essential components of all living organisms. The building blocks for these macromolecules are both purine and pyrimidine nucleotides. Most mammalian cells have the ability to synthesize purine and pyrimidine bases de novo. Preformed bases as well as nucleosides can also be converted to nucleotides by salvage routes.

Pathways for salvage and the de novo synthesis of pyrimidines have been shown for S. mansoni (Hill et al., 1981; Iltzsch et al., 1984). Activities of all of the enzymes involved in the de novo synthesis of uridylic acid (UMP) have been identified in extracts of the parasites (Aoki & Oya, 1979; Hill et al., 1981; el Kouni et al., 1983). Enzymes of pyrimidine salvage pathways have also been identified (Senft et al., 1973; el Kouni et al. 1983). Activities of both thymidine and deoxycytidine kinases were present. However, parasite extracts have no uridine kinase or thymidine phosphorylase but contain a uridine phosphorylase. The preferred substrate for uridine phosphorylase is uridine; the enzyme also catalyzes the reversible phosphorolysis of deoxyuridine, and

deoxythymidine, but not cytidine, deoxycytidine, or orotidine (el Kouni et al., 1988).

S. mansoni, on the other hand, lacks de novo purine biosynthesis mechanisms (Senft & Crabtree, 1983) and so rely on the host for the supply of preformed purines for nucleotide synthesis in the salvage pathways. Studies using intact S. mansoni revealed that adenosine was primarily deaminated by adenosine deaminase and that the inosine so formed could be salvaged to adenine nucleotides by sequential actions of purine nucleoside phosphorylase, hypoxanthine phosphoribosyltransferase, adenylosuccinate synthetase and adenylosuccinate lyase (Senft, Senft & Meich, 1973; Senft et al., 1973).

In vitro uptake studies (Levy & Read, 1975a,b) indicated that both purine and pyrimidine bases enter the parasite through the tegument. They postulated that adenine, guanine, hypoxanthine, adenosine and uridine entered by diffusion and mediated transport through distinct sites. In contrast, the uptake of cytosine, thymine and uracil appeared to be by diffusion. Since these parasites consume large numbers of host erythrocytes, substrates for salvage mechanisms may also be contributed by these cells.

Studies by Simpson, Sher & McCutchan (1982) demonstrated that the haploid genome size of S. mansoni is about 2.7×10^8 base pairs, which is about a tenth of the size of mammalian species. Base composition studies of the parasite genome from buoyant density measurements (Hillyer, 1974) and a codon frequency table generated from published data (Meadows & Simpson, 1989), indicate about 34.3% G-C and a high A-T content (66%). The genome contains no detectable amounts of modified base pairs and has

both repetitive and unique sequences of DNA as in other eukaryotic organisms (Simpson, Sher & McCutchan, 1982). The highly repeated sequences were constituted mostly by the genes coding the two large ribosomal RNA (rRNA) molecules. The structural organization of the rRNA genes is similar to other eukaryotic organisms and they are encoded within 10 kb tandem repeats (Simpson et al., 1984; Van Keulen et al., 1985). Both class sizes of rRNA molecules have a sedimentation coefficient similar to other eukaryotic species. However, under denaturing conditions the 28S rRNA splits into two equal sized molecules about the size of the 18S rRNA suggesting, the existence of a nick in the large rRNA (Tenniswood & Simpson, 1982). The size of this nick has been determined to be about 200 bp by Van Keulen, et al. (1985).

B. Pathology of Schistosoma mansoni infection

At the invasive stage individuals exposed to the schistosome cercariae experience dermatitis (swimmer's itch). In the chronic phase of heavy infection most vital organs are affected. The presence of the adult parasites in the blood stream of the host is relatively harmless. However, most of the eggs produced by the female parasites are retained in various organs, eliciting vigorous host granulomatous responses (Bogliolo, 1967). Granuloma formation is initiated by antigens secreted by the miracidium through pores within the rigid egg shell (Stenger et al., 1967). Eggs deposited in the mesenteric plexus disseminate mainly into the liver and the intestinal tract, where each one evokes granuloma formation. With advanced pathology, collateral circulation is established; eggs reach the systemic circulation and are delivered to the

renal, pulmonary or central nervous system, where they initiate further granulomas (reviewed by Boros, 1989).

Microcirculation studies in the livers of mice infected with schistosomiasis showed that the granulomas that form around eggs lodged in the presinusoidal capillaries impede hepatic blood flow (Bloch et al., 1972). Symmers' fibrosis develops in humans around the branches of portal veins after many years of infection. This fibrosis is followed by portal vein obstruction, which contributes to portal hypertension, resulting in collateral circulation. In heavy infections, continuous granuloma formation and fibrosis together with elevated portal pressure and intense immunologic activity lead to the development of hepatosplenomegaly. Hepatosplenomegaly with deranged liver function is followed by ascites fluid formation in the peritoneal cavity and esophageal varices, which rupture and cause fatal episodes of esophageal and gastrointestinal bleeding.

C. Control of Schistosomiasis

Numerous approaches have been implemented in attempt to reduce the transmission of infection and morbidity due to schistosomiasis (WHO, 1985; Jordan, 1986; Sleigh et al., 1986; Klumpp & Chu, 1987). Health education, improved sanitation and water supply with focal molluscicide and chemotherapy have been recommended as an effective means of controlling the disease (WHO, 1985). However, such strategies have suffered various limitations, and have therefore been marginally successful (Jordan et al., 1978; Polderman, 1984; Fenwick, 1987). Vaccine development has been proposed as a cost effective means of controlling the transmission of the

disease. Although expectations for the development of schistosome vaccines are high (Butterworth & Hagan, 1987; Capron et al., 1987; Smithers, 1988), there is a little chance that the vaccine will be available in the foreseeable future. The complexity of host-parasite relationship, lack of knowledge of the host immune effector mechanisms against the parasite and possible evasion of the host immune system by the parasite have further tempered optimism towards schistosome vaccines (reviewed by Mitchell, 1989).

At present, chemotherapy is the most effective method for the short-term control of schistosomiasis (WHO, 1983; Liese, 1986). Since these parasites multiply outside the human host, the worm load and oviposition are crucial for the perpetual cycle of infection. Drugs reduce both worm burden and egg excretion, thus contributing to a decline in both the intensity and prevalence of infection by reducing the population of infected snails (Cook et al., 1977; Pugh & Teesdale, 1984; Sleigh et al., 1986). Currently, however, few chemotherapeutic agents are available for the treatment of schistosomiasis, and the future of this approach is threatened by the possible emergence of drug resistant strains of the parasites (Bruce et al., 1987; Coles et al., 1987a). Evidence for resistance to hycanthone and oxamniquine is well documented (Dias et al., 1982; Coles et al., 1987b; Yeang et al., 1987). The ultimate fate of the effective broad spectrum antischistosomal agent praziquantel is also uncertain. Thus, research has to be continued towards the development of new antischistosomal drugs to complement existing drugs or replace those that cease to be effective.

D. Background and Objectives of Proposed Research

A class of new antischistosomal compounds has been synthesized by the Hoffmann-La Roche CO., Basel, Switzerland (Stohler and Montavon, 1984). These 9-acridanone hydrazone compounds have been shown to be effective against the three principal species of schistosomes that cause human disease. The antischistosomal activity of one of these compounds, Ro 15-5458, in mice and hamsters infected with S. mansoni, S. haematobium and S. japonicum is superior to other standard antischistosomal drugs such as praziquantel, oxamniquine and amoscanate (Table 1). In addition, unlike praziquantel, which is active only against the invasive stages and adult parasites in vivo (Andrews, 1981), Ro 15-5458 is effective against all the stages of the parasites (Stohler & Montavon, 1984; Eshete, unpublished observation), a property that may be exploited for early intervention in the infection. A single oral dose of Ro 15-5458 (50, 25, or 15 mg/kg) effectively clears infected rodents and baboons of parasites and significantly reduces fecal egg excretion (Stohler & Montavon, 1984; Sturrock et al., 1985; 1987; Sulaiman et al., unpublished data).

Limited data are available on the toxicity of Ro 15-5458 to the host. In acute toxicity studies, two different single doses were given to mice (250 and 500 mg/kg) and rats (125 and 500 mg/kg) and the number of survivors was determined 14 days after drug administration. All animals survived at the lower dose, but all died at the higher dose, suggesting the existence of a high margin of safety between the effective dose (ED_{90} = 10.5 mg/kg) and lethal dose. In vitro mutagenicity tests performed using S. typhimurium strains TA97 and TA102 as well as repair

TABLE 1

Antischistosomal Activity Against S. mansoni, S. haematobium and S. japonicum in Hamsters and Mice[a]

Compound	Hamsters[b]			Mice[b]
	S. mansoni	S. haematobium	S. japonicum	S. mansoni
Ro 15-5458	11.1	8.5	41.5	10.5
Praziquantel	40.4	<50.0	74.2	272.0
Oxamniquine	126.8	inactive	>400	64.1
Amoscanate	7.0	active	active	>100

[a]Reproduced from Stohler & Montavon, 1984.

[b]ED_{90} - single oral dose in mg/kg reducing the number of surviving schistosomes by 90%

tests with E. coli showed no mutagenic activity (Stohler & Montavon, 1984). In collaboration with Dr. R.J. Wilkins (University of Otago, New Zealand), we also tested for site specific binding and/or inhibition of DNA polymerase I activity using a nick translation system (Wilkins, 1985). At 100, 33, and 10 μg/ml of Ro 15-5458 neither binding nor polymerase inhibition was observed. These results may not be surprising in light of the absence of in vitro schistosomicidal action of Ro 15-5458 (vide infra); the drug is probably converted to a biologically active product which may result in toxicity. If active metabolic products are responsible for schistosomicidal activity, all the tests need to be repeated.

Despite extensive parasitological evaluation of Ro 15-5458, no report is available on its mechanism of antischistosomal action. The present study was designed to examine the in vitro and in vivo effects of Ro 15-5458, with the intent of understanding the mode of its schistosomicidal action. The in vitro effects of Ro 15-5458 on S. mansoni musculature and its relevance to the in vivo schistosomicidal action were examined. In addition, effects on well characterized biological processes of the parasite were studied after dosing the host with a 15 mg/kg of the drug and retrieving parasites.

SUMMARY AND SIGNIFICANCE

Under proper supervision, it appears that chemotherapy will play an ever increasing and crucial role in the control of schistosomiasis. A major risk in the widespread application of anthelmintics is the appearance of schistosome populations exhibiting drug resistance. This

necessitates the development of other antischistosomal compounds to replace drugs that cease to be effective.

Knowledge of the biochemical/physiological processes of schistosomes is required for a "rational" design of these drugs. Unfortunately, it is still lacking for these parasites. Under such circumstances, one approach that could be used is to develop an understanding of the mode of antischistosomal action of currently available drugs and others in the process of development. Mode of action studies may provide new information on the parasite's metabolic processes, which in turn may reveal areas for the discovery of other antischistosomal drugs. In this context, we have initiated an investigation on the mechanism of action of Ro 15-5458. It is anticipated that these studies, in addition to providing insights into the mechanism of antischistosomal action of this novel drug, will also contribute to the limited knowledge available regarding parasite-drug interaction, opening potential areas for the control of schistosomiasis.

CHAPTER II

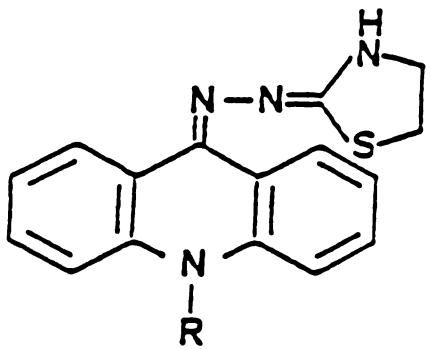
THE ABSORPTION KINETICS OF Ro 15-5458

INTRODUCTION

Knowledge of the pharmacokinetics of Ro 15-5458 is essential for its development as a potential broad spectrum antischistosomal agent, both for the meaningful interpretations of its biological effects and in the assessment of its toxicity to the host. In this section, the absorption kinetics of this drug have been described. A study of the absorption kinetics was performed after oral administration of the drug since it will probably be administered via this route. It is also important to note that oral administration of antischistosomal compounds would be most desirable for the treatment of schistosomiasis. Rabbits were used in this study due to their convenience, in model single dose pharmacokinetic studies.

MATERIALS AND METHODS

Ro 15-5458 [10-2-(diethylamino)ethyl-9-acridanone(2-thiazolin-2-yl)hydrazone] and its analogue Ro 15-9895 [10-2-(dimethylamino)ethyl-9-acridanone(2-thiazolin-2-yl)hydrazone] (Figure 3) were kindly supplied by Dr. H.R. Stohler (Hoffmann-La Roche Co., Basel, Switzerland).



CODE	R
Ro 15-9895	$-CH_2-CH_2-N(CH_3)_2$
Ro 15-5458	$-CH_2-CH_2-N(C_2H_5)_2$

Figure 3. Ro 15-5458 ($C_{22}H_{27}N_5S$) and Ro 15-9895 ($C_{20}H_{23}N_5S$).

A. Animal Studies

Female New Zealand rabbits weighing 2-3 kg (n=5) were fasted 12 h prior to dosing but had access to water. A single oral dose of 50 mg/kg Ro 15-5458 was given as a suspension in 25% glycerol plus 1% Cremophor EL. Five ml of blood was collected from ear veins of each rabbit before dosing and samples were similarly collected 20 min, 40 min, 1, 2, 3, 6, 12, 24, 48 and 72 h after dosing. Blood was left at room temperature to clot and plasma was separated by centrifugation at 12,000 g for 10 min. All plasma samples were frozen and assayed for Ro 15-5458 within 48 hours of collection.

B. Determination of Ro 15-5458 in Plasma

The plasma level of Ro 15-5458 was determined by high performance liquid chromatography (HPLC) (Waters Associates, Milford, MA), after a simple and rapid extraction of Ro 15-5458 and the internal standard (Ro 15-9895) from alkalinized (150 μl 1 N NaOH) 2-ml plasma samples and plasma-based standards with 10 ml hexane-butanol (9:1) with gentle rotating and mixing for 5 minutes. Plasma-based standards were prepared by spiking 2 ml of plasma collected before dosing at concentrations of 25, 50, 125, 500 and 1000 ng/ml of Ro 15-5458 and 100 ng/ml of Ro 15-9895. Nine ml of the organic phase was then re-extracted with 250 μl of 0.01 N HCl and 100 μl of the acidic phase was injected into a NOVA-PAK C_{18} column (Waters Associates). The mobile phase was 0.01 M ammonium acetate (pH 5.5) plus acetonitrile (4:6) containing 0.004 M triethylamine, run isochratic at a flow rate of 1 ml/min.

The column effluent was monitored with a variable wavelength spectrophotometric detector set at 254 nm. Data handling and plotting were performed by a computing integrator (Data Module, Waters Associates). The computing integrator programmed to store the standard calibration curve for each assay was used with the peak area ratio of Ro 15-5458 to internal standard to calculate the concentrations.

C. Mass Analysis

Ro 15-5458, the internal standard and other prominent HPLC peaks were collected and extracted with methylene chloride. Samples were then evaporated to dryness at 37°C under nitrogen and mass spectral analysis was performed by direct probe introduction of the compounds. Electron impact spectra were determined at 70 eV on Finnigan 3200 mass spectrometer with Riber SADR data system.

D. Pharmacokinetic Analysis

The half-life and the peak plasma concentration (C_{max}) were calculated from least squares regression analysis of the elimination phase of the plasma concentration-time curve (Gibaldi & Perrier, 1982). The area under the plasma concentration-time curve (AUC) was calculated using the trapezoidal rule. Other kinetic parameters were calculated using the equations described for one compartment absorption model after a single dose administration (Bates & Carrigan, 1975; Gibaldi & Perrier, 1982). The goodness of fit of the experimental data to one compartment open absorption models was tested using a non-linear least squares regression analysis program (PCNONLIN) (Metzler & Weiner, 1985).

RESULTS

A. Identification of Chromatographic Peaks

After HPLC separation, peaks of interest were confirmed by mass analysis (Figure 4). The retention times for the internal standard and Ro 15-5458 were 4.9±0.25 and 8.3±0.29 (mean ± SD) min, respectively. The recovery of the extraction procedure was 83±7% (mean ± SD), and the sensitivity of the assay is about 10 ng.

It is important to perform the extraction and injection of the sample within a short period, since the (-C=N-N=C-) bond in both Ro 15-5458 and Ro 15-9895 is acid labile (Figure 5). Both compounds were converted to the respective [10-2-(dialkylamino)ethyl-9-acridanone] product. Although the extent of hydrolysis was the same for both Ro 15-5458 and the internal standard, prolonged exposure of Ro 15-5458 to acid solutions resulted in a compound that interfered with the internal standard peak.

B. Elimination of Ro 15-5458

Plasma levels of Ro 15-5458 declined in a monoexponential fashion with time after a single oral dose of 50 mg/kg (Figure 6). This was determined from the regression analysis of the terminal phase of the plasma level-time curve (r=0.98). The same data was also fitted to a single exponential pharmacokinetic model with no time lag using the PCNONLIN program. The experimental data were well correlated (r=0.99) with the kinetic model. The elimination half-life, estimated from the average data, was 6 h. Individual elimination half-lives ranged from 3 to 7 h.

Figure 4. Mass spectra of Ro 15-9895, Ro 15-5458 and its hydrolysis product. Panel A is the mass spectrum of authentic Ro 15-5458, panel B is from the HPLC fraction collected at retention time (RT) of 8 min and panel C is a spectrum of Ro 15-9895, an HPLC fraction collected at RT of 5 min. Panel D is from authentic [10-2-(diethylamino)-ethyl-9-acridanone] while E is the mass spectrum of an HPLC fraction (RT = 3.6 min) from the hydrolysis of Ro 15-5458.

Figure 4

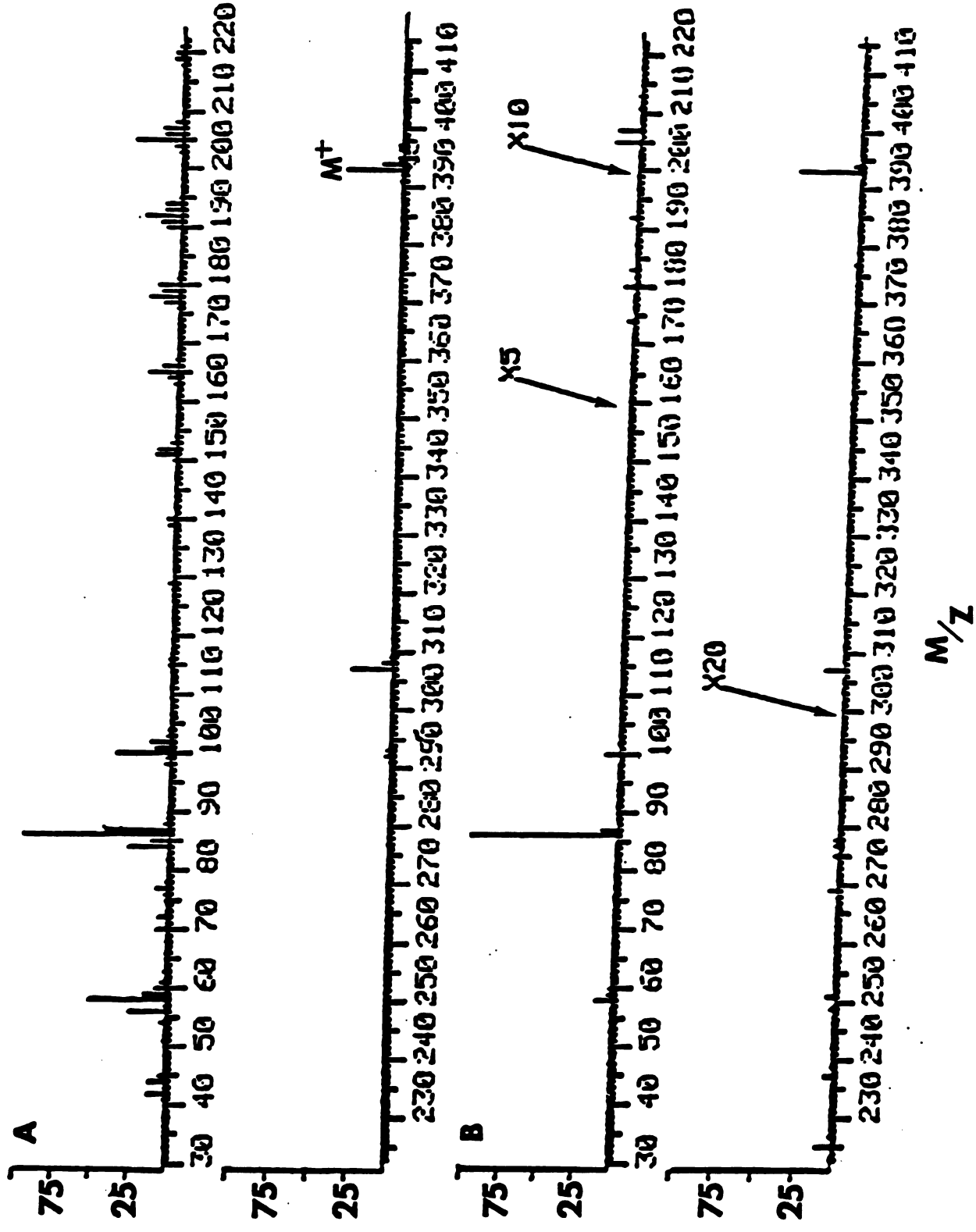

A
B
75
25
Relative Intensity
M+
X10
X5
X20
30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220
230 240 250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 400 410
M/Z

Figure 4 (continued)

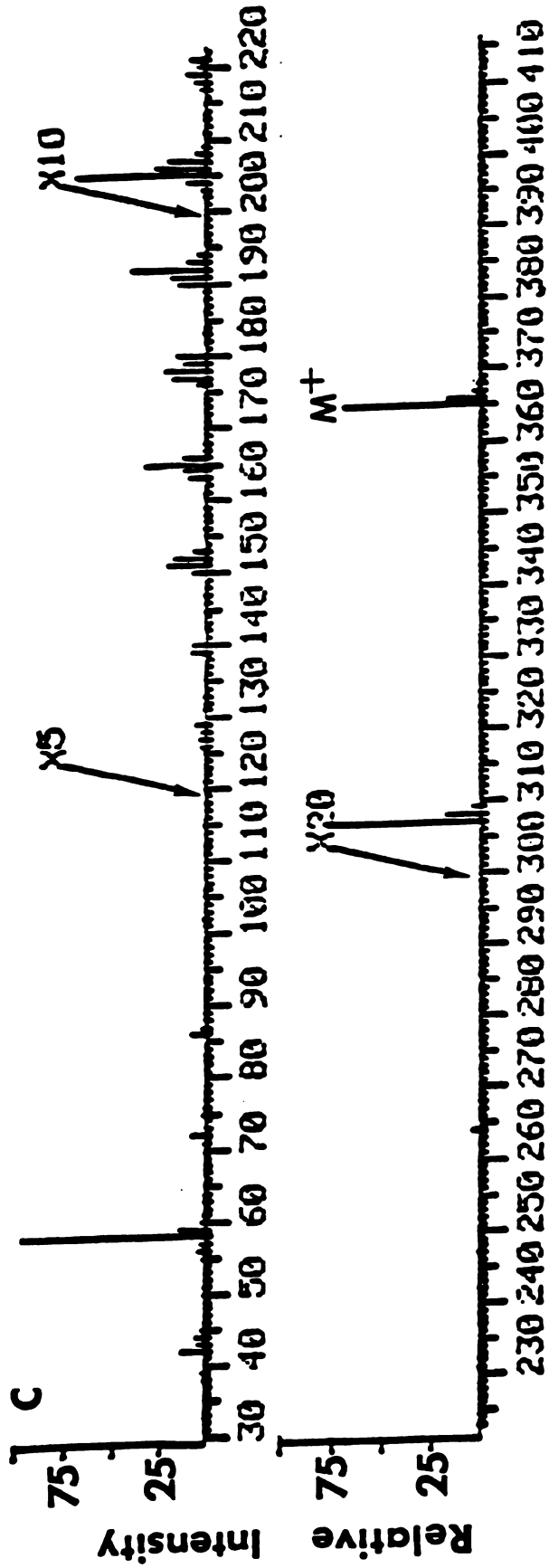
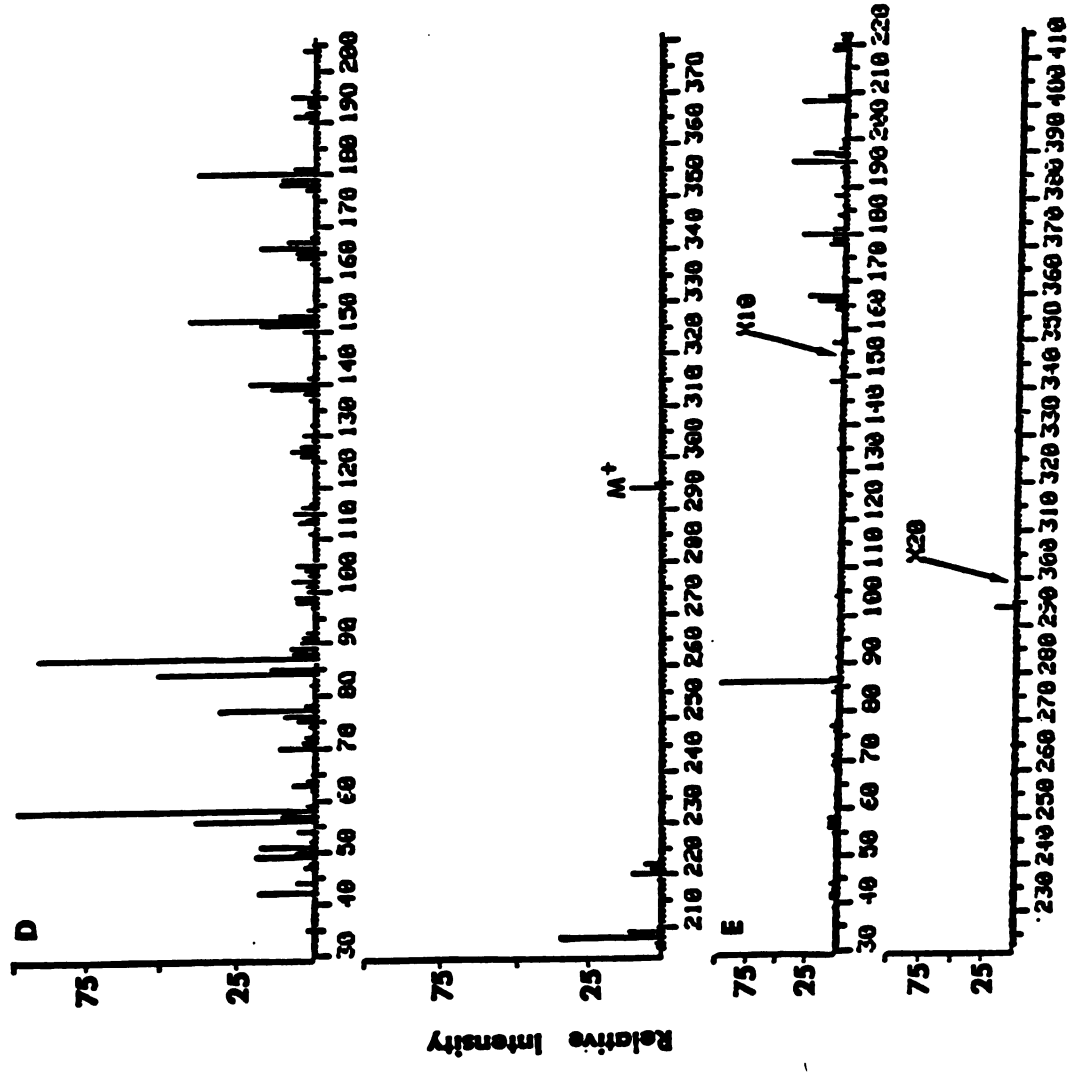


Figure 4 (continued)



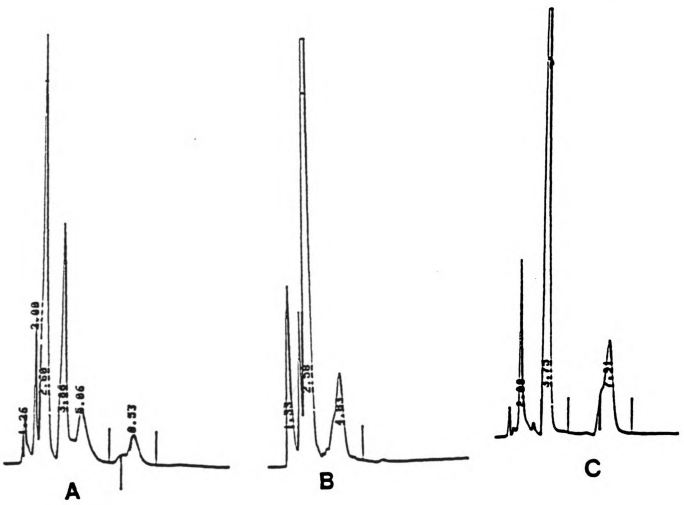


Figure 5. Chromatograms of Ro 15-5458 and Ro 15-9895. Chromatogram A is an extract from plasma of a rabbit. Peaks at retention times 5.06 and 8.53 min are from internal standard and Ro 15-5458, respectively. While peaks at retention times 2.6 and 3.86 are for the [10-2-(dialkylamino)-ethyl-9-acridanone] products of Ro 15-9895 and Ro 15-5458, respectively. Chromatogram B is for Ro 15-9895 and C is for Ro 15-5458 after exposure of both compounds at room temperature to 0.01 N HCl for 30 days.

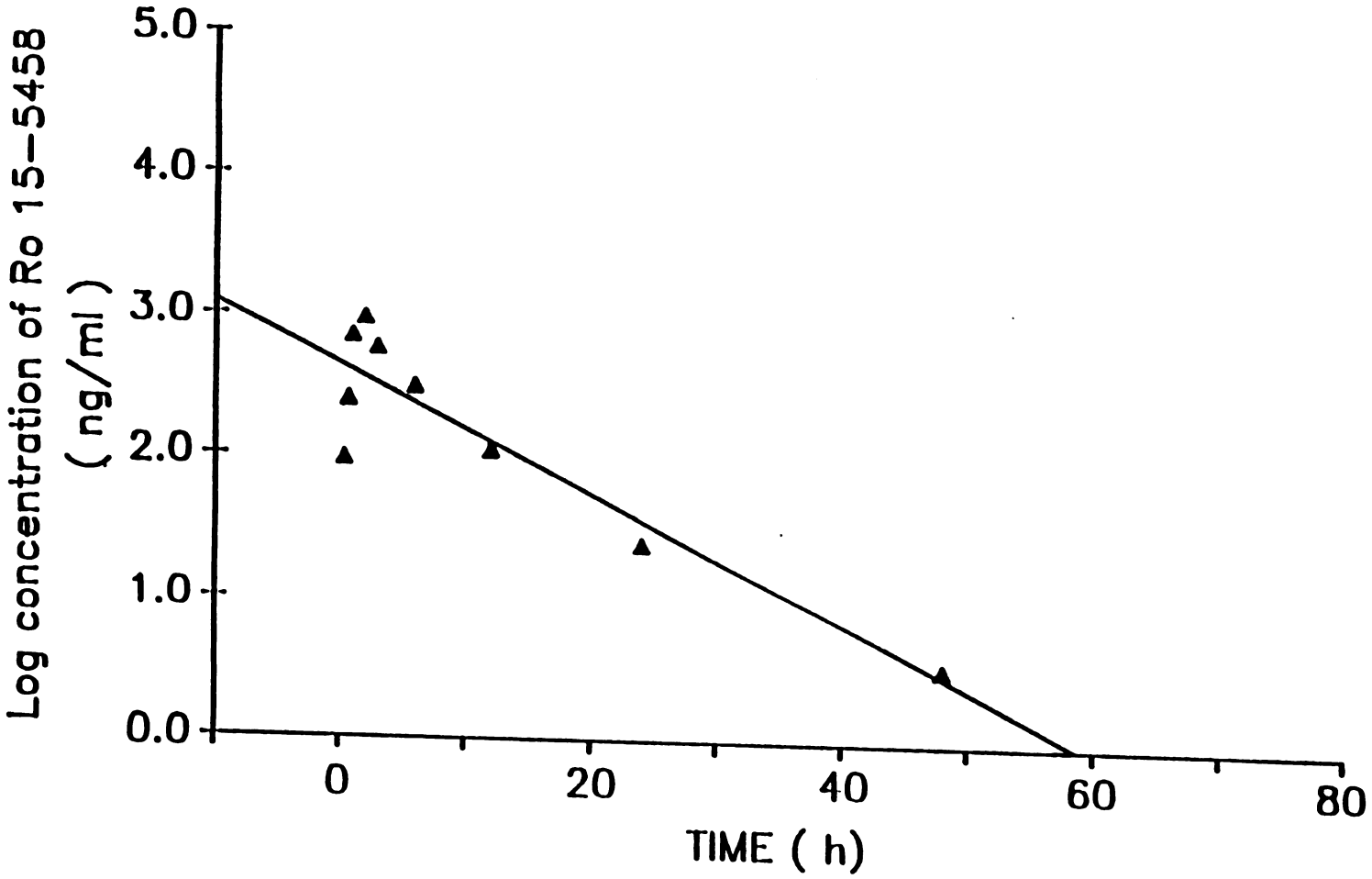


Figure 6. Average Ro 15-5458 concentration in plasma following a 50-mg/kg oral dose (n=5). The line was fitted by non-linear least squares regression (r=0.98).

C. Absorption of Ro 15-5458

Ro 15-5458 appeared in the general circulation abruptly and declined without a plateau (Figure 7; Appendix I). An average peak plasma concentration of 700 ng/ml was observed 2 h after dosing. However, results from individual rabbits suggested variable bioavailability of Ro 15-5458 after oral dosing. Maximum plasma levels ranged from 500 to 1200 ng/ml (1.3-3.1 μM).

Visual inspection of the plasma level-time pattern for individual animals suggested that the data were consistent with zero order absorption (Figure 8). To confirm this hypothesis, we fitted the average data to a one-compartment model, with either first-order or zero-order absorption, using the PCNONLIN program. Based on the correlation coefficients, the estimated pharmacokinetic parameters and their standard error of mean, the goodness of fit of the experimental data was better for the zero-order absorption kinetic model (Appendix II). Therefore, this model was used to calculate apparent kinetic parameters (Table 2) for the oral absorption of Ro 15-5458. The apparent rate of absorption (k_o/V), the lag time (t_o) and the duration of absorption (T) have been well estimated with this absorption model.

DISCUSSION

The absorption of drugs from the gastrointestinal tract after oral administration is a complex process. It is easier to study pharmacokinetics after intravenous infusion, as more reliable kinetic information

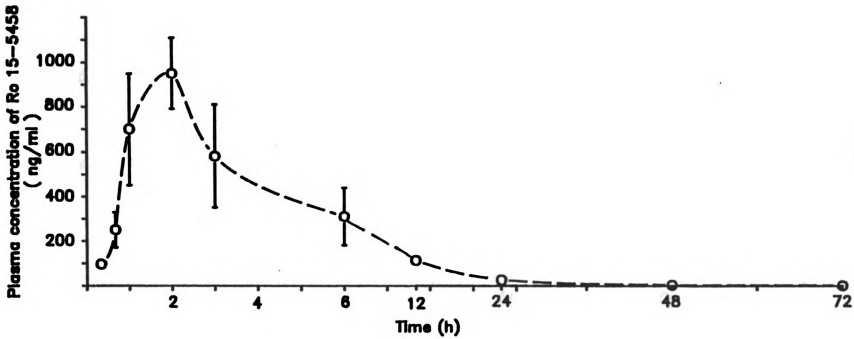


Figure 7. Plasma concentrations of Ro 15-5458 as a function of time following the oral administration of a 50-mg/kg single dose to rabbits (n=5). Result from individual rabbit is shown in Appendix I.

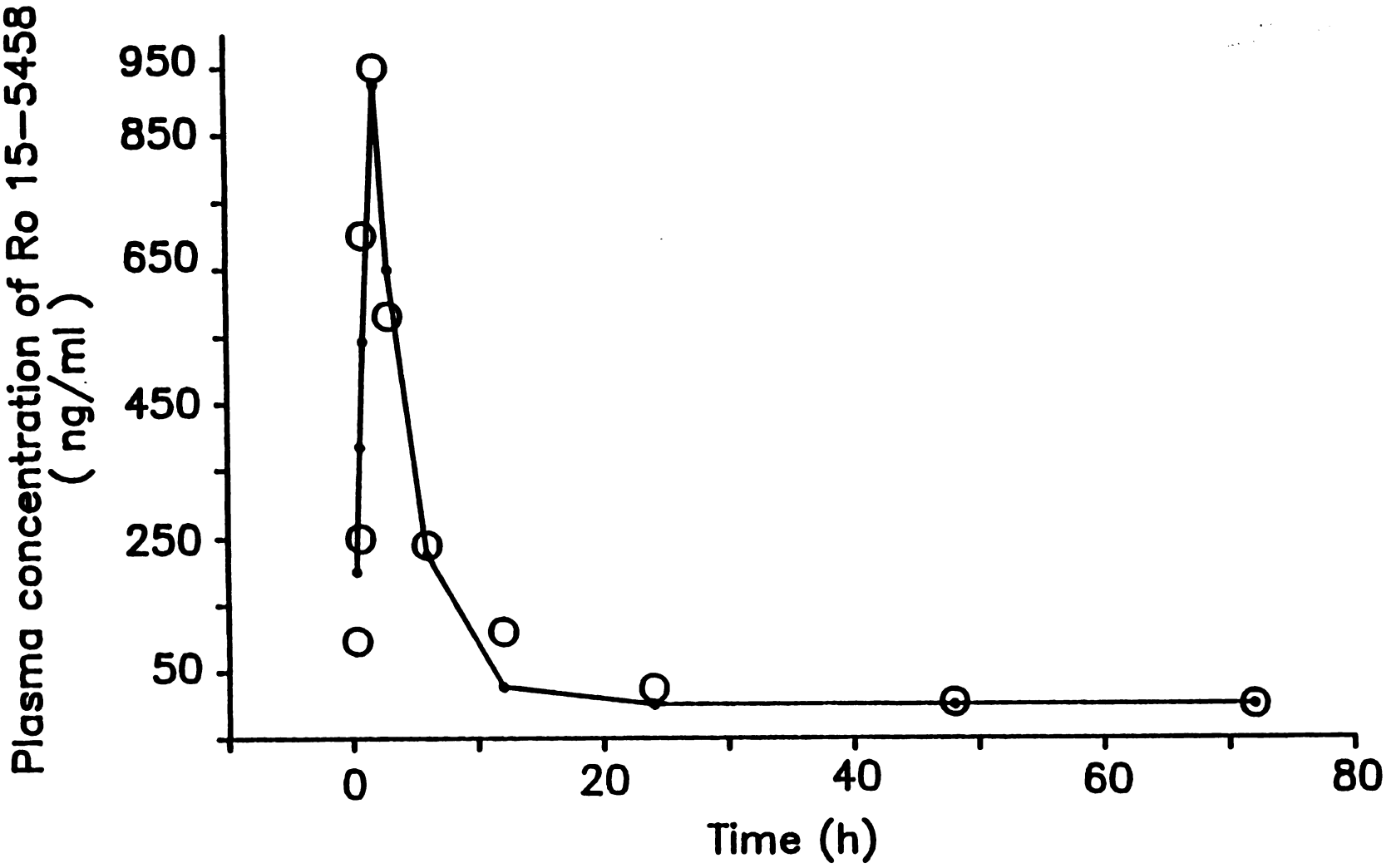


Figure 8. Plasma profile of Ro 15-5458 in rabbits (n=5) after a 50 mg/kg single oral dose. Key: ○○ , experimental points; and solid line, calculated value using a zero-order absorption kinetic model (r=0.97).

TABLE 2

Pharmacokinetic Data from Rabbits Following a 50-mg/kg Single Oral Dose of Ro 15-5458

Unless otherwise stated, parameters were calculated from the average plasma level (n=5).

Apparent elimination rate constant (k_e), h^{-1}	0.12
Maximum plasma level (C_{max}), μg/ml	0.7
Apparent rate of absorption (k_o/V), mg/h-L[a]	0.53
Area under plasma level time curve, (AUC) mg-h/L	5.8
Apparent onset of absorption (t_o), min[b]	6.0
Apparent duration of absorption (T), h[c]	1.5

[a]Average value calculated using plasma level and the corresponding time from each rabbit. The following equation was used:

$$C_{max} = k_o/Vk_e \quad 1\text{-}e^{-K_eT}$$

[b]Calculated using the following equation:

$$C = k_o/Vk_e \; 1\text{-}e^{-K_e(t-t_o)}$$

and plasma level and corresponding time (t) of the distribution phase, i.e. t_o is the mean lag time, calculated using each data point obtained during absorption (T).

[c]Calculated using the following equation:

$$AUC = k_oT/Vk_e$$

can be obtained from the plasma level-time data. In the absence of such data, however, the plasma level-time data obtained after oral administration are also useful. It is particularly important to obtain such data if it is intended to administer the drug by the oral route. The absorption of Ro 15-5458 from the gastrointestinal tract of rabbits was very rapid, the maximum concentration of the drug in the peripheral blood being reached within 1 to 2 h. However, Ro 15-5458 showed variability in the extent of appearance in the blood, as determined by the AUC and C_{max} values from the individual rabbits. The source of this variation is not clear. However, it is possible to make certain speculations based on some preliminary observations made in our laboratory. The presence of food in the gut of the rabbits significantly reduced the extent of absorption of Ro 15-5458. Therefore, the rate or extent of drug appearance in the blood could vary depending on the residual food in each rabbit after 12 hr of fasting. In this regard, it is important to note that food was observed in the stomach of some animals after 12 h of fasting. It was not possible to fast rabbits for longer periods, since toxic effects, including convulsions, were observed.

Another potential explanation for the observed variability could be inter-animal differences in excretion or metabolism. Urine samples collected from two rabbits between 0 and 2 h after dosing were analyzed by HPLC. During this period, about 6% and 11% of the measured maximum plasma level of the drug was excreted unmetabolized by these animals. Variations may thus arise from differences in the excretion of the drug during the time of absorption.

These observations suggest the need for modification of the dosage form before any therapeutic or toxicological evaluation of the drug can be performed in other species. It may also be necessary to examine the time interval between food intake and drug administration, since the amount of food in the gastrointestinal tract may be a factor in either the toxicity or the efficacy of the drug.

Schistosomes live as adults in the blood vessels of the host. Rational treatment of infection with these parasites requires maintenance of an optimal level of the chemotherapeutic agent in the general circulation, so that a balance is maintained between toxicity to the host and efficacy towards the parasite. Thus, understanding the plasma kinetics of Ro 15-5458 is important for its development as a potential broad spectrum antischistosomal agent. Extrapolation of the rabbit data to other definitive hosts of the parasite may not be straightforward. However, the overall absorption and plasma pattern of the drug in rabbits furnishes important preliminary information that will be required before human trials. The rabbit data are particularly interesting in terms of the toxicity of Ro 15-5458. A preliminary acute toxicity study reported previously by Stohler and Montavon (1984) described the death of all mice 14 days after treatment with a single oral dose of 500 mg/kg. On the other hand, a single oral dose of 15 mg/kg completely clears mice of worm burden (Eshete, this thesis). It is possible to expect low plasma levels of the drug in mice at this low dose, perhaps indicating a sufficient safety margin between the therapeutic dose and the toxic dose.

In treatment of plasma level-time data obtained after oral administration of drugs, the absorption kinetics have been invariably

assumed to be first-order. This notion has been challenged by evidence that, at least under certain conditions, the gastrointestinal absorption of several drugs, including ethanol (Cooke, 1970), sulfisoxazole (Kaplan et al., 1972), erythromycin (Colburn & Gibaldi, 1977), hydroflumethiazide (McNamara, Colburn & Gibaldi, 1978) and griseofulvin (Bates & Carrigan, 1975), is best described as an apparent zero-order (constant rate) rather than first-order process. The plasma level-time profile of orally administered Ro 15-5458 in rabbits also appears to follow a one-compartment pharmacokinetic model with zero-order absorption, first-order elimination. The lag time was short, which suggests an abrupt appearance of the drug in the circulation. Drug levels peak and decline without a plateau, consistent with zero-order absorption.

SUMMARY

The absorption kinetics of Ro 15-5458, a new antischistosomal drug, was studied in rabbits following the administration of a single 50 mg/kg oral dose as an aqueous suspension in 25% glycerol-1% cremophor EL. Ro 15-5458 was absorbed from the gastrointestinal tract rapidly with a lag time of about 6 min and declined without a plateau with a half-life of about 6 h. Maximum plasma levels ranged from 500 to 1200 ng/ml (1.3-3.1 μM). The plasma concentration-time profile of Ro 15-5458 after a single oral dose appears to follow a one-compartment pharmacokinetic model with zero-order absorption, first-order elimination.

CHAPTER III

THE *IN VITRO* EFFECTS OF RO 15-5458 ON ADULT *SCHISTOSOMA MANSONI*

INTRODUCTION

Three drugs have been recommended for the large-scale chemotherapy of schistosomiasis: metrifonate for *S. haematobium*, oxamniquine for *S. mansoni*, and praziquantel, which is effective against all the species of schistosomes responsible for the human disease. Metrifonate is inexpensive but requires several doses, and this makes its delivery expensive (Korte *et al.*, 1986). Oxamniquine is not well tolerated by patients, and strains of parasites resistant to the drug have been reported in Brazil (Dias *et al.*, 1982) and Kenya (Coles *et al.*, 1987b). One other drawback with drugs, which are only effective against a single species of the parasite, is that multidrug therapy may be required in areas where more than one species is endemic, which is undesirable for medical and economic reasons.

Praziquantel has a broad spectrum of activity against these parasites. Single oral dose administered for patients in most areas is well tolerated, but the cost of praziquantel continues to limit its use in developing countries. The possibility of appearance of drug-tolerant or resistant parasites must also be considered when treatment reaches a large sector of the infected population.

Another broad spectrum antischistosomal agent that is in the process of development is Ro 15-5458. The antischistosomal activity of this compound has been demonstrated to be superior, in experimental animals, to praziquantel and other standard antischistosomal drugs (Stohler & Montavon, 1984).

Preliminary work from our laboratory indicated that administration of a 15 mg/kg single oral dose of Ro 15-5458 to mice infected with S. mansoni effectively clears the infection. The drug was effective when administered i.p as well as i.m (Eshete, unpublished data). However, the in vitro incubation of adult schistosomes with reasonable concentrations of the drug did not result in parasite killing. In the meantime we observed significant stimulation of the parasite musculature after exposure to Ro 15-5458 in vitro.

Since the maintenance of the integrity of the neuromuscular system of schistosomes in vivo is critical for reproduction and perhaps in the feeding behavior of the parasites, in this section an attempt was made to characterize the effects of the drug on the musculature of schistosomes and to determine the relevance of this in vitro effect to the in vivo schistosomicidal action. Attempts were also made to generate active metabolites of the drug since the parent compound has no in vitro activity. Drug effects on some vital physiological processes of the parasite were also examined.

MATERIALS AND METHODS

A. Infection and Parasite Recovery

A Puerto Rican strain of S. mansoni maintained in laboratory-reared Biomphalaria glabrata and outbred female white mice was used in the study. The method of infection was by i.p injection of 200-300 schistosome cercariae. Adult parasites were isolated from infected mice 6 to 7 weeks post-infection as previously described (Bennett and Seed, 1977). Briefly, mice were killed with cervical dislocation and were dissected for parasite removal. The parasites collected from the mesenteric and intrahepatic veins were then placed in RPMI-1640 medium (Gibco, Grand Island, NY) buffered with 25 mM Hepes [4-N(-2-hydroxy ethyl)-1-piperazine ethane sulfonic acid] (Sigma Chemical Co., St. Louis, MO) to pH 7.4. Parasites from at least 5 mice were pooled and randomized into groups before any experimental manipulation. In experiments where single parasites were used, males and females were separated using a dissecting microscope and fine forceps after exposure in RPMI-1640 to 0.5 mg/ml sodium pentobarbital.

B. In Vitro Parasite Culture

Long-term parasite cultures were performed in Eagle's minimum essential medium (Dulbecco modified) supplemented with 20% calf serum containing 8 μg/ml of gentamicin and 1 μg/ml of amphotericin B at 37°C in 5% CO_2/95% air. Duplicate dishes containing 7 parasites in 3 ml medium were incubated with Ro 15-5458 at a final concentration of 1 or 10 μM. Control parasites were incubated in parallel with treated parasites. One group was incubated for 3 h and the other for 3 days. After a single

application of the drug and incubation for either 3 h or 3 days, the medium was replaced and incubation was continued for 20 days, changing the medium 3 times a week. Each parasite was viewed under a light microscope for contractile activity in a 10 μM 5-HT containing medium. The absence of response to the 5-HT was used as an index of parasite death.

C. Parasite Transfer

These experiments were performed using the method previously described by Cioli (1976). Twenty male parasites were exposed to 10 μM of Ro 15-5458 in RPMI-1640 medium buffered with 25 mM Hepes, pH 7.4 containing 20% calf serum. Parasites were washed and surgically transferred to the mesenteric vein of uninfected Nile rats (Arventicus niloticus) either after 3 h or 3 days of in vitro drug exposure. Parasites were retrieved from the recipient animals by perfusion 2 weeks following transfer and the number of viable parasites was determined.

D. Mechanical Activity Recordings

Contractile activity of paired parasites was measured as described by Bennett & Pax (1987). Parasites were randomized into 10x75 mm thin wall glass tubes (3 pairs/tube) containing 1.3 ml of Hepes-buffered RPMI-1640 medium and were preincubated for 15 min at 37°C. Stock solutions of all 9-acridanone hydrazone compounds were made in dimethyl sulfoxide (DMSO). Desired final concentrations of the drugs were achieved by transferring aliquots of the stock and the same volume of DMSO to treatment and control tubes, respectively. Incubations were continued for 30 min and the contractile activity was measured for 20 seconds with a

motility meter. Lactic acid analysis was also carried out on media aliquots by monitoring the rate of reduction of NAD^+ at 340 nm during the conversion of lactate to pyruvate by the enzyme lactate dehydrogenase (Schon, 1965).

Longitudinal muscle tone of male parasites was recorded as described by Fetterer et al. (1977, 1978). Briefly, parasites were placed in a recording chamber containing 2.5 ml of RPMI-1640 medium at 37°C. After the hook-up of parasites to the system, a 5 min equilibration period was allowed before the application of any treatment. Aliquots of 10 mM stock solutions of the 9-acridanone hydrazone compounds in DMSO were added to the recording chamber to desired concentrations such that the amount of DMSO did not exceed 1%. Tension was monitored by means of a non-flexible suction pipet made of polyethylene tubing (id 0.38 mm, od, 1.0 mm) attached to the tail end of the worm by mild suction applied with a syringe. The second pipet (flexible) with the same internal and external diameter was then attached 0.75 to 2.5 mm anterior to the non-flexible pipet. The attachment of the wire to the flexible pipet was such that any movement of the pipet produced an up or down movement of the flag. The flag was oriented such that it interrupted the light path between a light source and a photodiode whose output was attached to a chart recorder by way of a DC amplifier. With this arrangement, any contraction or relaxation of the muscle in the worm resulted in a change in the light reaching the photodiode and was consequently recorded as a voltage change by the chart recorder (Figure 9).

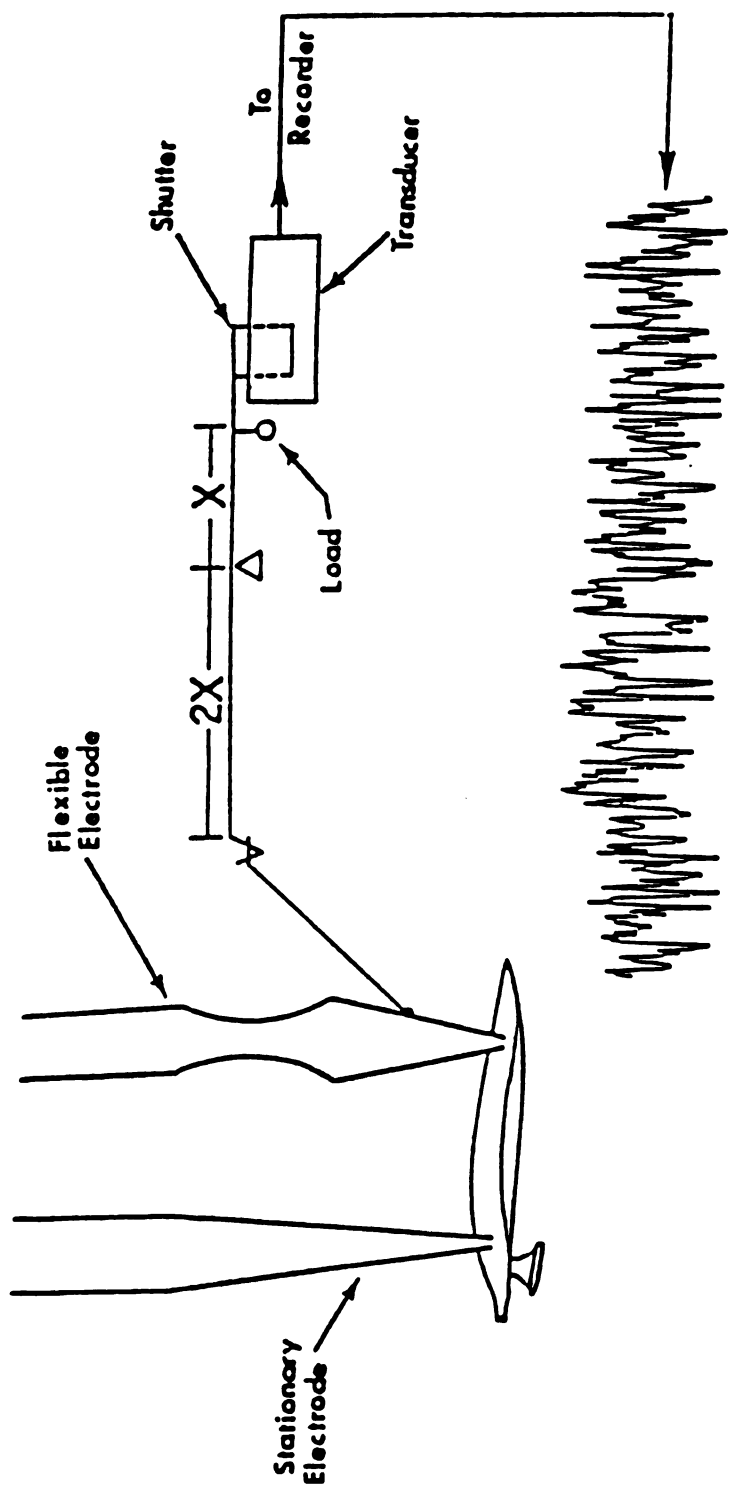


Figure 9. Schematic representation of the apparatus used to record the longitudinal muscle tension in adult male S. mansoni.

Since the force developed by the parasite depends on the distance between the two pipets, the tension developed is expressed in milligrams per millimeter of parasite length. Data presented in tables and elsewhere in the text, are the change in tension 10 min after addition of the agent compared to the tension just prior to its introduction or, for controls, after addition of the vehicle (DMSO). In cases where two or more pharmacological agents were introduced sequentially, data presented represents the difference in tension between the second and the first drug recorded for 10 min in both cases.

E. Egg Production Assay

The in vitro effect of Ro 15-5458 on the fecundity of S. mansoni was determined as described by Morrison et al. (1986). Briefly, 15 paired parasites were placed into sterile Erlenmeyer flasks containing 50 ml of RPMI-1640 medium described above containing 50% horse serum plus 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco, Long Island, NY) and 50 μM β-mercaptoethanol. Ro 15-5458 was dissolved in DMSO and was added in a volume not exceeding 50 μl. Control flasks received the same volume of drug-free DMSO. Parasites were then incubated at 37°C in an oscillating water bath for 72 h. After incubation, each flask was shaken and three 5 ml media aliquots were placed in a gridded petri plates and eggs counted under a dissecting microscope.

F. Uptake and Incorporation of Precursors

Parasites recovered from the same infection date were randomized into tubes (6 males/tube) containing 1 ml of RPMI-1640 medium. Parasites

were preincubated in a water bath at 37°C for 30 min and then Ro 15-5458 at the final concentration of 10 μM was added to treatment tubes. Both control tubes (containing 0.1% DMSO) and drug treated tubes then received 10 μCi/ml of either L-[4,5-^{3}H]leucine (50 Ci/mmole), [methyl-^{3}H]thymidine (74 Ci/mmole) or [5,6-^{3}H]uridine (45 Ci/mmole) (all from ICN Radiochemicals Irvine, CA). After a 1 h incubation, parasites were washed 3X with ice cold saline, homogenized and then sonicated in 1 ml of dH_2O. The homogenate was divided into two equal portions and both were treated with 5% trichloroacetic acid (TCA). The precipitate in one of the fractions was collected on glass fiber filters (Whatman GF/C) using a vacuum-operated filtration apparatus (Millipore Corp., Bedford, MA). The filters representing the TCA-insoluble portion, were transferred to a glass scintillation vial containing 250 μl of NCS tissue solubilizer (Amersham, Arlington Heights, IL) and 100 μl of glacial acetic acid and were incubated at 60°C for 20 min. After solubilization, the vials were brought to room temperature, 10 ml of ACS aqueous scintillant was added, and radioactivity was measured with a Beckman LS7600 scintillation spectrometer. The other fraction was centrifuged at 10,000 g for 10 min and the resulting supernatant was used to measure TCA-soluble radioactivity. The pellet was dissolved in 1 M NaOH for protein determination with the method of Albro (1975) using bovine serum albumin as standard.

G. Incubation of Parasites in Drug-Metabolizing System

1. Source of liver

Infected and uninfected ICR/BR Swiss Webster female mice (Harlan Sprague-Dawley, Indianapolis, IN) were treated either with sodium

phenobarbital (3x80 mg/kg, i.p) for 3 days or Ro 15-5458 (2x15 mg/kg, p.o) at 8 h interval. Mice from both treatment groups were killed 24 h after the last dose by cervical dislocation and the livers excised and placed in a sterile, ice-cold beaker.

2. Preparation of liver homogenate fraction

A liver homogenate fraction enriched in microsomal drug metabolizing enzymes was prepared as previously described (Ames et al, 1973). All the apparatus and solutions used were sterile and precooled to 4°C; the temperature of the tissue was kept below this temperature during the preparation. The livers were washed in 0.15 M KCl, blotted, minced with scissors in the same solution (3 ml/g of wet liver) and homogenized in a glass homogenizer with a Teflon pestle driven by a Con-Torque power unit (Eberbach Corp., Ann Arbor, MI). The homogenate was centrifuged for 10 min at 9000 x g and the supernatant (S_9) decanted and saved. One ml aliquots of the supernatant (microsomes from 0.4 g of wet liver) were quickly frozen in dry ice and stored at -80°C. On the same day, a portion of the supernatant was tested for activity by measuring aryl hydrocarbon hydroxylase (Van Canfort et al., 1977) and benzphetamine-N-demethylase (Prough and Ziegler, 1977) activity.

3. Preparation of NADPH-Regenerating System

Three different systems were used for drug activation; the standard 0.1 M phosphate, pH 7.5; RPMI-1640 buffered with 20 mM Hepes, pH 7.5; and Krebs-Ringers tris maleate (KRTM), pH 7.5. The composition of KRTM is 120 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 5 mM glucose (Read et al., 1963). All the systems contained 340 μM $NADP^+$, 81

μM NADPH, 300 μM NADH, 4.8 mM glucose-6-phosphate and 2 U/ml of glucose-6-phosphate dehydrogenase. The phosphate system also contained 6 mM $MgCl_2$.

4. Incubation of Schistosomes with Ro 15-5458

Ten pairs of parasites (n=3), were placed in a dish containing 3 ml of filter sterilized RPMI-1640 medium, 25 μM Ro 15-5458 and 1 ml of S_9 from uninfected or infected phenobarbital-treated mice. The dishes were then incubated at 37°C for 1 h. Simultaneously, 2 ml of phosphate system with 1 ml of S_9 and 100 μM Ro 15-5458 was incubated at the same temperature for 2 h without parasites. One ml of this mixture was added to another group of dishes containing 10 pairs of parasites in 7 ml of RPMI-1640 medium (14 mM phosphate and 12.5 μM of the original drug) and incubated for 1 h. Similar experiments were performed with S_9 from infected and uninfected Ro 15-5458 treated mice. Other dishes with only S_9, 25 μM Ro 15-5458 or 1 ml of S_9 and 1% DMSO (the vehicle used to dissolve drug) were also simultaneously incubated. After 1 h of preincubation parasites from all dishes were washed in RPMI-1640 and were incubated in 10 ml of RPMI-1640 medium containing 20% calf serum, 8 μg/ml of gentamicin and 1 μg/ml of amphotericin B up to 15 days changing the medium 3-times a week.

I. Incubation of Parasites in Plasma Recovered from Treated Rabbits

A 50 mg/kg single oral dose of Ro 15-5458 was administered to rabbits (n=2) and plasma was recovered 6 h after dosing by cardiac puncture. The plasma was divided into two equal volumes and one of the portions was heated at 55°C for 1 h to inactivate complement, while the other fraction was used for parasite incubations without further treatment. Fifteen pairs of parasites were incubated in RPMI-1640 medium

containing 50% plasma from dosed rabbits, 8 μg/ml gentamicin and 1 μg/ml of amphotericin B. Incubation was continued for 10 days after replacement of the rabbit serum with 10% calf serum in RPMI-1640 medium, changing the medium twice a week.

RESULTS

The effect of different concentrations of the drug on the motor activity of paired parasites is shown in Figure 10. The stimulatory effect of 100 μM of the drug was exhibited for a short time (ca 1 h) and declined dramatically within the next 10 h. With 1 μM of Ro 15-5458, a marginal stimulatory effect was observed, while 10 μM significantly stimulated the parasites, an effect which occurred quickly and lasted up to 48 h. Metabolic requirements of the parasites were also raised after incubation with Ro 15-5458; the amount of lactic acid produced by the parasites was correlated with the degree of muscular contraction (Table 3). The stimulatory effect of 10 μM Ro 15-5458 was reversed by the cholinomimetics, carbachol (100 μM), physostigmine (10 μM) and arecoline (1 μM) (Table 4).

Longitudinal muscle tension of adult male S. mansoni was also recorded in the presence and absence of Ro 15-5458 in the medium. Ro 15-5458 caused a dose-dependent increase in the muscle tone (Figure 11); drug concentrations greater than 50 μM cause acute toxicity to parasites, thus reducing the muscle response observed at these concentrations. A similar study was undertaken to compare Ro 15-5458 with other compounds which have been previously reported to have effects on schistosome longitudinal

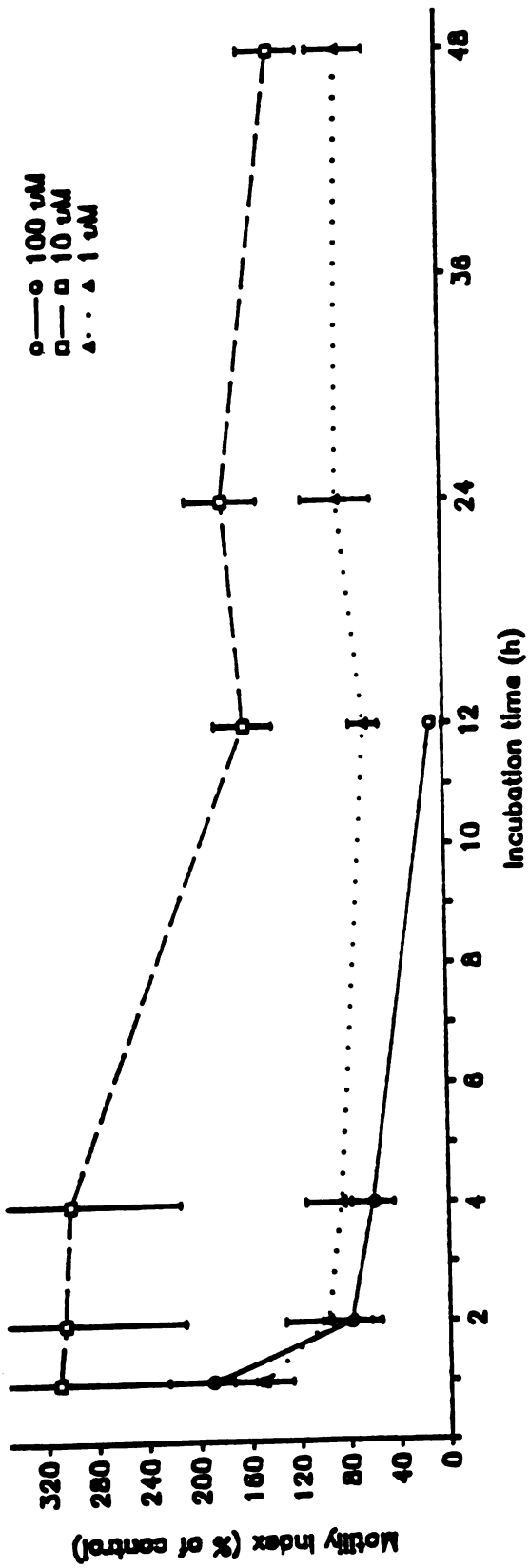


Figure 10. Effect of Ro 15-5458 on contractile activity of paired S. mansoni in vitro. Drug was introduced to tubes containing parasites in RPMI-1640 at the final concentration of 1, 10 and 100 µM and motility measured at times indicated after exposure. Each data point represents the mean ± SD (n=5).

TABLE 3

In vitro Effect of Ro 15-5458 on Lactic Acid Production by *S. mansoni*

Incubation Time (h)	Control 0.1% DMSO	Ro 15-5458 (μM) 10	1	0.1
24	2.4±0.7	7±1.0	2.7±0.3	3.3±0.1
48	5.7±0.7	11±2.0	6.3±0.3	6.7±0.7

Values are lactic acid excreted to medium, mean ± SD, in μmoles/pair (n=6).

TABLE 4

Effect of Ro 15-5458 on the Contractile Activity of Adult Paired *S. mansoni* and Reversal by Cholinergic Agonists

Treatment (μM)	Motility Index (arbitrary units)
Control (0.1% DMSO)	156±35
Ro 15-5458 (10)	242±49[a]
+ Carbachol (100)	25± 5[b]
Carbachol (100)	24±10
+ Ro 15-5458 (10)	81±24
Control (0.1% DMSO)	115±14
Ro 15-5458 (10)	295±48
+ Carbachol (10)	211±63
Carbachol (10)	36± 7
+ Ro 15-5458 (10)	238±31
Physostigmine (1)	37±13
+ Ro 15-5458 (10)	174±53

Motility of control parasites and parasites exposed to the first drug[a] was recorded. The second drug[b] was then introduced and the motility measured 30 min after the second drug's introduction.

Results are mean ± SD (n=5).

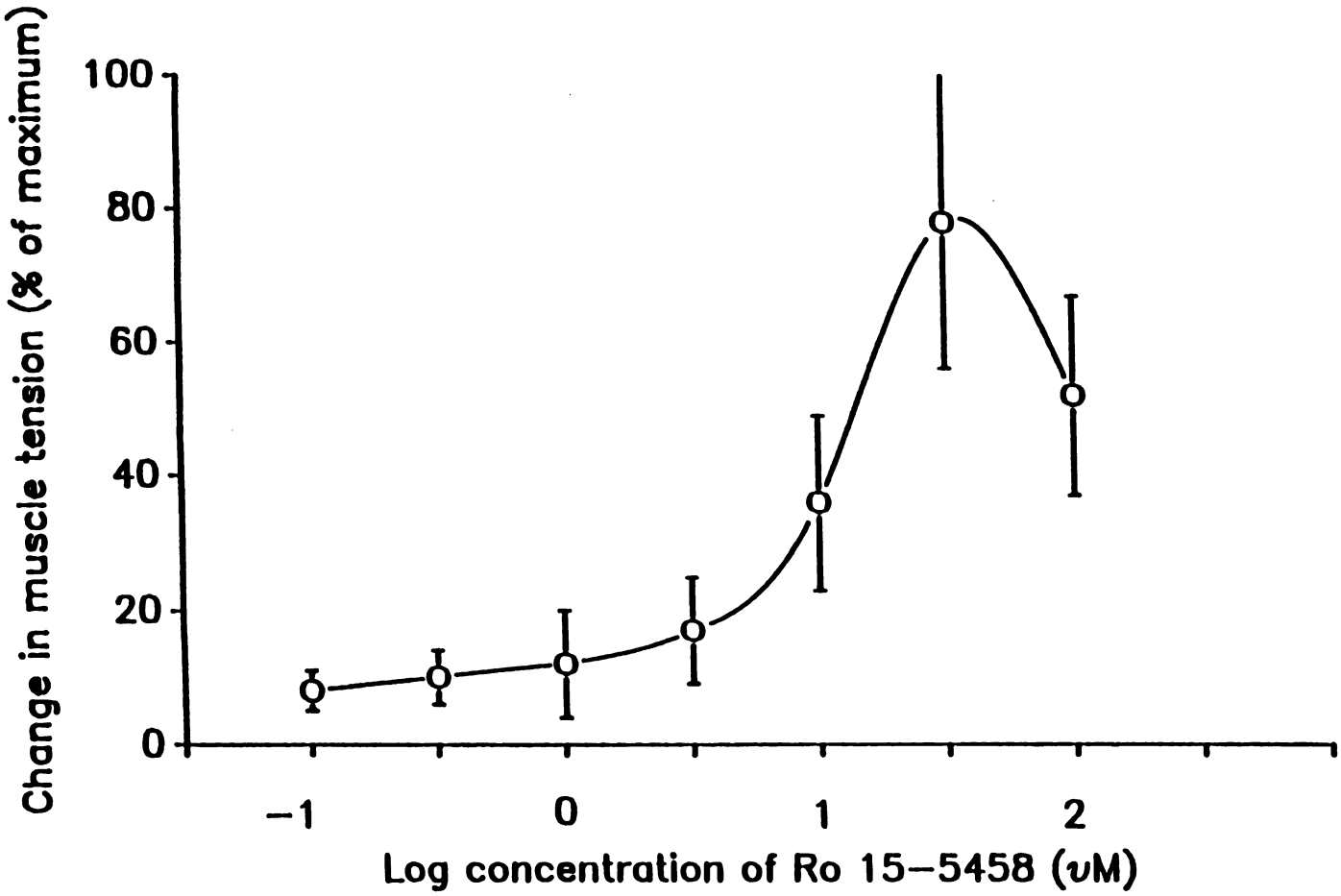


Figure 11. Dose-response relationship between Ro 15-5458 and muscle tension of adult male S. mansoni. The maximum value was determined from the double reciprocal plot of concentration vs. change in tension. This value (3.2 mg/mm) was taken as 100% change. Each data point represents the mean ± SD (n=5).

muscle tension (Mellin et al., 1983, Pax et al., 1984). The effects of atropine and 5-HT were (both 20 μM) compared to the same concentration of Ro 15-5458; Ro 15-5458 showed a more pronounced effect on longitudinal muscle tension than either atropine or 5-HT (Figure 12). The ionotropic effect of Ro 15-5458 was reversed by washing and was antagonized by 100 μM carbachol, 10 μM physostigmine, 1 μM arecoline and 100 μM of the 5-HT antagonist metergoline; physostigmine was more potent than carbachol in its action (Table 5). Carbachol causes relaxation and flaccid paralysis of schistosome longitudinal muscle. Ro 15-5458 altered the magnitude of the response to carbachol when it is simultaneously introduced in the recording chamber i.e., the dose of carbachol required to elicit a particular magnitude of response is increased by Ro 15-5458 (Figure 13).

The effect of other structural analogs of Ro 15-5458 on the musculature of male schistosomes was also studied using the same technique. A stimulatory effect on the parasite musculature was observed with all the compounds containing an aminoalkyl side chain at the 10 position of the acridine ring. Substitution of this functional group with $-CH_3$ (Ro 14-7918), $-CH_2-CH_2-CH_2-CH_3$ (Ro 15-3149) or $-CH_2-CH_2-O-CH_3$ (Ro 15-3455) diminished the muscular effects (Table 6).

The effect of the drug on in vitro egg production as well as on the uptake and incorporation of DNA, RNA and protein precursors into the parasite macromolecules was also examined. The results of the egg production experiments (Table 7) showed no difference in egg output between parasites treated with 10 μM Ro 15-5458 and control parasites.

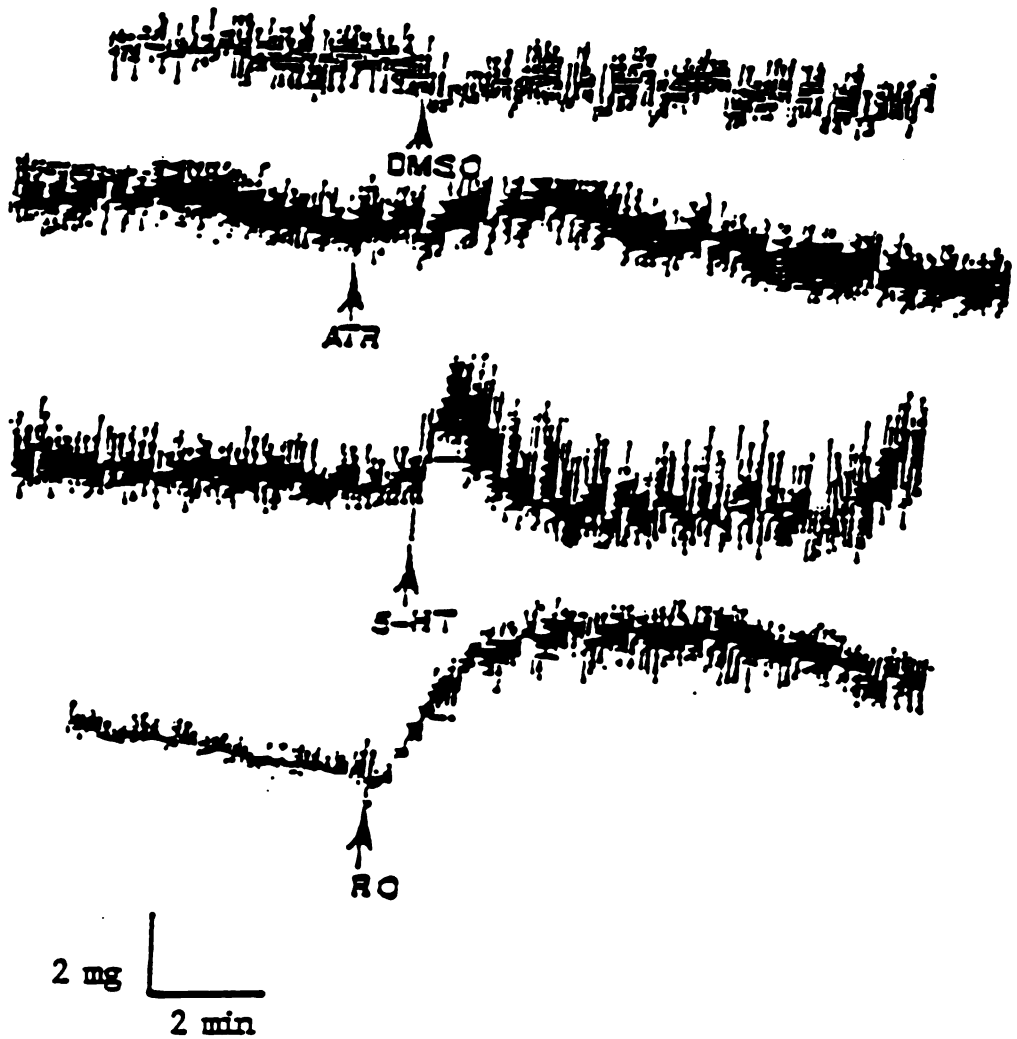


Figure 12. The effect of 20 μM Ro 15-5458, atropine and 5-HT on the longitudinal muscle tone of adult male S. mansoni. Drug was introduced at the arrow after 5-10 min of equilibration. ATR = atropine; 5-HT = 5-hydroxytryptamine; RO = Ro 15-5458.

TABLE 5

Effect of Ro 15-5458 on the Longitudinal Muscle Tone of Adult Male S. mansoni and Reversal by Cholinergic Agonists

Treatment (μM)	Change in Muscle Tone (mg/mm)
Ro 15-5458 (10)	2.5±1.2[a]
+ Carbachol (10)	0.6±0.7[b]
Ro 15-5458 (10)	2.1±0.8
+ Carbachol (100)	-4.7±2.3
Ro 15-5458 (10)	3.9±1.7
+ Physostigmine (10)	-0.7±0.3
Ro 15-5458 (100)	3.1±1.8
+ Arecoline (1)	-6.7±3.0
Ro 15-5458 (10)	3.3±1.8
+ Metergoline (100)	-1.2±0.6
Carbachol (10)	-1.6±0.1
+ Ro 15-5458 (10)	2.4±0.9
Carbachol (100)	-3.1±1.0
+ Ro 15-5458 (10)	2.0±0.7
Physostigmine (10)	-2.5±1.3
+ Ro 15-5458 (10)	-0.9±0.4

After recording the basal muscle tone, two drugs were introduced sequentially.

[a]Change in tension 10 min after introduction of the first drug as compared to the tension prior to treatment.

[b]Change in tension 10 min after introduction of the second drug as compared to the tension just prior to the second drug's introduction.

Results are the mean ± SD (n=5).

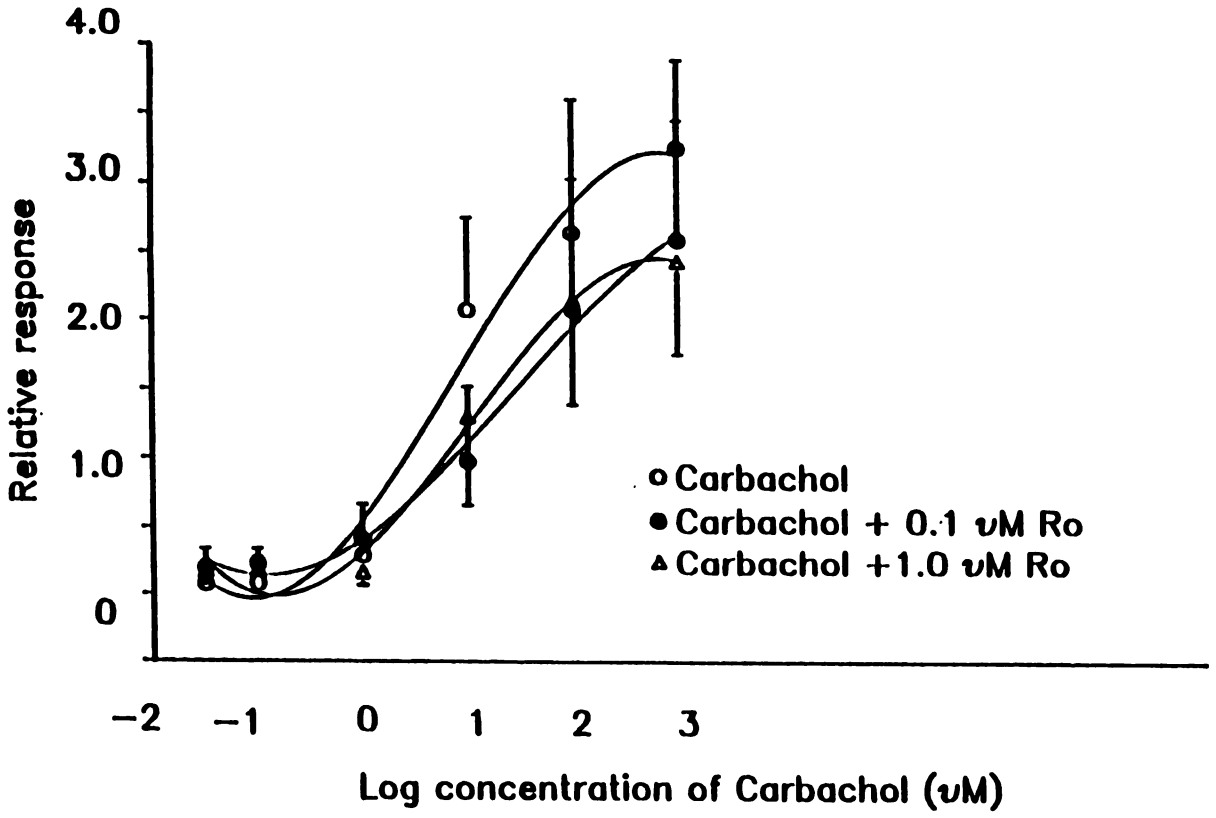


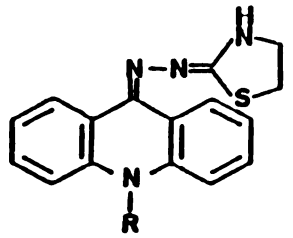
Figure 13. The effect of Ro 15-5458 on the response of schistosome longitudinal muscle tension to carbachol.
The response to carbachol or carbachol plus Ro 15-5458 was measured after recording the basal muscle tension.

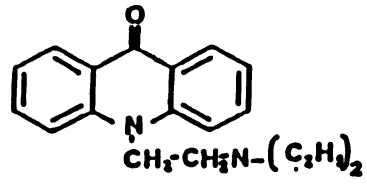
$$\text{Relative response} = \frac{\text{Change in tension due to carbachol}}{\text{Mean basal muscle tension}}$$

Each data point represents the mean ± SD (n=5).

Table 6. Effects of Ro 15-5458 and its analogs on the longitudinal muscle tone and contractility of adult S. mansoni[a]

[a]Longitudinal muscle tension of male and contractility of paired parasites was determined as described in Methods. [b]Control in 0.1% DMSO and 10 μM of the 9-acridanone hydrazone compounds were used. [c]ED_{90} was determined by the Hoffmann-La Roche Co.





Ro 21-6787

Ro CODE	R
14-7918	$-CH_3$
15-3149	$-CH_2-CH_2-CH_2-CH_3$
15-3455	$-CH_2-CH_2-O-CH_3$
15-9895	$-CH_2-CH_2-N(CH_3)_2$
15-5458	$-CH_2-CH_2-N(C_2H_5)_2$

TABLE 6

	Control	Ro 14-7918	Ro 15-3149	Ro 15-3455	Ro 15-5458	Ro 15-9895	Ro 21-6787
Change in muscle tone							
10 μM	---	0.32±0.2	0.28±0.12	0.16±0.13	1.1±0.2	0.82±0.23	1.7±0.1
100 μM	---	0.68±0.4	0.67±0.3	0.48±0.3	1.6±0.25	1.7 ±0.4	3.0±0.7
Motility Index[b]	248±81	182±53	171±22	169±43	556±120	555±120	507±130
ED_{50} in mice (mg/kg) single dose[c]	---	~100	>100	>100	10.5	16.3	>5x300

TABLE 7

The *In Vitro* Effect of Ro 15-5458 on Egg Production by *S. mansoni*

Exp. No.	Control (1% DMSO)	Ro 15-5458 (10 μM)
1	120±30	110±33
2	120±33	90±45
3	130±25	133±31

Results, eggs/female/72 h, are expressed as mean ± SD (n=4).

In addition, the continuous presence of 10 μM Ro 15-5458 during a 1 h incubation period has no effect on both the uptake and incorporation of precursors (Table 8).

Since the drug exerted a significant stimulatory effect on the parasite musculature, the relation between this phenomenon and the *in vivo* schistosomicidal action of the drug was examined. Three approaches were used to test this possibility:

(1) A 15 mg/kg dose of Ro 15-5458 was administered to infected mice and the shift of parasites from the mesenteric veins to the hepatic portal veins (hepatic shift) was examined after autopsy of mice, 1, 6, and 12 h, and 1, 3 and 4 days following drug administration.

(2) The contractile activity of parasites recovered at times indicated above was also determined using a micromotility meter. A slight increase in the motor activity of parasites was observed when mice were autopsied 1 h after dosing; otherwise neither the hepatic shift nor an increase in motor activity was observed at the other test periods.

(3) Ro 21-6787, the inactive analog of Ro 15-5458, caused a comparable increase in motor activity and muscle tension of *S. mansoni*. The ability of Ro 21-6787 to cause the same change on parasite muscle as Ro 15-5458, but its failure to kill parasites also argues against the possible involvement of the muscular effect on the schistosomicidal action of Ro 15-5458.

The *in vitro* schistosomicidal action of Ro 15-5458 was studied after exposing parasites in culture to the drug and subsequent long-term

TABLE 8

Uptake and Incorporation of Radioactive Precursors into Adult *S. mansoni* Exposed to Ro 15-5458 *In Vitro*

	^{3}H-Thymidine		^{3}H-Uridine		^{3}H-Leucine	
	Acid Insoluble	Acid Soluble	Acid Insoluble	Acid Soluble	Acid Insoluble	Acid Soluble
Control	128.6±59	470.6±89	136.5±20.3	6659.6±74	3.96±1.9	11.9±3
Ro 15-5458 (10 μM)	126.8±75	484.6±67	102.7±18	6961.6±62	4.14±1.3	12.8±3

Parasites (6 pairs/tube) were incubated for 1 h in RPMI-1640 containing 10 μCi/ml of tritiated precursor. TCA-soluble and TCA-insoluble radioactivity was determined as described in the Methods. Results (dpm/mg protein x 10^{-3}) are expressed as the mean ± SD (n=10).

incubation or transferring to uninfected Nile rats. Exposure of parasites to 10 μM Ro 15-5458 for 3 h or 3 days has no lethal effect, while schistosomes recovered from treated mice and incubated in vitro were all killed. Similarly, when parasites treated in vitro were surgically transferred to uninfected host, incomplete killing was noted. To the contrary, when donor mice were treated with a 15 mg/kg Ro 15-5458 and parasites transferred after 1, 3 and 4 days of dosing, all were killed (Table 9).

Since Ro 15-5458 exerts no in vitro schistosomicidal action, the possibility of the drug being metabolized to an active antischistosomal was considered. A high concentration of phosphate is lethal to schistosomes, thus drug was preincubated in 0.1 M phosphate NADPH regenerating system with S_9 fraction. When aliquots of these were transferred to incubation medium containing parasites, no killing was observed up to 15 days of incubation. It was felt that the preincubation process without the parasites may have the possibility of not exposing the parasites to products that may have a very short life; we therefore used the RPMI-1640- and KRTM-NADPH-regenerating system, in which case the incubation of parasites was possible in a system containing Ro 15-5458 and the S_9 fraction. Exposure of parasites in the latter system for 1 h did not result in parasite killing. Taken together the results indicate the absence of schistosomicidal product(s) in the systems. This does not preclude the possibility of formation of products that could be schistosomicidal but unstable in vitro.

Administration of SKF 525-A (proadifen hydrochloride), a compound that reduces the level of cytochrome P450 enzymes, prior to dosing with

TABLE 9

Percent Survival of Parasites Treated with 10 μM Drug *In Vitro* or After *In Vivo* Treatment with Ro 15-5458

Time[a]	CONTROL		IN VITRO		IN VIVO	
	Transfer[b]	Culture[c]	Transfer	Culture	Transfer	Culture
3 h	N.D.[d]	100	60	100	N.D.	93
1 day	N.D.	N.D.	N.D.	100	0	0
3 days	85	100	10	100	0	0
4 days	N.D.	100	N.D.	100	0	0

[a]Represents time of exposure to the drug *in vitro* or time of recovery from donor mice after dosing.

[b]20 male parasites were transferred to either Nile rats or hamsters (n=2).

[c]Triplicate dishes containing 7 worms were incubated for 20 days in Eagle's medium (Dulbecco modified) or RPMI-1640 containing 20% calf serum. Medium was changed 3 times a week. Absence of contractile activity in a 10 μM 5-HT containing medium was used as an index of parasite death.

[d]N.D. = not determined.

Ro 15-5458 was also without effect. In these experiments, a 75 mg/kg i.p. dose of SKF 525-A in saline was administered to infected mice at 8 h intervals for 24 h. A single dose of Ro 15-5458 (15 mg/kg) was then given to the same group of mice after 36 h of the last SKF 525-A dose. Mice were autopsied after 12 days for parasite count.

In addition, serum recovered from dosed rabbits and used for parasite incubation in vitro had no schistosomicidal effect.

DISCUSSION

The in vitro studies involved incubation of parasites with 10 μM of Ro 15-5458, which is about 3 times the maximum plasma concentration of the drug measured after a single oral dose of 50 mg/kg given to rabbits (Eshete & Bennett, 1990). The effects of 10 μM Ro 15-5458 were studied at this concentration since it exerted a significant effect on muscle without acute toxicity. Both contractile activity and longitudinal muscle tension were increased after exposure of parasites to this concentration of Ro 15-5458. Ro 15-5458 also blocked the paralytic effects of cholinomimetic agents on the parasite musculature. Characteristics of this blockade and the muscle effects resembled that of hycanthone, another antischistosomal agent that interacts with the cholinergic system of schistosomes (Hillman & Senft, 1975).

The mechanism or site of action of Ro 15-5458 on the parasite musculature was not determined. However, speculations can be made in light of the structural similarity between Ro 15-5458 and the anti-psychotics, chlorpromazine and trifluoperazine.

These phenothiazines have been shown to inhibit certain Ca^{++}-calmodulin-dependent enzymes such as cyclic nucleotide phosphodiesterase and Ca^{++}-Mg^{++}-ATPase (Levin & Weiss, 1977, 1979; Roufogalis, 1982). Inhibition of the schistosome calmodulin-dependent bovine heart adenosine 3',5'-cyclic monophosphate phosphodiesterase by these compounds (Thompson et al., 1986) has also been demonstrated. In addition, these investigators observed acute inotropic effects on the longitudinal musculature of parasites after exposure to the phenothiazines.

The existence of Ca^{++}-Mg^{++}-ATPase in whole worm homogenates and tegument fractions of S. mansoni has been reported by several investigators (Nechay et al., 1980; Cesari et al., 1981; Podesta & McDiarmid, 1982). The schistosome enzyme is inhibited by phenothiazines but is activated by cholinomimetic agents (Semeyn, 1987) and exogenous calmodulin (Cesari et al., 1981). The parasite enzyme is presumed to function like the mammalian enzyme in the extrusion of Ca^{++} from the cytosol. It is thought that the manner by which cholinergic agents exert their inhibitory action on schistosome mechanical activity is through the activation of a Ca^{++}-Mg^{++}-ATPase, which brings about a net efflux of Ca^{++} from the parasites (Semeyn, 1987). Phenothiazines by inhibiting the enzyme, increase the internal [Ca^{++}] and increase tone. Ro 15-5458 may act in a similar fashion on the parasite musculature, i.e., it may inhibit the Ca^{++}-Mg^{++}-ATPase, an enzyme presumed to be important for the actions of cholinomimetic agents. This inhibition would increase intracellular [Ca^{++}] and promote contraction.

Antischistosomal compounds, such as praziquantel, the benzodiazepine derivative, Ro 11-3128 (Pax et al., 1978) and alkyldibenzylamines (Bueding

& Penedo, 1957), cause paralysis of the parasite musculature. The resultant immobilization of the parasite suckers results in the dislodgment of parasites by the host blood flow (Andrews, 1981; Mehlhorn et al., 1981). Drugs that affect parasite musculature are thought to cause slow death by depriving parasites of nutrition. However, the muscular effect of Ro 15-5458 does not appear to be related to its schistosomicidal action. Evidence for this observation comes from the following:

(1) The inactive analog, Ro 21-6787 (ED_{90} > 5 x 300 mg/kg), is as potent as Ro 15-5458 (ED_{90}, 10.5 mg/kg) in increasing the muscle tone and motor activity of the parasites.

(2) The concentration-response curve of Ro 15-5458 on parasite musculature suggested that the threshold concentration, required to produce significant changes, is above 5 μM. It is doubtful that this concentration can be achieved after dosing mice with a 15 mg/kg, judging from the absorption kinetics data in rabbits (Eshete & Bennett, 1990).

(3) Absence of schistosomicidal activity upon long-term in vitro incubation and the absence of a hepatic shift even 4 days after dosing also suggest that other mechanism(s), unrelated to the muscular effect, must be responsible.

Nevertheless, a report on the ED_{90} of the 9-acridanone hydrazone compounds (Stohler & Montavon, 1984; Stohler, personal communication), together with the in vitro experiments on the parasite musculature (Table 6) suggest the existence of a relationship between the schistosomicidal activity and presence of the aminoalkyl side chain. Low antischistosomal activity was observed when this group was substituted with hydrogen or a

methyl group (Sturrock et al., 1987). The reason for this observation is not known at this time. However, it appears that the thiazolinyl group at position 9 of the acridine ring is vital for the schistosomicidal action of these compounds, although the relative potency may depend on the presence of an aminoalkyl side chain, perhaps due to pharmacokinetic reasons.

Studies on vervet monkeys in Sudan (Sulaiman et al., unpublished data) and on baboons (Sturrock et al., 1985; 1987) demonstrated the efficacy of Ro 15-5458 in reducing both the output and viability of eggs. Prompted by these results, the effect of this compound on the incorporation of precursors into parasite macromolecules was examined, since the synthesis of these macromolecules has a direct bearing on egg production and hatching ability. The presence of Ro 15-5458 in the incubation medium has no influence on either the uptake or incorporation rate of the precursors; this is in contrast to parasites treated in vivo, where the opposite result was observed. The results of the in vitro incubation and parasite transfer experiments suggest the absence of in vitro schistosomicidal activity of Ro 15-5458.

Why the drug needs the host system to exert its lethal action is not clear. It may be converted to an active metabolite(s), although this could not be simulated in the in vitro experiments. It is also possible that drug may be converted to an active form by stomach acid, since we noted in vitro schistosomicidal effect with Ro 15-5458 hydrolyzed with 0.01 N HCl and pH adjusted to 7.4. Another speculation is that the drug could bind to the host erythrocytes, ingestion of which by the parasites in sufficient amount would cause toxicity. This possibility was examined

in vitro, but the parasites did not consume cells in culture. Attempts to induce the cytochrome P450 enzymes by prior administration of sodium phenobarbital or inhibit them by the administration of SKF 525A also did not affect the activity of the drug.

Parasite elimination *in vivo* may also require additional host-dependent immunological events. However, in light of the absence of any structural or functional damage to the parasite surface membrane (Chapter IV) after dosing with Ro 15-5458, there is skepticism about the contribution of the cellular immunological response of the host to parasite killing at the initial phase of parasite drug interaction.

SUMMARY

No schistosomicidal effect could be demonstrated when pairs of *S. mansoni* isolated from mice were transferred into a medium containing a solution of Ro 15-5458 (10 μM) for 72 h. However, the drug caused a significant stimulation of the parasite musculature at this concentration, increasing both the contractile activity and longitudinal muscle tension. This property of the drug appears to depend on the presence of the aminoalkyl functional group at the position 10 of the acridine ring, since analogs lacking this group did not cause similar effects. An attempt was made to characterize the drug effects on the parasite musculature and determine the contribution of this effect to the *in vivo* schistosomicidal action of the compound. It appears that the drug actions on the parasite musculature are dependent on processes normally modulated by the cholinergic system, since concentration-dependent mutual antagonism was observed between Ro 15-5458 and cholinomimetic agents. Although drugs

that affect parasite musculature are known to cause slow death of parasites, the muscular effect of Ro 15-5458 does not appear to be related to its schistosomicidal action.

It appears that the drug requires host factors to exert its lethal effects. Conventional attempts to demonstrate bioactivation of the drug were without effect, perhaps due to the instability of the putative active product at the experimental condition. It is also important to note that the liver homogenate system utilized in the present study may not be responsible for the generation of the active product. Also, the possible involvement of the host immunological system in parasite killing cannot be overlooked.

CHAPTER IV

THE ANTISCHISTOSOMAL EFFECTS OF RO 15-5458 ADMINISTRATION TO THE MOUSE HOST

INTRODUCTION

The *in vitro* studies discussed previously indicated the absence of intrinsic schistosomicidal activity of Ro 15-5458 on *Schistosoma mansoni*. On the other hand, the administration of a 15 mg/kg single oral dose of Ro 15-5458 to mice infected with the parasites completely clears the infection within 7 days after dosing. Thus, the drug appears to be converted to an active antischistosomal compound *in vivo* or requires uncharacterized host factors to exert its lethal effects. Conventional attempts to detect the host-generated active product(s) or demonstrate bioactivation of the drug were not successful. Therefore, an approach was chosen in which parasites were exposed to the drug in the host before their removal for *in vitro* studies.

The present study utilized a form of bioassay where the survival of parasites *in vitro* and effects on various biochemical and physiological parameters were examined in parasites recovered from dosed mice. In most studies, drug effects were evaluated by comparison with parasites from the same group of infected mice which only received the vehicle. One problem with parasites treated *in vivo* is choosing when to remove them from the host to examine the effects of chemotherapy. To detect drug effects of

primary therapeutic significance, parasites have to be retrieved before the commencement of secondary morbid and host-mediated changes. For this purpose, in the S. mansoni-mouse model parasites are traditionally recovered prior to the hepatic shift (Jewsbury, Homewood & Gibson, 1974).

In the present study, the effects of Ro 15-5458 were examined after recovery of parasites before the commencement of the hepatic shift. The specific objective was to determine the earliest detectable drug-induced damage to the parasite, with the ultimate goal of understanding the mode of antischistosomal action of Ro 15-5458. To this end, the effects of in vivo treatment on vital parasite biochemical/physiological processes were examined. Attempts were also made to characterize the effects of Ro 15-5458 on the biosynthesis of parasite macromolecules.

MATERIALS AND METHODS

A. Chemicals

All chemicals used in this study were analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MO), Mallinckrodt (St. Louis, MO) or Boehringer-Mannheim (Indianapolis, IN). Chemicals for SDS-polyacrylamide gels were from Bio-Rad Labs (Richmond, CA). Agarose (pure grade) was from Bethesda Research Laboratories (Gaithersburg, MD). [2,8-^{3}H]adenine (39 Ci/mmole), [α-^{32}P]CTP (3000 Ci/mmole), [α-^{32}P]dCTP (3000 Ci/mmole), L-[4,5-^{3}H]leucine (50 Ci/mmole), L-[methyl-^{14}C]methionine (44 mCi/mmole), L-[^{35}S]methionine (1088 Ci/mmole), [methyl-^{3}H]thymidine (74 Ci/mmole) and [5,6-^{3}H]uridine (45 Ci/mmole) were from ICN Biomedicals (Irvine, CA).

B. Drug Administration

A stock of Ro 15-5458 was prepared as an aqueous suspension in 25% glycerol - 1% cremaphor EL and a 15 mg/kg single dose was administered by gavage to infected mice 5-7 weeks postinfection. Mice infected on the same day with the same batch and number of cercariae, but which only received the vehicle, were used as the source of control parasites.

C. Schistosomicidal Action of Ro 15-5458

A 15 mg/kg single oral dose of the drug was administered 5-7 weeks postinfection to mice infected with Puerto Rican strain of S. mansoni. Control and treated mice were autopsied 2 weeks after dosing and the number of parasites in the mesenteric and intrahepatic veins counted. Drug effects on hycanthone-sensitive and -resistant parasites were determined by the same protocol. Parasites selected for hycanthone resistance in the laboratory were provided by Dr. Alan Sher (National Institutes of Health, Bethesda, MD), Dr. John Bruce (University of Lowell, Lowell, MA), and Dr. Donato Cioli (Institute of Cell Biology, Rome, Italy).

D. In Vitro Culture and Parasite Transfer

For in vitro culture, paired adult schistosomes were isolated from mice and washed in sterile medium. Incubations were performed at 37°C in 10 ml of RPMI-1640 medium (Gibco, Long Island, NY) buffered with 25 mM Hepes, containing 20% calf serum plus 8 μg/ml of gentamicin and 1 μg/ml amphotericin B. Triplicate dishes containing 8 paired parasites in each group were incubated up to 15 days, changing the medium 3 times per week.

Parasite transfer experiments were performed as described by Cioli (1976). Donor mice were treated with a 15 mg/kg single oral dose of Ro 15-5458 and parasites were isolated 1,3 and 4 days after dosing. Twenty male parasites were then transferred to uninfected hamsters (Mesocricetus auratus). Worms were retrieved from recipient animals by perfusion 2 weeks following transfer.

E. Measurement of Surface Membrane Changes and Muscular Activity

Parasite surfaces were scanned with a JOEL 35CF electron microscope operated at 15 kv to identify drug-induced changes in the morphology of the male parasite surface membrane. Parasites removed from the veins by careful incision and pressing were collected with a soft brush and were transferred to saline isotonic to mouse plasma with the addition of sucrose (320 mOsm). Worms were carefully washed with the same saline and were processed for microscopical examination as previously described (Weisberg et al., 1983). Resting surface membrane potentials were recorded from the dorsal surface of male parasites as described by Thompson et al. (1982). Motor activity of parasites (3 pairs/ml RPMI-1640) was measured with a micromotility meter (Bennett & Pax, 1987).

F. Measurement of Parasite Gut Pigment

Twenty paired parasites were transferred to 250 μl of 25 mM potassium phosphate buffer, pH 7.6. Parasites were homogenized at room temperature at 1000 RPM for 3 min in a glass homogenizer with a teflon pestle driven with a motor unit (Talboys Engineering Corp., Emerson, NJ) and 100 μl of the homogenate was mixed with 2.5 M NaOH to a final volume

of 2 ml. The optical density of the samples was then determined at 580 nm using a double-beam spectrophotometer (Beckman UV-5260 Palo Alto, CA). Since schistosomes contain other porphyrins that may form ferrihemochromes that absorb at this wavelength, the same number of parasites were incubated in RPMI-1640 medium plus 5% calf serum for 72 h at 37°C to regurgitate their gut contents (Mercer & Chappell, 1985a; Senft & Senft, 1962). These parasites were treated as described above and the resulting absorbance value was subtracted from the initial determination to account for the non-hematin absorbance.

The dark pigment in the gut of schistosomes is composed mainly of hematin (Rogers, 1940). Thus, an attempt was made to compare its level in control and drug treated parasites using bovine hematin (Sigma Chemical Co.) as a standard.

G. Determination of Parasite Weight and Protein Content

The weight of 30 male parasites (n=6) was determined after drying at 110°C for 15 h. Total protein was measured by the method of Albro (1975), using bovine serum albumin as standard.

H. Determination of Glycogen, ATP, Lactate and Utilization of Glucose

Glycogen was determined by comparing the concentration of glucose in neutralized aliquots of the parasite homogenate in 0.5 M KOH, as described by Tielens & Van den Bergh (1987), before and after hydrolysis with 2.2 U/ml amyloglucosidase for 30 min at 48°C. Glucose was measured

by the method of Raabo & Terkildsen (1960). To reduce glycogen degradation in culture, schistosomes were transferred promptly from the veins to ice-cold medium before each experiment.

For ATP measurement, 50 pairs of parasites collected in ice-cold medium were homogenized in 300 μl of equal parts of saline and 12% TCA. The homogenate was centrifuged at 10,000 g for 5 min. ATP was measured in 100 μl aliquots of the supernatant as previously described (Jaworek et al., 1974).

Glucose utilization was measured as the sum of the reduction in glucose content of the medium plus the glycogen loss of the parasites during incubation. Briefly, 10 pairs of parasites were transferred simultaneously from each mouse into medium either at room temperature for in vitro culture or in an ice bath for determination of glycogen before incubation. Parasites in the ice bath were transferred to 200 μl of 0.5 M KOH for homogenization and the homogenate was stored at 4°C overnight. The other group of parasites were incubated for 21 h in RPMI-1640 medium containing 20% calf serum plus antibiotic-antimycotic solution. These parasites were homogenized in the same manner as the non-incubated parasites and the level of glycogen as well as media glucose was compared to preincubation levels. Lactic acid analysis was also carried out in media aliquots after treatment with 8% perchloric acid by the method of Schon (1965).

I. Measurement of the Uptake of Metabolic Precursors

The transtegumental uptake of metabolic precursors was determined by incubating male parasites (10/tube) at 37°C for 5 min. Parasites were

incubated in Krebs-Ringers saline containing 5.5 mM of glucose buffered with 25 mM Tris-maleate to pH 7.4 (Read et al., 1963) for leucine and methionine or Hepes buffered RPMI-1640 medium for adenine, uridine and thymidine. After equilibration at 37°C, tritiated precursor (10 μCi/ml) diluted with cold precursor was added at the final concentration of 5X the K_t (concentration of the solute yielding a half-maximum velocity of transport) value as previously determined (Isseroff et al., 1976, Levy & Read, 1975) to a specific activity of 33, 5.4, 44.5, 29.2 and 31.4x10^7 cpm/μmole of leucine, methionine, adenine, uridine and thymidine, respectively. After incubation, parasites were washed 3 times with cold medium and transferred to a glass vial containing 2.0 ml of 70% ethanol and extracted for 24 h. The amount of precursor transported was calculated from the specific activity at the start of each incubation.

J. Measurement of the Incorporation of Leucine into Parasite Protein

To determine the incorporation of [^{3}H]leucine into parasite proteins, 60 male parasites were incubated in minimum essential medium without leucine, buffered (pH 7.4) with 25 mM Hepes containing 20% calf serum and 400 μCi of [^{3}H]leucine (50 Ci/mmole). Following 24 h incubation at 37°C, parasites were washed and then frozen and thawed to remove the surface membrane. Detegumented worms were homogenized in 1.0 ml of 0.05 M Tris buffer (pH 7.5) and centrifuged at 100,000 g for 45 min. The resulting pellet (100k pellet) was solubilized for 10 min (3% SDS in Tris buffer at 100°C) and proteins (60 μg/well) were separated using 7.5% SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Five mm fractions of the gel were cut, dried overnight at room temperature and solubilized in 30%

H_2O_2:NH_4OH (99:1) at 60°C for 5 h and counted with a Beckman LS7600 liquid scintillation spectrometer after addition of 100 μl glacial acetic acid and 10 ml of ACS aqueous scintillant (Amersham).

K. DNA and RNA Extraction

Isolation of nucleic acids was performed essentially as described by Chirgwin et al. (1979). Parasites were ground into a paste in liquid nitrogen and the paste was transferred to 4.4 M guanidine thiocyanate containing 0.9% β-mercaptoethanol. After 1 h of denaturation, the homogenate was layered on a 5.7 M and 3 M preformed CsCl step-gradient and centrifuged at 29000 rpm for 22 h in a Beckman SW41 rotor at 19°C. Livers of uninfected mice were extracted simultaneously for comparison in the same manner. The DNA at the CsCl interface was collected with wide-bore pasteur pipets and dialyzed against 10 mM Tris HCl, pH 7.5, 1 mM EDTA and 0.1% SDS for 1 h. The DNA solution was made 50 μg/ml in DNase-free RNase A (Sigma Chemical Co., St. Louis, MO) and dialyzed for another hour against 10 mM Tris HCl, pH 7.5. Proteinase K (Boehringer-Mannheim, Indianapolis, IN) was added to 50 μg/ml and the dialysis continued overnight with several changes of the dialysis buffer. The DNA solution was then extracted 3X in equal volumes of Tris-saturated phenol, 2X in chloroform and 2X in ether. At this point, the solution was transferred to acid-washed Corex tubes and precipitated with 2.5 volumes of ethanol and 0.5 M ammonium acetate, pH 5.5, and stored at -20°C. The RNA at the bottom of the tubes was suspended in diethyl pyrocarbonate-treated water and extracted 3X with butanol/chloroform (1:4) before storage. Poly(A^+)

RNA was isolated using Oligo(dT)cellulose (BRL, Gaithersburg, MD) as described by Jacobson (1982).

L. In Vitro Translation

Total RNA (10 μg), after checking for integrity by electrophoresis on a 1.2% denaturing agarose gels, or 1 μg poly(A$^+$) RNA was translated in a rabbit reticulocyte system according to the supplier's protocol (Promega, Madison, WI). Translation was measured as the incorporation of [^{35}S]methionine (1088 Ci/mmole) in a 2 μl aliquot of the 25 μl translation mixture as alkali-resistant, TCA-precipitable counts. Samples that incorporated label 5-fold or more over zero-message controls were loaded on 10% SDS-polyacrylamide gels (Laemmli, 1970) after 5-fold dilution in sample loading buffer containing 2% SDS and heating at 95°C for 2 min. After electrophoresis, gels were fixed and stained with 0.02% Coomassie brilliant blue R in 50% methanol, 7% acetic acid overnight. The gels were dried, prepared for fluorography with EN ^{3}HANCE (New England Nuclear) and exposed at room temperature to Kodak X-OMAT x-ray film for 3-5 days. The resulting fluorograms were quantitated with an LKB UltraScan XL Laser densitometer (Pharmacia, Piscataway, NJ).

M. Recombinant Plasmids and Probe Synthesis

Bacteria harboring plasmids with schistosome ribosomal RNA genes (rDNA), pSM r3.0 and pSM r4.7 (Van Keulen *et al.*, 1985), and superoxide dismutase (SOD), pSM 12.38 (Simurda *et al.*, 1988), were obtained from Dr. Philip LoVerde (SUNY-Buffalo). The insert of pSM r3.0 is 3.0 kb and contains the coding region of the 28S rRNA gene. The insert of pSM r4.7

is 4.7 kb and contains a sequence that codes for the 18S rRNA. pSM 12.38 contains a 320 bp insert that encodes the carboxy terminal of schistosome SOD. The recombinant plasmid containing sequences for the β-actin gene was provided by Dr. Harold Weintraub (The Fred Hutchison Cancer Research Center, Seattle, WA). The insert (ca.1 kb) is from chicken brain (Cleveland et al., 1980) and is subcloned into the Sma I site of plasmid pSP64 in the antisense orientation. Bacterial culture and plasmid preparations were performed using standard methods (Davis et al., 1986).

β-actin riboprobes (ca. 10^8 dpm/μg RNA) were synthesized after linearization of the clone with Pvu II in a transcription system containing 50 μCi of [α-^{32}P]CTP (3000 Ci/mmole) according to the manufacturer's protocol (Promega, Madison, WI). Random primed synthesis of the ribosomal DNA and SOD probes was performed as described by Feinberg & Vogelstein (1983) after electroelution of the bands of interest from agarose gels (Figure 14). Probe synthesis was performed using a kit obtained from Boehringer-Mannheim (Indianapolis, IN) with 50 μCi of [α-^{32}P]dCTP (3000 Ci/mmole) as a labeling nucleotide to a specific activity greater than 10^8 dpm/μg DNA.

N. Dot-Blot Analysis

Total RNA (5 or 10 μg) was denatured with 1.2 M glyoxal in 10 mM phosphate buffer, pH 6.8, by incubating for 1 h at 50°C. Suitable dilutions of the samples in 0.1% SDS were applied to Zeta probe nylon membranes (BioRad Labs, Richmond, CA) using a manifold (BRL, Gaithersburg,

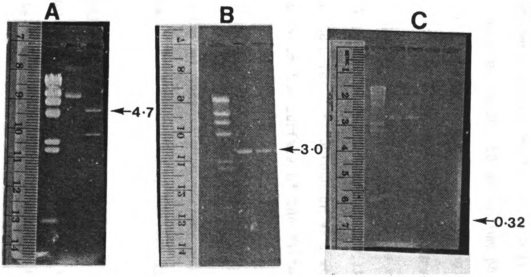


Figure 14. Recombinant plasmids. Plasmid DNA after extraction was digested with EcoRI (pSM r4.7 and pSM r3.0) and PST I (pSM 12.38) before electrophoresis. Separation was performed on 0.8% agarose gel except for pSM 12.38 where a 14% gel was used. A = pSM r4.7, B = pSM r3.0, and C = pSM 12.38.

MD) after increasing the total volume to 100 μl with 2xSSC (SSC is 150 mM NaCl, 15 mM trisodium citrate). The filters were baked at 80°C for 2 h and residual glyoxal was removed by placing the filters in 20 mM Tris HCl, pH 8.0, containing 1 mM EDTA, preheated to 100°C. Prehybridization and hybridization of the blots were performed as previously described (Thomas, 1980). Briefly, filters were hybridized for 18-24 h at 50°C in prehybridization buffer (50% formamide, 5XSSC, 50 mM sodium phosphate buffer, pH 6.8, 0.2% each of ficoll, bovine serum albumin and polyvinyl pyrolidine, 100 μg/ml yeast tRNA, 100 μg/ml of sonicated denatured salmon sperm DNA and 0.1% SDS) containing 10% dextran sulfate. Blots were washed for two 1-h periods in 2XSSC/0.1% SDS and subsequently in 0.1XSSC/0.1% SDS twice for the same period at 68°C for a riboprobe or 42°C when a DNA probe was used. The radioactivity on the surface of the filters was quantitated with a TRACE 96 direct counting system (Inotech Biosystems International, Lansing, MI), or the filters were exposed briefly to Kodak X-OMAT x-ray films at -80°C using a DuPont intensifying screen, and the density of the autoradiograms was measured as described by Mariash et al. (1982).

O. Northern Blot Analysis

Fifteen or 20 μg of total RNA or 1 μg poly(A^+) RNA was electrophoresed on 1.2% agarose-formaldehyde gels in MOPS buffer (20 mM [3-(N-morpholino)propanesulfonic acid], 5 mM sodium acetate and 1 mM EDTA), pH 7.0, and transferred to Zetaprobe membranes by capillary action using 10XSSC for 16-24 h. After baking the filters for 2 h at 80°C, hybridization and filter washing were performed as described above. Autoradiograms were prepared by exposing the filters to a Kodak X-OMAT film at -80°C. In

order to determine the relative intensity of hybridization of a specific probe in control vs. treated parasites, densitometric analysis was performed with a LKB UltraScan XL laser densitometer.

P. Southern Blot Analysis

Genomic DNA fragments were electrophoresed on 0.8% agarose gel in TBE buffer (50 mM Tris base, 50 mM boric acid and 1 mM EDTA), pH 8.0 after digestion with either EcoRI or Hind III enzymes. After electrophoresis gels were stained for photography with 0.5 μg/ml ethidium bromide and were transferred to Zetaprobe membranes using standard methods (Maniatis et al., 1982). Hybridization and filter washing was performed as described above except when Southern blots were hybridized with DNA probes; in which case the filters were washed for 1 h with four changes of 2XSSC/0.1% SDS at room temperature and subsequently in 0.2XSSC/0.1% SDS for two 20 min at 42°C. Autoradiograms were prepared by exposing the filters to a Kodak X-OMAT film at -80°C.

RESULTS

Administration of a 15 mg/kg single oral dose of Ro 15-5458 to mice infected with S. mansoni completely cleared parasites from mice after dosing. The drug was also effective against younger parasites, 10-20 days postinfection, when the same dose was administered to mice. The drug exerted similar schistosomicidal activity on wild-type parasites and parasites selected in the laboratory for hycanthone resistance (Table 10).

TABLE 10

Effect of Ro 15-5458 on Worm Count in Mice with Hycanthone Resistant Strain of S. mansoni[a]

	Control		Ro 15-5458	
	HYC SEN	HYC RES	HYC SEN	HYC RES
1)	39±16 (24)	19± 12 (23)	0 (11)	0 (19)
2)	37±20 (6)	24± 14 (11)	0 (6)	0 (10)
3)	120±61 (7)	169±173 (5)	4±5 (5)	0.4±0.5 (5)

[a]Mice 42-60 days post-infection were given a 15 mg/kg single dose of Ro 15-5458. Mice were autopsied 2 weeks after dosing and worms counted. Parasites were from the National Institute of Health, USA (row 1), University of Lowell (row 2) and Institute of Cell Biology, Rome (row 3). Results are mean number of parasites ± SD from the number of mice shown in parentheses.

A major sign associated with the action of any antischistosomal drug is the drug-induced shift of parasites from their normal niche, the mesenteric veins, to the liver. The time course of parasite shift after a 15 mg/kg single dose of Ro 15-5458 was determined by counting the number of parasites in the mesenteric and hepatic veins. The drug induced a significant shift of parasites 4-5 days after dosing. Since Ro 15-5458 is not lethal to schistosomes maintained in culture, we first determined the minimum amount of time required for lethal damage to accrue in the host.

Table 11 contains the results from an experiment in which mice were dosed with Ro 15-5458 and the parasites retrieved at various intervals after dosing and then maintained in culture. A latency period of about 12 h is required for the drug to irreversibly damage parasites in vivo. This effect was more pronounced on male than female parasites. In addition, when donor mice were treated with the drug and parasites removed from them for transfer to hamsters 1, 3, and 4 days after dosing, parasites from treated mice did not survive, while 85% of parasites transferred from untreated mice were recovered from the recipient animals (Chapter III).

Since drug-induced changes in the parasites were clearly detectable 4 days after dosing, we attempted to evaluate the drug's action on a number of well characterized physiological and biochemical processes of the parasite before the 4th day following dosing.

Parasites recovered from mice 3 days after dosing maintained a normal rate of motility and their surface membrane potential was also

TABLE 11

Survival of S. mansoni In Vitro After Removal of Parasites from Infected Mice Treated with a 15 mg/kg Single Dose of Ro 15-5458

Time of Recovery After Dosing (h)[a]	% Survival Compared to Control[b] Male	Female
3	93	100
12	20	39
24	5	28
72	0	3

[a]Parasites were retrieved after exposure to the drug in the mouse host and maintained in culture up to 15 days.

[b]Absence of contractile activity in a 10 μM 5-HT-containing medium was used as an index of parasite death.

TABLE 12

Effects of Ro 15-5458 on the Muscular Activity and Surface Membrane Potential of Adult Male *S. mansoni* 3 Days After Dosing

	Motility Index[a]	Surface Potential (mv)[b]
Control	103±22	-60.3±4.2
Ro 15-5458	132±35	-59.2±4.2

[a]Result is the mean ± SD for 3 experiments.

[b]Three different recordings were made on the dorsal surface of each male parasite. Result is mean ± SD (n=10).

normal (Table 12). It was also noted that in the early phase of in vitro incubation, parasites adhered well to the culture flask and remained in copula.

Scanning electron micrographs of the surfaces of drug treated male parasites were compared with those of control parasites, no drug-induced damage was apparent on parasites recovered from the mesenteric and portal veins up to 5 days after dosing (Figure 15).

However, when parasites retrieved from mice after dosing with Ro 15-5458 were examined in vitro for egg production as previously described (Chapter III), a biphasic effect was observed. Treatment significantly stimulated egg production 12 and 24 h after dosing, which was followed by reduced egg output 72 h after dosing (Figure 16).

The glycogen content of male schistosomes freshly dissected from the mesenteric and portal veins of mice 1, 2 and 3 days after dosing was not different from control parasites (Table 13). The glycogen reserve in treated parasites can be mobilized for the generation of lactate, although less efficiently than in controls. Paired parasites recovered from the host 3 days after dosing incubated in vitro for 21 h lost 56% of their glycogen content while control parasites lost 81%. Parasites retrieved from mice 3 days after drug administration were capable of utilizing glucose from the medium (Table 14) and the ATP level of the parasites was not significantly affected by the drug (Table 13). However, the amount of lactate excreted into the medium by treated parasites was significantly less than control parasites (Table 14).

Figure 15. Scanning electron micrographs of the dorsal surface of male S. mansoni after dosing the host. Parasites were recovered from a mouse given vehicle (A), or Ro 15-5458. Worm (B) was recovered 3 days after dosing, while worm (C) is after 5 days. Bar = 10 μm.

Figure 15

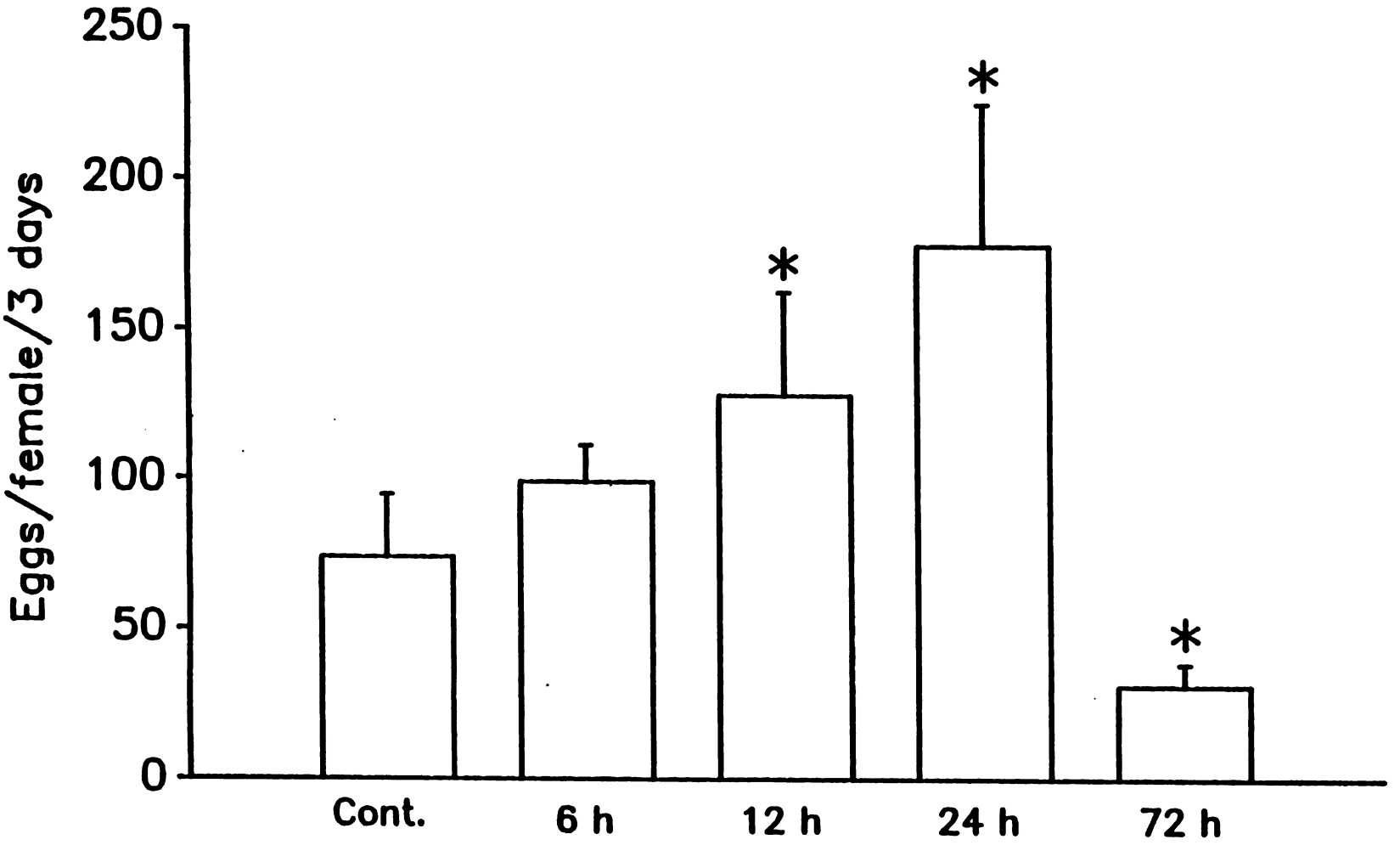


Figure 16. Effect of <u>in vivo</u> administered Ro 15-5458 on the <u>in vitro</u> egg production by <u>S. mansoni</u>. Paired parasites were removed from mice at the times indicated after drug administration and assayed for egg production. Results are the mean ± SD of 4 different experiments. *Significantly different from controls, $p \leq 0.05$ (ANOVA).

TABLE 13

Effects of Ro 15-5458 on Gut Pigment, Glycogen and ATP Content of S. mansoni[a]

Day of Parasite Recovery	Gut Pigment (μM)	Glycogen (μg/male)	ATP (nmoles/pair)
Control	1.7 ±0.20 (0.49±0.06)	12.7±0.26 (420±10)	1.2±0.2 (39.0±6.1)
1	2.4 ±0.11[b] (0.73±0.01)	12.3±0.23 (430± 8)	N.D.[c]
2	2.0 ±0.20 (0.63±0.06)	13.1±0.90 (480±23)	N.D.
3	2.0±0.01 (0.65)	12.4±0.25 (450±10)	1.1±0.4 (36±10.0)
4	1.8 ±0.16 (0.62±0.05)	11.4±0.50 (480±28)	N.D.

[a]Results are expressed as the mean of 2 experiments ± SD (n=20). ANOVA was performed to determine the significance of differences between the means.

[b]Significantly different from control group, ANOVA (Newman-Keul's), $p \leq 0.05$.

[c]Not determined.

NOTE: Results shown in parentheses are gut pigment (μmoles/mg protein), glycogen (μg/mg protein) and ATP (nmoles/mg protein).

TABLE 14

Effect of Ro 15-5458 on Carbohydrate Utilization and Lactic Acid Production of *S. mansoni* 3 Days After Dosing

	Carbohydrate Utilized (nmoles of glucose/pair/h)			Lactate (nmoles/pair/h)
	Medium	Glycogen	Total	
Control	68± 3 (1.8±0.1)	18±4 (0.46±0.01)	86.7±4 (2.2±0.1)	87±0.1 (2.23±0.01)
Ro 15-5458	70±12 (2.1±0.2)	11±4[a] (0.3 ±0.01)	80±8[a] (2.4±0.3)	73±0.1[a] (2.15±0.01)

Values are the mean ± SD of 2 experiments (n=14).

[a]Significantly different from controls (ANOVA, $0.1 \leq p \leq 0.05$).

NOTE: Values shown in parentheses are nmoles/mg protein/h (Mean ± SD).

Hematin forms a ferrihemochrome, characteristic of porphyrins with either -OH or H_2O on coordination position 5 and 6 in alkaline solution. A linear relationship ($r=0.99$) was observed between the optical density at 580 nm and the concentration of bovine hematin in the range of 1 to 100 μM. The result of gut pigment level comparison experiments showed no reduction in the gut pigment content of treated schistosomes. We actually observed significantly elevated pigment levels for parasites recovered one day after treatment (Table 13).

A drug-induced loss in total protein content and biomass of parasites was observed 3 days after dosing (Table 15). Male parasites showed change before females which was significantly lower for both sexes at later times of parasite recovery.

Because parasites lost protein at the time when no effect on glycogen content, ATP and gut pigment was apparent, the uptake of some important metabolic precursors into parasites retrieved 3 days after dosing was evaluated. The rate of uptake of leucine, methionine, adenine, uridine and thymidine was significantly reduced in drug exposed parasites (Table 16).

When the incorporation of [^{3}H]leucine, [^{3}H]thymidine or [^{3}H]uridine into TCA insoluble fractions of the parasites was determined as previously described (Chapter III) using parasites recovered 1, 2 or 3 days after dosing, it was found that the incorporation of leucine and thymidine, but not uridine, was significantly reduced ($p<0.05$) for parasites recovered 3 days after dosing compared to untreated parasites (Figure 17, panel A).

TABLE 15

Effect of Ro 15-5458 Treatment on the Protein Content and Weight of *S. mansoni*

Days After Dosing	Protein/worm[a] Male	Protein/worm[a] Female	Weight[c] Control	Weight[c] Ro 15-5458
1	96±2 (3)	100±3 (3)	0.143±0.02	0.137±0.04 (n=6)
2	92±5 (3)	98±4 (3)	N.D.[e]	N.D.
3	74±8[b] (5)	83±2[b] (2)	0.124±0.03	0.103±0.01[d] (n=6)
4	58±9[b] (3)	65±7[b] (3)	N.D.	N.D.

[a]In each experiment, control and treated parasites from the same group were used in parallel for protein measurement. The protein contents of control groups taken as 100%, were used for comparison. Results (percentage of untreated control) are mean ± SD of the number of experiments shown in parentheses.

[b]Significantly different from controls, $p \leq 0.05$ (ANOVA).

[c]Values are dry weight, mean ± SD, in mg/male.

[d]Significantly different from control group (Student's t-test, $p \leq 0.05$).

[e]Not determined.

TABLE 16

Effect of Ro 15-5458 on the Uptake of Nucleic Acid and Protein Precursors of S. mansoni Recovered 3 Days After Dosing

Precursor (mM)	Control	Ro 15-5458
Adenine (0.25)	0.026±.003	0.020±0.001[a]
Thymidine (1.0)	176±23	124±29[a]
Uridine (1.0)	132±42	89±25[a]
Leucine (0.0015)	18±5.0	15±4.2[a]
Methionine (0.0015)	8.1±2.0	6.9±1.2[a]

[a]Significantly different from control group, $0.1 < p < 0.05$ (ANOVA). Values are uptake rates, mean ± SD, in nmoles/male/hr, for 2 experiments (n=20). Uptake values are not corrected for diffusion.

Figure 17. Effect of Ro 15-5458 on the incorporation of tritiated precursors into the total acid-insoluble fractions of S. mansoni. Parasites recovered from mice at the times indicated after dosing were labeled for 1 h in RPMI-1640 medium containing 10 μCi/ml of tritiated leucine, thymidine and uridine and incorporation was measured as acid-precipitable radioactivity (panel A). In panel B, acid-soluble and -insoluble radioactivity was measured in the same group of parasites after labeling with [^{3}H]leucine as described in Methods. In each experiment, parasites recovered from mice which received the vehicle were labeled in the same manner and were used for radioactivity measurement. Average values (dpm/mg protein) from these controls, taken as 100%, were used to compare the radioactivity in treated parasites. *Significantly different from controls, $p \leq 0.05$. Student's t-test was performed after square root transformation of the data (n=10).

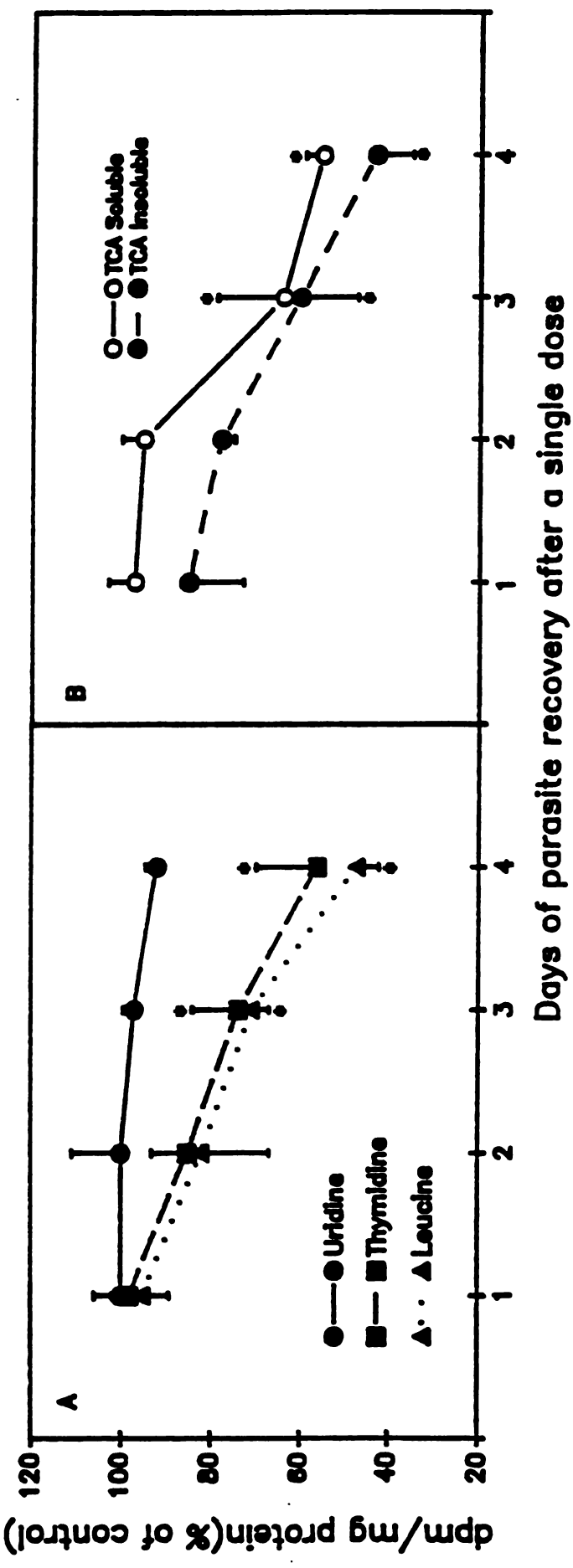

dpm/mg protein(% of control)
120
100
80
60
40
20
A
Uridine
Thymidine
Leucine
1
2
3
4
B
TCA Soluble
TCA Insoluble
1
2
3
4
Days of parasite recovery after a single dose

Figure 17

The specificity of drug action on the incorporation of leucine was tested by comparing the radiolabeled proteins separated by electrophoresis. Ro 15-5458 treatment resulted in reduced incorporation of [^{3}H]leucine into the 100k pellet proteins of male S. mansoni retrieved from mice 3 days after dosing. A 45-50% reduction in the incorporation of leucine was observed for proteins ranging in apparent molecular mass from 36 to 96 kD (Figure 18). Drug effect on the incorporation of leucine preceded the drug-induced reduction in the internal pool of the amino acid (Figure 17, panel B) suggesting the possibility of drug affecting the synthesis of parasite proteins.

Prompted by this result, RNA extracted from parasites retrieved from mice 20 h after dosing with Ro 15-5458 or vehicle was translated in a rabbit reticulocyte in vitro translation system. When 10 μg total RNA from each group was translated, a significant reduction ($p<0.05$) in the incorporation of [^{35}S]methionine was observed for RNA from treated parasites (Figure 19, panel A). However, RNA extracted from uninfected yet treated host liver supported translation to the same degree as RNA from non-treated livers (Figure 19, panel B). In addition, a 75 mg/kg single dose of the inactive structural analog, Ro 21-6787 was administered, to infected mice and RNA extracted from parasites recovered 48 h after dosing. RNA extracted from these parasites supported translation as efficiently as control parasites, while RNA from parasites treated with 15 mg/kg Ro 15-5458 exhibited a time-dependent reduction in the incorporation of [^{35}S]methionine (Table 17). On the other hand, when the same amount (1 μg) of poly(A^{+}) RNA from control parasites and parasites

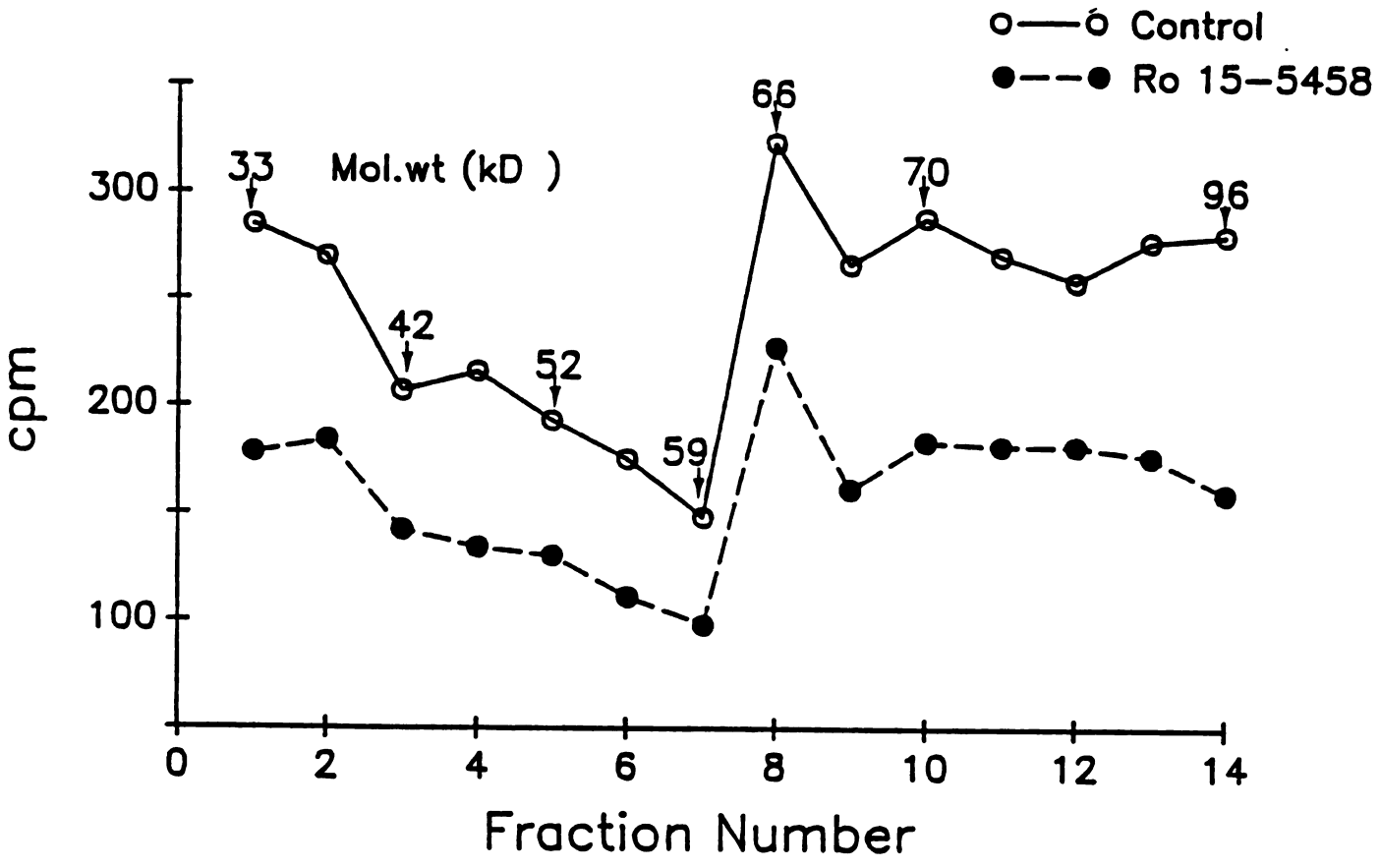


Figure 18. Effect of Ro 15-5458 on the incorporation of [^{3}H]leucine into the 100K pellet proteins of S. mansoni. Male parasites were incubated in vitro in leucine-free medium containing 20 μCi/ml [^{3}H]leucine. Incorporation was measured in gel fractions after solubilization as described in the Methods section.

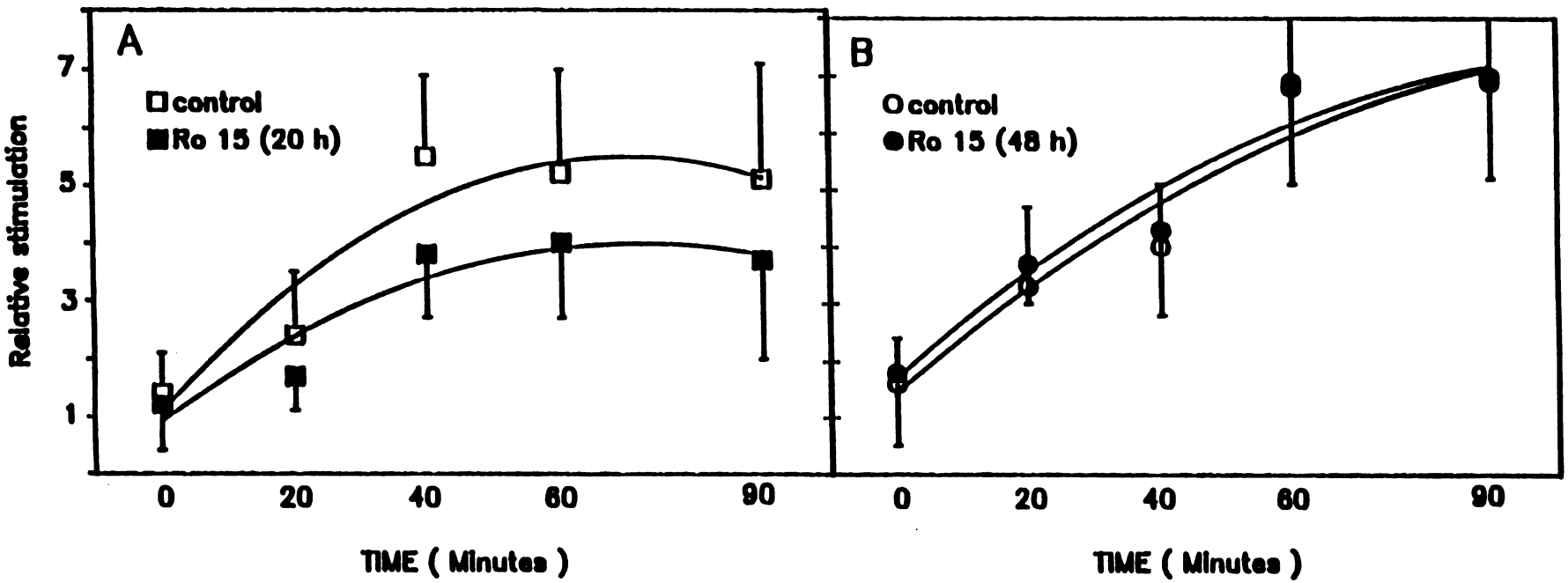


Figure 19. Time course of the incorporation of [^{35}S]methionine into proteins in translation system after the addition of schistosome (panel A) or mouse liver (panel B), RNA. Ten μg of total RNA was translated in a 25 μl rabbit reticulocyte system and 2 μl aliquots were removed at times indicated for measurement of alkali-resistant, TCA-insoluble counts. RNA was isolated from control or drug-treated parasites removed from the host at times indicated in parentheses. Results shown (mean $\pm$ SD) are stimulation of synthesis over zero-message controls. Experiments were performed using RNA from different batches of infection (n=3). Curves were fitted by non-linear regression analysis.

TABLE 17

In Vitro Translation of RNA Isolated from *S. mansoni*

Source of RNA[a]	^{35}S-Methionine Incorporated (cpm x 10^{-3})[b]	Stimulation Relative to zero Message
Control	15.2	5.6
Ro 15-5458 (6 h)	16.5	6.1
Ro 15-5458 (12 h)	8.7	3.2
Ro 15-5458 (48 h)	7.3	2.7
Ro 21-6787 (48 h)	15.0	5.5
No RNA	2.7	1.0

[a]RNA was extracted from control or drug-treated parasites and translated in a rabbit reticulocyte system. Parasites from treated hosts were retrieved after the time shown in parentheses.

[b]Results are alkali-resistant TCA-insoluble counts in a 1-μl aliquot of the reaction mixture.

recovered 3 days after dosing was translated, the same efficiency of translation was observed (Figure 20).

Electrophoresis and subsequent fluorographic analysis of the translation products showed a similar pattern for both treated and control parasite mRNA (Figure 21), with subtle quantitative and qualitative differences (Figure 22) as determined by densitometric measurement of the resulting fluorograms. The level of some low molecular weight (Mr) proteins was reduced in products directed by mRNA from treated parasites (stars in Figure 22). One striking observation in these results is that higher Mr peptides were synthesized in more abundance from treated parasite mRNA than from control mRNA.

Since the same amount of total RNA from Ro 15-5458-treated parasites was less efficient than control RNA in directing peptide synthesis, the possibility that the drug reduced the relative amount of mRNA was tested. Thus, first the effect of treatment on the total RNA content of adult schistosomes was examined. The same number of control and treated parasites (500-1000), were used for extraction and the resulting RNA compared by measuring the optical density of aliquots of the samples using a double-beam spectrophotometer (Beckman UV-5260, Palo Alto, CA). Administration of a 15 mg/kg single oral dose of Ro 15-5458 to the host resulted in a time-dependent reduction in the RNA content of parasites (Table 18). A 14%, 30% and 41% reduction was observed for parasites recovered from mice 12, 72 and 96 h after dosing, respectively.

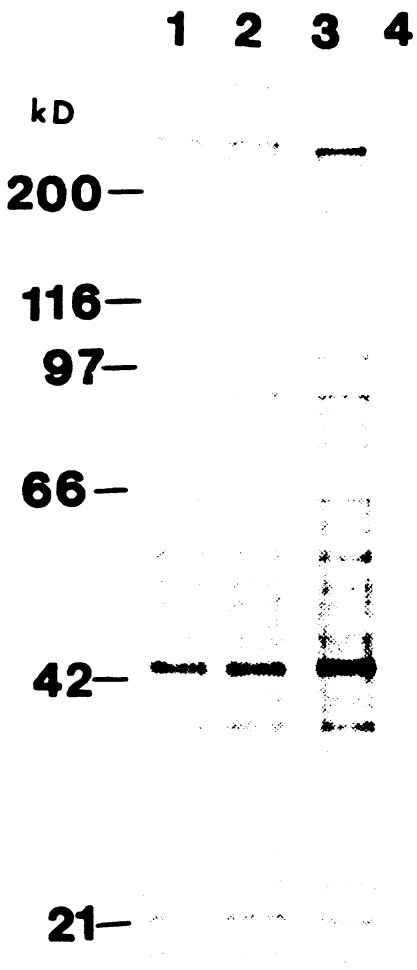


Figure 20. Fluorogram from the in vitro translation of poly(A⁺) RNA. Translation products of 1 μg poly(A⁺) RNA from control schistosomes (lane 1) and schistosomes recovered 3 days after dosing (lane 2). Lane 3 is from 10 μg total RNA of control parasites and lane 4 is without any added RNA.

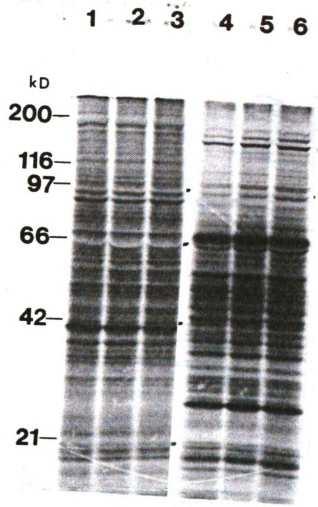


Figure 21. Fluorogram showing the polypeptide pattern of the in vitro translation products of schistosome and liver RNA before and after treatment with Ro 15-5458. RNA from control schistosomes (lane 1) and schistosomes recovered from the host 12 h (lane 2) and 72 h (lane 3) after dosing as well as control mouse liver (lane 4) and liver from mice sacrificed 8 days after administration of a single 15 mg/kg dose of Ro 15-5458 (lanes 5 and 6) were translated in a rabbit reticulocyte system. The products were separated on a 10% SDS-polyacrylamide gel for fluorography.

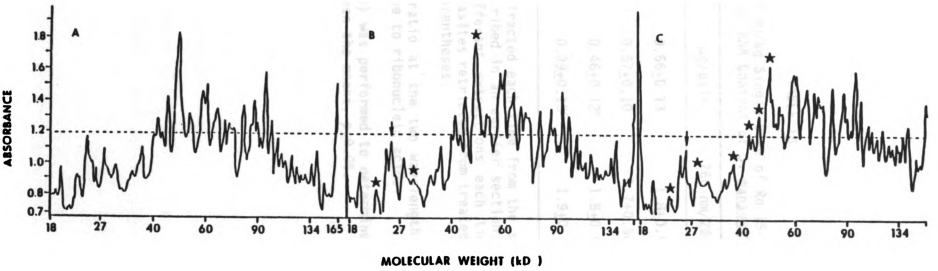


Figure 22. Densitometric scans from fluorogram of translation products. A = control parasite RNA, B = parasites retrieved 12 h, and C = 3 days after dosing with Ro 15-5458. Star = Band is smaller in that group compared to control. Arrow = extra band not seen in control.

TABLE 18

Effect of a 15-mg/kg Single Dose of Ro 15-5458 on the Total RNA Content of *S. mansoni*[a]

Source	μg/pair	260 nm/280 nm[b]	% Reduction
Control	0.66±0.13	1.8±0.55	
Ro 15-5458 (12 h)	0.57±0.10	1.7±0.50	14±8.0
Ro 15-5458 (72 h)	0.46±0.12[c]	1.8±0.50	30±8.4
Ro 15-5458 (96 h)	0.39±0.15[c]	1.9±0.20	41±7.8

[a]RNA was extracted each time from the same age and batch of infection as described in an earlier section. Results are mean ± SD from five different extractions each time involving control parasites and parasites retrieved from treated mice after dosing at times shown in parentheses.

[b]The O.D. ratio at the two wavelengths suggested that the absorption was due to ribonucleic acids.

[c]ANOVA (LSD) was performed to determine the significance of differences between the means, $p \leq 0.05$.

Furthermore, the effect of treatment on actin mRNA was studied by hybridization with a ^{32}P-labeled probe. Dot-blot analysis was performed after spotting serially diluted total RNA samples onto nylon membranes. Hybridization of the blots with a β-actin RNA probe from chicken brain under stringent conditions (0.025XSSC, 68°C) showed that treatment with Ro 15-5458 reduced the actin mRNA of schistosomes retrieved from treated animals but not that of the corresponding mouse liver (Figure 23). A pronounced drug effect was observed on RNA isolated from parasites more than 24 h after drug administration. On the other hand, no reduction was seen for the actin mRNA of parasites exposed to a 15 mg/kg dose of the inactive analog, Ro 21-6787. Hybridization of the same amount of RNA immobilized on nylon membranes with probes from pSM r3.0 and pSM r4.7 also indicated a reduction in the level of both the 18S and 28S rRNA in parasites exposed to Ro 15-5458, while low hybridization was observed for mouse liver RNA with the parasite rDNA probe (Figure 24).

The effect of the drug on the relative abundance of SOD and actin mRNAs and the 18S rRNA was also studied by northern hybridization. Fifteen to 20 μg of total RNA was electrophoresed in a denaturing agarose gel and stained with ethidium bromide to ensure that the same amounts of RNA were loaded (Figure 25). When a reduction in the intensity of rRNA bands in any of the lanes was observed, the same filter was hybridized with the 18S rRNA probe and the mRNA of interest and the intensity ratios from the resulting autoradiograms compared.

The actin probe identified two bands, 1.4 and 1.9 kb, as previously reported (Davis et al., 1985). Although treatment reduced the level of

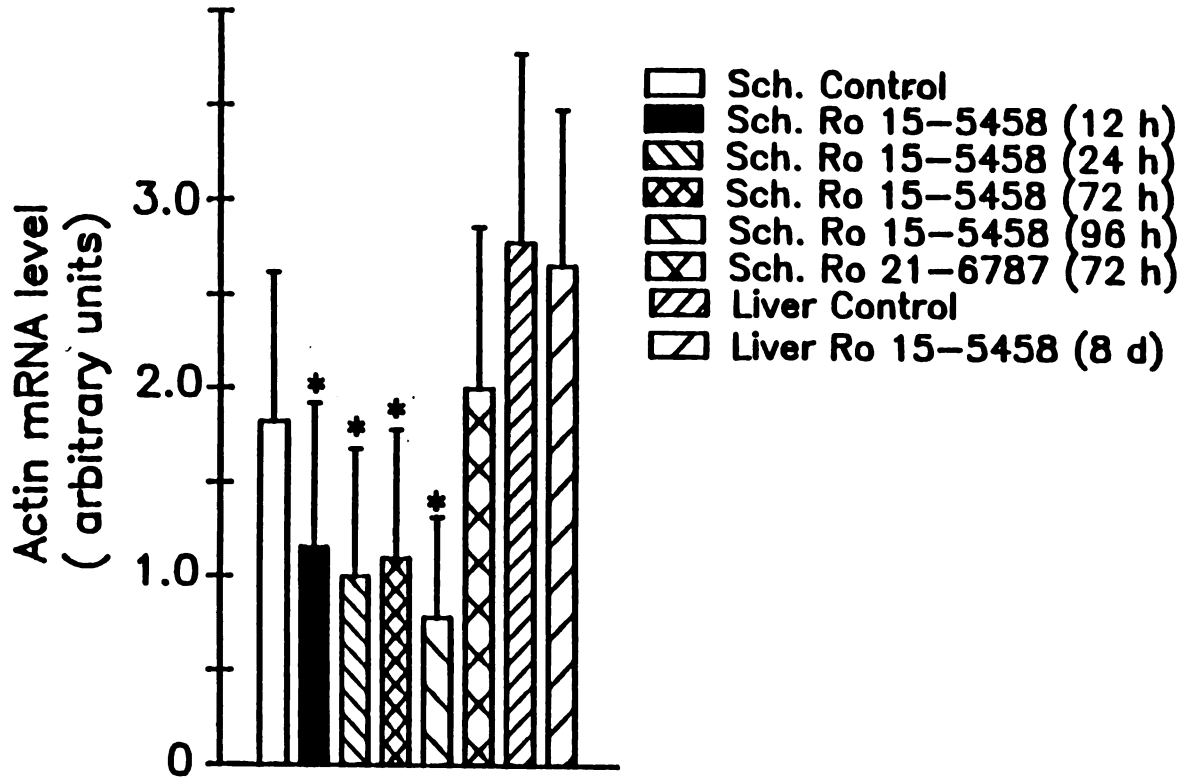


Figure 23. Effect of Ro 15-5458 treatment on the actin mRNA content of S. mansoni. Different amounts of RNA (2.5, 1.25, 0.625, and 0.313 μg) from each group were applied to nylon membranes and hybridized with a ^{32}P-labeled riboprobe complementary to actin RNA. Linear regression of the cpm vs. amount of RNA was performed for each sample and the slopes were compared for relative intensities of hybridization. Values are mean RNA levels $\pm$ SD (n=3). *Statistically different from controls (ANOVA), $p \leq 0.05$.

Figure 24. Effect of Ro 15-5458 treatment on the 18S and 28S rRNA levels of S. mansoni. Denatured RNA samples were serially diluted (5, 2.5, 1.25, 0.625 μg) and immobilized on nylon membranes and probed with either pSM r4.7 or pSM r3.0. Linear regression of O.D. vs. amount of RNA was performed for each sample and slopes were compared for the relative intensities of hybridization. Values are mean RNA levels ± SD (n=3). *Statistically different from controls (ANOVA), $p<0.05$.

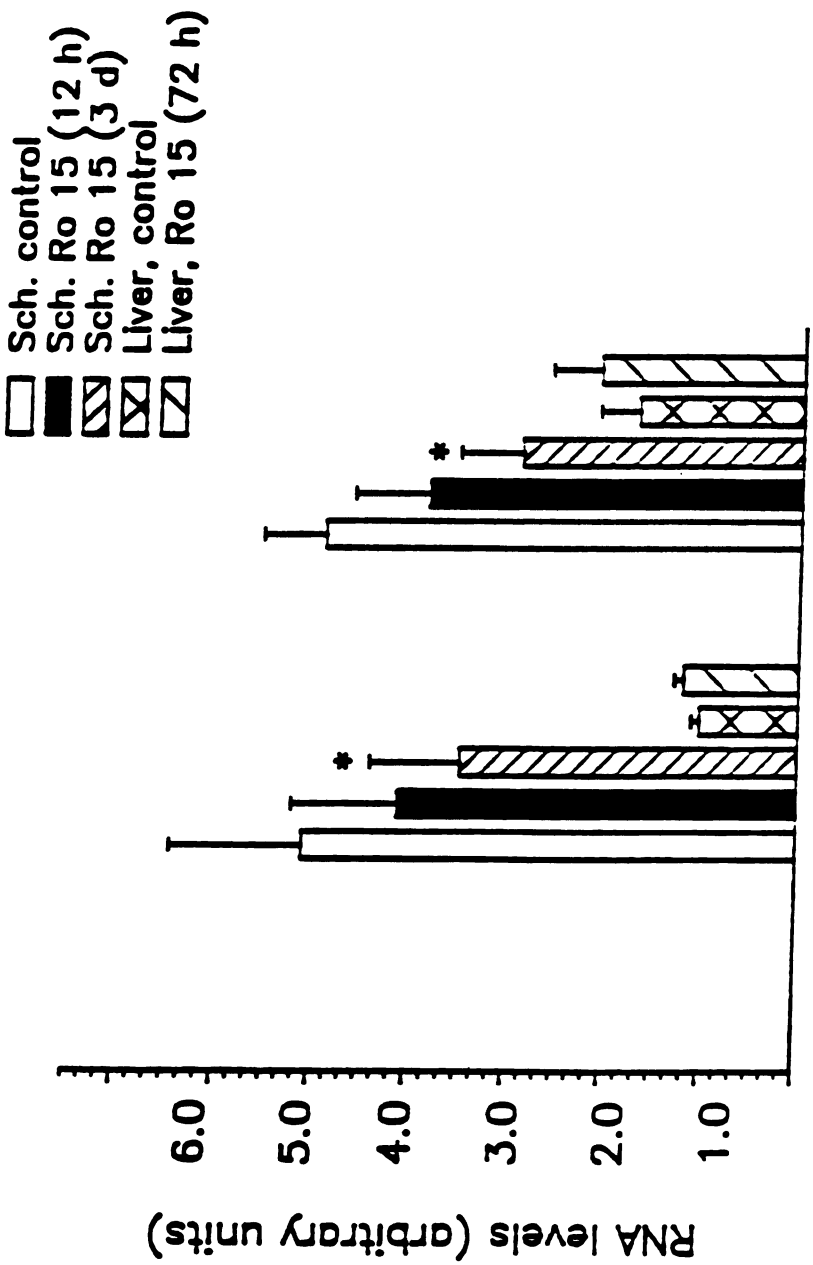

Sch. control
Sch. Ro 15 (12 h)
Sch. Ro 15 (3 d)
Liver, control
Liver, Ro 15 (72 h)
RNA levels (arbitrary units)
6.0
5.0
4.0
3.0
2.0
1.0
*
*
pSM r4.7
pSM r3.0

Figure 24

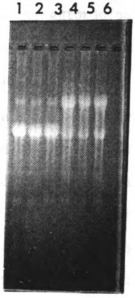


Figure 25. Ethidium bromide-stained RNA gel. 1.2% agarose gel containing 0.7 μg/ml ethidium bromide was used for RNA separation. RNA is from control schistosomes (lane 1), or schistosomes recovered 12 h after dosing with Ro 15-5458 (lane 2), 3 days after dosing with Ro 15-5458 (lane 3). RNA on lane 4 is from control mouse liver, while lanes 5 and 6 are from Ro 15-5458-treated mice sacrificed 8 days after dosing.

both messages, it appeared that the 1.9 kb species was affected to a greater extent (Figure 26). This was confirmed by electrophoresis and northern analysis of 1 μg poly(A⁺) RNA from control and treated parasites retrieved from the host 12 and 72 h after dosing. The level of the 1.4 kb actin mRNA was dramatically reduced in parasites recovered 3 days post-dose (Figure 27). A mRNA (ca. 0.78 kb) that hybridized with the SOD probe from schistosomes was also reduced after treatment with Ro 15-5458; a 12 and 44% reduction in the content of SOD mRNA was observed for parasites recovered 12 and 72 h after dosing, respectively (Figure 28).

Figure 29 depicts the autoradiogram and ethidium bromide stained gel picture from the same experiment. The RNA was transferred and simultaneously hybridized to both the β-actin and 18S rRNA probes. Result from this experiment indicated that treatment lowered the level of the rRNA's in light of the amount of RNA applied to the gel as can be evidenced from the ethidium bromide-stained pictures.

To check the possibility that Ro 15-5458 treatment altered the restriction fragment pattern of parasite DNA, a comparison was made between DNA extracted from control and treated parasites using ^{32}P-labeled probes that hybridize to either of the DNAs that code for the mRNAs described above.

Four to eight μg of genomic DNA was digested to completion with either EcoRI or Hind III restriction enzymes. The resulting fragments were electrophoresed and transferred and each filter was sequentially hybridized with probes for SOD, actin and rRNA by stripping the filter in water containing 1% glycerol at 85°C and rehybridization. Hybridization

Figure 26. Effect of a 15-mg/kg dose of Ro 15-5458 and Ro 21-6787 on the actin mRNA content of S. mansoni. 15 μg of total RNA was electrophoresed and transferred to nylon membranes. The filters were hybridized with ^{32}P-labeled riboprobe complementary to actin mRNA. RNA was isolated from control parasites (lane 1), parasites recovered 72 h from Ro 21-6787-treated mice (lane 2) and parasites recovered from Ro 15-5458-treated mice after 24 h (lane 3), 72 h (lane 4) and 96 h (lane 5). Numbers refer to sizes (kb). Mouse ribosomal RNA bands stained before transfer were used as size markers. The relative intensities of hybridization of both sizes of mRNA from each lane on the autoradiogram are shown as bar plots.

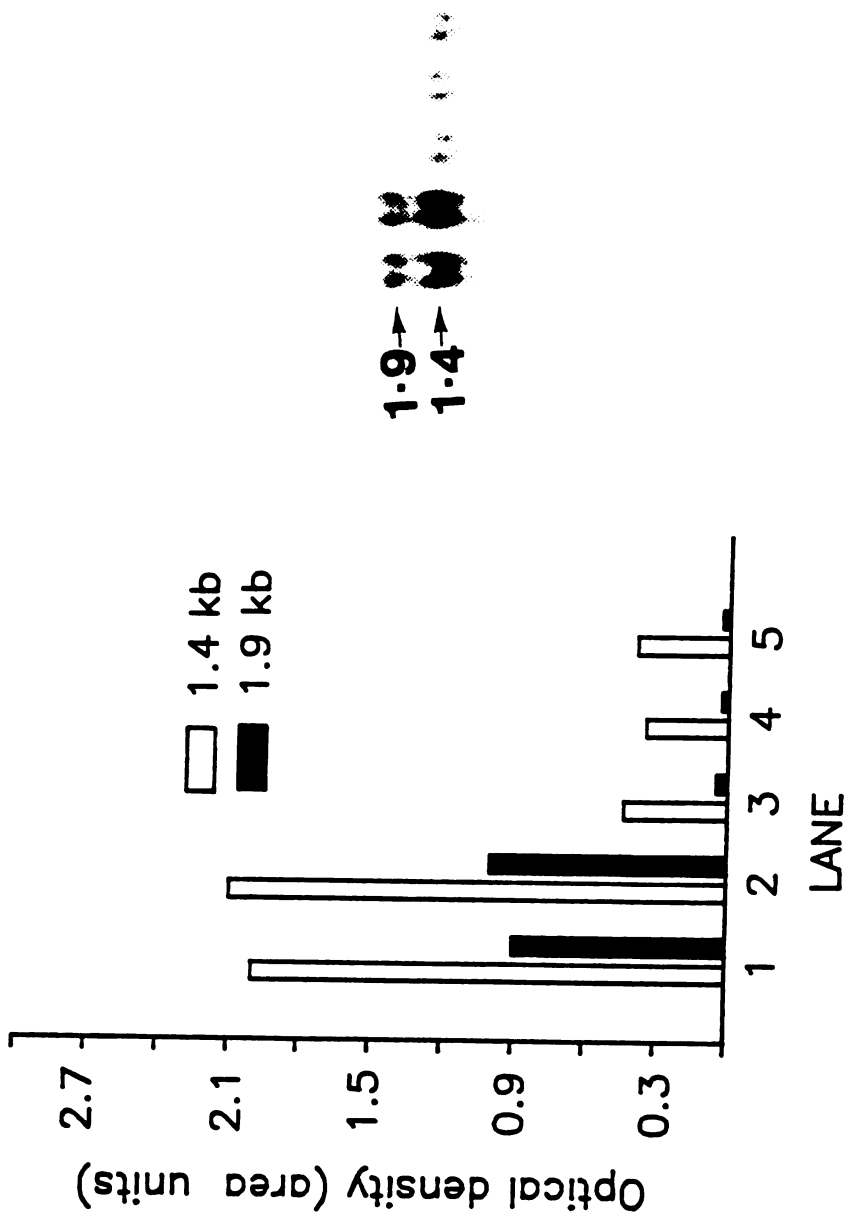

1 2 3 4 5
1·9
1·4
1.4 kb
1.9 kb
Optical density (area units)
2.7
2.1
1.5
0.9
0.3
1 2 3 4 5
LANE

Figure 26

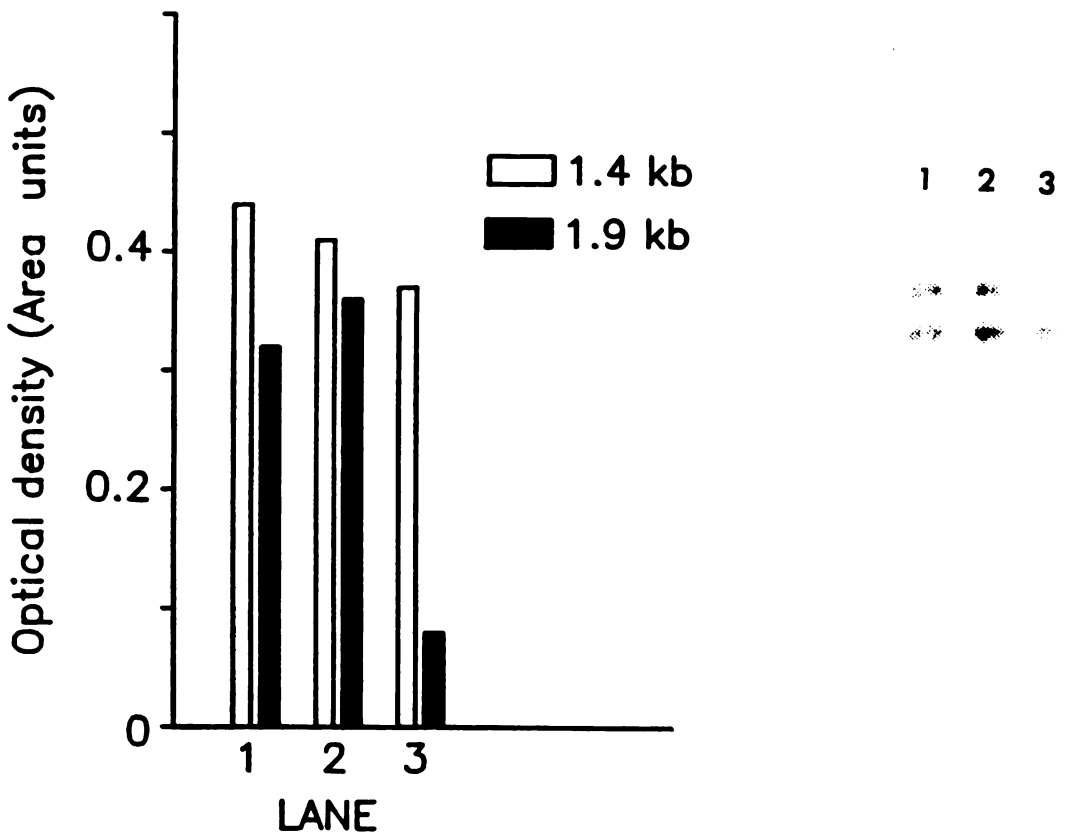


Figure 27. Effect of treatment with Ro 15-5458 on the two sizes of S. mansoni actin mRNA. 1 μg poly(A⁺) RNA was electrophoresed, transferred and hybridized with an actin riboprobe. Lane 1 is RNA from control schistosomes, lanes 2 and 3 are from treated parasites recovered 12 and 72 h after dosing, respectively. The relative intensities of hybridization are given as bar plots.

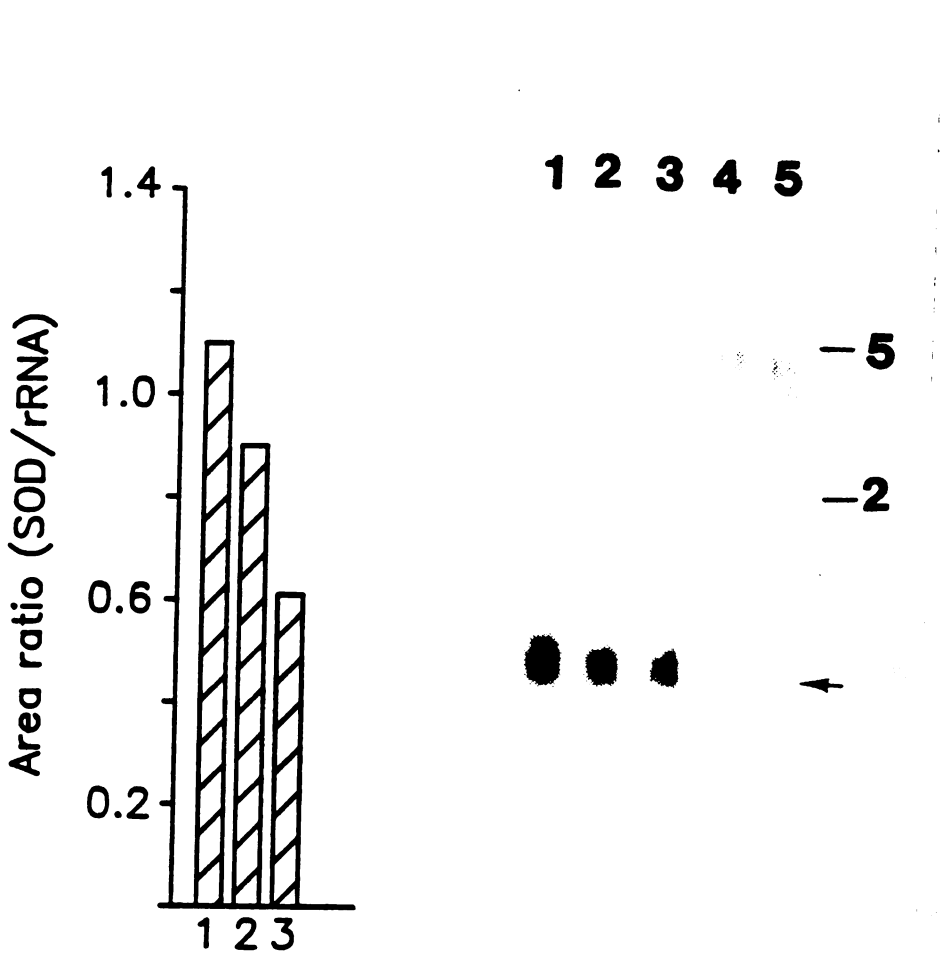


Figure 28. Effect of treatment with Ro 15-5458 on the SOD mRNA content of S. mansoni. 20 μg total RNA was electrophoresed, transferred and probed with a ^{32}P-labeled 320 bp DNA sequence from part of the SOD gene. RNA was isolated from control schistosomes (lane 1), schistosomes recovered from treated mice 12 h (lane 2), 72 h (lane 3) after dosing. Lane 4 is from control mouse liver, while lane 5 is from mouse liver recovered 15 days after dosing with 15 mg/kg Ro 15-5458. Arrow is for the SOD mRNA (0.78 kb), numbers are the size for mouse rRNAs (kb). The relative intensity of the bands is shown on the plot.

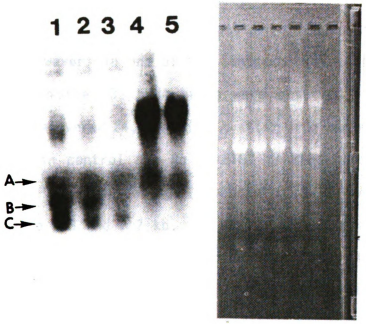


Figure 29. Effect of treatment with Ro 15-5458 on the 18S rRNA level of S. mansoni. RNA shown on the right panel was transferred to nylon membranes and hybridized with pSM r4.7 and β-actin probes simultaneously. RNA in lane 1 is from control schistosomes, lanes 2 and 3 are from Ro 15-5458-exposed parasites recovered 12 h and 3 days after dosing, respectively. Lane 4 is RNA from control mouse liver, while RNA on lane 5 is from Ro 15-5458-treated mouse sacrificed 3 days after dosing. A = rRNA; B = 1.9 kb actin mRNA; C = 1.4 kb actin mRNA.

of filters with an actin riboprobe identified four EcoRI fragments of size 6.5, 3.7, 2.4 and 1.8 kb (Figure 30, panel A) and Hind III fragments of size 7.2, 4.4, 3.1 and 1.8 (Figure 31, panel A). In both cases, the same pattern was observed in control and treated parasite DNA. The SOD fragment hybridized with a 5.6 EcoRI fragment for all DNAs tested (Figure 30, panel C). Hybridization of the same blot with a probe for the 18S rRNA gene revealed a 4.7 and 1.6 kb EcoRI fragment (Figure 30, panel B) and Hind III fragments of size 3.0, 1.5 and 0.51 kb (Figure 31, panel B), which also showed a similar size pattern in both control and treated parasites. However, in one of the Southern blots, large fragments that hybridized with the 18S rDNA probe were observed in EcoRI digests of parasite DNA recovered 24, 72 and 96 h after drug administration that were not detected in control and Ro 21-6787 treated parasites or parasites recovered 12 h after dosing (Appendix III). The additional EcoRI fragments were 9.3 and 7.7 kb. In addition, a 5.5 kb Hind III fragment was detected in DNA from parasites recovered 72 h after dosing (Appendix III). The sizes indicate that there was incomplete digestion of the genomic DNA in these samples (for restriction map of schistosome rDNA, see Appendix IV). However, rehybridization of the same filter with actin probe did not reveal any difference between the groups.

DISCUSSION

One problem with using parasites treated in their host for studying drug effects is the timing of removal for *in vitro* examination. For *S. mansoni*, the hepatic shift of the parasites has traditionally been used

Figure 30. EcoRI restriction analysis pattern of DNA isolated from Ro 15-5458-exposed S. mansoni. Genomic DNA digested with EcoRI (15 U/μg/μl) were subjected to Southern analysis as described in an earlier section and hybridized with ^{32}P-labeled probes from actin (panel A), 18S rDNA (panel B) and SOD (panel C). DNA was isolated from control schistosomes (lane 1, panels A, B and C), treated parasites retrieved 12 h (lane 2, panels A, B and C), 24 h (lane 3, panels A and C), 72 h (lane 4, panels A and C; lane 3, panel B), and 96 h (lane 5, panels A and C; lane 4, panel B) after dosing with Ro 15-5458. Lane 6, panels A and C are parasites retrieved 48 h after treatment of the host with Ro 21-6787. Numbers at the arrow refer to sizes in kb. Ethidium bromide-stained lambda DNA cut with Hind III was used as size marker.

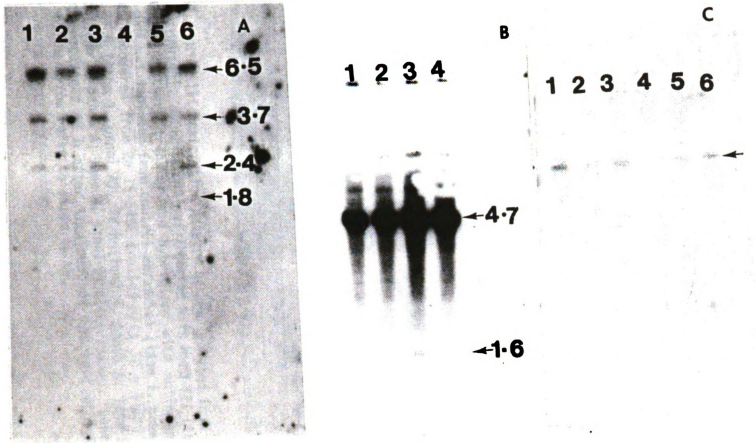


Figure 30

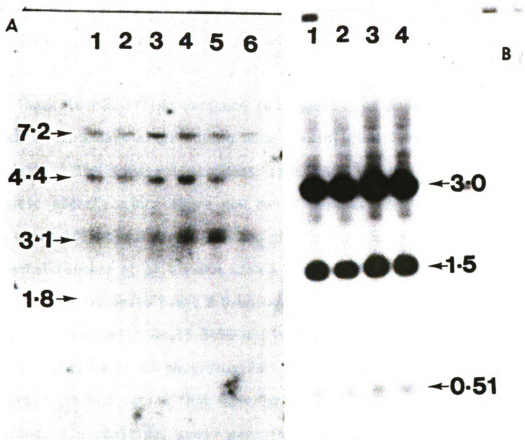


Figure 31. Hind III restriction pattern of DNA isolated from Ro 15-5458-treated S. mansoni. Genomic DNA was digested with Hind III (15 U/μg/μl), subjected to Southern transfer and hybridized with actin and 18S rDNA probes. DNA was isolated from control parasites (lane 1, panel A and B), parasites recovered from Ro 15-5458-treated mice 12 h (lane 2, panel A and B), 24 h (lane 3, panel A), 72 h (lane 4, panel A; lane 3, panel B) and 96 h (lane 5, panel A; lane 4, panel B) after dosing. Lane 6A is from parasites recovered 48 h after treatment of the host with Ro 21-6787. Numbers at the arrow refer to sizes in kb.

as an indicator of parasite morbidity. Important information on the effects of chemical agents may be obtained from the in vitro examination of parasites recovered prior to hepatic shift. However, utilization of this information in the interpretation of the primary actions of the compound may not be possible, unless further work qualifies that secondary biochemical effects and host immune responses have not already commenced.

Thus, first, drug-induced changes on selected biochemical and physiological processes of the parasites were examined before the hepatic shift. There is compelling evidence to suggest that parasites recovered from mice up to 3 days after dosing were physiologically fit for the host environment. The parasite tegument seems particularly vulnerable to therapeutic attack, since there now exist several unrelated schistosomicides which affect the morphology and physiology of this tissue, namely, praziquantel (Becker et al., 1980; Shaw & Eramus, 1983), oxamniquine (Kohn et al., 1982), amoscanate (Voge & Bueding, 1980) and BW484C (Watts, 1986). However, treatment with Ro 15-5458 did not alter the parasite tegument, either morphologically or physiologically, and no host cell accumulation was obserevd, an indication that host immune effects were not involved at this period. In addition, these parasites maintained normal tegumental potential, which is an indirect measure of the electrochemical gradient between the parasite's syncytium and its environment. Ro 15-5458 treatment did not affect the parasite's motor activity, a parameter which is altered by other antischistosomal drugs such as praziquantel and metrifonate.

Similarly, Ro 15-5458 had no effect on the parasite gut pigment, which is an indirect indicator of the number of erythrocytes ingested by

the parasites. However, treatment had a dramatic biphasic effect on parasite egg production. A 3-fold increase in egg output was observed 24 h after drug administration, which was followed by a 60% reduction 72 h after dosing. We have observed similar patterns of fecal egg excretion in vervet monkeys (Cercopithecus aethiops) after treatment with the drug (Sulaiman et al., unpublished data); about a 5-fold increase in the excretion of viable eggs was seen 2 days after treatment with either a 15 mg/kg or 25 mg/kg single oral dose, which declined significantly 2 weeks after treatment.

Treated parasites utilized glucose from the medium as efficiently as non-treated parasites. Since the in vivo turnover of schistosome glycogen is thought to be very rapid (Tielens et al., 1989a), maintenance of normal glycogen levels during this period also suggested the proper utilization of glucose within the host.

Glycogen constitutes about 15% of the dry weight of adult male S. mansoni (Bueding & Koletsky, 1950; Lennox & Schiller, 1972). The function of the glycogen reserve in schistosomes is not well understood. It may serve as a transient source of energy (Mercer & Chappell, 1985a; Tielens & Van den Bergh, 1987). In addition, when schistosomes are incubated in a glucose-free medium, they are able to utilize glycogen and generate lactic acid (Bueding, 1950; Bueding & Fisher, 1982).

Ro 15-5458-treated parasites exhibited a defect in catabolizing glycogen in vitro. This defect may be responsible for the reduction in the amount of lactate excreted into the medium and may explain the overall reduction in the utilization of glucose, since both groups of parasites were able to utilize glucose from the medium to the same extent.

The glycogen store of S. mansoni has also been reported to be metabolically active while the parasites are in the host (Tielens et al., 1989a). Maintenance of a constant level of glycogen in the parasites thus must require a steady state equilibrium in which synthesis and degradation proceed at equal rates. A defect in either of the pathways will disturb the equilibrium, generating either elevated or lower values. Since the level of glycogen in treated parasites was similar to controls up to 3 days after drug treatment, the effect of Ro 15-5458 on glycogenolysis seen after in vitro incubation (Table 14) may not reflect the state of glycogen metabolism in vivo.

Earlier reports indicated that when S. mansoni are maintained in culture, they lose glycogen (Mercer & Chappell,1985a). The decline in glycogen stores upon in vitro incubation of parasites is not prevented by increasing the amount of glucose in medium (Bueding, 1950; Mercer & Chappell, 1986a). It appears that removal of parasites from the host itself is sufficient reason for parasites to lose their glycogen deposit. On the other hand, since Ro 15-5458 treated parasites showed a defect in catabolizing glycogen in vitro, the effect of the drug may be to reduce the levels of certain metabolic enzymes in the parasites.

The most interesting observation made in this study was that parasites recovered from treated mice as early as 12 h after dosing failed to survive in culture (Table 12). This suggested that the drug exerted irreversible changes on the schistosomes at earlier periods, although parasites remained in the mesenteric veins and did not exhibit structural defects. Thus, the effect of treatment on other parameters was studied

with the intent of detecting early changes in the parasite's metabolic processes.

The protein content of parasites exposed to Ro 15-5458 dropped by about 26% on the 3rd day after dosing. This decrease in protein content was accompanied by a significant decrease in parasite weight and a reduction in the incorporation of [^{3}H]leucine into proteins. Analysis of the amount of label incorporated into different fractions after SDS-polyacrylamide gel electrophoresis clearly suggested a non-specific reduction in the incorporation of leucine into the proteins of treated parasites. This may result from a drug-induced reduction in the internal pool of the amino acid as a result of inhibition of the uptake of the amino acid into the parasites.

In order to determine the contribution of such a defect to the observed phenomenon, we studied the uptake (TCA-soluble) and incorporation (TCA-insoluble) of leucine in the same sample. A drug effect on the incorporation of leucine into parasite proteins was manifested earlier than the effect on its uptake (Figure 17, panel B). In addition, in parasites retrieved 3 days after dosing, when the TCA-soluble pool was significantly lower than controls, the reduction in the ethanol-extractable pool of the amino acid (Table 16) was of lower magnitude (17%) than the reduction in the incorporation of the amino acid (40-50%). These data suggest that the reduction in the incorporation of leucine was not a result of a decreased uptake of the amino acid. Since leucine is not metabolized prior to incorporation into schistosome proteins (Chappell & Walker, 1982), the amount inside the parasite can be used as an index of the available pool of the amino acid for synthesis. A decline in the

magnitude of the TCA-insoluble pool that is not associated with a drug-induced reduction in the soluble pool thus suggests a defect in protein synthesis.

Although Ro 15-5458 treatment resulted in the loss of parasite protein and weight, this effect of the drug does not appear to be related to the energy metabolism of the parasites. However, it is plausible that the decline in protein content of the parasite may be secondary to a drug effect on the synthesis of proteins. Inhibition of protein synthesis may result in reduced levels of metabolic and/or membrane transport proteins which ultimately results in reduced utilization of metabolic precursors from the host. The in vitro studies on the uptake of protein and nucleic acid precursors (Table 16), albeit at a gross level, supported this possibility.

Although the specific contribution of the tegument in the nutrition of schistosomes while parasites are in the host has not yet been defined, the surface membrane is apparently absorptive in vivo and likely contributes to the nutrition of these parasites. On the other hand, the tegument has been proposed to be the primary site of absorption of nutrients during short-term in vitro incubations (Asch & Read, 1975a,b; Chappell, 1974; Levy & Read, 1975; Rogers & Bueding, 1975; Uglem & Read, 1975) . Thus, results of the uptake experiments suggest a reduced contribution of the parasite's surface in its nutrition at later stages of parasite recovery following dosing.

When the absorptive function of the tegument is compromised, parasites will be deprived of nutrients which will culminate in a loss of parasite protein and other constituents. The decline in parasite protein

would be expected to be accompanied by a decline in parasite weight, since protein constitutes about 50% of the dry weight of adult S. mansoni (Mercer & Chappell, 1985a).

Treatment with Ro 15-5458 also decreased schistosome RNA-directed incorporation of [^{35}S]methionine, supporting the earlier observation that the drug interfered in the synthesis of parasite proteins. Translation of the same amount of poly(A$^+$) RNA from control and treated parasites indicated that treatment did not alter the ability of the mRNA to support synthesis but caused a reduction in the relative amount of message which was manifested as reduction in the incorporation of the amino acid. Electrophoretic and fluorographic analysis of the products after the translation of the same amount of total RNA also showed that treatment with the drug mainly caused quantitative rather than qualitative changes.

This effect of Ro 15-5458 appeared to be selective for the parasites, since RNA extracted from uninfected yet treated host liver supported translation to the same degree as RNA from non-treated livers. Acid hydrolysis of the thiazolinyl group from position 9 of the acridine ring renders Ro 15-5458 inactive, and RNA extracted from parasites treated with 75 mg/kg of the resulting compound (Ro 21-6787) supported translation as efficiently as control parasites, perhaps implicating the association of the observed effect with the schistosomicidal action of Ro 15-5458.

The only limiting factor in the translation system used in these studies was the schistosome mRNA, thus, the possibility of the drug decreasing the abundance of parasite messages was tested. Studies performed in this direction indicated a drug-induced decline in the total RNA content of the parasites. This effect of the drug was time-dependent,

in that a 41% reduction in RNA was observed for parasites retrieved 96 h postdose compared to 13% for those recovered 12 h after drug administration. Since poly(A^+) RNA constituted only 2-3% of the total cellular RNA of these parasites (unpublished observation), the 13 to 41% reduction in RNA content cannot be accounted for by a simple reduction in the mRNA content of the parasite as was suggested by the translation experiments. These results rather suggested a drug-induced reduction in the amount of total RNA, particularly the rRNA, since it is the most abundant species. Northern blot analysis of RNA extracted from treated and control parasites by probing with 18S rRNA genes from S. mansoni also indicated a reduction in the rRNA levels.

Actin proteins are widely distributed in many eukaryotic cells. Actin filaments have also been identified in the muscle fibers and surface spines of schistosomes (Cohen et al., 1983; Davis et al., 1985; Zhou & Podesta, 1989). Hybridization of actin DNA and RNA from S. mansoni with a β-actin DNA probe from chicken brain has been demonstrated (Simpson et al., 1983; Davis et al., 1985). Our work confirms this observation. This probe identified a 1.4 and 1.9 kb mRNA in adult parasites, consistent with that reported previously (Davis et al., 1985). The levels of both actin mRNAs were reduced in adult parasites after treatment of the host with Ro 15-5458.

A more pronounced effect was observed on the 1.9 kb message, suggesting a selective effect of treatment on this mRNA. A possible explanation is that the drug inhibited the expression of both sizes of actin mRNA equally, but the selective effect was seen due to differences

in the rate of turnover of the two actin mRNAs; the treatment effect would be manifested earlier on a message with a short half-life.

This reduction in the schistosome actin message content was not observed when mice were dosed with the inactive structural analog, Ro 21-6787; nor did Ro 15-5458 affect the actin mRNA level of the host liver when examined 48 h and 8 days after drug administration, suggesting selectivity in the actions of Ro 15-5458. Drug effect on the parasite was non-specific; in addition to reducing the actin mRNAs, treatment also reduced the relative abundance of parasite SOD mRNA and rRNAs.

Drugs that alter the abundance of a specific RNA may affect either the synthesis or breakdown of the RNA of interest. Ro 15-5458 treatment caused a decline in the steady-state level of all the RNAs examined. Thus, either an inhibition of RNA synthesis or enhancement of degradation by the drug may occur. It appears that the drug reduced the RNA level of parasites by inhibiting synthesis rather than enhancing degradation. This was evident from the in vitro translation of the same amount of poly(A$^+$) RNA. Using the same amount of poly(A$^+$) RNA, it was possible to attain the same degree of [^{35}S]methionine incorporation; fluorographic analysis of the translation products also indicated that the size of the resulting polypeptides and their electrophoretic pattern was similar for control and treated parasites. If the drug enhanced the breakdown of mRNAs, the resulting polypeptides should show heterogeneity in size for control and treated parasites and the northern blots would appear smeary. In addition, the appearance of high molecular weight polypeptides in abundance in the treated mRNA translates argues against a drug-induced enhancement of message degradation.

More support for this possibility comes from the relative drug-induced reduction of the two sizes of actin mRNAs in schistosomes. Comparison of the actin mRNA content of treated parasites with the controls revealed a proportionally higher reduction in the 1.9 kb than the 1.4 kb mRNA, which may suggest inherent differences in the rate of synthesis of the two sizes of mRNA. The 1.9 kb message appears to have a higher turnover rate than the 1.4 kb message, because a dramatic reduction (75%) was observed in the 1.9 kb message in parasites recovered 72 h after dosing. The observed variability in the drug effect on the two classes of actin mRNA is more likely a result of differences in their rate of synthesis, which along with observations discussed above, proposes that the effect of Ro 15-5458 treatment is to inhibit the expression of parasite genes.

A reduction in RNA content is the earliest phenomenon observed in parasites after treatment of the host. Treatment reduced the steady-state level of actin and SOD mRNA as well as rRNA, in parasites retrieved as early as 12 h after dosing. This was at a time when vital physiological and biochemical processes of the parasites were not altered, and coincided with our earlier observation which indicated that irreversible damage to parasites occur 12 h after dosing. A drug-induced reduction in the parasite RNA content would be a lethal event, since it would result in the reduced production of parasite proteins, which ultimately leads to an unfavorably altered host-parasite relationship.

How the drug reduces the RNA content of the parasite is not clear at this time. One possibility is that the drug or its product(s) may interact with the DNA of the parasite. Drugs such as actinomycin D, which

intercalate between DNA bases, have been shown to inhibit all RNA polymerases (Waksman Conference on actinomycins, 1974; Glaubiger & Ramu, 1982). Since the levels of all the mRNAs and rRNAs tested were reduced, this might explain the action of Ro 15-5458. However, further investigation will be required to characterize the actions of the drug or its product on parasite RNA content.

One antischistosomal drug which is known to interact with parasite DNA is hycanthone (Cioli et al., 1985). Ro 15-5458, like hycanthone has a tricyclic ring structure. Thus, as a potential future antischistosomal agent, it was necessary to determine the efficacy of Ro 15-5458 against strains of parasites known to be resistant to hycanthone. Ro 15-5458 cured infections with hycanthone-resistant parasites, as well as infections with wild-type of S. mansoni suggesting the absence of cross-resistance between the two drugs and a difference in their mode of antischistosomal action.

SUMMARY

A variety of biochemical and physiological parameters were evaluated in S. mansoni isolated from mice before the commencement of hepatic shift of parasites. While no drug effect could be demonstrated on the surface membrane potential, surface morphology, glycogen content, utilization of media glucose, gut pigment or ATP levels of the parasites, a significant reduction ($p<0.05$) in parasite weight and protein content was observed in parasites recovered 3 days after dosing. Possible drug actions that may contribute to the loss in parasite protein and perhaps ultimately result in parasite death have been investigated. A significant reduction in the

incorporation of [^{3}H]leucine into acid-insoluble fractions of the parasites was noted. The reduction in the incorporation of leucine into parasite proteins was non-specific and preceded the drug's action on the uptake of the amino acid. Treatment decreased schistosome RNA-directed protein synthesis, total RNA content, actin and superoxide dismutase mRNA levels and both the 18S and 28S rRNA content of the parasites. However, administration of the same or higher dose of Ro 21-6787, an inactive structural analog of Ro 15-5458, had no effect on either the in vitro translation of parasite mRNA or actin mRNA content. Ro 15-5458 did not have similar actions on treated but uninfected mouse liver RNA extracted after the administration of the same dose, suggesting selectivity in its action. We postulate that the effect of Ro 15-5458 and/or its products is to inhibit the expression of parasite genes. Reduction in the steady-state levels of both poly(A^+) and poly(A^-) RNA was observed, suggesting non-specificity in the action(s) of the drug. These defects were observed in parasites recovered as early as 12 h after dosing, the time when other parameters, including weight and protein content, were not different from control parasites. The reduction in parasite RNA content may be the mechanism by which Ro 15-5458 and/or its product exert schistosomicidal action, since maintenance of the level of these RNAs is critical for parasite viability.

GENERAL DISCUSSION AND CONCLUSIONS

Results obtained in these studies provide insights into the mode of antischistosomal action of Ro 15-5458. Most of the work focused on the evaluation of drug effect on parasites recovered after dosing the host since drug lacked activity *in vitro*. Apart from effects of primary therapeutic significance, this approach is liable to confusion arising from secondary biochemical effects, parasite morbidity, and the contribution of the immune response of the host.

Although these possibilities have to be taken into account in the evaluation of the *in vivo* actions of Ro 15-5458 on the parasites, results of experiments presented in Chapter IV suggest that parasites retrieved from mice within 3 days of drug administration are physiologically fit for the host environment. Treatment did not alter the surface membrane potential and morphology, energy metabolism and the consumption of host erythrocytes up to 3 days after drug administration. There was some disparity in the reported results in these experiments depending on the way the results were expressed. When expressed on the basis of parasite protein, most values for treated parasites were not different from, or exceeded values for control parasites.

These results have to be expected since treatment reduced the amount of protein in these parasites. It is also possible to argue that the drug did not alter parameters such as lactic acid production, glucose

consumption and the uptake of precursors for the synthesis of macromolecules. Despite the discrepancy in the reported results on the basis of comparison, the results in both cases support the notion that these parameters were not affected after treatment of the host in the time-frame of parasite recovery for in vitro examination.

On the other hand, a drug-induced defect in parasite protein synthesis and a reduction in the amount of parasite RNA was observed. A decline in actin and SOD mRNA as well as rRNA was evident by 12 h after dosing, 48 to 72 h before the parasites showed changes in any of the other parameters measured.

The mechanism by which treatment with Ro 15-5458 results in decline of parasite RNA was not investigated. Drug could be activated in the host to species that will inhibit the RNA polymerase enzymes or the active product(s) may interact with the parasite DNA to inhibit the transcription of the genes. It is also possible that the drug could bind to the host erythrocytes, ingested by the parasites in sufficient amount to interfere in the biosynthesis of parasite macromolecules.

Until one understands how the drug interacts with the host system to eliminate the parasites, it is not possible to define with certainty the mode of schistosomicidal action of the drug. However, taking into consideration the efficeint synthetic capability of schistosomes and the importance of these processes in the maintenance of the host-parasite relationship, we postulate that treatment effect on the parasite RNA is responsible for parasite killing.

Altogether, the results of these studies are consistent with this mechanism. Decline in the RNA content of the parasite was observed which

was followed by reduced synthesis of parasite proteins which ultimately resulted in reduced utilization of nutrients from the host, since protein-mediated mechanisms are required in both the gut and tegumemental absorption of nutrients from the host (see Pappas, 1989 for review). The defect in parasite nutrition which was demonstrated in this study in parasites recovered on the 3rd day after dosing would further inhibit synthesis by depriving the organism of metabolic precursors. Thus, the cycle will continue until a stage is reached when the parasite is no longer fit for the host environment.

The proposed mechanism may explain the time-dependent decline in parasite weight and protein content, the chronology of events observed in the parasite after administration of the drug to the host and perhaps the slow death of parasites after dosing the host with Ro 15-5458.

RECOMMENDED AREAS FOR FUTURE STUDIES

Future studies designed to characterize the effects of Ro 15-5458 on Schistosoma mansoni must first focus on the isolation of the active metabolite(s) of the drug. This task requires the production of drug labeled with isotopes of C, N, or H atoms at various parts of the molecule. This may be important due to the complexity of the structure of Ro 15-5458; it has the potential to give rise to various products.

After the successful isolation of the active product, its interaction with parasite nucleic acids and other putative sites must be investigated. For instance, the binding of the active product to parasite DNA by determining its effect on thermal denaturation profile and absorption spectrum could be examined.

In the absence of such a product, however, other possibilities, such as the role of the host-immune system and drug delivery via erythrocyte ingestion in the action of Ro 15-5458 have to be considered. Since the drug is active against all stages of the parasite, it may be prudent to perform these experiments on the parasite larvae (schistosomula) due to their susceptibility to the host-immune mechanisms; unlike the adult parasites the larvae also feed on host erythrocytes in vitro.

It may also be important to point out other aspects of the actions of Ro 15-5458 that could be a potentially useful tool in understanding the

parasite's molecular processes associated with growth, reproduction and protection against the host-immune mechanisms.

(1) Results reported in this thesis indicate that the administration of Ro 15-5458 to the host stimulates egg production by the parasites. This action of the drug has not been investigated in detail, but it does not increase the activity of parasite HMG-CoA reductase, an enzyme recently reported to regulate egg production (VandeWaa et al., 1989) nor did it induce the expression of genes reported to be expressed specifically by the female parasite (Bobek et al., 1986; Johnson, Taylor & Cordingley, 1987). Further work to unravel the stimulatory effect of treatment on egg production may shed light on the processes associated with egg production in these parasites.

(2) Studies on the sequence of mRNA encoding the SOD from S. mansoni (Simurda et al., 1988) indicate that the parasite enzyme shares homology with cytosolic SODs of 10 other species and the extracellular form of human SOD. The same workers have postulated that the SOD associated with the parasite tegumet, could be a mechanism of defense by the parasite against the cellular immune mechanisms of the host. In our experiment, a 0.78 kb mRNA hybridized with a probe from the carboxy third of schistosome SOD cDNA. Treatment with Ro 15-5458, also reduced the amount of this mRNA. Ro 15-5458 may thus be a valuable tool in characterizing the role of this protein in the parasites.

(3) In general, the rate of synthesis of rRNA in various species depends on the requirement for ribosome synthesis. There is considerable protein synthesis in the early stages of parasite development; synthesis of ribosomes is also high, which necessitates production of rRNA at a high

rate, thus drugs that interfere in the production of these RNAs must bring retardation of parasite growth and sexual maturity. The administration of subcurative doses of Ro 15-5458 (7.5 and 10 mg/kg) to mice 20 days after infection with S. mansoni arrested the growth of parasites compared to controls. Parasites from treated mice were small in size, had no pigmentation in their gut, did not lay eggs as evidenced by the absence of host liver pathology, but were found in copula. Further investigation on the effect of Ro 15-5458 on juvenile parasites would thus improve our understanding of the growth and sexual maturation of the parasites. Ro 15-5458 reduced the level of both the 18S and 28S rRNA in the adult parasites within 12 h of drug administration, suggesting a high turnover rate of these RNA species in adult S. mansoni. This may also be another area where the property of Ro 15-5458 or its products could be exploited.

(3) Various investigators (Cohen et al., 1983; Davis et al., 1985; Zhou & Podesta, 1989) have localized actin in the parasite musculature and surface spines. Two types of actin protein have been observed in male S. mansoni, but only one in female worms (Atkinson & Atkinson, 1982). Work by Davis et al. (1985) demonstrated that female parasites contain low levels of the 1.9 kb message compared to males. Incorporating their results with other studies (Hockley, 1973; Miller et al., 1972) that indicated fewer spines on the surface of the female compared to the male worms, Davis et al. (1985) suggested that the 1.9 kb message may direct the synthesis of spine-specific actin.

Since Ro 15-5458 treatment resulted in a proportionally higher reduction of the 1.9 kb actin message, it may be important to determine the actin content of surface spines after treatment with Ro 15-5458. A

high turnover rate of the parasite surface membrane has also been demonstrated (Kusel & MacKenzie, 1975; Tavares et al., 1980). If this actin protein could be localized exclusively to the spines, it may have a relatively higher rate of turnover, which may explain the observed action of Ro 15-5458 on its mRNA.

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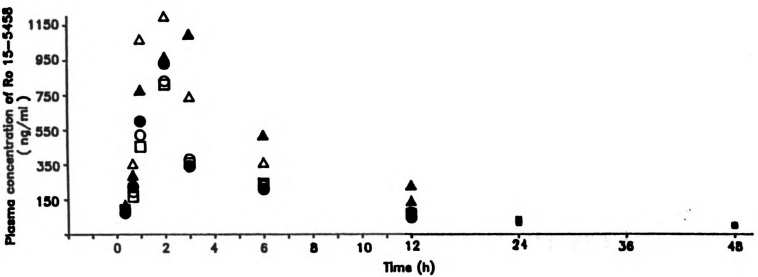
APPENDICES

APPENDIX I

Concentration of Ro 15-5458 in the Plasma of Rabbits Following the Administration of a 50 mg/kg Single Oral Dose



Each symbol represents the concentration in the plasma of an individual rabbit (n=5).

APPENDIX II

Pharamcokinetic Parameters Obtained After Fitting the Plasma Level-Time Data to a One-Compartment Model with Either First-Order Absorption or Zero-Order Absorption Using the PCNONLIN Program

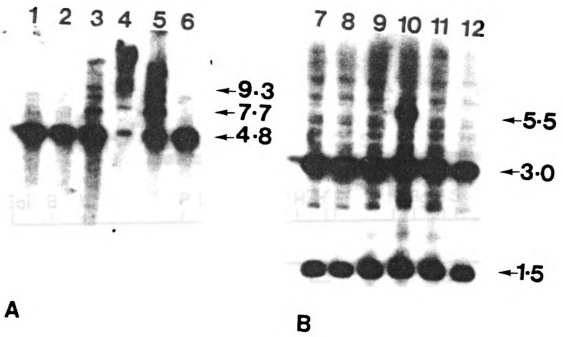
Parameter	First-Order Model	Zero-Order Model
Volume of distribution	25±473[a] (-175-175[b])	47.7±5.2 (35.6-60)
Apparent absorption rate constant	0.46±861 (-320-320)	---
Apparent elimination rate constant	0.46±862 (-200-200)	0.21±0.06 (0.09-0.34)
AUC	4.4±1.3	4.9±0.93
C_{max}	0.90	0.74±0.09
Correlation (r)	0.92	0.96

[a]Value ± SE

[b]95% confidence limits

APPENDIX III

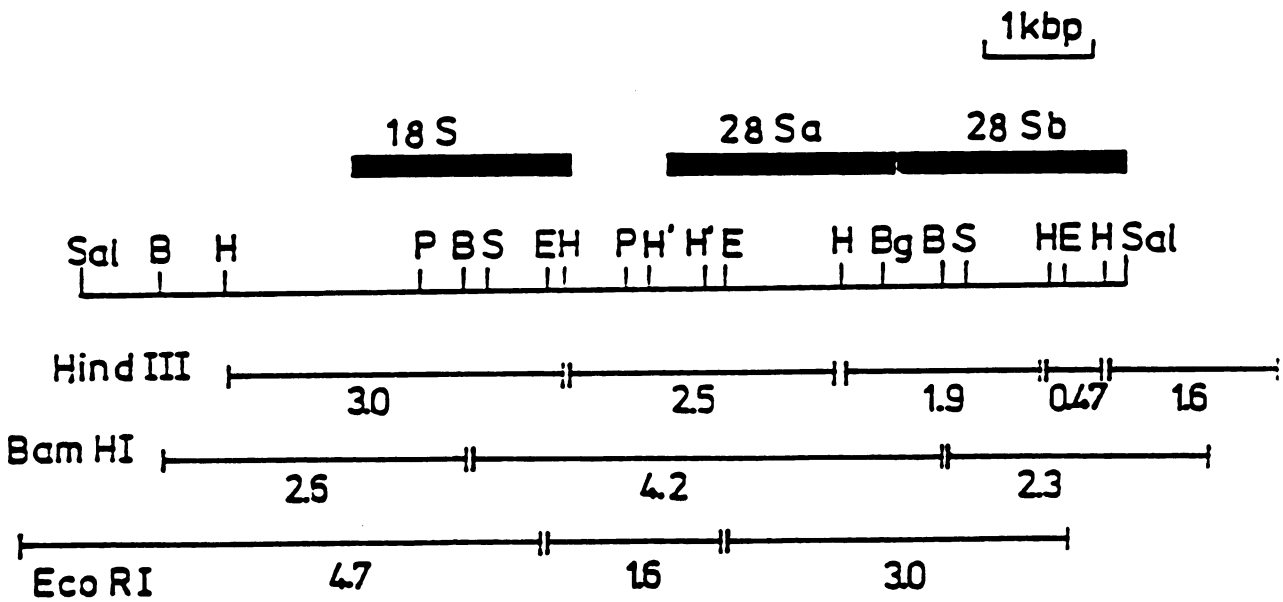
EcoRI (A) and Hind III (B) Restriction Pattern of DNA Isolated from Ro 15-5458-treated S. mansoni



DNAs were isolated from control parasites (lanes 1 and 7), parasites recovered from Ro 15-5458-treated mice after 12 h (lanes 2 and 8), 24 h (lanes 3 and 9), 72 h (lanes 4 and 10) and 96 h (lanes 5 and 11). Lanes 6 and 12 are DNA from parasites exposed to Ro 21-6787 (15 mg/kg) in the mouse and retrieved 48 h after dosing. Numbers at the arrow refer to sizes (kb).

APPENDIX IV

Restriction Map of rRNA Gene from S. mansoni



Reproduced from Van Keulen et al., 1985 (Mol. Biochem. Parasitol. 15: 215-210).