DEVELOPMENTAL CHANGES IN PHYSIOLOGICAL RESPONSES TO DRUGS AND IMMUNE SUBSTANCES IN *SCHISTOSOMA MANSONI*

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ABSTRACT

Developmental Changes in Physiological Responses to Drugs and Immune Substances in Schistosoma mansoni

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Immature post-transformational stages of Schistosoma mansoni (schistosomula) were studied using physiological and biochemical techniques to examine developmental changes in their responsiveness to drugs and immune substances.

Electrical activity in schistosomula is highly sensitive to a number of agents believed to affect the neuromuscular system, metabolism or the host/parasite interface of adult S. mansoni. Among the wide range of substances tested, measurable effects due to potassium cyanide and immune serum plus complement were restricted to freshly transformed schistosomula. Schistosomula, however, were less responsive than adults to the putative neurotransmitters 5HT and dopamine, as well as pentobarbital-sodium and the anthelmintic, oltipraz. Skin- and mechanically-transformed schistosomula exhibited similar responses to all treatments.

To examine more closely the conversion to a cyanide-insensitive state, immature and adult S. mansoni were studied electrophysiologically and by monitoring the fate of exogenous [^{14}C]glucose. Electrical recordings revealed that the parasite becomes insensitive to 10^{-3}M

cyanide by 24 hr after transformation. This finding may be explained on the basis of metabolic changes that occur between the 3 hr and 24 hr stages resulting in a 77% drop in CO_2 evolution and an 84% increase in lactate excretion. The timecourse of this conversion was similar in skin- and mechanically-transformed schistosomula, and was unaffected by inhibition of protein synthesis. No Pasteur effect is detected in immature or adult schistosomes.

Based on physiological recordings, 3 hr schistosomula are highly sensitive to 24 hr incubations in antiserum plus complement, while adult schistosomes are refractory to this treatment. Cytochalasin B (10^{-5}M) and colchicine (10^{-4}M) effect varying degrees of tegumental disruption in adult S. mansoni, but no measurable effects on tegument or muscle physiology. Furthermore, their effects, do not appear to be exacerbated by concurrent exposure to immune serum plus complement.

to Linda, Trevor and Trent

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

GENERAL INTRODUCTION

The freshly transformed schistomulum represents a stage in the life-cycle of Schistosoma mansoni that is of extreme clinical importance. In addition to being the only mammalian stage that is not refractory to host immune effectors, the freshly transformed parasite is also characterized by a number of structural and biochemical features that could render it a distinct alternative target for chemotherapeutics. Despite its importance, little is known of the schistosomulum's physiological or biochemical properties. In light of this, I have developed an objective and highly quantitative electrophysiological assay for examining responses of the immature parasite to a wide range of substances. In addition, correlations are obtained between the electrophysiological parameter and energy metabolism in various developmental stages of the schistosome.

Schistosoma mansoni: Parasite, Life-Cycle and Disease

Schistosoma mansoni is a digenetic trematode capable of infecting humans during one stage of its polymorphic life-cycle. The parasite is diecious, with male and female exhibiting extensive morphological and physiological differences at maturity within the definitive host.

The life-cycle of schistosomes (Figure 1) comprises the passing of ova from the gastrointestinal tract of a definitive host; their hatching

Figure 1. Life-cycle of S. mansoni.

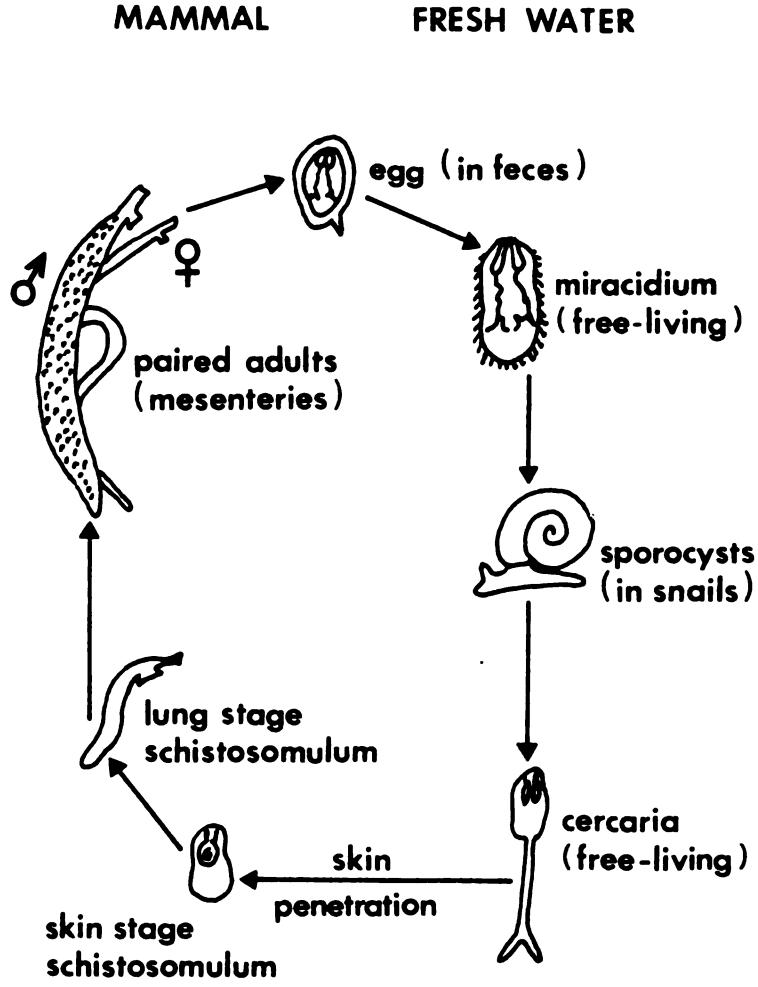
MAMMAL
FRESH WATER
egg (in feces)
miracidium
(free-living)
♂
♀
paired adults
(mesenteries)
sporocysts
(in snails)
lung stage
schistosomulum
cercaria
(free-living)
skin
penetration
skin stage
schistosomulum

Figure 1

in fresh water with the liberation of free-swimming miracidiae; the penetration of a suitable species of snail; the metamorphosis of the larvae into sporocysts then cercariae in the snail; the shedding of free-swimming cercariae into fresh water; the penetration of human skin by the cercariae; and the migration and growth of the immature worms in the liver and mesenteric vasculature (Noble and Noble, 1976).

Man is the most important definitive host for S. mansoni, although the parasite infects all primates and some rodents (Belding, 1965). The agitation of infested water during such activities as bathing, planting, fishing, or recreation stimulates cercarial activity. Recent evidence indicates that fatty acids in the skin, particularly linolenic and linoleic acid attract the cercariae and promote penetration (Haas and Schmitt, 1982). During the penetration process, the cercaria attaches itself to the skin by its oral sucker, which everts while mucus is secreted from the postacetabular glands of the cercaria onto the skin of the host. This mucus substance appears to penetrate the layers of the squames and eventually swells, thereby forcing the epithelial cells apart and opening points of entry in the keratin layer along natural cleavage planes (Stirewalt and Dorsey, 1974). Bruce et al. (1970) describe fragmentation and granulation of the keratin layer, indicating that penetration may also be aided by chemical lysis. Stirewalt and Dorsey (1974) provide evidence that preacetabular gland secretions are released during the parasite's migration through subsequent layers of the epidermis and dermis. The basement membrane of the dermo-epidermal junction appears to constitute the major barrier to migration, possibly requiring the concerted effort of many cercariae for successful penetration (Stirewalt and Dorsey, 1974).

After penetrating the skin, the parasites enter the schistosomulum stage of development. They travel along the lymph ducts into the veins and lungs, and eventually the liver, where they mature into adult schistosomes within 5-6 weeks. The female normally lies enclosed within the ventrally located gynecophoric canal of the larger male parasite, leaving only for brief periods to penetrate the narrowest capillaries of the intestine and rectum during oviposition (Belding, 1965).

Most pathology associated with the disease, schistosomiasis, is due to the backflow of deposited ova which do not successfully penetrate into the intestinal or rectal lumen for fecal expulsion. In the tissues, particularly the liver and spleen, the ova produce multiple foci of inflammatory cellular infiltration, ultimately producing extensive granulomatous pseudotubercles. These reactions are characterized by early eosinophilic and neutrophilic responses and late epithelioid cell and fibroblast involvement. The resulting granuloma formation ultimately leads to fibrosis of the liver and spleen, with accompanying extensive hydrodynamic impairment of hepatic-portal circulation.

The number of persons suffering from schistosomiasis in various parts of the world today is estimated at 250-400 million, with approximately 1.5 billion at risk to the disease. It thus ranks second only to malaria in occurrence among infectious diseases. During recent decades, the disease has been steadily on the increase, particularly in areas where major efforts have been made to improve irrigation of the soil. As a result, regions in which schistosomiasis had previously been endemic are showing a tendency to become hyperendemic zones.

Additionally, the wide and ever-increasing distribution of the disease is being accentuated by the increase in migration of infected people from endemic regions to the cities and other urban areas. Unfortunately, currently available anthelmintics are either marginally effective or too expensive to be made available to third world inhabitants on a population-wide basis. While a vaccine for the disease has been the aim of intensive research during the past 15 years, its completion is probably many years away (Taylor, 1980).

Anatomical and Physiological Characteristics Relevant to the Present Study

The cercaria and early stage schistosomulum exhibit a body shape that is roughly pear-shaped with rounded ends. The cercaria also possesses a long tail with a terminal furca (Figure 2). The body dimensions of the parasites at these developmental stages are approximately 20x50 μm (Belding, 1965). The mass of the cercaria minus its tail, or the early stage schistosomulum, is approximately 0.1% as great as the adult parasite's. Adult male S. mansoni are approximately 1 cm long and 0.2 cm wide, with a wet weight of 0.6 mg. Females are slightly longer than males, but only about 10% as wide and 25% as massive (Cornford et al., 1979). Both sexes possess rostrally located anterior and ventral suckers that are involved in locomotion and stabilization.

The gross morphology of schistosomes is relatively simple. The tegument and underlying muscle bundles form a shell within which intestinal cecae, protonephridia, and gonads are suspended in a parenchymal matrix composed largely of lipid and glycogen particles and

Figure 2. Micrographs of cercaria (left) and adult S. mansoni. The cercaria's tail is shed during transformation to the schistosomulum stage, however, the gross morphology of the body remains the same until after parasites reach the lungs on days 5-8. Calibration bars: 25 μm (left), 500 μm (right).

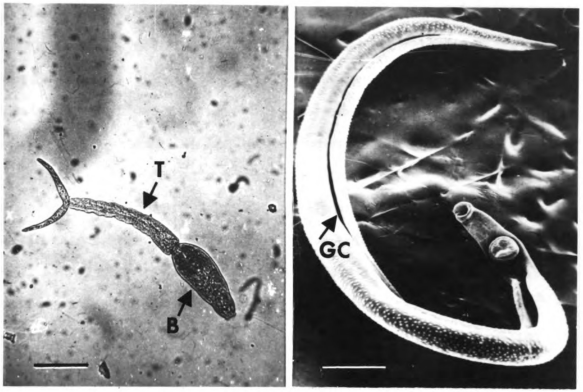

Figure 2

extracellular space. The nervous system is composed of two paired circumesophageal ganglia within which most nerve nuclei are located, and four lateral nerve trunks. From these trunks extend smaller fibers that synapse with muscle bundles, and may innervate putative sensory receptors located within the tegument (Morris and Threadgold, 1967; Silk and Spence, 1969a) (Figure 3).

The outermost layer of the schistosome (i.e., the host-parasite interface) is a syncytium known as the tegument (Morris and Threadgold, 1968; Hockley, 1977). In all stages of the parasite, the tegument contains: (1) spines that have thin filaments packed in a paracrystalline array and extend from the surface membrane of the tegument to the basal lamina; (2) a few mitochondria; and (3) various membrane bound inclusions known as membranous, or multilaminate, or spherical bodies and elongate bodies (Morris and Threadgold, 1968; Wilson and Barnes, 1974). The tegumental syncytium is connected by way of internuncial processes to cell bodies, called cytons, which contain nuclei, Golgi apparatus, and endoplasmic reticulum (Silk and Spence, 1969a; Wilson and Barnes, 1974). Internuncial processes pass through several layers, including: (1) a basal lamina, (2) an interstitial layer, and (3) an outer circularly arranged and an inner longitudinally arranged muscle layer in order to connect the tegumental syncytium with the cytons (Silk and Spence, 1969a).

The membranes bounding the tegument are complex. On the basal surface there is a classical trilaminate 8-10 nm membrane which is continuous with the trilaminate membrane of the internuncial processes and the cytons (Hockley and McLaren, 1973; Wilson and Barnes, 1974).

Figure 3. Schematic of tegument and muscle layers in S. mansoni. T, tegument; OM, outer double membrane of tegument; BL, basal lamina; IM, internal membrane of the tegument; S, spine; MP membranous particles; EB, elongated bodies; IMa, interstitial matrix; CM,, circular muscle; LM, longitudinal muscle; TC, tegumental cyton; N, nucleus; CC, cytoplasmic channel; JP, junctional process; L, lipid granule; ECS, extracellular space.

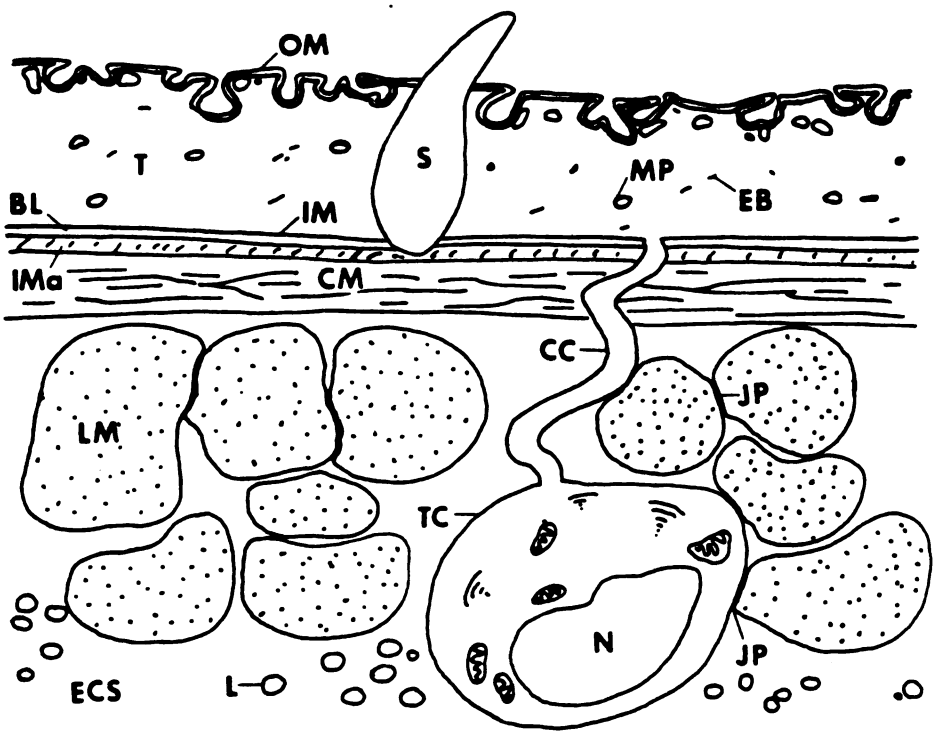

Figure 3

The morphology of the surface membrane depends on the stage of the life-cycle of the parasite (Smith et al., 1969; Hockley and McLaren, 1973). The cercarial membrane is trilaminate and is covered by a 1 μm thick extracellular coat which resembles the glycocalyx covering the brush border of intestinal epithelium (Hockley and McLaren, 1973). Upon penetration through the skin of the definitive host, most of this surface coat is lost (Stirewalt, 1963), but a few microvilli with some of the coat material remain on the surface of the freshly transformed schistosomulum (Brink et al., 1978). It has been suggested that the cercarial membrane is shed by means of the microvilli, but how much of the surface membrane of the cercaria is lost by this mechanism is unclear (McLaren and Hockley, 1976). Within 3-5 hr after penetration most of the trilaminate membrane is replaced by a heptalaminate membrane which consists of two adjacent lipid bilayers (Hockley et al., 1975).

The surface membrane of the schistosome is important because it is the target of antibody-dependent cell mediated cytotoxic reactions which have been shown to kill the parasites in vitro (Smithers et al., 1977). The tegument of S. mansoni has also been implicated in several physiological processes. In addition to providing a barrier that is highly resistant to the host's degradative enzymes, it appears to be an important site of entry for glucose (Fripp, 1967) and several amino acids (Senft, 1968). A thin layer of material coats the outer surface of the tegument that is partially composed of acid mucopolysaccharides (Stein and Lumsden, 1973), and may contain enzymes involved in the absorption and digestion of nutrients (Lumsden, 1975; Pappas and Read, 1975; Ernst, 1977). There is also anatomical evidence suggesting that

innervated sensory structures are located in the tegument that may function to detect the direction of flow of the surrounding medium (Morris and Threadgold, 1968).

Recent work conducted by Fetterer et al. (1980) has demonstrated that a well defined tegumental potential (E_{teg}) of about -60 mV exists that can be altered by changing physical and/or chemical qualities of the parasite's environment. Elevated concentrations of external K^+, reduced Ca^{++}, cold, and ouabain all induce depolarization of the tegument, indicating that some form of active Na^+-K^+ transport must be involved in maintaining this potential (Fetterer et al., 1980). Fetterer et al. (1980) employed horseradish peroxidase (HRP) as a marker for determining the location of the recording electrode while gathering these data. During all penetrations in which potentials in this range were recorded, HRP iontophoretically injected was subsequently found to occupy only those compartments directly associated with the tegument (e.g., cytons cytoplasmic channels, and the outer tegument itself).

Recordings made in the tegument of unanesthetized parasites revealed spontaneous depolarizations that ranged from 3 to 15 mV in amplitude with 20 to 100 msec durations. Most of this activity was abolished by anesthesia (Fetterer et al., 1980). Other evidence suggesting that the tegument may actively propagate electrical activity has been reported in microelectrode (Thompson et al., 1982a) and surface electrical (Fetterer et al., 1977; Semeyn et al., 1982) recordings. Alternatively, the transients recorded in these studies could be volume conducted depolarizations of underlying muscle elements which,

based on microelectrophysiological studies, are electrically coupled to the tegumental syncytium, with a coupling ratio of 0.85 (Thompson et al., 1982a). A possible morphological substrate for electrical coupling between these tissues has been observed in electron micrographs. Plasma membranes of integumentary cells appear to form junctional complexes with neighboring muscle cells that closely resemble those connecting smooth muscle cells (Silk and Spence, 1969a).

The majority of the muscle system in S. mansoni is located immediately beneath the basement membrane of the tegument, where it is arranged in an outer circular and an inner longitudinal fashion (Silk and Spence, 1969b). All muscle appears to be of the smooth variety with no striations, and in that sense it resembles most invertebrate muscle. Thick (180-400 Å diameter) myofilaments are surrounded by 8-14 thin (50 Å diameter) filaments, a ratio very near that observed in vertebrate smooth muscle preparations (Perry and Grand, 1979). The thick filaments are arranged in parallel, while thin filaments exhibit a great deal of branching and cross-linking with other thin as well as thick filaments. Ovoid nuclei are normally separate from and deeper than the fiber bundles, and are connected to them by cytoplasmic processes (Silk and Spence, 1969b).

The sarcoplasmic reticulum in schistosomes is poorly developed, and appears only at scattered intervals. Mitochondria appear in sac-like distensions of the sarcoplasm along myofibril bundles. Lipid globules as well as α and β glycogen particles are distributed throughout the muscle cells (Silk and Spence, 1969b). Junctional complexes are observed between the outer layers of adjoining sarcolemmas. These

junctions separate apposing cell membranes by about 7-9 nm, and are up to 400 μm long. They appear to be similar to those at the interface between cyton cells and neighboring muscle bundles (Silk and Spence, 1969b).

Fetterer et al. (1977) have developed a technique for monitoring schistosome muscle activity directly, using suction electrodes in circuit with a force transducer. This method is, in some cases, as much as 100X more sensitive than those previously reported for measuring mechanical responses in the parasite. In addition to serving as a sensitive monitor for the effects of various pharmacological agents, this method has demonstrated that the contractile properties of the schistosome musculature are much like those reported for other invertebrate as well as vertebrate muscle preparations. For example, elevated K^+ (Fetterer et al., 1977) and hyperosmotic sucrose solutions (Pax et al., 1981) both induce paralysis.

Bricker et al. (1982), using HRP as a marker, have recently identified a second compartment of electrical potential in the schistosome as being endemic to muscle tissue. Resting membrane potentials recorded intramuscularly are generally about -30 mV, or about one-half as great as those recorded in overlying tegumental regions (Bricker et al., 1982; Thompson et al., 1982a). Electrophysiological recordings using multiple electrodes have revealed that the muscle compartment in schistosomes, like the tegument, is an electrical syncytium; and that low resistance pathways connect these two tissue compartments in a nonrectifying manner (Thompson et al., 1982a).

Developmental Changes in the Parasite within the Definitive Host

Schistosomes undergo several important changes during the infection process in man prior to their habitation of the liver and hepatic portal vasculature as mature organisms. There are three stages, in particular, during which the parasite undergoes marked changes that are easily identified: (1) during penetration of host skin, cercariae shed most of their surface coat and quickly adapt to new osmotic conditions (Stirewalt, 1963); (2) shortly after penetration, the parasite begins to show a decline in immunogenicity, so that by 24 hours it is refractory to most immune effectors (Sher, 1982); concomitantly, the energy metabolism of the parasite begins to change from an aerobic to an anaerobic one (Coles, 1972a; Von Kruger et al., 1978); and (3) almost immediately upon entering the hepatic portal vasculature, the worms enter a period of exponential growth; this follows a period of 8 to 15 days of mitogenic dormancy which characterizes the lung stage of development (Lawson et al., 1980).

A number of theories have been advanced which attempt to define the conditions which precipitate the important changes that occur after transformation. Adaptation to the saline environment is thought to occur primarily as a result of physical changes cercariae undergo during penetration of host tissue. Three changes occur at the time of penetration that may be specifically related to osmoregulation in the new environment: (1) a rapid reduction in the carbohydrate-rich filamentous coat (glycocalyx) (Hockley et al., 1972; Cousin et al., 1981), (2) a transient appearance of microvilli at the surface of the tegument (McLaren et al., 1976; Brink et al., 1977; Cousin et al., 1981), and

(3) a rapid reduction of the bladder and collecting tubules (Cousin et al., 1981). Because these changes are believed to be mediated by mechanical penetration itself, together with the osmotic pressure of the new environment, it is unlikely that they could be altered by chemotherapeutic intervention.

Changes relevant to metabolism and the gradual loss of susceptibility to host immune reactions occur more gradually than the permeability changes associated with penetration. The precise timecourse as well as the mechanisms mediating these changes are not well understood. Previous in vivo studies have shown that damage mediated by antischistosome antibody in the presence of complement or immune effector cells is possible only within the first day or two after a transcutaneous cercarial infection (McLaren, 1982; Sher et al., 1982). There are at least three processes that occur at the level of the tegument that could account, in part, for the development of immune refractoriness in schistosomes: (1) a transition in the outer tegumental membrane from a trilaminate to a heptalaminate (double membrane) formation (Hockley et al., 1973), (2) the masking of endogenous surface antigens with host or host-like antigens (Clegg et al., 1972; Dean et al., 1977; Goldring et al., 1977; Samuelson et al., 1980), and (3) the rapid sloughing of tegumental membrane components from the surface of the parasite (Samuelson et al., 1982).

Processes relevant to the development of the double outer tegumental membrane begin to occur immediately after the parasite enters the host. Multilaminate vesicles are transported from the cytons to the syncytium where they fuse with the outer tegumental membrane, gradually replacing the original trilaminate one. This process appears

to be completed within 3 to 5 hr after penetration (Hockley et al., 1973). The fact that refractoriness to the cytotoxic effects of antibody plus complement begins to increase within 4 hr after transformation (Sher et al., 1982) suggests that the double membrane structure may be important in conferring protection to the parasite. However, the parasite concomitantly shows reduced levels of antibody and C-3 binding (Samuelson et al., 1980), and these factors may be more important in the development of refractoriness. Furthermore, neutrophils will adhere to and phagocytise extensive areas of tegumental membrane in all stages of the schistosome, yet these effector cells are not cytotoxic to the parasites (Vadas et al., 1979).

Evidence supporting the theory that schistosomes evade host immune mechanisms by disguising their surface antigens is provided in a number of separate studies. Adult schistosomes share many surface antigens with their hosts (Smithers et al., 1969; Clegg et al., 1970; Damian et al., 1973). While some of these antigens appear to be produced by the parasite, others are apparently of host origin. This is supported by findings that schistosomula maintained in vitro and in vivo passively adsorb ABO bloodgroup (Goldring et al., 1976), Forssman (Dean et al., 1972) and histocompatibility (Sher et al., 1978) antigens. Furthermore, these antigens are serologically identical to host antigens found on adult worms. Thus, incorporation of host or host-like material into the tegument may confer protection from the host immune system by disguising endogenous parasite antigens, thereby rendering them antigenically inert (Clegg et al., 1972). In addition, recent in vitro tests demonstrate that significant amounts of host cholesterol and

triglycerides are taken up by the schistosome during the transformation process (Rumjanek, 1982). These additional cytochemical changes in the tegument could also contribute to the development of refractoriness by disguising the parasite with host material.

Other attempts to define more specifically the biochemical changes that occur in the tegument which impart protection to the parasite from immune attack have implicated inherent processes that may occur in addition to or independent of the formation of double membrane or the masking phenomena. Wilson and Barnes (1977) have demonstrated that the outer tegument of schistosomes is continuously replaced, with a half-life of 2-4 hours in control medium *in vitro*. Thus, through a process of continuous turnover of the tegumental membrane, the parasite may effectively avoid lysis by host complement by detaching itself from its own antibody-bound epitopes. Wilson and Barnes (1977) have further demonstrated that the plant lectin concanavalin A appears to stabilize the vesicular fusion process which continuously provides new membrane to the host-parasite interface of the schistosome. Because of this effect, it was thought that concanavalin A might somehow interfere with the normal repair process of the outer tegumental membrane, thus rendering the parasites more susceptible to immune attack. However, while concanavalin A as well as lectin from *Ricinus communis* do exert profound tegumental disruption in adult *S. mansoni*, the effects do not depend on the presence of immune substances, and freshly transformed schistosomula are unaffected by these treatments (Simpson *et al.*, 1982). In fact, incubation of freshly transformed schistosomula for 5-6 hr in culture medium containing 4 μg/ml concanavalin A may render this stage of the parasite more resistant to the lethal effects of antibody

plus complement, even though binding of anti-parasite antibody does not change from control levels (Van Pijkeren et al., 1982). In another study designed to determine the effects of inhibiting a tegumental process on the immune response, Kemp et al. (1978) showed that schistosomes actively shed antischistosome antibody from their surface with a half-time of less than 20 min, and that this process is inhibited by 10^{-5}M cytochalasin B, implicating a role for microfilaments in this process. No data are reported in their study, however, regarding possible changes in cytotoxicity brought about by drug-induced inhibition of antibody elimination.

It is also possible that changes in the distribution of tegumental integral membrane proteins (IMP) in schistosomes is important in the process of immune evasion (McLaren et al., 1978). Freeze fracture studies indicate that the IMP of the outer tegumental membrane of schistosomes cultured in vitro or recovered from mice undergo dramatic changes in number and distribution during the early stages of transformation. During the first 4 hr, the outer bilayer is mostly lipid with few IMP. By 6 hours after penetration, the number of IMP in the outer tegumental membrane begins to increase, reaching a peak in the E2 face of the outer bilayer by day 4, after which they decline (McLaren et al., 1978). The extent to which these IMP are targets for specific or nonspecific immune mechanisms has not been determined.

Another important stage in the development of the schistosome occurs while the parasite occupies the lungs of the vertebrate host. After reaching the lungs, the young parasites slowly increase up to 4 times in length, but not at all in mass. This change results in a 50%

increase in the surface to volume ratio of the organism, and probably facilitates migration through the lumina of blood capillaries (Wilson et al., 1977; Miller et al., 1979). Also linked to mobility enhancement and occurring during the lung stage is the regression of tegumental spines and sensory papillae, so that only those at the rostral and caudal extremes of the parasite remain. These organs are observed to reappear immediately after the hepatic migration which begins to occur on day 8 or 9 (Miller et al., 1979).

Coincident with the above changes are a series of superficial tegumental enfoldings that appear to pinch off, incorporate, and degenerate surrounding host lung tissue (Bruce et al., 1974). This process begins immediately upon the schistosomulum's arrival in the lungs, and is probably linked to the appearance of additional host antigens on the tegumental surface, which by day 5 completely obscure identifiable worm surface antigens (Goldring et al., 1977). By this time, neither hyperimmune serum from a previous infection (Smithers et al., 1965; McLaren et al., 1975) nor cell mediated immune mechanisms (Sher et al., 1982) exert significant effects on the schistosomula in vitro or in vivo.

Within 5 days after penetration, the schistosomula begin to migrate from the lungs to the liver and hepatic portal vasculature where they remain. This migration may require several trips through the systemic circulation. Most parasites will reach the liver by day 12 or 13 (Miller et al., 1979). Prior to reaching the liver, immature worms appear to be in a semi-quiescent metabolic state during which: (1) no mitosis occurs, (2) their mass is slightly reduced and (3) no net N_2 incorporation occurs (Lawson et al., 1980). Immediately upon entering

the hepatic portal system, some unknown mechanism(s) signal the initiation of a period of rapid growth and metabolic activity. Wilson et al. (1978) have suggested that this process may be a direct response to the nutrient-enriched environment. Also apparent at this time is a doubling in the duration of the peristaltic contractions of the parasite (Wilson et al., 1978), and an enhanced level of responsiveness to the drug hycanthone (Tomosky et al., 1977), which stimulates mechanical activity.

After the schistosomulum enters the liver, additional changes in its development appear to be primarily quantitative. However, Coles (1973) points out that several drugs tested in the past have exhibited a range of effectiveness on schistosomes at various developmental stages. Most thoroughly studied have been the antimonial derivatives, which are extremely effective at killing worms in the first week of infection (Schubert, 1948), but less so on 14-21 day old parasites (Standen, 1955). Stohler et al. (1963) demonstrated that the drug becomes effective once again by day 34. Lucanthone HCl also shows a biphasic pattern of effectiveness, being lethal to 0-24 hr worms (Stohler et al., 1963) and worms older than 28 days. Other drugs tested showing a similar biphasic pattern of effectiveness include maleic acid-mono-4(3'-chloro-4'-methylphenyl)piperazide (Lammler, 1958), nitrothiazole derivative (Sadun et al., 1966), 2-aminomethyl-tetrahydroquinolone derivatives (Foster et al., 1971), and nitrovinyl furane (Lennox et al., 1972). It appears from these studies that worms in the 3rd and 4th weeks of an infection are particularly resistant to drugs. It has not yet been determined how much of this is due to

changes in worm metabolism, changes in position within the host, or to other developmental factors.

Metabolic Changes Occurring in S. mansoni within the Definitive Host

Coles (1973) has suggested that changes in the schistosome's energy metabolism may contribute to the observations that *in vivo*, both antimony (Coles, 1972) and cyanide (Bueding *et al.*, 1953) show lower levels of depression when administered to 2-3 week old infection stages than when administered to mature stages. These changes are important because major differences in the metabolism of the parasite and its host could hold the key to the development of successful chemotherapeutics.

A number of previous studies have examined energy metabolism in various stages of the parasite's life-cycle. Oliver *et al.* (1953) showed that the free-living cercaria of *S. mansoni* is an aerobic organism with the potential to obtain some energy by fermentation. Results of inhibition studies suggest that a functional citric acid cycle is present in the cercaria (Bruce *et al.*, 1969), and a number of the enzymes of aerobic carbohydrate metabolism have been assayed quantitatively (Coles, 1972b; Shapiro *et al.*, 1983). Oxygen uptake in the cercaria has been measured using Clark electrodes by Coles (1972) and Von Kruger *et al.* (1978) and found to be on the order of 80-100 µl O_2/mg protein/hr. Cercariae incubated in 2×10^{-4}M cyanide showed an 83% inhibition of oxygen uptake (Coles, 1972). Furthermore, Coles (1972) has shown that cercariae made anaerobic or treated with 10^{-3}M cyanide or 10^{-3}M fluoroacetate excrete low levels of lactic acid and demonstrate a Pasteur effect; i.e. increased glycogen usage under anaerobic

conditions. Quantitative determinations of glycogen utilized (measured as glucose) and lactic acid formed after cercariae had been kept for 2 hours in 10^{-3}M NaCN showed that all of the glucose used under these conditions could be accounted for by lactic acid formed; i.e. cercariae are homolactic fermenters under anaerobic conditions. However, Coles (1972) further reported that exposure of the cercariae to 10^{-3}M cyanide for 4 hours appeared to result in mortality, based on visual observations of their motility levels. This effect was not attenuated by the addition of high levels of glucose to the incubation medium.

After penetrating the mammalian skin, the newly transformed schistosomulum begins to excrete lactic acid under both aerobic and anaerobic conditions (Coles, 1972), although respiration continues to be of some importance in energy production (Coles, 1972). The time-course and the mechanism of the switch from the fully aerobic metabolism of the cercaria to the anaerobic one of the adult schistosome are unknown. Shiff (1972) has suggested that the trigger which initiates penetration of the cercaria, believed to be host fatty acids, may also initiate the metabolic change. Coles (1972) found that by raising the osmotic pressure of their incubation media, cercariae were induced to transiently excrete lactate, suggesting that the metabolic shift may be a response to elevated osmotic pressure. Alternatively, Von Kruger et al. (1978) have shown that mere separation of the cercarial body from its tail initiates lactate excretion.

The freshly transformed schistosomulum takes up oxygen at a rate of 20-30 µl O_2/mg protein/hr (Von Kruger et al., 1978), or about one-third of that recorded in the cercaria. This level is reduced by 85% in the presence of 2×10^{-4}M cyanide (Coles, 1972). Other metabolic

inhibitors shown to depress respiration in the schistosomulum include rhotenone, sodium arsenite, and sodium malonate (Von Kruger et al., 1978). Much of the reduction from cercarial levels in oxygen uptake recorded in the freshly transformed schistosomulum is attributed to the loss of the cercaria's tail, which accounts for 30-35% of its total protein content (Coles et al., 1972).

The outstanding feature of carbohydrate metabolism in the adult schistosome is the rapid rate of glucose uptake and utilization, and the subsequent high levels of lactic acid produced. The adult may use up to 26% of its dry weight in glucose per hour in lactate fermentation (Bueding, 1950). Most energy needs of the adult schistosome are met anaerobically, so the adult stage does not appear to have a strict requirement for oxygen in energy production. Evidence for the lack of a role for oxygen in the energy metabolism of adult schistosomes is presented primarily in the work of Bueding et al. (1950, 1959, 1969, 1972, 1982) and Schiller (1975). Their studies have shown that the survival of S. mansoni in vitro is not adversely affected by the absence of oxygen (Schiller et al., 1975; Bueding et al., 1972). Glucose utilization and production of lactic acid has been shown to be similar under aerobic and anaerobic conditions, indicating the absence of a Pasteur effect. Furthermore, ATP levels in the parasite are not reduced after incubation under nitrogen, even after periods of increased carbohydrate metabolism induced by exposure to $5x10^{-5}$M 5HT (Bueding, 1972). In previously tested vertebrate and invertebrate preparations that show a Pasteur effect, phosphofructokinase activity, a rate-limiting factor in glycolysis, is inhibited by citrate, an important

intermediate of respiration (Passenneau and Lowry, 1964). It is believed that the mechanism underlying the Pasteur effect is based on the reduction of citrate brought about by anaerobiosis and the subsequent disinhibition of PFK. Unless the organism possesses a cyanide-insensitive terminal oxidase, a Pasteur effect is also brought about by inhibition of the terminal oxidase of electron transport by cyanide. Alternate oxidases have not been reported in schistosomes (Coles, 1972).

Production of ATP by glycolysis is inhibited in schistosomes by the presence of trivalent antimonials (Coles, 1978). Schistosomes exposed *in vitro* or *in vivo* to trivalent antimonials show elevated levels of glucose-6-phosphate and fructose-6-phosphate and reduced levels of fructose-1,6-diphosphate. These sugar phosphate levels return to normal when the drug is withdrawn or the worms are transferred to untreated hosts (Bueding and Fisher, 1966). This information has been used to argue that antimony compounds are toxic to schistosomes because of their inhibition of PFK (Bueding and Fisher, 1966; Bueding, 1959). More recent studies using worm extracts have corroborated this view (Coles and Chappell, 1979). Shen *et al*. (1959) have demonstrated that the antimonials also inhibit glycolysis in *S. japonicum*. Their studies, however, indicate that they may also inhibit schistosome glutamic-pyruvic transaminase, suggesting another possible mode of action for these compounds.

OBJECTIVES

The general goal of the present study is to gain a better understanding of the biochemical and physiological properties of the schistosomulum of Schistosoma mansoni. To date, most research on the parasite has focused on the adult stage. Because of their size and the difficulties involved in their recovery from host organisms, the schistosomulum stage of the parasite has been studied on an extremely limited basis. Recent findings, however, indicating that the freshly transformed parasite is the most important and perhaps the only viable target for immune mechanisms in vivo (McLaren et al., 1982; Sher et al., 1982; Samuelson et al., 1982) have stimulated a great deal of interest in the schistosomulum.

Previous work on the schistosomulum stage has focused on the morphology of the parasite or its responses to various immune substances. These studies have relied primarily on assays based on patterns of recovery (Periera et al., 1975; Mamoud, 1979), microscopic evaluation (Torpier et al., 1979; Kassis et al., 1979), or release of ^{51}Cr (David et al., 1977); all of which are time-consuming, difficult to quantify, and of questionable validity (McLaren, 1982). A major objective of the present study is, therefore, to develop an objective and quantifiable assay for detecting physiologiccal responses in the immature stages of S. mansoni.

Studies on the immature stages of the schistosome have been aided by the development of techniques for artificially transforming large numbers of cercariae to the schistosomulum stage by means of mechanical decaudation in serum-containing media (Ramalho-Pinto et al., 1974). These organisms develop the outer double membrane in the same manner, and over a similar timecourse as schistosomula which have penetrated through the skin of a mouse (Brink et al., 1977). Furthermore, the in vitro development of artificially transformed schistosomula parallels that of skin penetrated organisms, and when the two forms are injected intravenously into mice, comparable percentages of the organisms develop to maturity. The only morphologically detectable differences appear to be the longer retention of acetabular gland contents and elements of the glycocalyx by the mechanically transformed schistosomula (Brink et al., 1977). Both types of schistosomula bind antischistosome antibodies, as visualized by immunofluorescence, but the binding is slightly stronger in artificially transformed worms (Brink et al., 1977; Bickle and Ford, 1982).

Based on the above studies, it appears that the artificially prepared schistosomula fulfill all of the main criteria for transformation from cercaria to schistosomulum (Stirewalt, 1974). Since they can be prepared in large numbers, uncontaminated by host material, they appear to constitute an excellent source of organisms for experimental analysis. It should be noted, however, that because the mechanically-transformed parasites do not have the opportunity to acquire surface molecules from the host skin they may present a significantly different surface configuration from that of the skin-transformed organisms. In

this context it is relevant to note that both Tavares et al. (1978) and McLaren (1982) have reported enhanced susceptibility of in vitro derived schistosomula to the effects of complement-dependent mouse anti-schistosome antibody in an immune assay system. However, because the artificial transformation techniques yield large quantities of organisms with relative ease, it is likely that they will be used extensively in future studies that require large numbers of parasites. It will be extremely important, therefore, to determine more precisely the extent to which these organisms are suitable material for study. Thus, a second major objective of the present study is to determine the extent to which biochemical and physiological responses in the artificially transformed schistosomulum parallel those of the skin penetrated schistosomulum.

A third major objective of the present study is to elucidate more precisely the timecourse of the metabolic conversion undergone by the parasite during its development from the cercarial stage, which is aerobic (Coles, 1972), to the adult stage, which is predominantly anaerobic (Bueding et al., 1950, 1959, 1982). Because the adult schistosome exhibits an energy metabolism which differs significantly from that of its vertebrate host, this metabolic conversion is extremely important, as enzymes and intermediates involved in energy formation are likely targets for chemotherapeutics (Coles, 1972).

A final major objective of the present study is to obtain additional information on the mechanisms by which the adult schistosome evades the complement mediated effects of anti-schistosome antibody in vitro. If the principal mechanism(s) by which immune refractoriness is accomplished are linked to the parasite's capacity to insert new

membrane into its outer tegumental membrane (Hockley *et al.*, 1973) or to rapidly slough off anti-schistosome antibodies (Kemp *et al.*, 1980), then inhibition of these processes during *in vitro* incubations should render the parasites more susceptible to immune mediated damage.

The present study is divided into three sections, each of which directly addresses one or more of the preceding objectives. Each section contains a Summary, Introduction, Materials and Methods, Results, and Discussion. It is hoped that the information obtained through these studies will make possible increasingly rational approaches to the study of, and ultimately the clinical control of schistosomiasis.

SECTION I

Schistosoma mansoni: A Comparative Study of Schistosomula Transformed Mechanically and by Skin Penetration. Electrophysiological Responses to a Wide Range of Substances

Summary

Volume conducted electrical potentials were recorded from schistosomula of Schistosoma mansoni transformed mechanically (MS) and by skin penetration (SS). The spontaneous electrical activity recorded consisted of bi- and triphasic transients ranging from 20-200 μV in amplitude and 10-300 msec in duration. Low amplitude potentials occurred at a much greater frequency than large amplitude potentials, which appeared to correlate with peristaltic-like contractions of the schistosomulum's musculature. Electrical activity in the schistosomulum was highly sensitive to a number of agents believed to affect metabolic pathways, the neuromuscular system or the host/parasite interface of adult schistosomes. Among the most reactive substances were potassium antimony tartrate, eserine, poly-l-arginine and potassium cyanide. Over a wide range of experimental treatments, electrophysiological responses in schistosomula transformed from cercariae by mechanical decaudation and skin penetration were remarkably similar, supporting the notion that MS are suitable material for in vitro immunochemical, biochemical and physiological study. Some treatments, however, were

more or less effective in altering electrophysiological activity and motility in the schistosomulum than in adult S. mansoni. This suggests that significant physiological atlerations may occur during development from skin stage to adult parasites concomitant with immunochemical and morphological changes already known to occur.

Introduction

Schistosomes undergo a number of structural and biochemical changes during transformation from cercaria to schistosomulum. Within three hours after host penetration, the glycocalyx is lost, the outer tegumental membrane becomes heptalaminate (Hockley and McLaren, 1973), the parasites become sensitive to hypo-osmotic conditions (Stirewalt, 1963), and produce lactic acid under aerobic conditions in vitro (Coles, 1972). In addition, the newly transformed schistosomula undergo a rapid, nearly linear decline in susceptibility to rejection by passively transferred immune serum (Sher, 1977), in vitro culture with antibody plus complement (Dean, 1977), or in vitro exposure to complement plus eosinophils (Dessein, Samuelson, Butterworth, Hogan, Sherry, Vadas and David, 1981). This development of refractoriness to antibody-dependent killing appears to be an inherent process that occurs in the absence of host macromolecules and is complete within 24 hours after transformation (Dean, 1977; Moser et al., 1980; Samuelson et al., 1980), although some recent studies suggest that the parasite becomes susceptible again between 6 and 14 days post-infection (Smithers and Gammage, 1980; Georgi et al., 1983).

Techniques for transforming large numbers of cercariae in vitro using isolated skin penetration (Clegg and Smithers, 1972) or mechanical

decaudation (Ramalho-Pinto et al., 1974) have made the schistosomulum readily accessible for study. Most studies indicate that parasites prepared by these methods fulfill the main criteria of transformation from cercaria to schistosomulum (Stirewalt, 1964), and are therefore appropriate material for immunochemical and physiological studies (Brink et al., 1977). Some recent findings of Tavares et al. (1978) and McLaren and Incani (1982), however, suggest that schistosomula derived mechanically exhibit an enhanced susceptibility to the effects of complement-dependent anti-schistosome antibody. This difference could be due to the absence of host material on the surface of mechanically-transformed worms that is acquired by skin penetration worms during the penetration process (Smithers et al., 1969). That is, host antigens specific for the glycocalyx of skin epidermis which are found on skin penetrated parasites (Smith and Kusel, 1979) could mask exposed epitopes of the schistosomulum. Alternatively, the differences in immune sensitivity could be due to changes in parasite lipid composition which accompanies skin penetration (Rumjanek, 1982) or to exposure of the parasite to its own lytic substances secreted during the penetration process (Bruce et al., 1970).

Because the freshly transformed schistosome is the principal target for immunological control, any changes undergone by the parasite during this period are potentially important. While numerous transformation-related changes in the parasite's structure and immunochemistry have been documented, very little is known about the physiological properties of the schistosomulum. In this paper, we describe an objective and quantifiable technique for analyzing the short-term in vitro

effects of experimental substances on electrophysiological properties of the schistosomulum. Electrical responses to a wide range of substances were measured in mechanical and skin transformed parasites in order to determine whether or not significant physiological difference exist between the two groups.

Materials and Methods

Preparation and culture of schistosomula. A Puerto Rican strain of S. mansoni was maintained by passage through Swiss Webster mice and Biomphalaria glabrata snails. Schistosomula, the larval or skin stage parasites, were prepared from the same batch of cercariae by 2 methods. Mechanically-transformed schistosomula (MS) were obtained by a modification of the method of Ramalho-Pinto et al. (1974). Cercariae were collected in dechlorinated water, placed in 15 ml centrifuge tubes and cooled to 4°C for 10 min to reduce motility. After centrifugation at low speed for 1 min the supernatant was decanted and replaced by a cold 50:50 mixture of RPMI-1640 (Gibco, Grand Island, New York) and heat inactivated horse serum. The packed cercariae were resuspended and stirred for 1 min at moderate speed in a Vortex mixer. Following a 30 min incubation at 30°C, transformed parasites observed to be motile under a dissecting microscope were individually transferred by way of suction pipettes into vials containing RPMI-1640 alone, and maintained at 37°C. Skin penetration schistosomula (SS) were prepared by a modification of the method of Cousin et al. (1981). Approximately 1,000 cercariae in dechlorinated water were pipetted onto the inner surface of the ears of ether-anesthetized Swiss Webster mice and allowed to

penetrate for 45 min. The mice were killed by cervical dislocation; nonpenetrating cercariae were removed by cotton swabs; the ears were removed and finely minced in a solution of RPMI-1640. After mincing, motile schistosomula were individually transferred into vials containing RPMI-1640 and maintained at 37°C. For both groups, transformation was verified by means of the methylene blue dye exclusion test (Clegg and Smithers, 1968).

Apparatus and recording procedures. Endogenous electrical activity was recorded from schistosomula between 3 and 6 hr after transformation by a modification of the method described by Fetterer et al. (1977). Glass suction electrodes were manufactured from 1.25 mm capillary tubing (W.P. Instruments, New Haven, CT) that was pulled to a fine tip on a horizontal electrode puller (Narishige, Tokyo, Japan). The electrodes were modified by cutting back the tip with a diamond pencil so that the new tip was relatively flat, with an outside diameter of 10-15 μm. The electrode tip was then heated on a microforge (Aloe Corp., NY) which melted the glass from the inside. Heat was applied until the flattened tip of the electrode was smooth and the aperture was reduced to about 2 μm.

Electrodes were firmly attached to the parasite's surface during electrical recordings by way of negative pressure created by a 10 ml syringe (Figure 4). Prior to attachment of the parasite, Hanks' Balanced Salt solution (HBS) contained in a separate vial was drawn into the electrode until it made contact with a silver wire which led to an amplifier (Model P-15, Grass Instruments, Quincy, MA). Electrical signals from the schistosomula were filtered with the low pass set

Figure 4. Schematic of apparatus used for recording endogenous electrical transients from the surface of schistosomes. Volume conducted potentials were detected by way of a microforged glass suction electrode constructed from 1.25 mM capillary tubing. Electrical signals were filtered at the first amplifier with the low pass set at 0.3 Hz and the high pass set at 1 KHz. Signals were displayed on an oscilloscope or a chart recorder and passed onto an analog-to-digital converter prior to computer analysis.

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Pg. 37

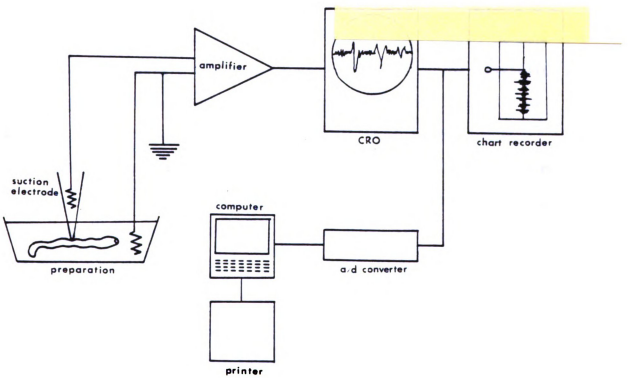


Figure 4

at 0.3 Hz and the high pass at 1 KHz, displayed on an oscilloscope (Model 5113, Tektronix Inc., Beaverton, OR) or a chart recorder (Narco-Biosystems Inc., Houston, TX), and passed onto an analog-to-digital converter prior to computer analysis (Alis I-10 Data Acquisition and Control System, Ecotech, Inc., Lansing, MI).

After electrode attachment, a 2 min equilibration period was allowed prior to electrical recording. During this time the parasite was observed and its level of motility recorded as being above control level, (+); control level, (0); below control level, (-); or nomotile (=). The recorded electrical activity was quantified by counting all negative field potentials in excess of 20 μV over six 10-sec intervals. Data from these intervals were then averaged. Potentials under 20 μV were not counted as they were within the noise range of the system. Electrical responses were measured in at least 6 control parasites and 6 parasites exposed to each experimental substance. Statistical analysis for significance of differences between means used Student's t-test non-paired.

Experimental treatments. Praziquantel (Bayer AG), oltipraz (Rhone-Poulenc) and cytochalasin B (Sigma) were initially dissolved in dimethylsulfoxide (DMSO, Mallinckrodt) at such concentrations that the final incubation media never contained more than 0.1% DMSO. An appropriate amount of DMSO was placed in all controls. Concanavalin A (Pharmacia), potassium antimony tartrate (KSB, kindly supplied by Dr. E. Bueding, Johns Hopkins University, Baltimore, MD), aminophylline, carbachol, 2,4-dinitrophenol (2,4-DNP), dopamine (DA), eserine, 5-hydroxytryptamine (5HT), linolenic acid, ouabain, sodium pentobarbital

(Na-PB), poly-l-argninine, poly-l-lysine, potassium cyanide (KCN) and puromycin (all from Sigma) were dissolved in double-distilled water. In all experiments RPMI-1640 alone, or with an appropriate amount of DMSO, served as the control medium. Most drugs were tested in the range of concentrations demonstrated to be most effective in altering endogenous electrical activity in adult parasites. The incubation period, in most cases, was 1 hr; exceptions included 5HT, linolenic acid (15 min), cytochalasin B, concanavalin A (3 hr) and puromycin (6 hr).

In altered ion experiments, HBS served as the control medium. Elevated potassium HBS was made by replacing NaCl with KCl to bring the final potassium concentration to 60 mM. Ca^{++}-free HBS was made by eliminating $CaCl_2$ from the solution and adding 0.5 mM ethyleneglycol-bis(β-aminoethylester), N,N'-tetraacetic acid (EGTA, Sigma). Parasites were individually pipetted into HBS or altered ion media 15 min prior to recording.

Monoclonal antischistosomulum antibody (kindly supplied by Dr. Michael Phillips, University of Pennsylvania) was prepared as described by Zodda and Phillips (1982). The monoclone was added to a solution also containing fresh guinea pig complement and a 50:50 mixture of RPMI-1640 plus heat inactivated horse serum (RPMI/HS) in a volume ratio of 1:3:30. Motile MS and SS were individually pipetted into vials containing the monoclonal solution 1 hr after the completion of transformation and incubated at 37°C for a period of 24 hr prior to testing. Controls consisted of schistosomula incubated for a similar period in solutions containing: (a) RPMI/HS alone, (b) RPMI/HS plus fresh

complement, or (c) RPMI/HS plus monoclonal antibody. Incubation media also contained 100 units/ml penicillin and 100 μg/ml streptomycin.

Results

Characteristics of spontaneous electrical activity. Endogenous electrical activity recorded from the surface of schistosomula of S. mansoni consisted of multi-phasic potentials ranging from 20-200 μV in amplitude and 10-300 msec in duration. Under control conditions (in RPMI-1640, 37°C) these potentials occurred at a rate of 31.5±1.3/sec for MS and 31.4±1.6/sec for SS with an average amplitude of 35.8±1.4 μV and 34.3±0.7 μV, respectively. Rise times for potentials in both MS and SS were on the order of 3-8 μV/msec. There was an approximately exponential decrease in the frequency of potentials with increasing amplitude (Figure 5). All features of electrical activity recorded from mechanical and skin transformed schistosomula under control conditions were essentially identical.

After a brief (1-2 min) episode of enhanced activity which normally followed electrode attachment, the frequency of low amplitude potentials was relatively constant over a period of up to 30 min of recording in both MS and SS. Large amplitude potentials (>50 μV) occurred more sporadically and appeared to correlate with gross peristaltic-like contractions of the parasite, suggesting that the origin of these transients may be underlying muscle bundles.

To verify that the electrical activity originated from within the parasite, controls were conducted by recording from worms before and after the addition of 50% ethanol to the recording medium. This treatment immediately immobilized the parasites and eliminated nearly all

Figure 5. Electrical activity recorded from the surface of mechanically-transformed 3 hr schistosomula under control conditions and after the addition of 50% ethyl alcohol to the recording medium. Upper: slow speed chart recording showing decline in electrical activity after alcohol exposure. Middle right: high speed oscilloscope traces obtained from the same parasite before (upper) and after (lower) exposure to alcohol. Note the elimination of all large amplitude multiphasic potentials. Lower: computer-generated histogram showing the frequency distribution of endogenous electrical transients of various amplitudes. Open bars represent electrical activity recorded from schistosomula incubated in HS/RPMI; hatched bars represent electrical activity recorded from the same parasites 1 min after the addition of alcohol. Vertical lines are $\pm$ 1 SE; N=8.

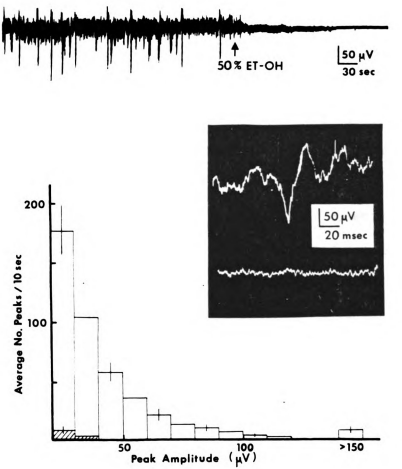

50 % ET-OH
50 μV
30 sec
50 μV
20 msec
200
100
Average No. Peaks / 10 sec
50
100
>150
Peak Amplitude (μV)

Figure 5

electrical transients (Figure 5). No distinct regional variation in the frequency or amplitude of electrical activity was detected. The level of electrical activity was similar for schistosomula incubated in RPMI-1640 alone or in a 1:1 mixture of RPMI-1640 plus heat-inactivated horse serum. After a 15 min incubation in HBS, however, the frequency of activity was reduced by about 25%, while the average amplitude of potentials was unchanged.

Ion substitution experiments. Recordings obtained from schistosomula incubated in HBS containing 60 mM potassium or 0 mM calcium plus 0.5 mM EGTA indicate that both ions are important for generation of the electrical transients recorded. The frequency of potentials recorded after 15 min in 0 mM Ca^{++}-HBS plus 0.5 mM EGTA was reduced by 63% in MS and 53% in SS. The average amplitude of potentials was also reduced by this treatment; 25% in MS and 27% in SS. The frequency of potentials was also reduced in parasites exposed to elevated K^{+}; 21% in MS and 49% in SS; average potential amplitude, however, was unaffected in both groups. Motility in the schistosomulum appeared to be unaffected by exposure to 60 mM K^{+}-HBS, while the absence of Ca^{++} in the medium resulted in a gradual reduction in motility and a significant decrease in the frequency of large (>50 μV) amplitude potentials in both MS, down 75% and SS, down 94%.

Drug responses. A number of substances tested exerted significant effects on the motility and electrical activity of the schistosomulum (Table 1). MS and SS responded qualitatively in a similar fashion to all drugs which exerted significant effects on motility and endogenous electrical activity. Treatments producing highly significant ($p<.01$) changes in schistosomulum activity were: $1x10^{-5}$M

TABLE 1

Changes in Electrical Activity and Motility in Mechanical- and Skin-Transformed Schistosomula Brought About by Experimental Substances

Experimental Treatment	Concentration Tested	Time of Exposure	Change from Control Levels: % Electrical Activity; (Motility)*	
			Mechanically Transformed	Skin Transformed
Ethanol	50%	2 min	-99%[a] (=)	-100%[a] (=)
Aminophylline	$1x10^{-3}M$	1 hr	+10% (0)	-4% (0)
Antimony Tartrate	$1x10^{-5}M$	1 hr	-83%[a] (=)	-93%[a] (=)
Carbachol	$1x10^{-4}M$	1 hr	-44%[b] (0)	-53%[b] (-)
Concanavalin A	100 µg/ml	3 hr	+9% (0)	-1% (0)
Cytochalasin B	$1x10^{-5}M$	3 hr	-14% (0)	+16% (0)
Cyanide	$1x10^{-3}M$	1 hr	-79%[b] (-)	-82%[a] (-)
2,4-Dinitrophenol	$1x10^{-4}M$	1 hr	-43%[b] (-)	-52%[b] (-)
Dopamine	$1x10^{-4}M$	1 hr	+5% (0)	-2% (0)
Eserine	$1x10^{-6}M$	1 hr	-89%[a] (=)	-93%[a] (=)
Linolenic Acid	$3x10^{-5}M$	15 min	+9% (0)	+13% (0)
Oltipraz	$3x10^{-5}M$	1 hr	+1% (0)	+8% (0)
Ouabain	$1x10^{-4}M$	1 hr	-62%[b] (-)	-87%[b] (-)
Pentobarbital	$1x10^{-4}M$	1 hr	-14% (0)	-13% (0)

CONTINUED ON NEXT PAGE....

TABLE 1 (continued)

Experimental Treatment	Concentration Tested	Time of Exposure	Change from Control Levels: % Electrical Activity; (Motility)*	
			Mechanically Transformed	Skin Transformed
Poly-L-Arginine	$1x10^{-6}M$	1 hr	N.D.	-85%[a] (=)
Poly-L-Lysine	$1x10^{-6}M$	1 hr	-91%[a] (=)	-89%[a] (=)
Praziquantel	$1x10^{-6}M$	1 hr	-64%[b] (-)	-46%[b] (-)
Puromycin	200 µg/ml	6 hr	-2% (0)	-13% (0)
Serotonin (5HT)	$1x10^{-5}M$	15 min	+11% (+)	+27%[b] (+)
0 Ca^{++}-HBS	---	15 min	-63%[b] (-)	-45%[b] (-)
60 mM K^{+}-HBS	---	15 min	-21% (0)	-49%[b] (0)
Complement + Antibody	3:1:30 (v/v)	24 hr	-77%[a] (-)	-65%[a] (-)
Complement (alone)	1:30 (v/v)	24 hr	+2% (0)	+15% (0)
Antibody (alone)	1:30 (v/v)	24 hr	-25% (0)	-18% (0)

[a] $p<0.01$

[b] $0.01<p<0.05$

*Motility level in parentheses: (+) above control level; (0) control levels; (-) below control level; (=) nonmotile.

potassium antimony tartrate, an inhibitor of parasite phosphofructokinase; $1x10^{-6}M$ eserine, an anticholinesterase; $1x10^{-6}M$ poly-l-arginine and $1x10^{-6}M$ poly-l-lysine, compounds similar to the cationic proteins of eosinophils; and $1x10^{-3}M$ potassium cyanide, an electron transport blocker. These substances, which probably act at a number of different sites in the parasite, reduced motility and eliminated 80% or more of the electrical potentials recorded. These drugs exert similar effects on adult male S. mansoni (Semeyn et al., 1982), except for KCN, which does not affect motility or electrical activity in adults when tested under similar conditions, i.e., after a 1 hr incubation in $1x10^{-3}M$ KCN (D. Thompson, personal observation).

Recent investigations by Moser et al. (1980) and McLaren and Terry (1982) have demonstrated that eosinophils attach and degranulate onto skin stage and lung stage parasites in identical numbers, yet kill only the former. To investigate the possibility that lung stage parasites might be refractory to the lytic action of major basic protein of eosinohpils, electrophysiological recordings were obtained from 6 day lung schistosomula exposed to various concentrations of poly-l-arginine, a compound that mimics the action of major basic protein contained in eosinophil granules. We observed a rapid, dose-dependent inhibition of electrical activity and motility induced by this substance in lung stage parasites similar to that recorded in freshly transformed MS and SS.

Several drugs tested which have inhibitory effects on electrical activity recorded in adult S. mansoni (Semeyn et al., 1982) did not affect motility or electrical activity in the freshly transformed

schistosomulum. These included dopamine ($1x10^{-4}M$) and pentobarbital sodium ($1x10^{-3}M$), drugs believed to act by altering ionic conductances in other nerve or muscle preparations. A putative excitatory neurotransmitter in schistosomes, 5HT (Bennett et al., 1969), while stimulating motility in both groups of freshly transformed parasites, significantly increased the total number of potentials recorded after a 15 min incubation at $1x10^{-5}M$ only in the SS. The drug did, however, increase the frequency of large (>50 μV) potentials ($0.01<p<.05$) in both groups. The apparent lack of a significant increase in the frequency of low amplitude potentials recorded in the MS exposed to 5HT may be an artifact introduced by our method of counting the potentials. That is, large potentials with comparatively long time courses may obscure many of the smaller, high frequency potentials.

Concanavalin A, which binds to the surface of schistosomula (Murrell et al., 1978) and may impart some degree of protection from the lethal effects of antibody plus complement (Van Pijkeren et al., 1982) did not affect electrical activity or motility in either MS or SS after a 3 hr incubation in 100 μg/ml. Incubation for 3 hr in $1x10^{-5}M$ cytochalasin B, which interferes with microfilament assembly and secretory function in adult schistosomes (Wilson and Barnes, 1974), also failed to affect electrical activity or motility in the freshly transformed schistosomulum. Puromycin, an inhibitor of protein synthesis in schistosomes (Kusel, 1972) did not alter motility or electrical activity after a 6 hr incubation in 200 μg/ml of the drug.

Both ouabain, a Na,K-ATPase inhibitor and 2,4-DNP, which blocks oxidative phosphorylation, reduced motility and electrical activity in

MS and SS after 1 hr incubations in $1x10^{-4}M$ of the drugs. These drugs exert similar effects on the adult parasite (Fetterer et al., 1980; Fetterer and Pax, 1981). However, aminophylline, a phosphodiesterase inhibitor which slowly increases longitudinal muscle tension in adult S. mansoni (D. Thompson, personal observation), affected neither motility nor electrical activity in the schistosomulum after a 1 hr incubation at $1x10^{-3}M$.

Linolenic acid, a potent stimulant for cercarial penetration in vitro (Haas and Schmitt, 1982) was without effect on motility or electrical activity in the freshly transformed schistosomulum when tested at $3x10^{-5}M$, an effective concentration for inducing cercarial penetration of agar plates.

Praziquantel, an important antischistosomal compound which causes rapid spastic paralysis and depolarization of tegument and muscle in adult schistosomes (Fetterer et al., 1980) as well as a reduction in electrical activity (D. Thompson, personal observation) produced a gradual reduction in motility and electrical activity in freshly transformed schistosomula. After a 1 hr incubation in $1x10^{-6}M$ praziquantel, total electrical activity was reduced by about 50% and motility was almost completely eliminated. Oltipraz, another important antischistosomal agent, did not affect motility or electrical activity in either MS or SS after a 1 hr incubation in concentrations as high as $3x10^{-5}M$, even though similar exposure results in a significant reduction in the electrical activity recorded from adult schistosomes (D. Semeyn and D. Morrison, personal communication).

Responses to antischistosomulum antibody. Freshly transformed schistosomula incubated for 24 hr in the presence of a monoclonal antischistosomulum antibody plus complement exhibited electrophysiological and motility responses which indicated that most worms were significantly affected by this treatment. In the MS group, 80% of the parasites tested were essentially nonmotile after a 24 hr incubation in solution containing the monoclonal antibody plus fresh complement. Electrical recordings obtained from this group indicated a 77% reduction in total electrical activity, while the average potential amplitude dropped by 30%. Results from the SS group were similar; 70% were nonmotile and the parasites exhibited a 65% reduction in total electrical activity while the average amplitude of potentials dropped 35%. Electrical activity and motility of MS and SS incubated in media containing either fresh complement or the monoclonal, but not both, were similar to RPMI/HS controls. Parasites preincubated 24 hr in control medium alone prior to addition of the monoclone and complement exhibited the same level of motility and electrical activity after 24 hr as schistosomula incubated 48 hr in the control medium alone.

Discussion

The electrical potentials recorded from the surface of immature S. mansoni are qualitatively similar to those recorded from the adult parasite (Fetterer et al., 1977; Semeyn et al., 1982). The precise origin of electrical potentials recorded in this study has not been determined. Previous studies have demonstrated that both the tegument and underlying muscle fibers in adult schistosomes are electrical

syncytia, and that these two tissue compartments are connected by low resistance pathways (Thompson et al., 1982a). Active membrane responses can be initiated by injecting positive current through microelectrodes into either tegument or muscle. These active responses spread with little decrement from tegument to muscle and vice versa (Thompson et al., 1982a). Therefore, electrical potentials recorded from the surface of schistosomula could originate in muscle bundles beneath the tegument, and spread by way of low resistance pathways to the surface. Alternatively, they could originate in the tegument itself, or in nerve elements within the parasite. The wide range of potential amplitudes and rise times recorded suggests that they may arise from more than one source.

Previous studies have demonstrated that measurement of surface electrical potentials provides a sensitive assay for agents affecting the neuromuscular system of adult schistosomes (Fetterer et al., 1977; Semeyn et al., 1982; Mellin et al., 1983). By this technique, the inhibitory effects of carbachol and dopamine on the schistosome can be detected at concentrations as low as $1x10^{-8}M$, while the stimulatory effects of 5HT are detectable at $1x10^{-7}M$ (Semeyn et al., 1982). These drug concentrations are 100X lower than those required to measure detectable changes by previously used techniques which depend on visual observation (Tomosky et al., 1974), ultrasound (Brown et al., 1973) or photoelectric effects (Hillman and Senft, 1973).

In the present study, measurements of endogenous electrical activity provided a sensitive assay for the effects of a wide range of substances on mechanical- and skin-transformed schistosomula. A highly significant reduction in the frequency and mean amplitude of electrical

potentials was recorded from SS incubated for 1 hr in $1x10^{-6}$M poly-l-arginine. Butterworth et al. (1979), using release of ^{51}Cr as an assay for damage to schistosomula, were able to detect significant changes only after an 18 hr incubation in $1x10^{-5}$M poly-l-arginine. Furthermore, the release of ^{51}Cr, long used as any assay for drug- or immune substance-induced damage to schistosomula, has proven to be unreliable, as human neutrophils induce a pronounced release of the element without killing the parasites (Glauert and Butterworth, 1977). Other in vitro assays for detecting damage to the schistosomula in response to short-term exposure to various substances, such as membrane disruption at the electron microscope level or motility changes alone are neither objective nor quantifiable. They may also require a number of time-consuming preparatory steps, as is the case for electron microscopy, and generally depend on exposure to the substances being tested for periods significantly longer than the periods required for detecting electrophysiological changes.

Results of this study indicate that schistosomula transformed from cercariae by in vitro mechanical techniques or by in vivo skin penetration exhibit electrical activity which is indistinguishable under control conditions. The frequency of electrical potentials, their distribution over various amplitudes, patterns of occurrence and waveforms were essentially identical for MS and SS in control medium. In response to the wide range of experimental substances tested, every treatment that induced a significant change in the number of transients recorded from MS had a qualitatively similar effect on SS. The lack of a significant difference between MS and SS in the reduction of electrical activity brought about by antischistosomulum antibody plus

complement supports the notion that inherent processes mediate the parasite's response to this treatment. This notion is further supported by the observation that both groups become refractory to this treatment if they are preincubated in control medium 24 hr before exposure to antischistosomulum antibody plus complement. That is, in terms of the parameters measured in this type of in vitro assay after short-term exposures, the presence of host molecules on the surface of the parasite does not appear to be an essential element in the development of refractoriness to the effects of antibody plus complement. These results support the notion that schistosomula derived from cercariae by mechanical techniques are, in fact, appropriate material for immunochemical and physiological studies. Our results do not rule out the possibility, however, that schistosomula transformed by alternative techniques may respond differently to immune effectors or other treatments. Bickel and Ford (1982), for instance, found that schistosomula transformed by the isolated skin preparation differed from those isolated in vitro in terms of surface antigenicity and susceptibility to antibody-dependent, eosinophil-mediated killing.

Of the substances tested, other than antibody plus complement, only cyanide exerted a greater effect on the freshly transformed schistosomulum than it does on the adult parasite, where this compound is totally ineffectual in reducing electrical transients (D. Thompson, personal observation). The activity of cyanide at this early period following transformation may be due to the parasite's continued reliance on electron transport for energy production, which appears, based on electrophysiological evidence, to persist for a period of less

than 12 hr following transformation. That is, surface electrical potentials recorded from 12 hr old MS and SS after a 1 hr incubation in $1x10^{-3}$M KCN are statistically similar to control levels (Thompson et al., 1982b).

A number of substances were less effective in altering electrical activity in the freshly transformed schistosomulum than they are in the adult schistosome. These included carbachol, which reduced the total number of potentials by 40-50% in the schistosomulum as opposed to an 80% reduction in the adult (Semeyn et al., 1982); dopamine, which eliminates all potentials greater than 40 μV in the adult when applied at $1x10^{-5}$M, but had no effect on motility or electrical activity of MS or SS at a concentration 10X as great; and oltipraz, which significantly reduces electrical activity in the adult, but is without effect on the schistosomulum.

Age-related differences in schistosome drug sensitivity could depend on developmental changes in membrane permeability, receptor proteins or metabolism. The reorganization of tegumental intramembraneous particles (IMP) which occurs during early development (McLaren et al., 1978) could account for the differential responsiveness to some drugs. That is, if some macromolecules embedded in the outer tegumental membrane could serve as receptors or carriers for certain drugs, the lack of responsiveness to those drugs in freshly transformed schistosomula could simply be a function of the low IMP density observed during that period. Alternatively, responses in the immature parasites could be influenced by aerobic enzymes or metabolites which may persist for some time after transformation.

Because the schistosome is well adapted for avoiding host immune responses under normal conditions, it is likely that future vaccines, to be successful, may depend on the synergistic effects of chemotherapeutics. That is, it may be necessary to disrupt the normal processes schistosomes use to evade immune effectors before the advantages of a vaccine can be realized. In order to rationally approach the development of vaccine-enhancing drugs or other chemoprophylactics for schistosomiasis, additional knowledge of basic physiological processes in the immature schistosome will be required.

SECTION II

Changes in Glucose Metabolism and Cyanide-Sensitivity in Schistosoma mansoni During Development

Summary

Early post-transformational and adult Schistosoma mansoni were studied using biochemical and electrophysiological techniques to determine the timecourse of the conversion to a physiologically cyanide-insensitive state, and the extent to which metabolic changes can be detected by electrophysiological analysis. Volume conducted electrical potentials recorded from the parasites indicate that 3 hr post-transformational schistosomula are highly cyanide-sensitive, as a 1 hr incubation in 1 mM cyanide eliminated 70-80% of the activity recorded from that group. Evolution of CO_2 in the 3 hr schistosomulum was reduced by 85% in the presence of 1 mM cyanide. By 24 hr after transformation, evolution of CO_2 under control conditions was reduced by 77% from 3 hr levels, while lactic acid excretion rose by 84%. Incubation of the 24 hr schistosomulum in cyanide no longer affected the frequency or magnitude of endogenous electrical transients, but did eliminate 83% of the already reduced levels of CO_2 produced. Electrophysiological analyses indicate that the timecourse of metabolic changes in skin- and mechanically-transformed schistosomula are similar, and incubation of schistosomula in 200 $\mu g \cdot ml^{-1}$ puromycin does not alter the onset of

cyanide-insensitivity. The adult parasite evolved a low level of CO_2 which was reduced by 88% in the presence of 1 mM cyanide. No significant Pasteur effect was detected, however, and endogenous electrical activity as well as mechanical responses of the adult musculature were unaffected by cyanide exposure. Our results indicate that schistosomula continue to rely on cyanide-sensitive respiratory components for at least 3 hr after transformation; by 24 hr, however, the parasites are metabolically similar to the adult stage, i.e. they depend on lactate fermentation for most of their energy requirements.

Introduction

Fermentation of glucose to lactate in the adult trematode, Schistosoma mansoni occurs both under aerobic and anaerobic conditions. Glycolysis is the principal source of energy and no Pasteur effect is observed (Schiller et al., 1975; Bueding et al., 1982; Bueding, 1950). During the cercarial stage of the parasite's life cycle, however, most energy is derived by oxidative metabolism (Oliver et al., 1953; Coles, 1972). Cercariae are killed within 4 hr in the presence of 1 mM cyanide, and addition of glucose (1 $mg \cdot ml^{-1}$) does not prolong their life (Coles, 1972b). However, Coles (1972b) reported that cercariae do exhibit a Pasteur effect under nitrogen, and that lactic acid is excreted under anaerobic conditions only.

Between the aerobic cercaria and anaerobic adult stages of the parasite's life cycle, there is a period of transition during which the recently penetrated schistosomulum undergoes a number of important changes. Morphologically, the parasite loses its tail and develops a

heptalaminate outer tegumental membrane (Hockley et al., 1973). Immunologically, the worm undergoes a rapid, nearly linear decline in susceptibility to rejection by passively transferred immune serum (Sher, 1977), possibly due to surface changes which inhibit recognition and/or binding by host immunoglobulins (Dean, 1977). Most evidence suggests that these morphological and immunological changes occur rapidly and are, for the most part, completed within 24 hr after the parasite enters the host.

The conversion from the aerobic metabolic state of cercariae to the predominantly anaerobic one of adult schistosomes is important because of its possible functional relationship with changes in immuno- and chemosensitivity. Still, the timecourse of this metabolic conversion is not well documented. Previous studies that have indirectly addressed this subject have failed to provide quantitative data correlating metabolic conditions with the physiological status of the parasite. Additionally, most of these studies fail to measure evolved CO_2, the major byproduct of oxidative metabolism, and rely primarily on enzymatic assays which are less sensitive than readily available chromatographic techniques.

In the present study, [^{14}C]glucose is used to study the fate of exogenous glucose in the metabolism of 3 hr and 24 hr schistosomula, as well as adult S. mansoni. $^{14}CO_2$ and [^{14}C]lactic acid produced in vitro by various stages of the parasite during incubations in Warburg vessels are measured under control conditions and in the presence of 1 mM potassium cyanide or 0.1 mM potassium antimony tartrate. An electrophysiological assay is described which provides a sensitive measure for

correlating perturbations in metabolism with a quantifiable physiological parameter of the schistosome.

Materials and Methods

Parasite preparations and incubation media. A Puerto Rican strain of S. mansoni was maintained by passage through Swiss Webster mice and Biomphalaria glabrata snails. Cercariae were collected in dechlorinated water by exposing 100-150 infected snails to light for 1-2 hr. The cercarial suspensions were passed through a wire grid to remove snail feces and other debris. Cercariae were counted under a microscope, and maintained in dechlorinated water at 30°C prior to use. Bacterial contamination in all suspensions of organisms was reduced by using sterilized glassware and by adding 100 units·μl^{-1} penicillin plus 100 $\mu g \cdot ml^{-1}$ streptomycin to media prior to sterile filtration.

Schistosomula, the larval or skin stage parasites, were prepared from the same batch of cercariae by two methods. Mechanically transformed schistosomula were obtained by a modification of the method of Ramalho-Pinto et al. (1974). Cercariae were placed in 15 ml conical centrifuge tubes and cooled to 4°C for 10 min to reduce motility. After centrifugation at low speed for 1 min, the supernatant was decanted and replaced by a cold 50:50 mixture of filtered horse serum and RPMI-1650 (HS/RPMI; both obtained from Gibco, Grand Island, NY). To remove the parasite's tails and thereby initiate transformation to the schistosomulum stage, the packed cercariae were resuspended and stirred for 1 min at a modearte speed on a vortex mixer. After the suspension was centrifuged, the tail-rich supernatant was discarded and the

body-rich pellet resuspended in additional HS/RPMI. This process was repeated 6 times, so that the final suspension of parasites contained fewer than 6% tails or nontransformed cercariae.

Skin penetrated schistosomula were prepared by a modification of the method of Cousin et al. (1981). Approximately 1,000 cercariae in dechlorinated water were pipetted onto the cleaned inner surface of the ears of uninfected, ether-anesthetized Swiss Webster mice and allowed to penetrate for 45 min. The mice were killed by cervical dislocation and any nonpenetrating cercariae were removed by cotton swabs. The ears were removed and finely minced in a 20 ml petri dish containing 5 ml of the HS/RPMI medium. After a 1 hr incubation at 37°C with continuous mechanical agitation, motile schistosomula were individually transferred by pipette into vials containing HS/RPMI and maintained at 37°C. In subsequent experiments, 3 hr schistosomula consisted of parasites transformed from cercariae 3 hr prior to testing; 24 hr schistosomula were transformed and maintained in the HS/RPMI medium 24 hr prior to testing. For both groups, transformation was verified by means of the methylene blue dye exclusion test (Clegg et al., 1968).

To determine the effects of protein synthesis inhibition on the metabolic conversion of the schistosomulum, 200 $\mu g \cdot ml^{-1}$ puromycin (dissolved in distilled water; Sigma Chemical Co.) was added to cercarial preparations 30 min prior to mechanical- and skin-transformation. These parasites were maintained in a similar concentration of puromycin during all subsequent incubations and electrophysiological recordings which were conducted 24 hr after transformation.

Adult parasites were obtained according to the method of Fetterer et al. (1977) by extracting worm pairs from the portal veins of

ervically dislocated mice 55 days after infection. Extracted worm pairs were separated and the males were placed in vials containing HS/RPMI and maintained at 37°C prior to use.

Electrophysiological and mechanical recordings. Endogenous electrical activity was recorded from the parasites by a modification of the method described by Fetterer et al. (1977). Glass suction electrodes were manufactured from 1.25 mm capillary tubing (W.P. Instruments, New Haven, CT) that was pulled to a fine tip on a horizontal electrode puller (Narishige, Tokyo, Japan). The electrodes were modified by cutting back the tip with a diamond pencil so that the new tip was flat, with an outside diameter of 15-30 μm. The electrode tip was then heated on a microforge (Aloe Corp., New York, NY) which melted the glass from the inside. Heat was applied until the flattened tip of the electrode was smooth, and the aperture was reduced to approximately 2 μm for recording from schistosomula, or 15-20 μm for recording from adults. Electrodes were firmly attached to the parasite's surface during electrical recordings by way of negative pressure created by a 10 ml syringe. Prior to attachment of the parasite, Hanks' Balanced Salt solution (HBS) contained in a separate vial was drawn into the electrode until it made contact with a silver wire which led to an amplifier (Model P-15, Grass Instruments, Quincy MA). Electrical signals from the parasites were filtered with the low pass set at 0.3 Hz and the high pass at 1 kHz; displayed on an oscilloscope (Model 5113, Tektronix Inc., Beaverton, OR) or a chart recorder (Narco Biosystems, Inc., Houston, TX), and passed onto an analog-to-digital converter prior to computer analysis (Alis I-10 Data Acquisition and Control System, Ecotech Inc., Lansing, MI).

All recordings of endogenous electrical potentials from parasites exposed to potassium cyanide (CN^-; Sigma Chemical, St. Louis, MO) or potassium antimony tartrate (Sb^{+++}; Fisher Scientific, Fair Lawn, NJ) were obtained either 1-2 hr or 24-28 hr after the parasites were preincubated in the presence of the drug. Parasites isolated identically, but incubated in the presence of vehicle served as controls.

After electrode attachment, a 2-4 min equilibration period was allowed prior to electrical recording. Electrical activity was quantified by counting all negative field potentials in excess of 20 μV over six 10-sec intervals. Data from these intervals were then averaged. Potentials under 20 μV were not counted as they were within the noise range of the system. Electrical responses were measured in at least 8 control parasites and 8 parasites exposed to each experimental substance. Statistical analysis for significance of difference between means used Student's non-paired t-test.

Muscle tension recordings were obtained from adult schistosomes using the method described by Fetterer et al. (1977). The effects of 1 mM CN^- and 0.1 mM Sb^{+++} on the energy-dependent muscle tension changes induced by exposure to elevated potassium (60 mM; increases muscle tension in schistosomes) or the acetylcholine analog, carbachol (0.1 mM; reduces muscle tension in schistosomes) were assessed by preincubating the parasites for 1-2 hr or 24-28 hr in CN^-- or Sb^{+++}-containing media prior to exposure to the tension-altering treatments. Mechanical responses were measured in at least 5 control parasites and 5 parasites preincubated in drug.

Incubation in [^{14}C]glucose-containing media and analysis of metabolites. In biochemical experiments involving immature stages, parasites were concentrated to 12,000/ml in HS/RPMI containing antibiotics. Only mechanically-transformed schistosomula were used in biochemical studies, since the skin-transformed parasites were difficult to obtain in sufficient quantities. Aliquots of 400 µl (containing 5,000 schistosomula) were pipetted into the center wells of 15 ml Warburg reaction vessels. Drug-treated parasites were concentrated in incubation media containing 1 mM CN^- or 0.1 mM Sb^{+++} 1 hr before testing. In experiments involving adult schistosomes, 6 males were placed into the Warburg vessels along with 400 µl HS/RPMI. Heat-killed controls of immatures and adults were preincubated for 15 min at 56°C immediately prior to testing.

After placement of parasites, uniformly labelled D-[^{14}C]glucose (1.4 µCi, specific activity = 348.2 $Ci \cdot mol^{-1}$; New England Nuclear, Boston, MA) was added to the center well of each vessel. To capture evolved $^{14}CO_2$, 1 ml of 1 N sodium hydroxide (NaOH), freshly prepared in boiled distilled water, was added to the base of the Warburg vessels. The vessels were then placed on a shaker bath and maintained at 37°C for a period of 4 hr. Following incubation, the vessels were cooled on ice for 15 min prior to sample collection. For measuring evolved $^{14}CO_2$, three 200 µl aliquots of the 1 N NaOH were pipetted from the base of each vessel into scintillation vials containing 50 µl glacial acetic acid and 6 ml aqueous scintillant (Amersham Co., Arlington Heights, IL), and counted on a Beckman LS-7,000 liquid scintillation system (Beckman Instruments, Fullerton, CA).

Acid-soluble metabolic endproducts were fractionated with a modification of the method of Tielens et al. (1981). A 350 µl sample of incubation medium was recovered from the center well and 20 µl cold 71% perchloric acid was added. The samples were centrifuged at 1,000 g for 15 min and radioactivity was determined in the supernatant by scintillation counting. Fifty µl samples of the supernatant were loaded onto BioRad AG 1-X10, 100-200 mesh anion-exchange columns (12 x 0.75 cm; BioRad Labs, Los Angeles, CA) in chloride form using 25 mM Hepes, pH=11.0 as the primary solvent. The columns were eluted with 10 ml of 25 mM Hepes, pH=11.0 (F1), followed by 5 ml of 5 mM HCl (F2), and 5 ml of 500 mM HCl (F3). Over 99% of the radioactivity applied was recovered. Five hundred µl aliquots of the eluted material were placed into vials containing 10 ml aqueous scintillant for counting. Results were converted from disintegrations per minute (dpm, counts per minute divided by efficiency of counting) to nmol CO_2 or lactic acid produced. Labelled substrates from the column fractions were identified by descending paper chromatography, with butanol/water/acetic acid (25:10:4) as developing solvent. In control and CN^- experiments, only 10-12% of the glucose was degraded over the 4 hr incubation periods. Over 90% of the acid-soluble fraction co-migrated with and exhibited the expected retention factor of L-lactic acid. Total protein was determined by the method of Albro (1975) using bovine serum albumin standards. All tests were run in triplicate on at least 3 separate occasions ($N \geq 9$), except studies involving Sb^{+++} ($N \geq 4$). Variation among triplicate tests was usually less than 15%. Statistical analysis of these results were based on comparison of dpm per mg protein for each sample, using Student's non-paired t-test.

Results

Characteristics of volume conducted electrical potentials. Endogenous electrical activity recorded from the surface of schistosomula and adult S. mansoni consisted of multiphasic potentials ranging from 20-200 μV in amplitude and 10-300 msec in duration (Figure 5). Under control conditions these potentials occurred at a rate of 30-40·sec^{-1} and exhibited rise times of 3-8 μV·msec^{-1}. There was an exponential decrease in the frequency of potentials with increasing amplitude. Characteristics of electrical activity recorded from all stages of development were essentially identical under control conditions, although the adult parasite showed some regional variation in the frequency of potentials.

After a brief (1-3 min) episode of enhanced activity that normally followed electrode attachment, the frequency of low amplitude potentials was relatively constant over a period of up to 30 min of recording. Large amplitude potentials (>50 μV) occurred more sporadically and appeared to correlate with peristaltic-like contractions of the parasite, suggesting that these transients may originate in underlying muscle bundles. To verify that the electrical activity originated from within the parasite, controls were conducted by recording from worms before and after addition of 0.5 ml 50% ethanol to the recording medium. This treatment immediately immobilized the parasites and eliminated nearly all electrical transients (Figure 5).

Developmental changes in substrate utilization. The results presented in Figure 6 demonstrate that the schistosome undergoes a pronounced metabolic alteration during development from the 3 hr schistosomulum to the adult stage. Most of this change appears to be complete

Figure 6. Developmental changes in S. mansoni in the distribution of metabolic endproducts after 4 hr incubations in D-[^{14}C]glucose-containing media under control conditions. Values presented are means of at least 3 separate experiments performed in triplicate ($N \geq 9$); vertical lines are 1 SE.

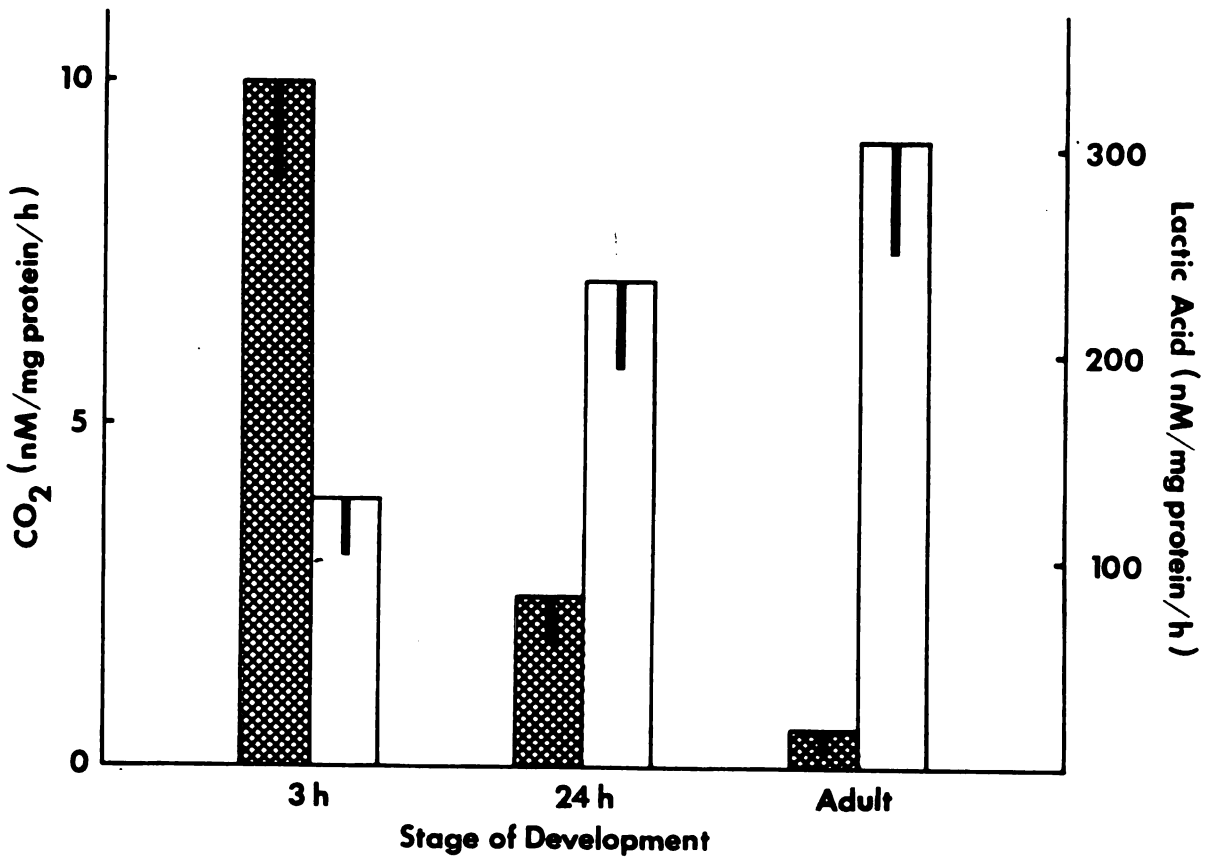

10
5
0
CO_2 (nM/mg protein/h)
300
200
100
Lactic Acid (nM/mg protein/h)
3 h
24 h
Adult
Stage of Development

Figure 6

by 24 hr after transformation, at which point CO_2 evolution is reduced by 77% from 3 hr levels, while lactic acid excretion nearly doubles, to a level only 18% less than that measured in the adult.

Developmental changes in physiological responses to metabolic inhibitors. Electrophysiological recordings indicate that schistosomes undergo a significant development alteration in responsiveness to CN^-, while their response to Sb^{+++} is consistent throughout the course of development (Figure 7). The frequency and mean amplitude of endogenous electrical transients recorded from 3 hr mechanically- and skin-derived schistosomula were significantly depressed after a 1 hr incubation in 1 mM CN^-. The degree of CN^--induced depression was essentially identical to that induced by a 1 hr incubation in 0.1 mM Sb^{+++}, indicating that the 3 hr post-transformational stage, may rely on a CN^--sensitive terminal oxidase for generating energy to sustain membrane electrical events.

Electrical recordings obtained from 24 hr mechanically- and skin-derived schistosomula exposed to 1 mM CN^- indicate that inhibition of the terminal oxidase no longer results in a significant depression of the spontaneous potentials. Since electrical activity in both groups was unaffected by 1 mM CN^-, it appears that the method of transformation does not significantly alter this conversion process. Additionally, based on electrophysiological responses, the expression of CN^--insensitivity by 24 hr is not affected by the presence of 200 $\mu g \cdot ml^{-1}$ puromycin, indicating that new protein synthesis is not required for this conversion.

Figure 7. Frequency of endogenous electrical potentials recorded from developmental stages of S. mansoni under control conditions (hatched bars) or after 1 hr incubations in media containing 10^{-3}M cyanide (open bars) or 10^{-4}M antimony (shaded bars). Values obtained from skin- and mechanically-transformed (shown here) schistosomula were similar. The frequency of potentials recorded from 24 hr schistosomula incubated in 200 µg/ml purmoycin (P) was similar to control levels. Adult parasites incubated 24 hr in 10^{-3}M cyanide (X) exhibited control levels of electrical potentials. Values presented are mean number of potentials recorded over a 10-sec interval. Vertical lines are 1 SE; $N \geq 8$.

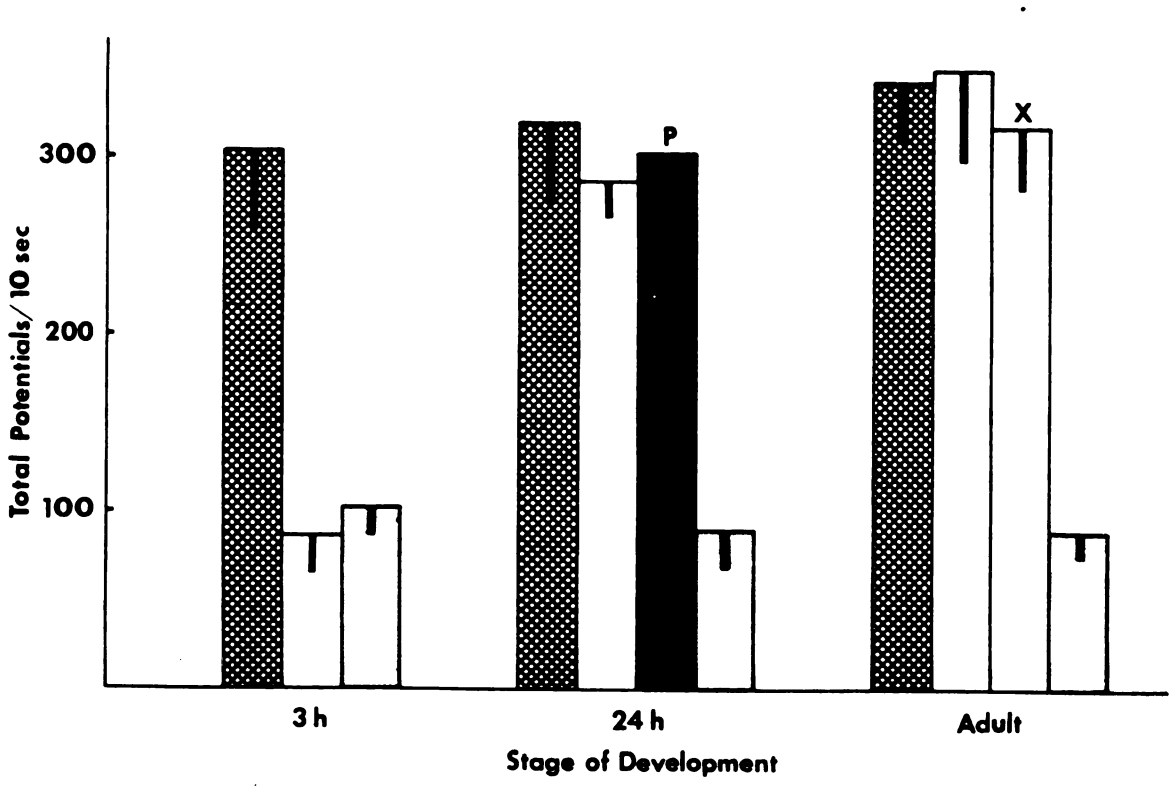

Total Potentials/10 sec
300
200
100
P
X
3 h
24 h
Adult
Stage of Development

Figure 7

The adult parasite, like the 24 hr schistosomulum, exhibited no change in electrical activity in response to 1 mM CN^-, even after 24 hr incubations. Inhibition of glycolysis by 0.1 mM Sb^{+++}, however, eliminated 80% of the potentials within 1 hr, and by 24 hr the electrical activity was totally eliminated.

Muscle tension recordings obtained from adult schistosomes indicate that energy obtained from respiration is not essential for muscle function in the mature organism. Introducing medium containing 60 mM potassium to the recording chamber resulted in a rapid increase in longitudinal muscle tension, which occurred at approximately the same rate and was similarly sustained in parasites preincubated in control medium or in medium containing 1 mM CN^- for either 1 hr or 24 hr (Figure 8, upper graph). Similarly unaffected by preincubation in 1 mM CN^- was the relaxation of longitudinal muscle induced by exposure to 0.1 mM carbachol (Figure 8, lower graph). These mechanical responses were significantly diminished, however, when adult parasites were preincubated 1 hr in medium containing 0.1 mM Sb^{+++}.

Effects of metabolic inhibitors on substrate utilization. Results shown in Figure 9 illustrate the effects of metabolic inhibitors on patterns of substrate utilization in various stages of the parasite. CO_2 production was approximately equally inhibited by 1 mM CN^- and 0.1 mM Sb^{+++} at all stages tested (upper graph). Lactic acid excretion, however, was significantly inhibited only by exposure to Sb^{+++}, which depressed levels by 80-90% at each stage of development (lower graph). No significant CN^--induced Pasteur effect was detected in any stages of *S. mansoni* tested.

Figure 8. Effects of incubation for 1 hr (open circles) or 24 hr (open squares) in 10^{-3}M cyanide, or 1 hr in 10^{-4}M antimony (triangles) on longitudinal muscle tension changes induced by exposure to 60 mM potassium (upper graph) or 10^{-4}M carbachol (lower graph) in adult male S. mansoni. Closed circles represent muscle tension recorded in control parasites exposed to the same tension-altering treatments. For all treatments, $N \geq 5$; vertical lines are 1 SE.

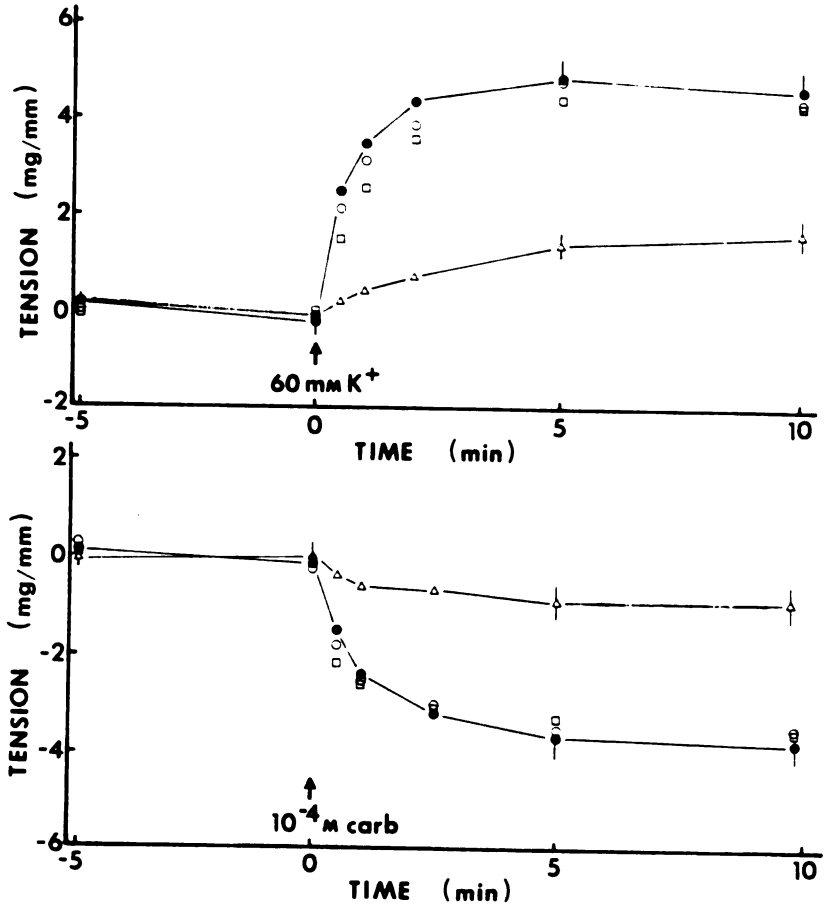

6
4
2
0
-2
TENSION (mg/mm)
60 mM K+
-5
0
5
10
TIME (min)
2
0
-2
-4
-6
TENSION (mg/mm)
10-4 M carb
-5
0
5
10
TIME (min)

Figure 8

Figure 9. Developmental changes in the effects of metabolic inhibition due to 10^{-3}M cyanide (open bars) or 10^{-4}M antimony (shaded bars) on CO_2 (upper graph) or lactic acid (lower graph) produced during 4 hr incubations in D-[^{14}C]glucose-containing media. CO_2 and lactic acid produced by 24 hr schistosomula incubated in 200 μg/ml puromycin (P) were similar to control levels (hatched bars). Values presented are means of at least 3 separate experiments performed in triplicate ($N \geq 9$), except for antimony experiments ($N \geq 4$).

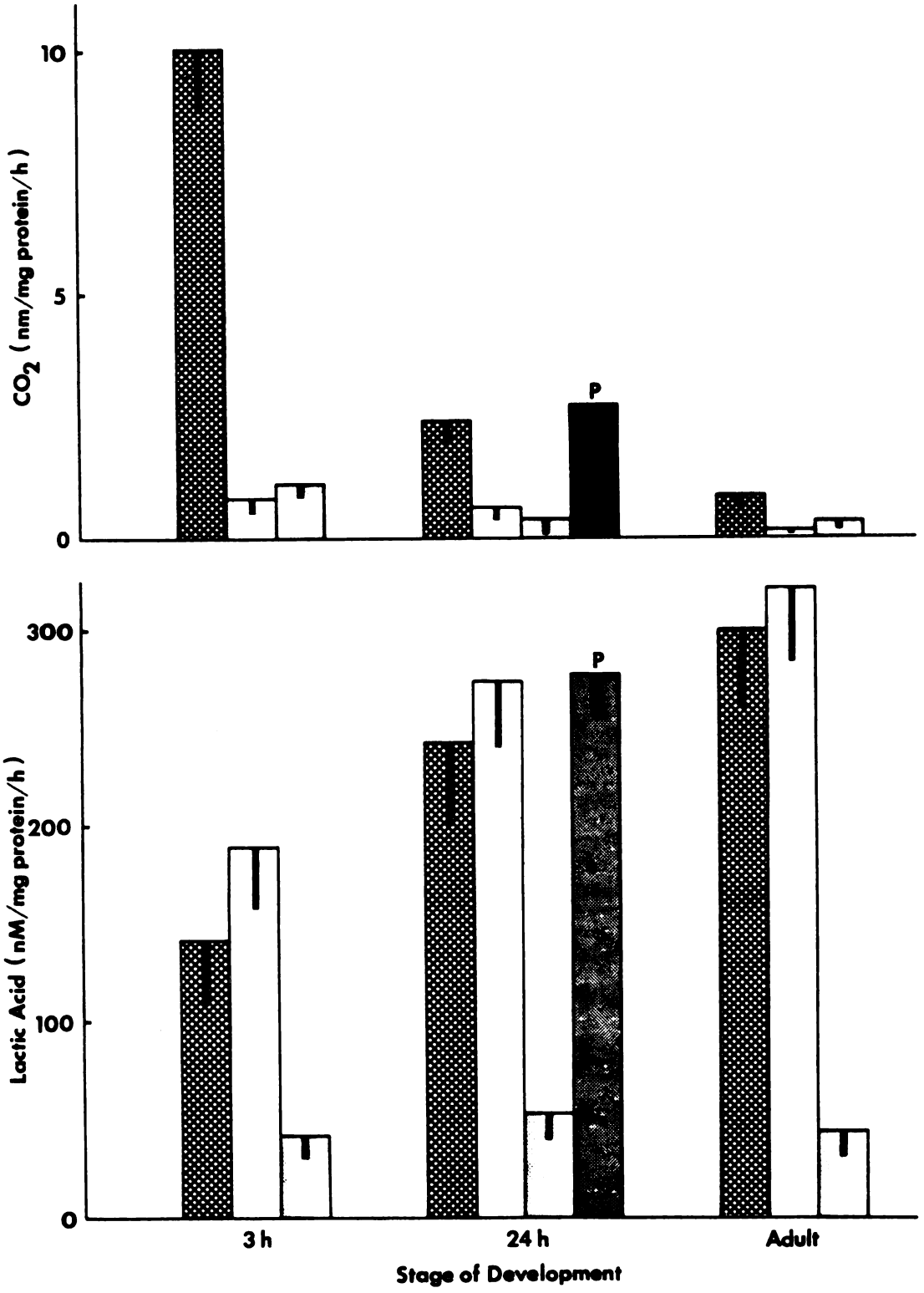

10
5
0
CO_2 (nm/mg protein/h)
P
300
200
100
0
Lactic Acid (nM/mg protein/h)
P
3 h
24 h
Adult
Stage of Development

Figure 9

Consistent with results obtained from electrophysiological recordings, the presence of 200 $\mu g \cdot ml^{-1}$ puromycin throughout the transformation process and incubation period of 24 hr schistosomula did not inhibit their conversion to a predominantly anaerobic metabolic state. This is evidenced by the low levels of CO_2 and high levels of lactic acid produced by these parasites.

Discussion

The timecourse of the conversion from a CN^--sensitive to a CN^--insensitive energy metabolism in S. mansoni was followed by correlating transitions in CO_2 and lactic acid formation with a fundamental physiological property of the parasite. Our results indicate that this metabolic conversion occurs rapidly after transformation of the cercaria to the schistosomulum stage; so that by 24 hr, most energy production in the schistosomulum occurs via lactic acid fermentation.

Previous studies have demonstrated that measurement of endogenous electrical potentials provides a sensitive assay for agents affecting the neuromuscular system of immature and adult schistosomes (Fetterer et al., 1977; Semeyn et al., 1982; Thompson et al., 1982b; Mellin et al., 1983). By this technique the inhibitory effects of carbachol and dopamine on the schistosome can be detected at concentrations as low as 10 nM, while the stimulatory effects of 5HT are detectable at 0.1 μM. These drug concentrations are 100X lower than those required to measure detectable changes by previously used techniques. Our results indicate that a high correlation also exists between endogenous electrical activity and the metabolic state of the parasite. That is, metabolic

inhibition at any stage of development is associated with a significant depression in endogenous electrical activity. Since the transmembrane electrical gradients which give rise to these potentials in schistosomes are maintained, in part, by energy-dependent processes (Fetterer et al., 1977), it is not surprising that they are sensitive to metabolic inhibition.

Based on electrophysiological recordings, 3 hr schistosomula were depressed equally by 1 mM CN^- and 0.1 mM Sb^{+++}. Evolution of CO_2 by the 3 hr schistosomulum was reduced by almost 90% during 4 hr exposures to these treatments. The high degree of CN^--sensitivity obtained in electrophysiological recordings of 3 hr schistosomula is somewhat surprising, in light of findings that this stage of the parasite already excretes nearly 60% as much lactic acid as 24 hr schistosomula under control conditions. Apparently, the energy requirements of the 3 hr stage cannot be met entirely by lactic acid fermentation alone. It is important to note, however, that interstage comparisons of metabolic end-products must be interpreted with extreme caution. That is, these measurements may also be a function of significant morphological and/or permeability differences between the immature and adult stages, which could not be adequately controlled in the present study.

A number of studies suggest that the in vivo skin penetration process effects changes in schistosomula which render them less susceptible than mechanically-derived parasites to the harmful effects of antibody-mediated mechanisms of immunity (Van Pijkeren et al., 1982; McLaren et al., 1978). During the skin penetration process, the cercaria's tail is shed and the contents of its preacetabular glands

secreted. This loss accounts for 35-50% of the parasite's total nitrogen content (Coles, 1972b). In the present study, 30% of the total protein per 5,000 cercariae was lost during the mechanical transformation process. An additional 17% was lost between the 3 hr and 24 hr stages, presumably due to delayed secretion of preacetabular gland contents and/or sloughing off of residual cercarial surface glycoproteins. It is not presently known what effects residual preacetabular gland enzymes or glycocalyx, both present to a greater extent in mechanically-transformed than skin-transformed schistosomula (Cousin et al., 1981), may have on metabolic processes in the parasite. Results of our physiological assays, however, indicate that the timecourse of the conversion to a CN^--insensitive metabolism is similar for both groups.

In the 24 hr schistosomulum oxidative phosphorylation appears to play a less significant role in energy production than in the 3 hr schistosomulum, since 1 mM CN^- did not eliminate a significant portion of the endogenous electrical potentials. The absence of a physiological response to CN^- in this case may be explained on the basis of the increase in lactic acid production which occurs between the 3 hr and 24 hr stages.

Several studies have attempted to elucidate the molecular mechanisms regulating the switch from an aerobic to an anaerobic metabolism in schistosomes. Lactate dehydrogenase has been carefully examined by Von Kruger et al. (1978) and found not to change during this metabolic shift. From results obtained in the present study after transforming and incubating schistosomula 24 hr in medium containing 200 $\mu g \cdot ml^{-1}$ puromycin, a potent inhibitor of protein synthesis in schistosomes

during all stages of development (Kusel, 1972; Nagai et al., 1977; Tavares et al., 1980), it appears that the conversion to CN^--insensitivity does not depend on synthesis of new proteins. The metabolic changes that occur concomitantly with transformation must therefore depend on a response from enzymes already present in the emerged cercaria.

Results of experiments on the adult schistosome presented in this study are consistent with the findings of Bueding and Fisher (1982) who report that the adult parasite exhibits no Pasteur effect and no dependence on oxygen for energy formation. Although some CN^--sensitive CO_2 was evolved during incubations of the adult parasite, the amount was less than 5% (on a per mg protein basis) of that evolved by the 3 hr schistosomulum. The ratio of lactic acid excreted to CO_2 evolved under control conditions clearly shows that the adult parasite relies much less on oxidative phosphorylation for its energy production than the 25% reported by Coles (1972). These findings are consistent with our observations that exposure to CN^- does not depress endogenous electrical activity or muscle responsiveness in the adult, and further substantiate the principal role of homolactic fermentation in the energy metabolism of adult S. mansoni. It is important to note, however, that significant strain differences may exist in the metabolism of carbohydrates by schistosomes. Such differences have already been demonstrated among various strains of cestodes (Ovington et al., 1981) and nematodes (Bryant et al., 1983) and, if present among S. mansoni, they could account for the differences between our data and that of Cole's regarding the role of aerobic energy production in the adult parasite.

SECTION III

Schistosoma mansoni: Physiological Effects of Concurrent In Vitro Exposure to Antiserum Plus Complement and Drugs that Affect Cytoskeletal Components

Summary

Standard electrophysiological and muscle tension recordings were obtained from adult male S. mansoni after 2 hr and 24 hr incubations in media containing drugs believed to inhibit protein synthesis, transport or assembly in the parasite. Recordings were also obtained from adult parasites exposed concurrently to the drugs and polyclonal anti-schistosome immune serum supplemented with fresh guinea pig complement. While 10^{-5}M cytochalasin B, a microfilament inhibitor, and 10^{-4}M colchicine, a microtubule inhibitor, effected pronounced morphological changes in the parasite's tegument, most physiological properties of the parasite remained intact. That is, no significant changes were detected in the distribution of electrical potential recorded from the tegument or muscle, the frequency or mean amplitude of endogenous electrical transients, the inotropic effects of exposure to elevated K^+ or carbachol on longitudinal muscle, or the rate at which bound [^{125}I]sulphanilic acid was shed from the parasite. Concomitant exposure to the immune substances neither exacerbated the morphological changes brought about by these drugs, nor acted synergistically with the drugs promote detectable physiological changes. Puromycin, a potent inhibitor of

protein translation, did not alter tegumental morphology or physiological responses in the parasite. No synergistic effects were detected when immune serum and complement were added to the incubations containing puromycin. Poly-l-arginine, an analog of the major basic protein contained in guinea pig eosinophil secretory granules, and trifluoperazine, an inhibitor of the Ca^{++}-regulatory protein calmodulin, both effected time- and dose-dependent physiological as well as morphological changes in the schistosome. These substances significantly depressed most physiological responses in the parasite at concentrations as low as 10^{-6}M after 24 hr incubations. No synergism was detected, however, when low concentrations of poly-l-arginine or trifluoperazine were added to media containing the immune serum plus complement.

Introduction

Adult Schistosoma mansoni evoke an immune response which renders their vertebrate host refractory to a secondary infection while apparently exerting little or no effect on themselves. The molecular basis for this condition, described by Smithers and Terry (1969) as concomitant immunity, has not been determined. Alterations in surface lipids (Rumjanek, 1982), carbohydrates (Simpson et al., 1983), or in the rate of incorporation and turnover of tegumental protein (Kusel and MacKenzie, 1975) might contribute to the development of immune-refractoriness in schistosomes. Alternatively, tegumental antigens released by the schistosome could modulate the host immune response by suppressing lymphocyte blastogenesis (Todd et al., 1979; Ottsen, 1980) and/or immediate hypersensitivity responsiveness (Hofstetter et al., 1983).

Regardless of the precise mechanisms involved, it is clear that the schistosome's tegumental covering must play an essential role in the immune-evasion process. Being the host/parasite interface, the tegument is both a barrier to immune invasion as well as the principal source of parasitic antigens. Knowledge of the tegument may, therefore, hold the key to understanding the parasite's ability to circumvent the acquired immune response, and provide essential clues for the development of a vaccine.

While a number of studies have examined changes in schistosome anatomy and biochemistry brought about by substances which affect the outer tegumental membrane or other cytoskeletal components of the schistosome, little is known of the parasite's physiological responses to these substances, or whether they act synergistically with immune substances. In the present study, adult male S. mansoni were exposed in vitro to several substances which act on membrane or cytoskeletal components in the schistosome as well as other preparations. The parasites were analyzed for drug-induced changes in morphology, membrane electrical properties, muscle contractility, and rate of surface-label turnover. The complement-mediated effects of polyclonal immune serum were similarly analyzed, alone or in conjunction with the drugs. The immune treatment used was one already shown to induce pronounced physiological changes in immature S. mansoni in vitro (Thompson et al., 1982b).

Materials and Methods

Parasites, incubation media, drugs and immune substances. A Puerto Rican strain of S. mansoni was maintained by passage through

Swiss Webster mice and biomphalaria glabrata snails. Adult parasites were obtained by the method of Fetterer et al. (1977) by extracting worm pairs from the portal veins of cervically dislocated mice 55 days after infection. Worm pairs were separated and females discarded; males were placed in vials containing a 50:50 mixture of horse serum and RPMI-1640 (HS/RPMI; Grand Island Biological Co., Grand Island, NY), and maintained at 37°C prior to use. Bacterial contamination was reduced by using sterilized glassware and by adding 100 units/ml penicillin plus 100 μg/ml streptomycin to all media prior to sterile filtration.

Puromycin, colchicine and poly-l-arginine (P-L-A) were dissolved in distilled water at a concentration of 10^{-2}M (except for P-L-A, 10^{-3}M) and pipetted into incubation media as required immediately prior to the addition of parasites. Cytochalasin B and trifluoperazine (TFP) were initially dissolved in dimethylsulfoxide (DMSO) at such concentrations that the final incubation media never contained more than 0.2% DMSO. All drugs were obtained from Sigma Chemical Co., St. Louis, MO.

Polyclonal anti-schistosome immune serum (IMS) was prepared by heat-inactivating serum obtained from Swiss Webster mice infected 56-70 days earlier with 250 cercariae of S. mansoni. IMS incubations also contained fresh guinea pig complement and RPMI-1640 in a volume ratio of 1:4:20. Controls in immune substance studies consisted of parasites similarly exposed to solutions where equal volumes of heat-inactivated serum obtained from uninfected mice replaced the IMS.

Electrophysiological and mechanical recordings. Endogenous electrical activity was recorded from the parasites by a modification of the method described by Fetterer et al. (1977). Glass suction

electrodes were manufactured from 1.25 mm capillary tubing (W.P. Instruments, New Haven, CT) that was pulled to a fine tip on a horizontal electrode puller (Narishige, Tokyo, Japan). The electrodes were modified by cutting back the tip with a diamond pencil so that the new tip was flat, with an outside diameter of 40-50 μm. The electrode tip was then heated on a microforge (Aloe Corp., New York, NY) which melted the glass from the inside. Heat was applied until the flattened tip was smooth, and the aperture was reduced to approximately 15-20 μm. Electrodes were firmly attached to the parasite's surface during electrical recordings by way of negative pressure created by a 10 ml syringe. Prior to attachment of the parasite, Hanks' Balanced Salt solution (HBS) contained in a separate vial was drawn into the electrode until it made contact with a silver wire which led to an amplifier (Model P-15, Grass Instruments, Quincy, MA). Electrical signals from the parasites were filtered with the low pass set at 0.3 Hz and the high pass at 1 kHz, displayed on an oscilloscope (Model 5113, Tektronix Inc., Beaverton, OR) or a chart recorder (Narco Biosystems Inc., Houston, TX), and passed into an analog-to-digital converter prior to computer analysis (Alis I-10 Data Acquisition and Control System, Ecotech Inc., Lansing, MI).

After electrode attachment, a 3-5 min equilibration period was allowed prior to electrical recording. The electrical activity was quantified by counting and averaging all negative field potentials in excess of 20 μV over six 10 sec intervals. Potentials under 20 μV in amplitude were not counted, as they were within the noise range of the system. All recordings of endogenous electrical potentials from parasites exposed to the various drugs were obtained 1-2 hr or 24-28 hr

after addition of drug to the parasite-containing vials. Incubation medium consisting of RPMI-1640 alone, or with DMSO vehicle served as the recording medium.

Methods of microelectrode recording from the tegument and muscle compartments were as described by Thompson et al. (1982a). Recordings were obtained from drug-treated and control parasites intermittently during the first 1-2 hr after drug exposure and again 24-28 hr later. All electrophysiological experiments were performed on at least 2 separate occasions, N=8. Statistical analysis for significance of difference between means used Student's t-test.

Muscle tension recordings were obtained using the method described by Fetterer et al. (1977) (Figure 10). Effects of the drugs on muscle tension changes induced by exposing the parasite to elevated potassium (60 mM; increases muscle tension in schistosomes) or the acetylcholine analog, carbachol (10^{-4}M; reduces muscle tension in schistosomes) were assessed by preincubating the parasites for 1-2 hr or 24-28 hr in drug-containing media prior to exposure to the inotropic treatments. For statistical analysis, changes in muscle tension recorded in drug-treated and control parasites 10 min after exposure to elevated potassium or carbachol were compared using Student's t-test non-paired; $N \geq 5$.

Turnover of [^{125}I]-labelled surface components. The conditions used for labelling schistosomes with [^{125}I]sulphanilic acid were similar to those described by Taylor et al. (1981). Parasites were preincubated 24 hr in HS/RPMI containing drugs prior to labelling. Radiolabelling was performed at 4°C by addition of 0.15 mCi diazonium [^{125}I]iodosulphanilic acid (specific activity, 7.3 Ci/mg; New England Nuclear, Boston, MA) to separate test tubes containing 80 parasites

Figure 10. Schematic of apparatus used to record longitudinal muscle tension in adult male S. mansoni.

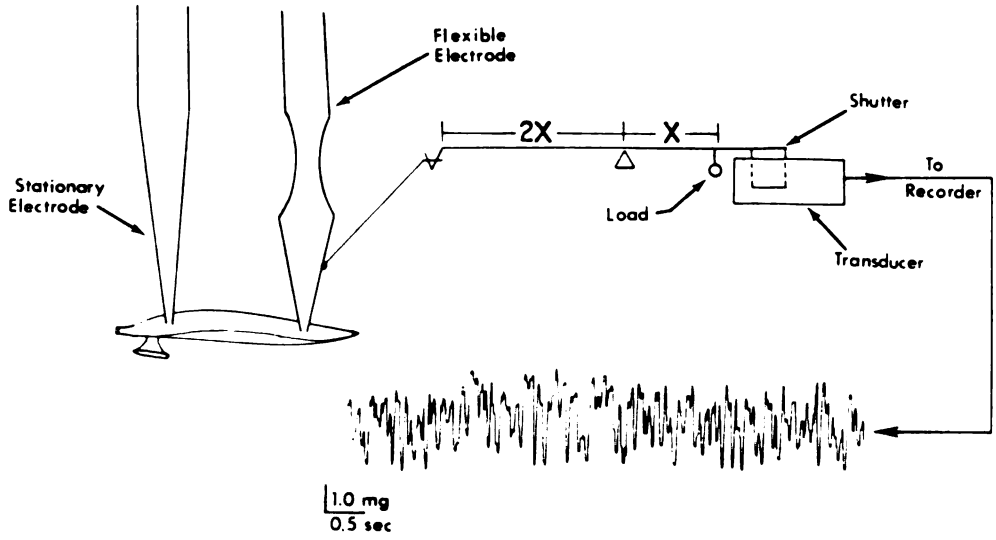

Flexible
Electrode
Stationary
Electrode
2X
X
Shutter
To
Recorder
Load
Transducer
1.0 mg
0.5 sec

Figure 10

each in 300 µl cold RPMI-1640, pH 7.4. Conversion of [^{125}I]iodosulphanilic acid to its diazonium salt was carried out immediately prior to the reaction. After 15 min, the labelling reaction was stopped by rinsing 2X for 5 min each with 2.5 ml cold RPMI-1640 containing 20% fetal calf serum (FCS/RPMI; Grand Island Biological Co.). Parasites were rinsed 5X for 2 min each with warm FCS/RPMI and distributed into flasks containing 20 ml HS/RPMI plus the specified concentration of drugs. The flasks were placed on a shaker bath and maintained at 37°C prior to testing. At the specified times, the parasites were rinsed 5X with warm FCS/RPMI and immediately counted on a γ-radiation detector (Searle Analytic, Des Plaines, IL).

To verify that the radiolabel was binding to surface components of the schistosome, a group of labelled parasites were detegumented after 1 hr of incubation according to the method of Knowles and Oaks (1979), whereby the worms were exposed for 5, 10 or 15 min to 1 ml cold 0.2% Triton X-100 prepared in HBS, pH 7.4, then gently vortexed for 1 min. Supernatants were decanted and the carcasses rinsed 2X with 1 ml warm FCS/RPMI. The original supernatant containing the tegumental fraction was combined with the 2 obtained in subsequent rinses and counted. Carcasses were counted separately.

To determine the relative distribution of label between protein and lipid, labelled parasites were rinsed and counted as previously described, then lipid-extracted using chloroform plus methanol, 2:1 (v/v). The lipid fraction was counted and compared to the whole-animal values previously obtained. All labelling experiments were performed in triplicate.

Scanning and transmission electron microscopy. At the specified times, the incubation media were decanted, 2% glutaraldehyde (Polysciences, Warrington, PA) in 0.1 M phosphate buffer, pH 7.3, was added, and the suspension kept for a minimum of 8 hr at 4°C. Thereafter, fixative was decanted, the specimens rinsed 2X in 0.2 M phosphate buffer and dehydrated through an ascending series (10% increments) of ethanol:water solutions to 100% ethanol. Scanning electron microscopy (SEM) specimens were critical point dried, mounted on stubs, sputter coated with gold, and examined in a JEOL JSM-35C scanning electron microscope at 15 kV. Transmission electron microscopy (TEM) specimens were postfixed in 1% osmium tetroxide, dehydrated, and embedded in Mullenhauer's resin, with acetone serving as an intermediate solvent. Sections were cut using glass and diamond knives on a Sorvall Porter-Blum ultramicrotome. Thin sections were collected on 300 mesh copper grids, stained with uranyl acetate and lead citrate, and examined on a Phillips 201 transmission electron microscope at 60 kV.

To determine the effects of drugs on tegumental uptake of macromolecules, parasites preincubated 20 hr in medium containing 10^{-5}M cytochalasin B, 10^{-4}M colchicine, 200 μg/ml puromycin or the specified immune treatments with proper controls were transferred into vials containing a solution prepared by adding 3 mg horseradish peroxidase (HRP, type VI, Sigma) to 1 ml RPMI-1640, and allowed to incubate for 30 min or 3 hr at 37°C. Thereafter, the specimens were rinsed 3X for 3 min each in warm HS/RPMI and individually transferred into vials containing fixative. After a minimum fixation period of 8 hr, the specimens were rinsed 3X in 0.2 M phosphate buffer, then incubated 20 min in an HRP reaction-intensifying solution consisting of 0.5% $CoCl_2$ in 0.1 M

Tris buffer, pH 7.4. The parasites were then rinsed 3X for 3 min each in 0.2 M phosphate buffer and reacted for 30 min in a freshly prepared solution containing 6 mg 3'3-diaminobenzidine-HCl (Sigma Chemical Co.), 20 ml 0.1 M Tris buffer pH 75, and 0.8 ml 4% H_2O_2. The reaction was terminated by rinsing the specimens 3X for 10 min each in 0.2 M phosphate buffer, pH 7.4. These specimens were then dehydrated and prepared for TEM and SEM without postfixation. Control parasites were preincubated 24 hr in RPMI-1640, then exposed 30 min or 3hr to HRP added to warm medium or cold medium that was maintained for the duration of the incubation period on ice. These experiments were performed on two separate occasions. The effects of P-L-A and TFP were not examined in this assay as preliminary results showed that these treatments removed most of the tegument from the parasite.

Results

Effects of drugs and immune substances on tegumental morphology. The teguments of adult male Schistosoma mansoni incubated 24 hr in medium containing 200 μg/ml puromycin appeared morphologically similar to control parasites incubated under similar conditions (Figure 11, A and B). Addition to the incubation medium of complement plus polyclonal IMS did not effect a significant change in the appearance of the tegument, nor did these substances act in conjunction with puromycin to alter tegumental morphology.

Cytochalasin B at 10^{-5}M effected a pronounced change in tegumental morphology, apparent in SEM micrographs as irregularly shaped porous fragments 0.5-5.0 μm in diameter that were randomly scattered on the surface of the parasite. Based on TEM micrographs, these fragments

Figure 11. Scanning and transmission electron micrographs obtained from adult male S. mansoni incubated 24 hr in 200 μg/ml puromycin (A and B) and $1x10^{-5}$M trifluoperazine (C and D). Puromycin-treated parasites were morphologically similar to controls. Note extensive disruption of tegument apparent in parasites exposed to trifluoperazine. Similar changes were apparent in parasites incubated for 24 hr in $1x10^{-6}$M poly-l-arginine. Calibration bars: A and C, 10 μM; B and D, 1 μM.

Figure 11

appeared to be evaginations of the surface membrane overlying large areas where the tegumental cytoplasm had become vacuolated. The extent of tegumental disruption brought about by this substance did not change appreciably over time, as parasites exposed 2 hr or 24 hr appeared similar. Parasites exposed to 10^{-4}M colchicine exhibited a lower level of tegumental disruption than that effected by 10^{-5}M cytochalasin B. At the SEM level, this disruption was apparent at 24 hr, but not at 2 hr.

Exposure of the schistosome to 10^{-6}M P-L-A or 10^{-5}M TFP for 24 hr resulted in pronounced disruption of the tegument (Figure 11, C and D). The nature and extent of disruption appeared to be similar in response to both drugs. Most of the syncytium appeared to be stripped away from the carcasses of parasites exposed to these treatments and components of the tegument which remained associated with the carcass appeared to form vacuoles. Exposure to these substances for briefer periods resulted in less extensive disruption of the tegument.

Parasites incubated in media containing the immune substances appeared morphologically similar to controls, both at the SEM and TEM levels. The presence of immune serum plus complement concurrently with the drugs tested did not exacerbate the morphological disruption brought about by cytochalasin B or colchicine, nor did they initiate disruption in the puromycin-treated schistosomes.

TEM micrographs obtained from control schistosomes incubated for 30 min or 3 hr in HRP-containing medium revealed that the enzyme marker was not taken up by the tegument. That is, there was no evidence of reaction product in the tegument, either in vacuoles or freely suspended in the syncytium. The marker was also absent from the teguments

of drug-treated parasites, including those showing tegumental disruption in response to 24 hr incubations in 10^{-5}M cytochalasin B or 10^{-4}M colchicine. Addition of complement plus polyclonal IMS to the incubation medium did not promote uptake of the marker in our studies, either by itself or in conjunction with the drugs tested.

Microelectrode and surface electrical recordings. Microelectrode recordings obtained from the teguments of parasites exposed 24-28 hr to 10^{-5}M cytochalasin B, 10^{-4}M colchicine or 200 µg/ml puromycin yielded values similar to the tegumental potential of control parasites of -51.8±0.7 mV. Tegumental potentials remained at control levels when the incubation medium was supplemented with complement plus polyclonal IMS (Table 2). Analogously, the endogenous electrical potentials recorded from the surface of these parasites, with or without the immune substances, remained similar to control values for which the mean frequency and amplitude was 32.1±1.1/sec and 41 µV, respectively (Figure 12).

Electrode penetrations of schistosomes incubated 24-28 hr in 10^{-6}M P-L-A indicated that the ionic gradients maintaining the tegumental potentials were significantly altered by this treatment. That is, the teguments of these parasites were depolarized in a dose-dependent manner. Concentrations of 10^{-5}M and above of this drug effected membrane depolarization so extensive there were virtually no distinguishable compartments of electrical potential remaining after 24 hr (Figure 13). Parasites incubated for shorter periods in P-L-A showed a gradual drug-induced depolarization of the tegument (Figure 14). The frequency and mean amplitude of endogenous electrical potentials were also reduced by this drug in a dose-dependent manner (Figure 15).

TABLE 2

Tegumental Potentials Recorded in Adult Male S. mansoni After 24 hr Incubations in RPMI-1640 Alone (A), with Drugs (B), or Concurrently with Drugs and Immune Mouse Serum plus Guinea Pig Complement (C)

	Condition		
	A (RPMI-1640)	B (with drug)	C (drug + immune serum)
Treatment	Tegument potential (mV)		
Cytochalasin B $1x10^{-5}M$	-51.8±1.5	-48.3±1.4	-55.0±1.3
Colchicine $1x10^{-4}M$	-54.6±1.2	-51.6±1.1	-53.9±1.6
Puromycin 200 µg/ml	-54.3±1.1	-56.8±0.7	-52.6±1.3
IMS + Complement 5%/10%	-53.2±1.3	---	-54.3±1.7

Values expressed are means ± 1 SE; N≥10.

Figure 12. Frequency of endogenous electrical potentials recorded from adult male S. mansoni showing absence of effects on this parameter due to drugs (open bars) or concurrent exposure to drugs plus 5% polyclonal immune mouse serum and 10% fresh guinea pig complement (shaded bars) after 24 hr incubations. Concentrations of drugs tested were: cytochalasin B, $1x10^{-5}$M; colchicine, $1x10^{-4}$M; puromycin, 200 µg/ml; P-L-A, $1x10^{-8}$M; TFP, $1x10^{-7}$M. Values expressed are means ± 1 SE; N≥6.

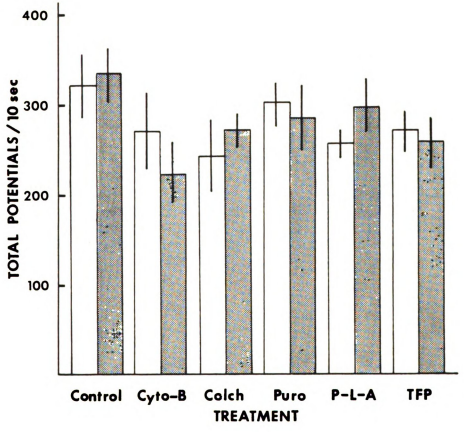

400
300
200
100
TOTAL POTENTIALS / 10 sec
Control
Cyto-B
Colch
Puro
P-L-A
TFP
TREATMENT

Figure 12

Figure 13. Dose-dependent effects of 24 hr preincubations in poly-l-arginine on tegument potential recorded in adult male S. mansoni (closed circles). Open circle is the potential recorded from control parasites in a separate experiment in which test groups were incubated 24 hr in 10^{-8}M P-L-A, alone (closed square) or concurrently with 5% immune mouse serum plus 10% fresh guinea pig complement (open triangle). Values expressed are means $\pm$ 1 SE; $N \geq 6$.

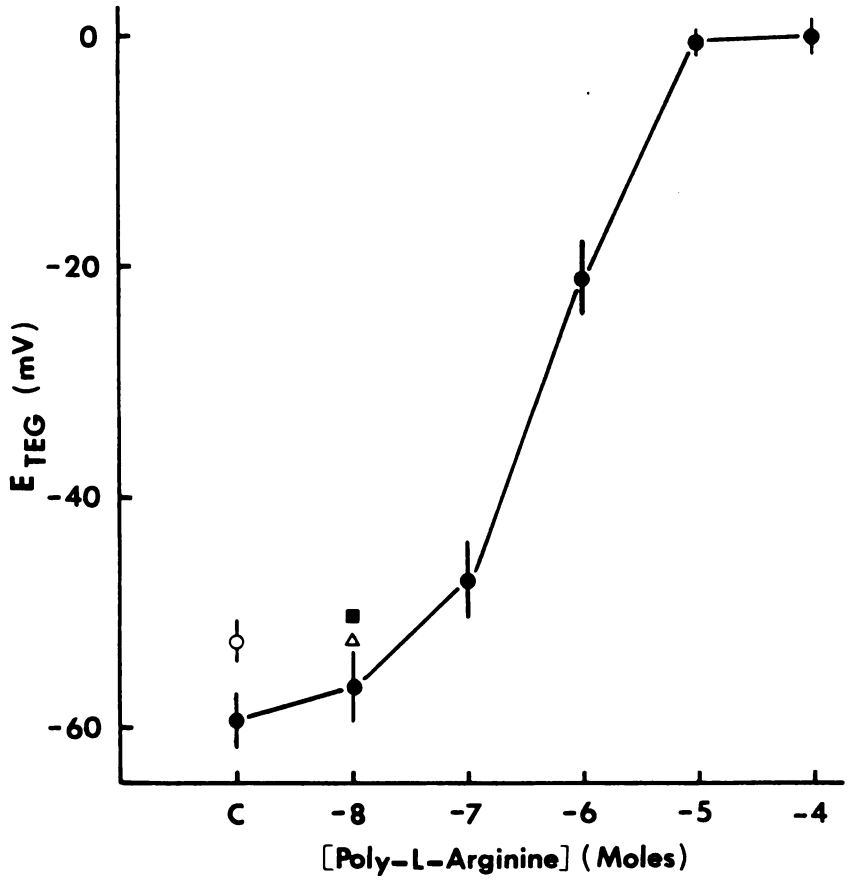


Figure 13

Figure 14. Acute effects of exposure (arrow) to $1x10^{-5}$M poly-l-arginine (closed circles) on tegument potential of adult male S. mansoni. Open circles are control values recorded from parasites incubated in RPMI-1640. Values expressed are means $\pm$ 1 SE; N=6.

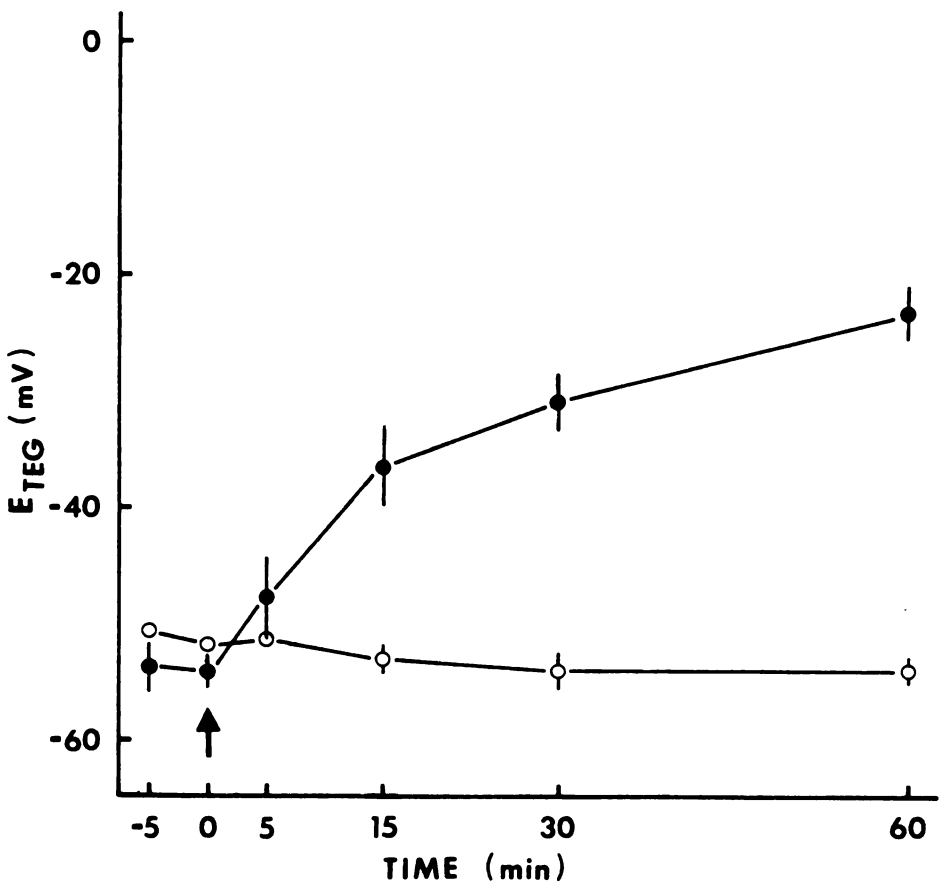


Figure 14

Figure 15. Frequency of endogenous electrical potentials recorded from adult male S. mansoni showing dose-dependent effects of 1-2 hr incubations in poly-l-arginine. Values expressed are means ± 1 SE; N≥6. For statistical analysis, potential frequencies of drug-treated parasites were compared with control (C) levels using Student's t-test non-paired; (a) P<.005, (b) P<.01, (c) P<.05.

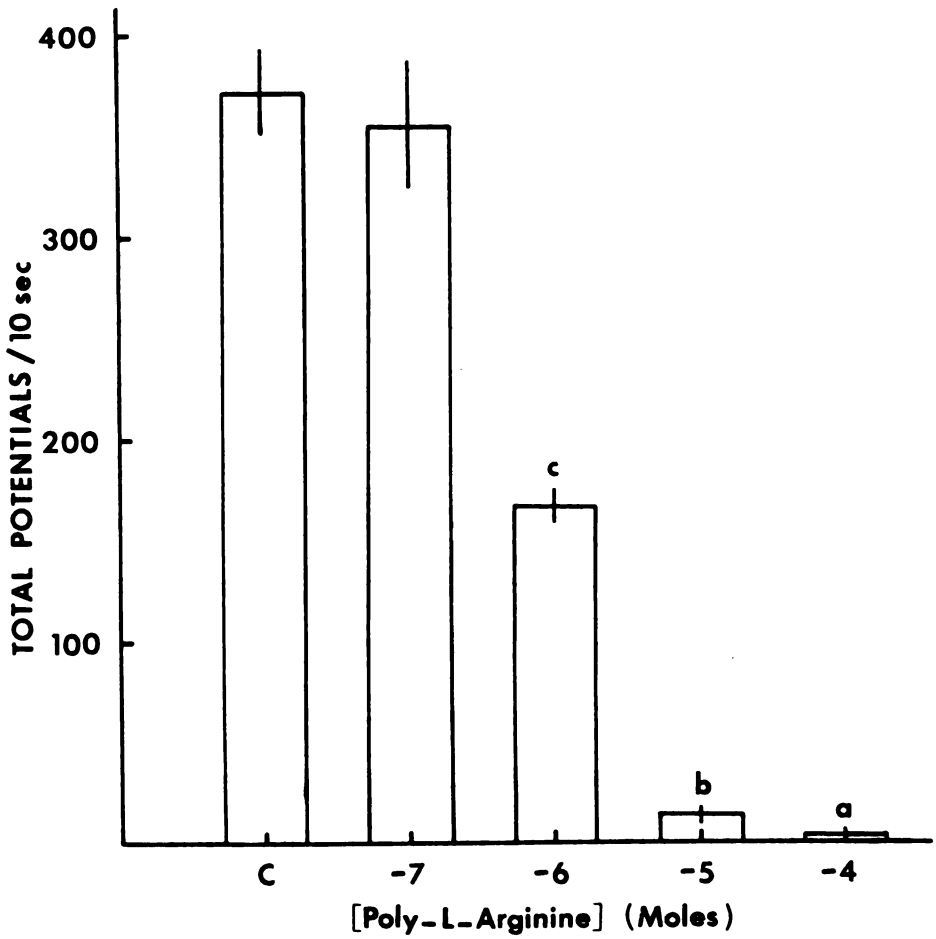

400
300
200
100
TOTAL POTENTIALS / 10 sec
c
b
a
C
-7
-6
-5
-4
[Poly-L-Arginine] (Moles)

Figure 15

Dose-dependent reductions in the tegumental potential and frequency of endogenous electrical potentials were also effected by TFP (Figures 16 and 17). The timecourse of tegumental membrane depolarization brought about by 10^{-5}M TFP was similar to that induced by 10^{-5}M P-L-A (Figure 18). That is, a significant depolarization of the tegument occurred within 15 min of drug-exposure, and by 1 hr, the tegumental potential was reduced by 43% ($p<.01$).

The presence of complement plus polyclonal IMS did not enhance the membrane-depolarizing effects or the depression of endogenous electrical activity brought about by 24 hr exposures to 10^{-8}M P-L-A or 10^{-7}M TFP, the highest concentrations of these drugs which, by themselves, exerted no significant effects on either physiological parameter.

Mechanical recordings. Elevated levels of potassium (60 mM) and 10^{-4}M carbachol induced a significant contraction and relaxation, respectively, of longidutinal muscle in control parasites, as well as those preincubated 24-28 hr in RPMI-1640 containing 10^{-5}M cytochalasin B, 10^{-4}M colchicine or 200 μg/ml puromycin (Figure 19). Addition of immune serum plus complement to the incubation media, alone or in conjunction with the above drugs, did not affect the responsiveness of schistosome longitudinal muscle to these inotropic treatments.

In contrast to the above treatments, parasites incubated for periods as brief as 1-2 hr in P-L-A or TFP exhibited significant dose-dependent changes in the responsiveness of their longitudinal muscle to the inotropic agents (Figures 20 and 21).

Turnover of [^{125}I]-labelled surface components. Preincubation of the schistosomes 24 hr in media containing 10^{-5}M cytochalasin B, 10^{-4}M

Figure 16. Dose-dependent effects of 24 hr preincubations in trifluoperazine on tegument potential recorded in adult male S. mansoni (closed circles). Open circle is the potential recorded from control parasites in a separate experiment in which test groups were incubated for 24 hr in 10^{-7}M TFP, alone (open square) or concurrently with 5% immune mouse serum plus 10% fresh guinea pig complement (open triangle). Values expressed are means $\pm$ 1 SE; N$\geq$6.

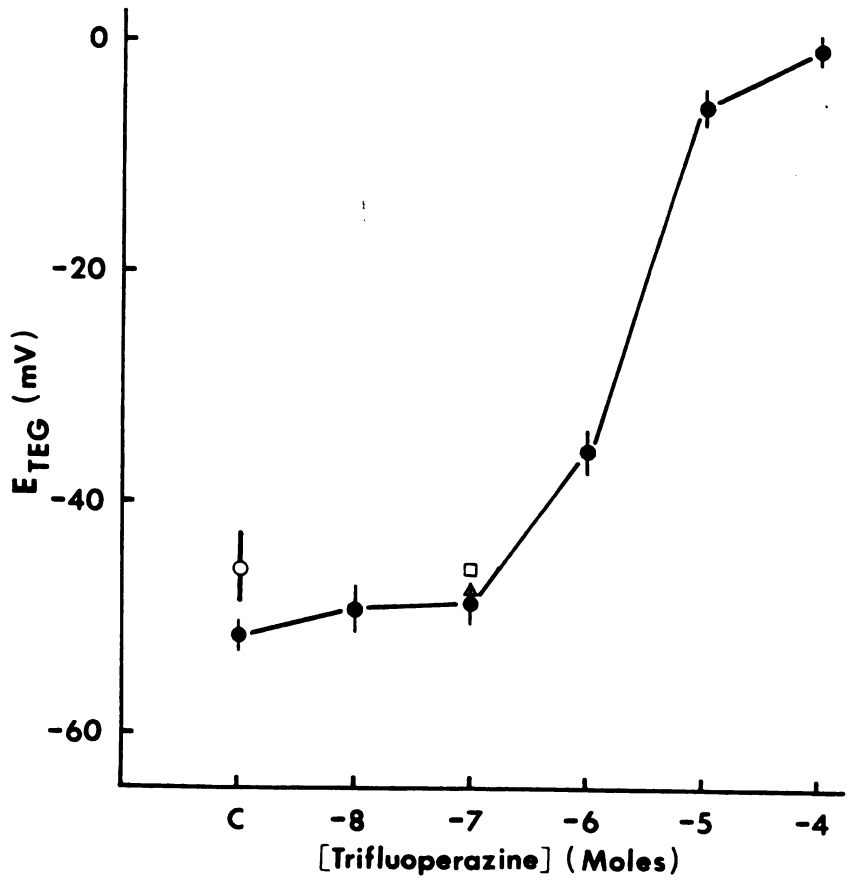


Figure 16

Figure 17. Frequency of endogenous electrical potentials recorded from adult male S. mansoni showing dose-dependent effects of 1-2 hr incubations in trifluoperazine. Values expressed are means ± 1 SE; N≥6. For statistical analysis, potential frequencies of drug-treated parasites were compared with control (C) levels using Student's t-test non-paired; (b) P<.01, (c) P<.05.

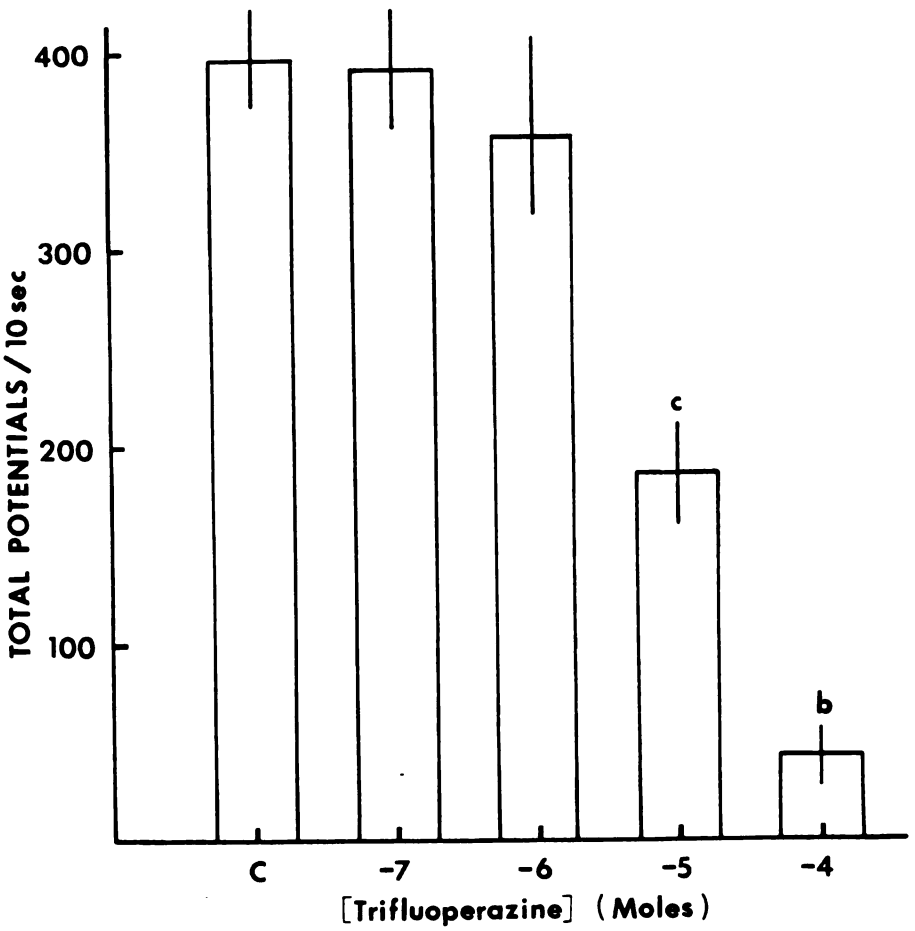

400
300
200
100
TOTAL POTENTIALS / 10 sec
c
b
C
-7
-6
-5
-4
[Trifluoperazine] (Moles)

Figure 17

Figure 18. Acute effects of exposure (arrow) to $1x10^{-5}$M trifluoperazine (closed circles) on tegument potential of adult male S. mansoni. Open circles are control values recorded from parasites incubated in RPMI-1640. Values expressed are means $\pm$ 1 SE; N=6.

Fig. 1 Pg. 109.

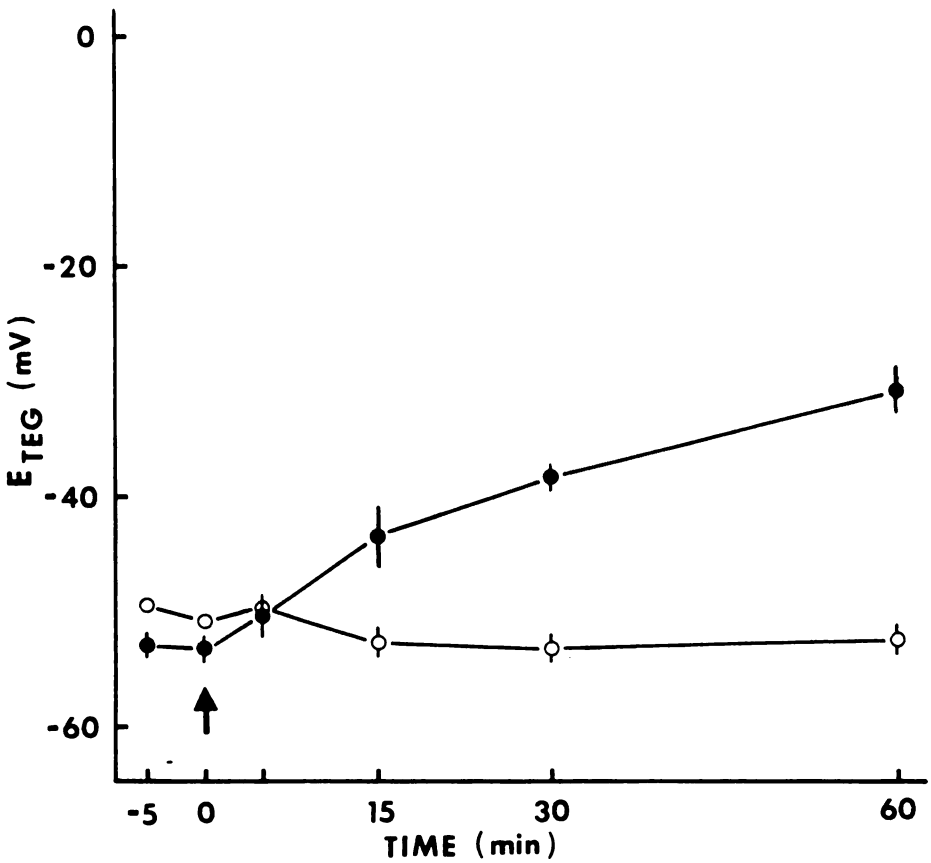


Figure 18

Figure 19. Effects of preincubation for 24-28 hr in control medium (closed circles), $1x10^{-5}$M cytochalasin B (closed triangles), $1x10^{-4}$M colchicine (open circles), or 200 μg/ml puromycin (open triangles) on longitudinal muscle tension changes produced by exposure (arrows) to 60 mM potassium (upper graph) or 10^{-4}M carbachol (lower graph). For all treatments, $N \geq 6$; vertical lines are $\pm$ 1 SE.

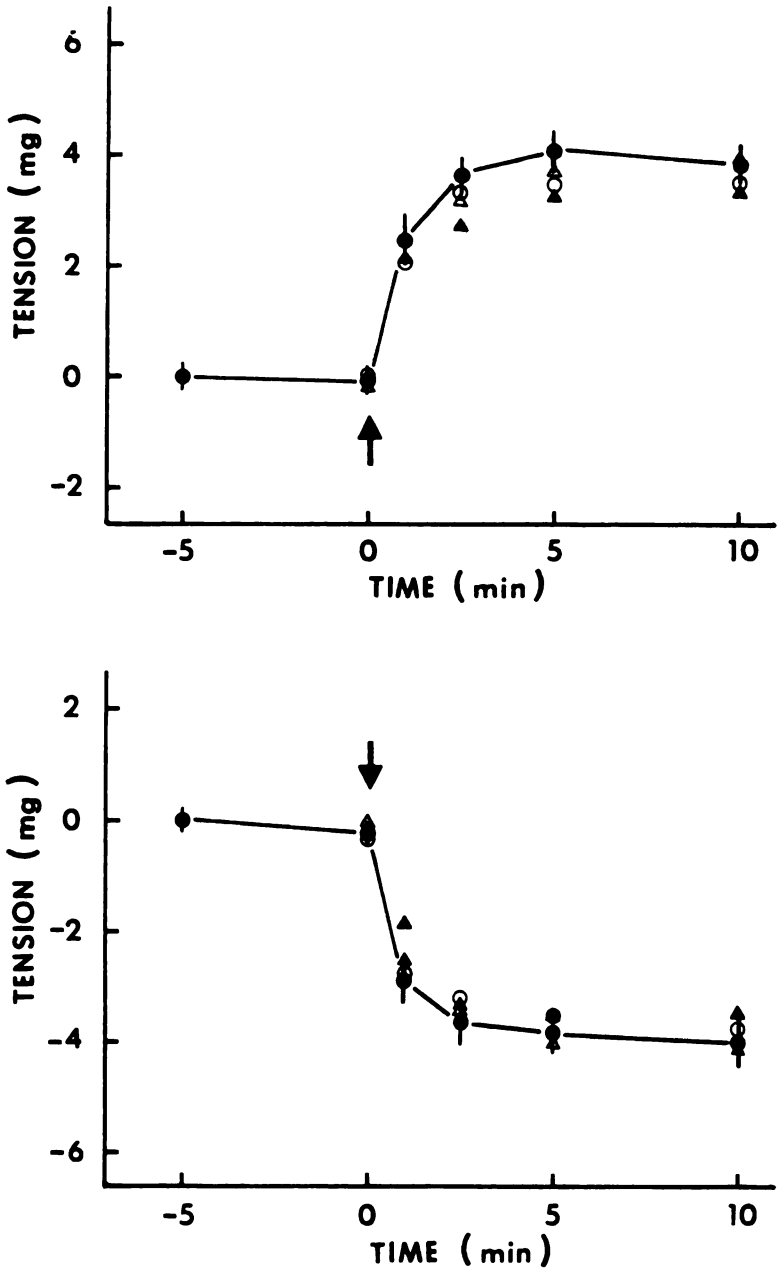
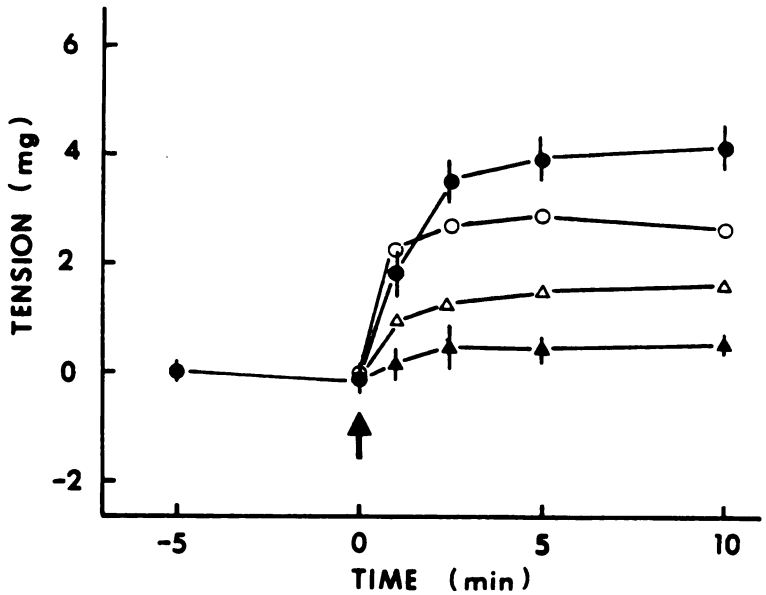

6
4
2
0
-2
TENSION (mg)
-5
0
5
10
TIME (min)
2
0
-2
-4
-6
TENSION (mg)
-5
0
5
10
TIME (min)

Figure 19

Figure 20. Dose-dependent effects of 1-2 hr preincubations in poly-l-arginine on longitudinal muscle tension changes induced by exposure to 60 mM potassium (upper graph) or 10^{-4}M carbachol (lower graph). Closed circles represent tension recorded in control paraistes incubated in RPMI-1640. Drug concentrations tested were: $1x10^{-7}$M (open circles), $1x10^{-6}$M (open triangles), and $1x10^{-5}$M (closed triangles). Values expressed are means $\pm$ 1 SE; N$\geq$5.


6
4
2
0
-2
TENSION (mg)
-5
0
5
10
TIME (min)

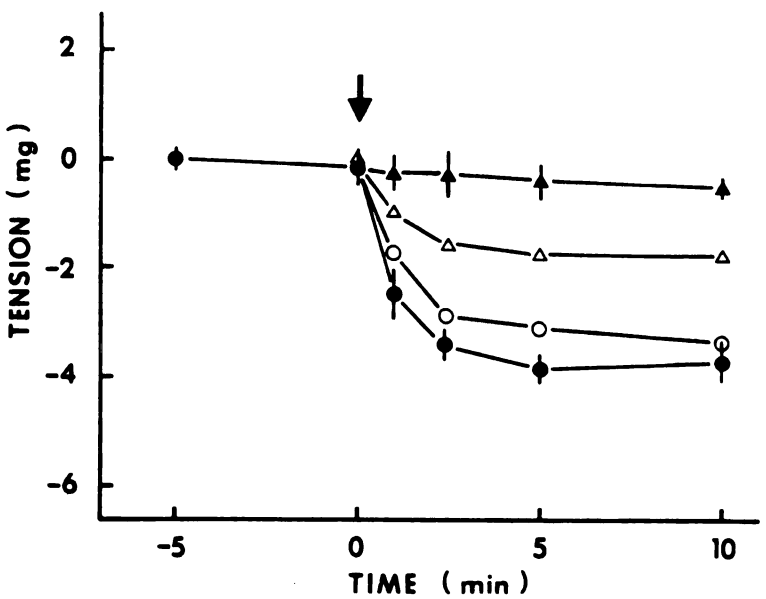

2
0
-2
-4
-6
TENSION (mg)
-5
0
5
10
TIME (min)

Figure 20

Figure 21. Dose-dependent effects of 1-2 hr preincubations in trifluoperazine on longitudinal muscle tension changes induced by exposure (arrows) to 60 mM potassium (upper graph) or 10^{-4}M carbachol (lower graph). Closed circles represent tension recorded in control parasites incubated in RPMI-1640. Drug concentrations tested were: $1x10^{-7}$M (open squares), $1x10^{-6}$M (open circles), $1x10^{-5}$M (open triangles), and $1x10^{-4}$M (closed triangles). Values expressed are means $\pm$ 1 SE; N$\geq$5.

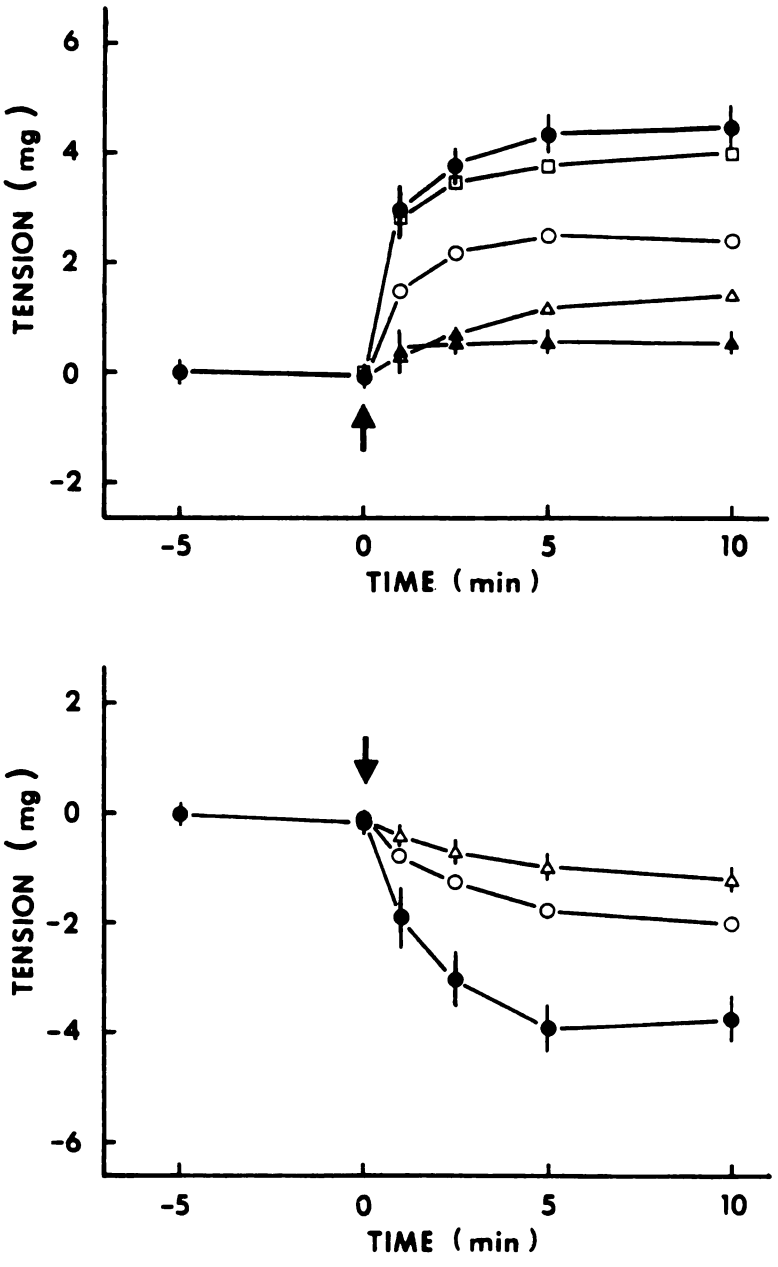

6
4
2
0
-2
TENSION (mg)
-5
0
5
10
TIME (min)
2
0
-2
-4
-6
TENSION (mg)
-5
0
5
10
TIME (min)

Figure 21

colchicine or 200 μg/ml puromycin did not significantly affect the rate at which [^{125}I]sulphanilic acid-labelled surface components were released from the parasite (Figure 22). Based on the results of our assay, labelled components of the schistosome's tegument were turned over with a half-time of 2-3 hr, even in the presence of protein synthesis or transport inhibitors. Experiments involving Triton X-100 indicated that the treatment described for removing the schistosome's tegument eliminates 84%, 87% and 91% of the surface label when the parasites are incubated for 5, 10 and 15 min, respectively, in cold 0.2% Triton-containing HBS prior to vortexing. Extraction with chloroform plus methanol revealed that less than 8% of the label recovered was associated with the lipid fraction.

Discussion

Results of the present study indicate that, over short-term incubations, no simple relationship exists in adult male S. mansoni between tegumental morphology and the ability of the tegument to function as a permeability barrier to ions and some immune substances. Drugs such as cytochalasin B and colchicine that are believed to inhibit function of schistosome microfilaments (Wilson and Barnes, 1974) and microtubules (Wessels, 1974), respectively, and also effect disruption of the parasite's tegument (Bricker et al., 1983), do not concomitantly produce changes in the electrophysiological or mechanical properties of the parasite. By all of our measurements, tegumental function remains intact even when the parasite is challenged concurrently with the above drugs and an immune treatment which, by itself, inhibits motility and electrophysiological activity in immature stages of S. mansoni

Figure 22. Loss of covalently bound ^{125}I from adult male S. mansoni during incubation in RPMI-1640 alone (closed circles) or with $2x10^{-5}M$ cytochalasin B (open circles), $1x10^{-4}M$ colchicine (open squares), 200 µg/ml puromycin (open triangles), or $1x10^{-6}M$ trifluoperazine (closed triangles). Parasites were preincubated in drugs 24 hr prior to labelling.

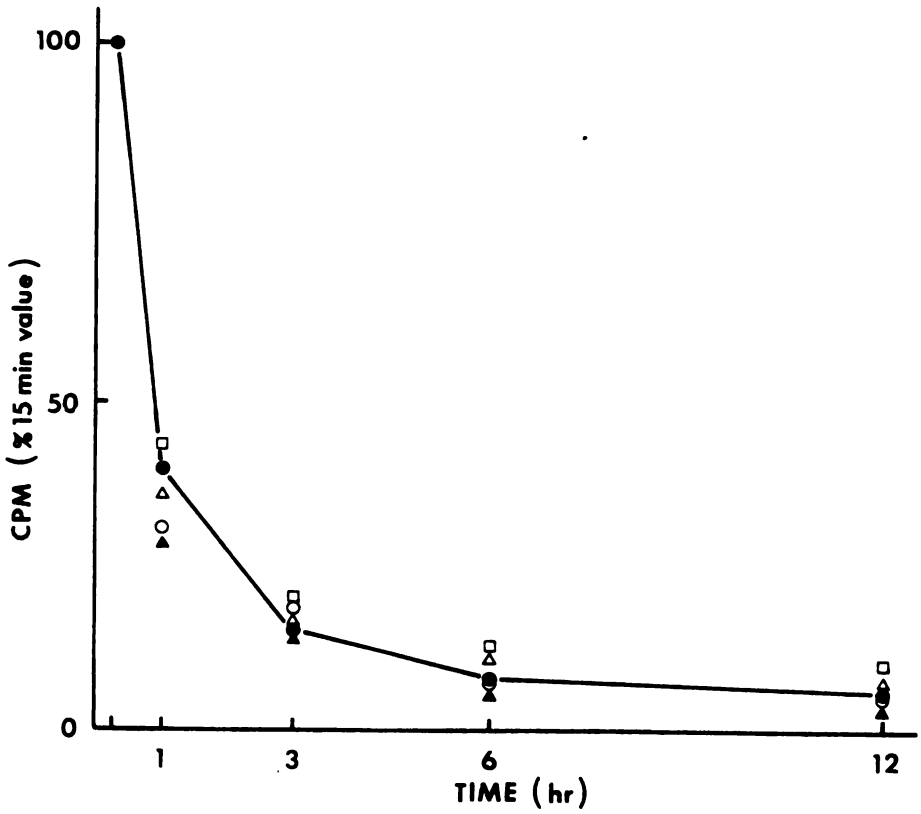


Figure 22

(Thompson et al., 1982b). Conversely, tegumental disruption effected by P-L-A or TFP was also accompanied by pronounced changes in tegumental membrane function, best evidenced by the rapid, dose-dependent depolarization of the tegument and muscle.

Based on the physiological responses we recorded, a 2 hr exposure to 10^{-6}M P-L-A or 10^{-5}M TFP results in a more extensive perturbation of the parasite than that effected by 10^{-5}M cytochalasin B, even though at that time point the degree of tegumental disruption induced by these three substances is morphologically indistinguishable. After 24 hr of incubation, however, major differences in the drug's capacities for disruption of schistosome tegument were readily apparent at the morphological as well as the physiological level. Parasites incubated in cytochalasin B showed no appreciable increase in morphological disruption from the levels observed at the 2 hr time point. Electrophysiological recordings showing healthy tegumental potentials in cytochalasin B-treated schistosomes indicated that the tegumental membranes of these parasites had remained functional as a permeability barrier to ions. The teguments of parasites exposed to P-L-A or TFP for 24 hr, however, exhibited a level of morphological disruption far more extensive than that recorded at the 2 hr time point, and the membrane-permeability changes detected during the first hour of exposure were even more pronounced.

The type and extent of tegumental disruption induced by 24 hr in vitro exposure to 10^{-6}M P-L-A or 10^{-5}M TFP appeared similar to that reported to occur after short-term incubations in 2×10^{-5}M major basic protein (MBP) isolated from human or guinea pig eosinophils (Butterworth et al., 1979). In the case of MBP, it is believed that disruption

is due to the basic properties of the molecule. MBP extracted from guinea pig eosinophil secretory granules is largely composed of polycationic peptides, especially poly-l-arginine (Lewis et al., 1978). Therefore, the disruption and concomitant physiological changes we observed on exposure to P-L-A could also be due, in part, to the membrane-destabilizing effects of this basic molecule. The effects do not appear to depend on specific properties of the arginine molecule itself, as incubations in poly-L-lysine elicited similar responses in the schistosome (D. Thompson, personal observations).

While the extensive tegumental disruption brought about by P-L-A and TFP may be directly linked to the physiological responses recorded, tegumental damage by itself is not sufficient for effecting the type of neuromuscular depression that we recorded. Recent studies in our laboratory have shown that the muscle fibers in schistosomes completely stripped of their teguments with Triton X-100 continue to remain functional in almost all respects (Depenbusch et al., 1983). That is, the parasite's musculature continues to respond mechanically to elevated potassium and carbachol, and no changes in the resting membrane potential is incurred by this treatment. These results indicate that changes in muscle function brought about by P-L-A and TFP in the present study are not explainable solely on the basis of the drug's capacities for tegumental disruption and removal.

The similarities we observed in the extent of tegumental disruption and physiological change brought about by exposure to P-L-A and TFP are probably coincidental. Most evidence indicates that TFP acts primarily by inhibiting calmodulin, a Ca^{++}-regulatory protein apparently ubiquitous to eukaryotes (Sheterline, 1983) and found in relatively

high concentrations in the schistosome (D. Thompson, personal observations). The wide range of effects we observed upon exposing schistosomes to TFP is not surprising, in light of the evidence indicating that calmodulin is a multifunctional protein, which is not only concerned with regulation by Ca^{++} of contractile processes, but confers Ca^{++} dependence on a wide range of enzymes in eukaryotic cells (Means and Dedman, 1980). Physiological responses similar to those recorded in TFP-treated schistosomes were also recorded in parasites similarly exposed to Calmidazolium (Janssen Pharmaceutica, Beerse, Belgium), another specific inhibitor of calmodulin.

If the TFP-induced inhibition of contractility in schistosomes was due specifically to inhibition of calmodulin-mediated assembly of F-actin, then one would have expected to see a similar response to cytochalasin B, which is generally thought to act on the same assembly process (McLean-Fletcher and Pollard, 1980). The fact that cytochalasin B did not affect muscle contractility suggests that the contractile mechanism in schistosomes may differ significantly from the numerous cardiac and smooth muscle preparations that are sensitive to cytochalasin B-induced inhibition (Wessels et al., 1971; Goodman and Miranda, 1978). It is also possible that, in schistosomes, TFP inhibits contractility by inhibiting calmodulin-mediated phosphorylation of myosin light chains by MLC kinase, as shown in other preparations (Amphlett et al., 1976). Alternatively, the drug could mediate its effects through interaction with numerous other calmodulin-dependent enzymes in the parasite.

In earlier reports (Thompson et al., 1982b, 1983) we have shown that the complement-mediated effects of polyclonal IMS and an

anti-schistosomulum monoclonal antibody on 3 hr skin- and mechanically-derived schistosomula are detectable by electrophysiological analysis after 24 hr incubations. Similar recordings obtained in the present study which show no tendencies for synergism between the immune treatments and the drugs tested suggest that the adult parasite's ability to avoid the harmful effects of the immune substances we tested may not be contingent on "normal" tegumental function. That is, over the course of short-term incubations, at least, inhibition of translation, microfilament and microtubule function or Ca^{++}-regulatory proteins does not render the parasite susceptible to physiological perturbations beyond those affected by the drugs or immune substances alone. We cannot on the basis of our results, however, rule out the possibility that synergistic effects might become apparent after longer periods of incubation. Neither can we dismiss the possibility that similar incubations with cellular components of the immune system might show significantly different patterns of effectiveness.

A number of mechanisms have been proposed to explain the apparent refractoriness to immune substances exhibited by adult schistosomes. One of the most attractive theories attributes this ability to the rapid rate at which the parasite can replace its outer tegumental membrane (McLaren, 1980). By most estimates, this process occurs with a half-time of 1.5-4.0 hr (Wilson and Barnes, 1977; Kusel et al., 1975). Furthermore, Kemp et al. (1980) have shown that fluorescent-labelled antibodies directed against surface antigens on schistosomes are actively shed from the surface of the parasite with a half-life of less than 20 min, implicating the existence of a specific turnover mechanism that operates even more rapidly. In their study, elimination

of potentially harmful antibodies was inhibited by 10^{-5}M cytochalasin B. This suggests a role for microfilaments in this process. No data are reported in their study, however, regarding changes in cytotoxicity resulting from drug-induced inhibition of antibody-elimination. The fact that cytochalasin B did not act synergistically with the immune treatments used in the present study argues against the importance of this process in immune evasion by the adult schistosome. Results of surface-labelling experiments that we present, showing cytochalasin B, colchicine and puromycin all to be ineffectual in altering the rate of tegumental protein turnover suggest that this process in the parasite may not be contingent on reactions inhibited by these substances. Alternatively, the cytochalasin B-sensitive mechanism which mediated the release of the antischistosome antibodies used by Kemp et al. (1980) may differ from that involved with the normal turnover of endogenous tegumental proteins.

Kusel (1972) has shown that 200 μg/ml puromycin inhibits approximately 90% of new protein formation in adult S. mansoni. Freshly transformed schistosomula incubated in the presence of this concentration of the drug showed less serum-induced protection against the lethal effects of antibody plus complement upon in vitro incubations than did controls (Tavares et al., 1978). This suggests that new protein formation is important in the immune-evasion process of the immature schistosome. Results of our incubations using 200 μg/ml puromycin in conjunction with the immune treatments, however, indicate that synthesis of new proteins may be less important as a mediator of immune-evasion to the adult stage of the parasite. Again, though, it is possible that inhibition of protein synthesis for longer periods

might result in changes that would render the parasite more susceptible to immune effectors. Future studies should address that possibility more thoroughly, and further explore the possibility that the schistosome's immune-evasion mechanisms might be disrupted by drugs that interfere with cytoskeletal components in the parasite.

GENERAL DISCUSSION

The Electrophysiological Assay

Electrophysiological techniques have proven to be powerful analytical tools for elucidating nerve, muscle, and epithelial cell function in a wide variety of vertebrate and invertebrate preparations. Prior to the work of Fetterer et al. (1977), however, no electrophysiological studies on the schistosome had been undertaken. In their 1977 study, Fetterer et al. reported that volume conducted electrical transients originating from within S. mansoni and recorded by way of suction electrodes appeared to correlate with the motility of the parasite. That is, in simultaneous recordings, peristaltic-like contractions of the parasite's longitudinal musculature were observed to be temporally associated with large multiphasic electrical potentials. This finding suggested that membrane electrical events recorded from the surface were originating from muscle tissue beneath the tegument. In a subsequent study, spontaneous membrane depolarizations were recorded using microelectrodes inserted directly into the tegumental syncytium (Fetterer et al., 1980). This finding suggested that the tegument itself may be an excitable epithelium capable of giving rise to the electrical potentials recorded from the surface. Other preparations in which excitable epithelia had been isolated include newt gut (Shiba, 1970),

insect salivary glands (Caveney, 1974), and the exumbrellar epithelium of hydromedusae (Josephson and Schwab, 1979).

Determination of the precise origin(s) of the electrical potentials recorded from the surface of adult S. mansoni has been further complicated by recent findings that the tegument and underlying muscle tissue in the parasite are electrically coupled in a nonrectifying manner, with a coupling ratio of 0.85 (Thompson et al., 1982a). Low resistance pathways connecting the two tissue compartments assure that electrical signals originating from within muscle will simultaneously be conducted into the tegument and vice versa. Those studies also revealed that both the tegument and muscle give rise to active membrane responses when injected with positive current, further suggesting that either tissue could be the locus of the spontaneous surface electrical potentials.

Regardless of their origin, however, the endogenous electrical potentials have been shown to be exquisitely sensitive to a number of agents that affect the neuromuscular system of adult S. mansoni. For example, serotonin, acetylcholine, and dopamine are putative neurotransmitters in the schistosome (Barker et al., 1966; Bennett et al., 1969, 1971, 1973; Hillman et al., 1973; Tomosky et al., 1974) that modify the surface transients in the adult worm (Pax et al., 1981; Semeyn et al., 1982; Mellin et al., 1983). Semeyn et al. (1982) have shown that these substances as well as some of their analogs significantly alter the frequency and/or magnitude of electrical potentials in the adult worm at concentrations as low as 1×10^{-8}M. These drug concentrations are 100 to 1,000 times lower than the levels at which their

effects can be detected by measuring motor activity. Other substances shown to modify this parameter in the adult parasite include anti-schistosomal compounds such as oltipraz (D. Morrison, personal communication) and praziquantel (D. Thompson, personal observations). While the ionic dependence of these potentials has not been determined, their frequency is significantly affected by alterations in the concentrations of inorganic ions shown to interfere specifically with synaptic transmission in other preparations (Katz and Miledi, 1965), i.e., decreased Ca^{++} (0.14 mM), increased Mg^{++} (30 mM), or the presence of Co^{++} (1 mM) (Semeyn et al., 1982).

In the present study, the method of Fetterer et al. (1977) was modified to facilitate the recording of electrical activity from the surface of immature S. mansoni. This activity closely resembled that recorded from the adult stage. Under control conditions, the frequency and mean amplitude of potentials recorded from the cercaria and schistosomulum stages were only 10-15% lower than those recorded from the adult. With few exceptions, drugs that altered electrical activity in the adult showed similar patterns of effectiveness on the immature stages.

The physiological assay developed in the present study for monitoring the effects of drugs and immune substances on the immature schistosome has several advantages over techniques previously used to study these organisms. First, and perhaps most important, the electrophysiological assay is extremely sensitive, and capable of detecting changes in membrane function much faster than conventional assays that generally measure endpoints that depend on parasite mortality, e.g., vital dye uptake, swelling, or membrane degradation. Using the in

vitro electrophysiological assay in a blind study designed to test the complement mediated effects of anti-schistosome monoclonal antibodies (produced in the laboratory of Dr. Michael Phillips, University of Pennsylvania), we were able to identify with over 90% accuracy monoclones with demonstrated *in vivo* effectiveness against immature *S. mansoni* (Zodda *et al.*, 1982). The *in vivo* assay used by Zodda *et al.* to determine the effectiveness of these monoclones was based on a recovery technique that required 8-12 days of exposure.

A second major advantage of the electrophysiological assay is its objectiveness. Assays used in a number of laboratories rely extensively on visual analysis of large numbers of parasites; swelling or the absence of parasite motility are generally used as criteria for drug or immune substance effectiveness (Kassis *et al.*, 1979; Torpier *et al.*, 1979; McLaren *et al.*, 1982; Van Pijkeren *et al.*, 1982). However, we have found that the motility level of immature stages of the parasite is often a poor indicator of the parasite's vitality. That is, unless disturbed, the cercaria and schistosomulum tend to attach themselves to the base of observation or recording chambers and remain relatively motionless for extended periods. Similar observations have been reported elsewhere (Haas and Schmitt, 1982). Once perturbed by electrode attachment, however, previously nonmotile parasites often show extremely high levels of motility concomitant with high levels of endogenous electrical activity. Similarly, minor changes in the temperature of the recording media often alter motility and/or the body shape of immature parasites, both of which could also lead to inaccurate subjective evaluations of parasite vitality.

The high potential for quantitative analysis offered by the electrophysiological assay is an additional advantage of this system. By passing the electrical signal recorded from the parasites directly to the computer (after analog-to-digital conversion), it is possible to analyze the raw data almost immediately in a quantitative fashion. Thus, we were able to ascertain the statistical significance of various treatment effects on the parasite while they were occurring. Furthermore, the inter-experiment consistency of the electrophysiological parameters in control parasites was remarkable, so it is possible to quickly obtain a fairly reliable indication of a treatment's effectiveness by measuring the activity in a small number of organisms.

A fourth advantage of the electrophysiological assay is that, unlike other assays used, it measures a fundamental and dynamic physiological property of the parasite. In other preparations where intracellular voltage clamping has been possible, it has been demonstrated that volume conducted electrical potentials recorded extracellularly represent current flow in response to specific ionic conductance changes across membranes (de No, 1952; Jack et al., 1975). Current flow which follows these conductance changes depends, in turn, on ionic gradients which appear to be energy dependent in the schistosome (Fetterer et al., 1980) as they are in other preparations. Distinguishing the effects on endogenous electrical activity in the schistosome brought about by changes in energy-dependent ionic gradients from those due to effects on specific ionic conductance channels will require additional studies. It is likely, though, that the effects of some agents on the electrophysiological activity in schistosomes reflect changes in both. Serotonin, for instance, is a putative

excitatory neurotransmitter in schistosomes (Bennett et al., 1969) that induces a significant increase in the frequency and magnitude of the surface potentials when administered in low doses (Semeyn et al., 1982). This substance has also been shown, however, to stimulate glycolysis and ATP production in homogenates of several organisms, including F. hepatica and S. mansoni (Abrahams et al., 1976). Therefore, the stimulatory effects of the serotonin on electrical activity in the adult schistosome could be due to a direct neurotransmitter action of the substance on muscle or nerve, or to indirect actions via the parasite's energy metabolism, or both.

In the context of possible ambiguities that may arise in interpretation of the surface transients, it is relevant to note that the volume conducted responses are less precise and therefore less informative, perhaps, than those recorded intracellularly using microelectrodes. However, while it has been possible to learn a great deal about the tegument and neuromuscular physiology of the adult parasite by intracellular techniques (Fetterer et al., 1980a,b; Bricker et al., 1982; Thompson et al., 1982a), the size of the cercaria and schistosomulum has made similar studies on these organisms much less feasible. To date, microelectrode studies on the schistosomulum have been limited to measurements of an internal potential (of unknown origin) and the input resistance associated with that electrical compartment (see Appendix for a summary of these data, Thompson et al., 1982b).

Implications of the Study

Results obtained in the present study that show skin- and mechanically-derived schistosomula to respond similarly to a wide range of

experimental substances corroborates previous morphological studies that suggest the artificially transformed parasites are suitable material for investigation (Brink et al., 1977; Cousin et al., 1981). The stage-dependent differences we recorded in response to some substances can be explained on the basis of developmental changes in the parasites already known to occur. For example, the schistosomulum showed a much smaller increase in potential frequency and magnitude in response to 10^{-5}M serotonin than that shown in the adult parasite (Semeyn et al., 1982). Abrahams et al. (1976) showed that 5HT levels in the adult worm are up to 5 times higher (per mg protein) than levels in the freshly transformed schistosomulum, indicating that this substance may play a less important role during the early stages of development. Similarly, the absence of detectable effects on the adult stage of the parasite brought about by incubations in antischistosome antibody plus complement correlates with previous reports that indicate the parasite rapidly loses surface antigens and C-3 acceptor sites during development (Samuelson et al., 1980), so that by 4 days after penetration very little binding of antibody or complement occurs (McLaren, 1978; Sher, 1982).

A number of important implications can be drawn from results obtained in the present study on exogenous glucose metabolism and cyanide sensitivity. Those pertaining to the timecourse of this conversion are especially important. For instance, the fact that the conversion away from a cyanide-sensitive metabolism appears to be complete by 24 hr after transformation would be an important consideration in the development of chemotherapeutics designed to interfere with

aerobic enzymes or intermediates in schistosomes. The fact that this conversion was not delayed or otherwise altered by inhibition of protein synthesis implies that it is regulated by mechanisms other than the synthesis of new proteins. A likely alternative might be the activation of already existing glycolytic enzymes and/or the inactivation of TCA cycle enzymes.

The metabolic studies also revealed that electrophysiological activity in the parasite is extremely sensitive to the energy state of the organism. Based on the relative levels of CO_2 and lactic acid produced, it would appear that the 3 hr schistosomulum derives less than 25% of its energy via oxidative phosphorylation, yet cyanide-induced inhibition of the terminal oxidase in this stage of the organism resulted in a 73% reduction in the frequency of the endogenous transients. Although the adult showed no cyanide-induced depression of physiological activity, biochemical analysis of metabolic byproducts showed that this stage of the organism derived less than 2% of its energy aerobically. Conversely, antimony-induced inhibition of glycolysis in the adult S. mansoni resulted in a pronounced depression of the physiological activity, equal in magnitude to that induced by 10^{-3}M cyanide or 10^{-4}M antimony in the immature stages. The results of mechanical recordings that showed 50-60% reductions in the responsiveness of longitudinal muscle to inotropic agents after a 1 hr incubation in 10^{-4}M antimony are consistent with the observed electrophysiological responses to metabolic inhibition.

Results pertaining to the physiological responses of adult S. mansoni to anti-schistosome antibody plus complement simply underscore the mature organism's ability to evade immune-induced disruption. The

fact that puromycin, cytochalasin B, and colchicine were all apparently ineffective in reducing the parasite's immune-evasion capabilities argues against the notion that processes inhibited by these drugs are essential components to the process, at least over short-term in vitro exposures. Therefore, the results we obtained indicate that several popular but untested theories regarding the mechanisms by which adult schistosomes evade host immune responses may be incorrect. That is, if the parasite relies on a cytochalasin B-sensitive mechanism for rapidly sloughing off anti-schistosome antibodies (Kemp et al., 1980), then the parasite should respond to concurrent exposure to these substances. Similarly, if the mechanism of evasion involves synthesis of host-like proteins which are inserted into the outer tegumental membrane (Damian et al., 1973), then inhibition of protein synthesis by puromycin (Kusel, 1972; Tavares et al., 1978) should also render the parasite more susceptible. Finally, if the parasite's ability to rapidly insert new membrane segments from multilaminate vesicles into its covering is an essential feature of the immune evasion process, then inhibition of this process by concanavalin A (Wilson and Barnes, 1978) should also render the schistosome more susceptible to the lethal effects of antibody plus complement. In our studies, however, none of these treatments appeared to compromise the adult parasite's ability to evade the effects of antischistosome antibody plus complement, which were readily apparent after similar incubations of the 3 hr schistosomulum.

It is important to recognize that there are a number of limitations associated with in vitro studies of the type used in this study. Previous studies have shown that, in vivo, schistosomes are challenged

by a number of innate, specific, and nonspecific host-protective mechanisms (McLaren, 1980), most of which were circumvented in the present *in vitro* study. For example, in the present study, parasites were not exposed to any of the cellular components of immunity which may play a major role in the *in vivo* response (McLaren, 1980). These factors may be even more important in considering results obtained in recordings from the artifically-transformed schistosomulum. That is, the mechanically-transformed schistosomula were not exposed at all to the innate mechanisms associated with the host's integument which normally damage or kill a number of the parasites during the penetration process. Clegg and Smithers (1968) reported that a large portion of invading schistosomula may become damaged or killed within 15 min after skin penetration in several laboratory hosts, including mice (20%) and rats (50%). Ghandour and Ibrahim (1978) have suggested that the early phase of high parasite mortality may be attributed to the exhaustion of energy reserves by the parasite during the penetration process. Consistent with that hypothesis is the work of Lewert and Mandlowitz (1963) that showed older mice with highly polymerized and therefore more enzyme resistant skin barriers were less suceptible to infection by *S. mansoni* than young mice. Alternatively, damage from the parasite's own degradative enzymes has also been implicated in the early phase of high parasite attrition (Bruce *et al.*, 1970). These potentially harmful factors were bypassed entirely by the mechanically-transformed schistosomula in the present study. However, the fact that skin- and mechanically-transformed schistosomula responded similarly to all of the substances tested argues against the notion that the penetration process *per se* promotes physiologically significant changes in

the parasite that are significantly different from those brought about by mechanical decaudation. Results of the comparative studies between these two groups of organisms must be interpreted with caution, however, as it is possible that by using only those skin-derived parasites which survived the penetration process, we were artificially selecting a subpopulation of organisms with distinctive characteristics.

More surprising, perhaps, than the apparent absence of drug-immune substance synergism in studies of the adult parasite was the fact that detectable physiological changes did not accompany extensive alterations in tegumental morphology brought about by exposure to cytochalasin B and colchicine. These results corroborate previous findings (Depenbusch et al., 1983) that showed normal muscle function in adult S. mansoni after removal of the parasite's tegument with Triton X-100.

In contrast to the lack of physiological responses shown by the adult parasite after incubations in cytochalasin B, colchicine, puromycin, and/or the immune treatments, the effects of trifluoperazine and poly-l-arginine were rapid and pronounced. The effects of trifluoperazine on the schistosome may be explained on the basis of that drug's capacity for inhibiting the function of the Ca^{++}-binding protein calmodulin, which may regulate a number of Ca^{++}-dependent processes in the parasite as it is believed to do in other preparations (Means and Dedman, 1980). Subsequent studies have revealed that this regulatory protein is present in schistosomes at a concentration similar to that found in mouse kidney, or about 0.7% of the parasite's total protein content (D. Thompson, personal observations).

SUMMARY

1) Spontaneous endogenous electrical activity can be recorded from the surface of immature stages of Schistosoma mansoni using micro-forged glass suction electrodes. This electrical activity is a complex of multiphasic potentials occurring at a rate of 30-40/sec, with spike amplitudes ranging from 20-200 μV and rise times on the order of 3-8 μV/msec. There is an exponential decrease in the frequency of these potentials with increasing potential amplitude, and the largest of these potentials appear to correlate with gross peristaltic-like contractions of the parasite's longitudinal musculature.

2) Electrophysiological activity in the schistosomulum is highly sensitive to a number of agents, including putative neurotransmitters, anthelmintics, metabolic inhibitors, and antiserum plus complement. A number of agents tested, however, including carbachol, dopamine, and serotonin are less effective in altering electrical activity in the immature parasite than they are in the adult.

3) Under control conditions as well as all experimental conditions tested in the present study, electrophysiological responses in mechanically-transformed schistosomula were similar to those recorded in skin-transformed worms, indicating that the

mechanically-derived parasites are appropriate material for physiological and biochemical investigation.

4) The freshly transformed schistosomulum is extremely sensitive to the effects of a 24 hr incubation in medium containing anti-schistosome antibody plus fresh guinea pig complement. Antibody or complement alone, however, do not effect the electrophysiological activity. Depression of the electrophysiological activity is not observed when schistosomula are preincubated 24 hr in control medium prior to their exposure to the immune substances.

5) Electrophysiological recordings indicate that the parasite undergoes a rapid conversion after transformation to the schistosomulum stage, from a state that is physiologically cyanide-sensitive to one that is insensitive to this respiratory inhibitor. While preincubation in 10^{-3}M cyanide for 1 hr depresses the frequency of electrical potentials by 70-85% in the 3 hr schistosomulum, this treatment does not affect the activity in 24 hr parasites. Electrical activity in all stages of the parasite is depressed by 65-75% after 1 hr incubations in 10^{-4}M antimony tartrate, an inhibitor of phosphofructokinase in schistosomes.

6) Biochemical analyses using ^{14}C-labelled glucose indicate that the 3 hr schistosomulum derives a significant portion of its energy aerobically. CO_2 levels at this stage of development are reduced by 85% in the presence of 10^{-3}M cyanide. By 24 hr after transformation, evolution of CO_2 under control conditions is reduced by 77% from 3 hr levels, while lactic acid excretion increases 84%, to a level approaching that measured in the adult parasite. The

presence of 200 μg/ml puromycin in the incubation medium does not retard or otherwise alter this biochemical conversion.

7) The adult schistosome evolves a low level of CO_2 which is reduced by 88% in the presence of 10^{-3}M cyanide. At this stage, however, exposure to cyanide for periods of 24 hr depresses none of the physiological parameters measured in the present study. Neither the adult parasite nor any of the immature stages tested in the present study exhibit a significant Pasteur effect in response to cyanide exposure.

8) Incubation of adult S. mansoni in 10^{-5}M cytochalasin B or 10^{-4}M colchicine for 24 hr results in anatomical disruption of the tegument that is more pronounced in parasites exposed to the former drug. This effect, however, is not accompanied by significant changes in a number of physiological parameters related to tegument and muscle function.

9) Incubation of the adult parasite in puromycin at levels sufficient to inhibit 95-98% of new protein synthesis does not alter tegument morphology or any of the physiological parameters measured.

10) Incubation of the adult parasite in antiserum plus fresh guinea pig complement for 24 hr does not induce morphological or physiological changes in the worm, and concomitant exposure to 200 μg/ml puromycin yields no additional changes. The tegumental disruption brought about by 10^{-5}M cytochalasin B or 10^{-4}M colchicine does not appear to be exacerbated by concomitant exposure to the immune substances, nor do these drugs appear to synergize with the immune substances to initiate detectable physiological changes.

11) The adult schistosome is highly sensitive to poly-l-arginine and trifluoperzine. Both substances induce a dose-dependent depression of electrophysiological activity and longitudinal muscle contractility. These changes may be explained on the basis of the ability of these drugs to alter membrane permeability in the parasite, as evidenced by the rapid depolarization of the tegument potential that they induce. Exposure to either of these drugs for 2 hr results in pronounced "blebbing" of the tegumental surface that is similar to that brought about by cytochalasin B. Exposure to these substances for 24 hr results in a more extensive disruption of the tegument, so that large portions of the syncytium appear to be removed from the parasite.

12) The physiological effects of low doses of poly-l-arginine and trifluoperazine do not appear to be exacerbated by concomitant exposure to antiserum plus complement.

APPENDIX

Microelectrode Recordings from Schistosomula

Treatment	Exposure	Membrane Potential (mV)	Input Resistant (MOhms)
Control (RPMI-1640)	1 hr	-24.6±0.7	6.2±1.1
60 mM K^+ HBS	1 hr	-20.0±1.4[a]	5.8±1.3
$1x10^{-4}$M Ouabain	1 hr	-17.1±1.1[a]	5.9±0.8
$1x10^{-4}$M Carbachol	1 hr	-22.5±1.3	ND
10% Antibody D-6	4-6 hr	-25.0±0.8	6.1±1.3
10% D-6 + Complement	4-6 hr	-16.8±3.0[a]	3.9±1.4[a]

For all treatments N=8; values expressed are means ± SE; (a) signifies $p<.05$.

Intracellular recordings were obtained from 3-6 hr mechanically-transformed schistosomula according to the methods of Thompson et al. (1982a). These recordings revealed the existence of an internal potential in these organisms which was sensitive to a number of agents including ouabain and immune serum plus complement. Because of the size and high level of motility exhibited by the parasites, microelectrode recordings were difficult to obtain, and the precise origin of the potential remains unknown.

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