THE BIOCHEMICAL CHARACTERISTICS AND PHYSIOLOGICAL FUNCTION OF PHENOL OXIDASE IN SCHISTOSOMA MANSONI

By

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ABSTRACT

THE BIOCHEMICAL CHARACTERISTICS AND PHYSIOLOGICAL FUNCTION OF PHENOL OXIDASE IN SCHISTOSOMA MANSONI

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The schistosome egg appears to be primarily responsible for the development of most of the pathology associated with schistosomiasis. One component of the egg which has been demonstrated to be critical to the development of the pathology of the disease is the eggshell. It is the purpose of this thesis to establish the mechanism by which the eggshell is formed in *Schistosoma mansoni* and to evaluate the effectiveness of inhibiting this process in order to alleviate schistosomal pathology.

Female *S. mansoni* were found to possess an enzyme which could be classified as phenol oxidase (E.C. 1.10.3.1). This enzyme was latent and could be activated by *in vitro* incubation in a tissue culture medium or balanced salt solution. Enzyme activity increased from non-detectable levels up to .41±.02 μmoles O_2/min-mg protein. The enzyme was found exclusively in the female schistosome. Activation of the enzyme was not inhibited by 0.1 mM antimony potassium tartrate, a potent inhibitor of ATP synthesis.

The enzyme sedimented in a 1,000 x g pellet and could not be solubilized by treatment with detergents, proteolytic and lipolytic enzymes,

and freezing and thawing. The enzyme was found to have a pH optimum of 7.0 and was inhibited non-competitively by diethyldithiocarbamate (DDC) and allylthiourea. L-Dihydroxyphenylalanine (L-DOPA) was the best substrate (Km = 0.5 mM; Vmax = .338 μmoles O_2/min-mg protein). L-tyrosine and dopamine were good substrates (Km = 0.5 mM and 2 mM; Vmax = .148 and .250 μmoles O_2/min-mg protein, respectively).

Phenol oxidase was localized within the eggshell globules of vitelline cells by fluorescence histochemistry. The fluorescence of the product formed by L-tyrosine methyl ester in this assay was identical to the fluorescence of the product formed from the reaction of phenol oxidase with this substrate *in vitro*.

The concentration of L-tyrosine in the female schistosome (252 ng/mg worm) was 3 times higher than the phenol oxidase Km for L-tyrosine while the concentrations of L-DOPA and dopamine (.954 and .790 ng/mg worm, respectively) were 100 and 500 times less than the Km for these substrates. L-DOPA was not actively accumulated by the female schistosome. The peptide tri-L-tyrosine was oxidized at <3% of the rate of L-tyrosine methyl ester and a tyrosine:lysine peptide was not oxidized at a significant rate. Female *S. mansoni* did not incorporate L-tyrosine preferentially into specific proteins and did not incorporate L-tyrosine into protein to a significantly greater extent than L-leucine. These results suggest that free L-tyrosine is the *in vivo* substrate for *S. mansoni* phenol oxidase.

Inactive analogs of phenol oxidase inhibitors, peroxidase inhibitors, autooxidation inhibitors and inhibitors of lipid peroxidation were incapable of inhibiting formation of the schistosome eggshell at 100

mg/kg *in vivo*. This dose of a phenol oxidase inhibitor caused 100% inhibition of eggshell formation. ^{3}H-Labeled proteins from female schistosomes were polymerized *in vitro* following incubation with *S. mansoni* phenol oxidase and excess ^{3}H-labeled L-tyrosine. Fluorescent substances found in eggshell hydrolysates were similar to those formed from the reaction of phenol oxidase generated quinones with lysine. These observations support a concept of phenol oxidase catalyzed eggshell formation in which phenol oxidase reacts with tyrosine to form a quinone which subsequently reacts non-enzymatically with lysine residues in adjacent proteins to form a cross-linked protein which is one of the major constituents of the *S. mansoni* eggshell.

DEDICATION

To my wife, Ann, without whose support, patience and understanding, this would not have been possible.

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF FIGURES

LIST OF FIGURES (continued)

Figure Page

LIST OF FIGURES (continued)

LIST OF ABBREVIATIONS

ADP - adenosine-diphosphate
ATP - adenosine-triphosphate
BME - Eagle's basal medium
BMEFC - Eagles' basal medium containing 10% fetal calf serum and Earle's salt buffered with .02 M Tris, pH 7.4
DA - dopamine
DOPA - dihydroxyphenylalanine
DOPAC - dihydroxyphenylacetic acid
DDC - sodium diethyldithiocarbamate
H & E - hematoxylin and eosin
HBSS - Hank's balanced salt solution
HVA - homovanillic acid
3MT - 3-methoxytyramine
NE - norepinephrine
NM - normetanephrine
PAS - periodic acid-shift
PCA - perchloric acid
PFP - pentafluoropropanol
PFPA - pentafluoropropionic anhydride
PTTU - 1-phenyl-3-(2-thiazolyl)-2-thiourea
S.E.M. - standard error of the mean
SDS - sodium dodecyl sulfate
SbK - antimony potassium tartrate
TCA - trichloroacetic acid
TCP - tranylcypromine
TME - tyrosine methyl ester
TX-100 - Triton X-100
UK-14624 - 1-phenyl-3-(2-thiazolyl)-2-thiourea

INTRODUCTION

Schistosomiasis is a parasitic disease which has been estimated to infect between 117 and 200 million people throughout the world (Wright, 1968) and infects countless numbers of other animals in the vertebrate kingdom. This disease is particularly prevalent in developing nations where the lack of adequate sanitational faciltles contributes to the maintenance of the life cycle of the schistosome. The life cycle of this parasite is complex, involving three morphologically distinct forms of the parasite and two hosts. In brief, eggs excreted in the urine or feces of the vertebrate host release a free-swimming larval form known as a miracidium, when the eggs come in contact with fresh water. These miracidia then penetrate a snail host wherein they multiply and undergo transformation to an intermediate form known as cercaria (Faust and Hoffman, 1934). The cercariae are subsequently released by the snail whereupon they penetrate a vertebrate host in the water and develop into mature schistosomes in the vertebrate host (Faust *et al.*, 1934).

The methods most commonly used in attempts to control the disease involve both destruction of the snail host and its habitat, and destruction of the parasite within the host (WHO, 1973). However, these attempts have often met with rather limited success (WHO, 1973). The snail has proven to be highly resistant to the effects of molluscidal agents (Duke and Moore, 1976). The effectiveness of chemotherapy in the host has been limited not only by the toxicity of most of the compounds currently in use (WHO, 1965; Katz, 1977; Bueding and Batzinger, 1977) but also by the predilection of the treated population for reinfection (WHO, 1965, 1973). In recent years, some interest has been expressed in the development of treatments which will either block the development of schistosomal pathology (Warren, 1968) or which will inhibit the process of egg production (Campbell and Cuckler, 1967). These two objectives are very similar inasmuch as they deal with the schistosome egg.

A. Importance of the Schistosome Egg to the Development of Schistosomal Pathology

The deposition of schistosome eggs within host tissues is the primary cause of the pathology of schistosomiasis (Lichtenberg, 1955). The formation of a granuloma around each egg deposited in host tissues results in considerable necrosis and scarring of the affected organs. The primary organs involved include the bladder (*S. hematobium*), liver and intestine (*S. mansoni*, *S. japonicum*). The development of these granulomas is linked to the secretion of a number of specific, soluble, maturation-dependent antigens by intact schistosome eggs (Pelley *et al.*, 1976; Hamburger *et al.*, 1976). These antigens are produced by the developing miracidium within the egg and appear to diffuse through the eggshell into the surrounding tissues, providing the stimulus for granuloma formation (Hang *et al.*, 1974). The proteinaceous shell which surrounds the miracidium does not possess antigenic activity and thus is not directly involved in granuloma formation (Boros and Warren, 1970; Lichtenberg and Raslavicious, 1967). However, the intact eggshell has been shown to be critical to the development of the granuloma since it protects the developing micracidium from immunolysis by the host immune system and permits the gradual release of schistosome antigens into host tissues (Boros and Warren, 1970; Hang *et al.*, 1974; Lichtenberg and Raslavicious, 1967).

A number of investigators have attempted to alleviate the necrosis and scarring caused by granuloma formation through immunosuppression of the granuloma (Domingo and Warren, 1967, 1968; Mahmoud and Warren, 1974). However, it has recently been reported that in the absence of granuloma formation, freely diffusing schistosome egg antigens have a cytotoxic action on host tissues (Byram and Lichtenberg, 1977). An alternative approach to the alleviation of this pathology is the elimination of the source of granuloma formation by suppression of egg production. Schistosomes which no longer produce eggs can no longer contribute to the miracidial pool which is essential to the maintenance of the life cycle (Duke and Moore, 1976). Thus, the suppression of egg production has the dual effect of alleviating the pathology associated with schistosomiasis and interrupting transmission of the disease.

One aspect of egg production which has proven to be particularly susceptible to drug action has been the process of eggshell formation. In recent years, thiosinamine (Machado *et al.*, 1970), dapsone (Katz, 1977), and disulfiram (Bennett and Gianutsos, 1978) have been noted to inhibit eggshell formation in *S. mansoni*. It has been suggested that these drugs inhibit eggshell formation by inhibiting an enzyme known as phenol oxidase. This enzyme is responsible for the hardening (sclerotization) of proteins in a wide variety of invertebrate species (Pryor, 1962) and has previously been suggested to be responsible for the process of eggshell formation in the trematodes (Stephenson, 1947; Smyth, 1954). However, at this time there is little evidence to support the concept of phenol oxidase catalyzed eggshell formation in schistosomes.

It is the purpose of this thesis to establish the mechanism by which the eggshell is formed in *S. mansoni* and to evaluate the effectiveness of inhibiting this process in order to alleviate the pathology of schistosomiasis.

B. Eggshell Formation

The morphology and biochemistry of eggshell formation has been described in a number of trematodes, including *S. mansoni* (Stephenson, 1947; Gonnert, 1955; Rao, 1959; Smyth and Clegg, 1959; Nollen, 1968). The bulk of this work has been done with the trematode *Fasciola hepatica* (Stephenson, 1947; Rao, 1959; Smyth and Clegg, 1959). Thus, much of the following description is based on observations of eggshell formation in *F. hepatica*, although it appears that this process is quite similar in *S. mansoni* (Gonnert, 1955).

1. Morphology

The anterior portion of the female schistosome (approximately one-fifth of its total length) contains the ovary, the ootype--an enlargement of the oviductal canal in the vicinity of Mehlis' gland, the uterus and the uterine canal (Figure 1) (Gonnert, 1955). The posterior four-fifths of the schistosome is composed primarily of the vitelline glands and the associated ducts responsible for the transport of vitelline cells to the uterus. In the process of eggshell formation, vitelline cells are combined with the ovum (egg) in the ootype whereupon

Figure 1. Anatomical features associated with egg production in female *S. mansoni* (adapted from Gonnert, 1955).

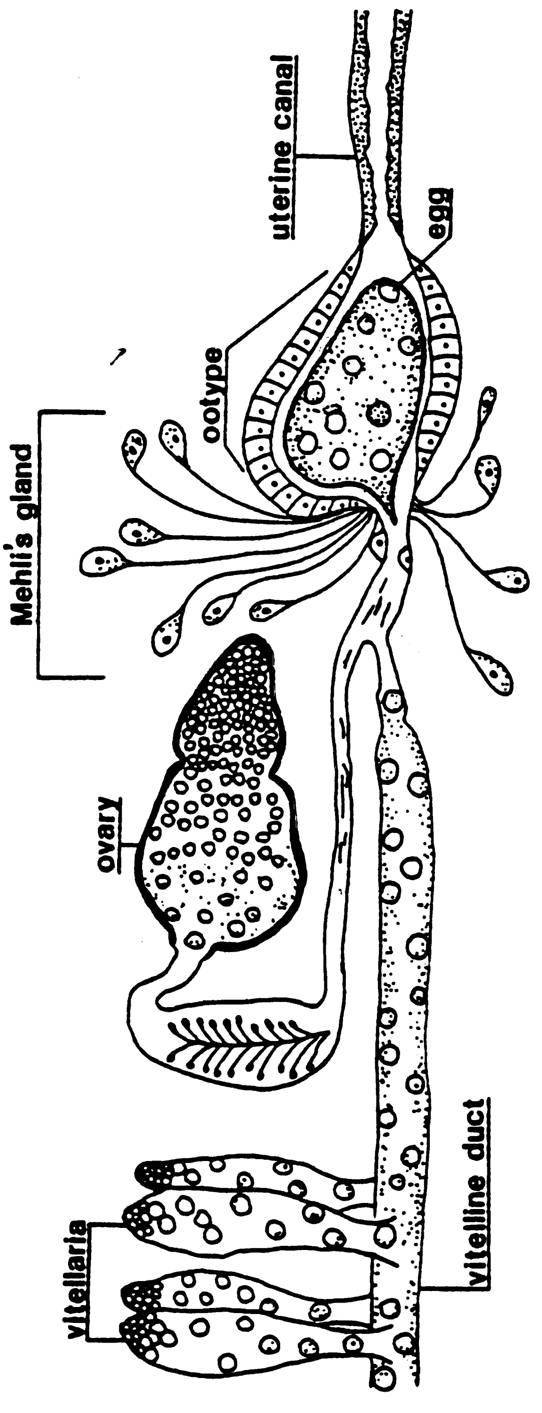

uterine canal
egg
ootype
Mehli's gland
ovary
vitellaria
vitelline duct

Figure 1

they release from their cytoplasm numerous subcellular particles which are often referred to as vitelline droplets. These particles contain large quantities of membranes which histochemically react with stains for phenolic substances (Stephenson, 1947; Smyth and Clegg, 1959). Mehlis' gland, which empties into the ootype, may be responsible for the release of the vitelline droplets, but its function has not adequately been defined (Rao, 1959; Clegg, 1965; Clegg and Morgan, 1966). Following their release, the droplets coalesce to form a thin membrane around the surface of the uterus. At this time, formation of the eggshell appears to occur. The formation of the hardened shell is associated with the appearance of a dramatic yellow fluorescence which is characteristic of the eggshell (Kelley and Lichtenberg, 1970). Acid hydrolysis of _F. hepatica_ eggshells has shown them to be composed almost entirely of amino acids (Rainsford, 1967). Thus, the eggshell appears to be composed primarily, of extensively cross-linked proteins to which some fluorescent chromagen may be attached. This cross-linked protein is highly resistant to immunolytic (Stenger _et al._, 1967) and chemical degradation (Stephenson, 1947). It is this resistance to immunolytic degradation which is responsible for the persistance of the egg within host tissues and which thus contributes significantly to the development of the granuloma.

2. _Biochemistry_

The mechanism by which the schistosome eggshell is formed has been proposed to involve the process of quinone-tanning of eggshell precursor proteins (Smyth and Clegg, 1959; Stephenson, 1947). According to this theory, phenol oxidase catalyzes the oxidation of an ortho-phenol to an ortho-quinone. The ortho-quinone subsequently reacts with basic amino acids in adjacent proteins to form a cross-link. This concept of quinone-tanning in the process of shell-hardening was first introduced by Pryor (1940). In this study, phenol oxidase, phenolic substances and basic proteins were demonstrated to be intimately associated with the process of cuticular hardening in the cockroach _Blatta_ _Orientales_. Mixing of the phenol (3,4-dihydroxybenzoic acid; Pryor _et_ _al_., 1946) with phenol oxidase produced a reddish color which is characteristic of the reaction of quinones with free amines (Pugh and Raper, 1927). The appearance of this reddish color was associated with the disappearance of histochemical staining characteristic of free amines (i.e., basic amino acids) in the cuticular proteins of _Blatta_, and was also associated with the onset of cuticular hardening. Considerable evidence has accumulated in the ensuing years which has strongly supported this concept of quinone-mediated protein cross-link formation. A brief review and discussion of this evidence follows.

a. _Reactions of quinones with amino acids and proteins_

The quinones are a group of highly reactive substances which readily react non-enzymatically with a number of nucleophilic moieties including primary and secondary amines and thiols (for a comprehensive review of this subject, see Mason, 1955). The reactions of the amines with quinones characteristically results in the generation of numerous colored products which can be either amino- or imino-quinones (Figure 2) (Pugh and Raper, 1927; Hackman and Todd, 1953; Jackson and Kendall, 1949). The apparent ease with which one quinone molecule reacts with several nucleophilic ligands (Figure 2) makes the quinones ideal intermediates in the formation of protein cross-links.

Figure 2. Reactions of benzoquinones with amines. A: Pugh and Raper, 1927. B and C: Hackman and Todd, 1953.

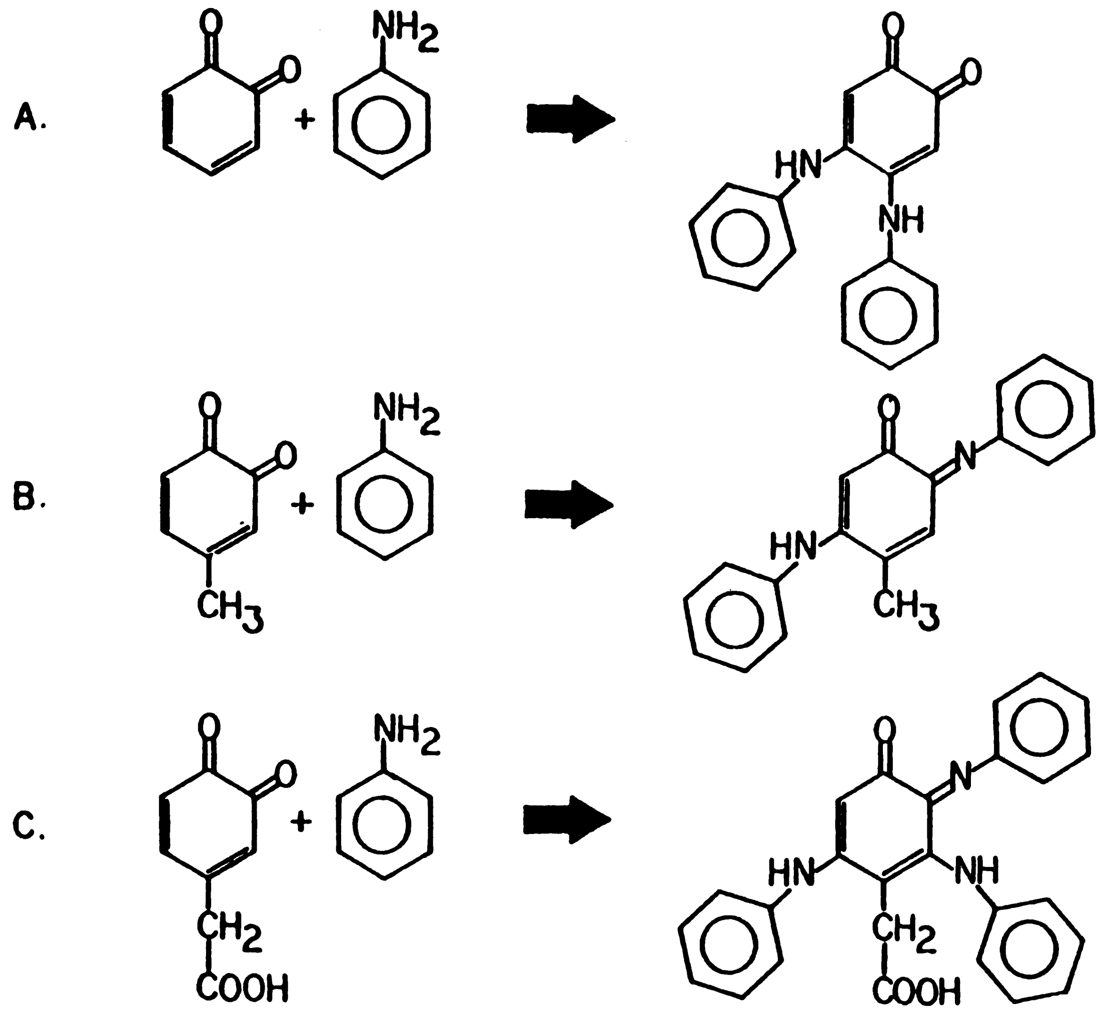


Figure 2

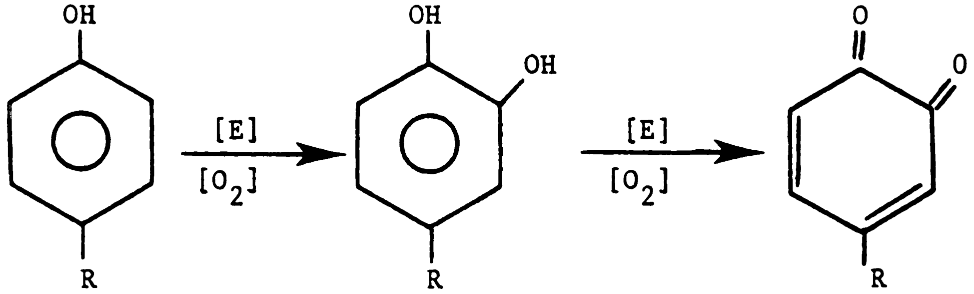
Following the reaction of a quinone with a nucleophilic side chain from one protein, the quinone reacts with a nucleophilic side chain from an adjacent protein in order to form a cross-linked protein. The types of nucleophiles which readily react with quinones ($R-NH_2$, R-SH) are commonly found in proteins (e.g., lysine, histidine and cysteine). In addition, proteins in solution (most notably insect cuticular proteins) develop color upon the addition of quinones (Mason and Peterson, 1965; Hackman, 1953). These quinoid proteins appear to have an absorption maximum around 480 nm which is characteristic of the products formed from the reactions of many amino acids with ortho-quinones (Mason and Peterson, 1965). In the absence of reactive nucleophiles, quinones often cross-react with other quinones to produce melanin, an insoluble, polymeric, acid-stable substance. However, not all reactive sites of the quinones are utilized in this polymer formation (Mason, 1955). Thus, the observation that melanin, _in vivo_, is invariably isolated in a protein bound form is consistent with the known reactivity of quinones with nucleophilic amino acids (Piatelli _et al_., 1962; Seiji _et al_., 1963).

In light of the multiple reactive sites of quinones and their abilities to react with amino acids and proteins, it is not surprising that it has been found that numerous proteins will form insoluble and non-reactive complexes in the presence of quinones generated enzymatically from the reaction of phenol oxidase with a phenolic substrate (Sizer and Brindley, 1951). With regard to the specific process of shell-hardening, Hackman (1953) has demonstrated that water soluble proteins extracted from unhardened insect cuticles will precipitate upon the addition of phenol oxidase and catechol.

Thus, it is apparent that quinone-tanning can be involved in the cross-linking of proteins and the hardening of insect cuticles. However, it is not certain that the presence of phenol oxidase is a necessary requirement for the generation of the quinones which are involved in the process of protein cross-linking.

b. Requirements for the generation of quinones

1) Enzymatic formation of quinones. The *ortho*-quinones, which appear to be capable of mediating protein cross-link formation, are formed from the oxidation of *o*-dihydroxyphenols. *Ortho*-quinones can be generated enzymatically from *o*-dihydroxyphenols by the enzyme phenol oxidase (also known as polyphenoloxidase, tyrosinase, *o*-diphenol:O_2 oxidoreductase, E.C. 1.10.3.1). The chemistry of this well-known enzyme has been extensively reviewed elsewhere (Nelson and Dawson, 1944; Lerner 1953; Brooks and Dawson, 1966). In brief, phenol oxidase catalyzes the following reaction:



This enzyme is a copper containing enzyme and is inhibited by copper chelating agents such as diethyldithiocarbamate and thiourea and is also inhibited by copper complexing agents such as cyanide and various sulfhydryl containing compounds (Dubois *et al.*, 1946; Flesch and Rothman, 1948). Phenol oxidase is somewhat unique in that it can catalyze both the hydroxylation of tyrosine and the oxidation of dihydroxyphenylalanine (DOPA). The only other enzyme which is capable of performing this dual function is peroxidase (Lerner, 1953). However, the phenol oxidase catalyzed hydroxylation of tyrosine is unique in that the end product of tyrosine hydroxylation is both a substrate for the second step of the reaction (DOPA oxidation) and a cofactor for the first step (tyrosine hydroxylation) (Hearing and Ekel, 1976; Pomerantz and Warner, 1967). In contrast, the peroxidative conversion of tyrosine to DOPA is not activated by DOPA and also requires the presence of peroxide. However, not all phenol oxidases possess the capability of converting tyrosine to DOPA. Karlson and Liebau (1961) were able to crystallize a soluble phenol oxidase obtained from the larvae of *Calliphora* which was copper containing and specific only for dihydroxyphenols. Relatively impure preparations of phenol oxidase from insect cuticles have also been found to oxidize only dihydroxyphenols while phenol oxidases purified from the haemolymph of these same organisms oxidizes both mono- and dihydroxyphenols (Mills *et al*., 1968; Ishaaya, 1972). In every case, if monophenol oxidase activity is present, it cannot be separated from diphenoloxidase activity. In those insects where monophenol oxidase activity has not been found, the onset of cuticular hardening has generally been associated with dramatic increases in the activity of enzymes such as tyrosine decarboxylase (Hopkins and Wirtz, 1976) and DOPA-decarboxylase (Wirtz and Hopkins, 1977). These enzymes are involved in the metabolism of tyrosine to a dihydroxyphenol which serves as a substrate for the phenol oxidase of the species in question (*Periplaneta americana*). The subject of tyrosine metabolism as it relates to the process of cuticular hardening in insects has been extensively reviewed by Brunet (1963). In general, it appears that the metabolism of tyrosine within insects is optimized to produce specific phenolic substrates for diphenoloxidases during the process of cuticular sclerotization. Those findings argue against a significant role for peroxidase (another enzyme capable of generating quinones) in the process of quinone-tanning in insects, since peroxidase can produce quinones from a wide variety of substrates (Mason, 1957). However, in cases where the major substrate appears to be tyrosine or DOPA, the situation is not as clear. Both of these compounds serve as substrates or are intermediates in other common metabolic processes such as protein synthesis and/or neurotransmitter synthesis and thus changes in the metabolism of these compounds during sclerotization may be obscured by other biochemical changes not associated with sclerotization. Furthermore, both peroxidase and phenol oxidase can catalyze the formation of quinones from either tyrosine or DOPA. Okun _et al_. (1975) and Patel _et al_. (1974) have obtained evidence which suggests that peroxidase may be primarily responsible for the generation of melanin from tyrosine in mouse melanomas. Thus, it is necessary to rule out this possibility in the elucidation of mechanisms of quinone-mediated protein cross-link formation in which tyrosine or DOPA are considered to be the major _in vivo_ precursors of the quinone. This task should not be difficult since phenol oxidase and peroxidase are readily distinguishable from each other. Peroxidase is an iron containing protein while phenol oxidase is copper containing. Peroxidase requires the presence of hydrogen peroxide in order to catalyze the hydroxylation of tyrosine and the oxidation of DOPA (Mason, 1957; Paul, 1959) while phenol oxidase requires molecular oxygen.

Other enzymes or enzyme systems which have been demonstrated to be able to produce quinones or melanin from phenolic precursors include the cytochrome oxidase system (Hesselbach, 1951) and laccase (Whitehead _et al_., 1960). However, these enzymes have not been adequately demonstrated to play a significant role in the generation of _o_-quinones during protein hardening and thus their role in this process has not been given much credence (Pryor, 1962).

2) _Non-enzymatic formation of quinones_. The _ortho_-quinones can be readily generated from the corresponding _o_-dihydroxyphenols (catechols) by autooxidation. The autooxidation of the catechols occurs rapidly in the presence of excess hydroxide ions, ferric ions, or any suitable hydrogen ion acceptor (Senoh _et al_., 1959: Harrison _et al_., 1968; Hawley _et al_., 1967). The monophenol tyrosine can also be converted to a quinone (DOPA-quinone) in the presence of L-DOPA and cupric or ferric ions (Foster, 1950). However, the autooxidative generation of quinones is also known to produce hydrogen peroxide (James and Weissberger, 1938), a feature which distinguishes it from the enzyme catalyzed formation of quinones. Since it has not been possible to correlate increases in autooxidation with the hardening of the cuticle in insects, the autooxidative formation of quinones is not thought to play a significant role in the quinone-tanning of insect cuticular proteins (Pryor, 1962).

However, it has not always been possible to detect all of the components which are required for the process of protein hardening by quinone-tanning. The component which has been most notably absent in some studies is the phenolic substrate which is necessary for the generation of a quinone (Brown, 1952; Blower, 1951). In such cases, one must consider several alternative hypotheses.

c. Other mechanisms of protein hardening

Protein cross-links can be found in a number of structural proteins including collagen, elastin and fibrin. However, these proteins do not have the same characteristics as the proteins involved in cuticular hardening. Many of these protein structures are somewhat elastic and contain a number of unique amino-acid sequences (Mahler and Cordes, 1971). In addition, the formation of cross links in these protein structures appears to be catalyzed by some very specific cross-linking enzymes such as lysyl oxidase (Siegal et al., 1970) or serum trans-glutaminase in fibrin (Matavic and Loewy, 1968). Thus, cross-links formed by these enzymes are readily distinguished from quinone-formed cross-links both in terms of the characteristics of the protein formed and the enzyme involved.

On the other hand, Brown (1950) and Blower (1951) have proposed that, in the absence of free phenolic substrates, protein bound tyrosine or DOPA is converted to protein bound DOPA-quinone by phenol oxidase. This protein bound quinone subsequently cross reacts with ε-amino groups from lysine molecules in adjacent proteins to form a hardened protein mesh. This mechanism eliminates the necessity of demonstrating free phenolic substrates for phenol oxidase and has been proposed in those cases where these substances have been difficult to identify, although most of the other components and characteristics of quinone-tanned proteins can be readily identified (Brown, 1952; Blower, 1951; Smyth and Clegg, 1959). Evidence to support this notion of protein tanning has come from studies by Hackman (1953) and Yasonobu *et al.* (1959) who have shown that tyrosine rich proteins and polypeptides can serve as substrates for phenol oxidase in the absence of exogenously added phenols. However, although proteins and peptides can serve as substrates for phenol oxidase, they have not been conclusively demonstrated to form cross-links following exposure to phenol oxidase. Pryor (1962) has suggested that the above mechanism is not feasible since steric hindrance would not permit effective attack of the ε-amino group at the reactive site of the protein bound quinone (Figure 3a). Furthermore, Gianutsos and Bennett (1977) have recently demonstrated the presence of high concentrations of the dihydroxyphenol, dopamine (DA), in the egg-producing regions of *F. hepatica* using a specific and sensitive radioenzymatic assay for this compound. Earlier workers had not been able to detect this substance histochemically (Smyth and Clegg, 1959) and had subsequently proposed a mechanism of auto-tanning similar to that proposed by Brown (1950). The recent findings of Gianutsos and Bennett (1977) make it unnecessary to postulate a mechanism of quinone-tanning which involves the action of phenol oxidase on a protein bound substrate. Thus, while it appears possible that phenol oxidase could be involved in the formation of direct protein cross-links, the bulk of the evidence strongly favors cross-link formation mediated by a quinone or quinone-derived polymer.

An alternative mechanism of protein cross-link formation involves the enzyme peroxidase. Peroxidase has previously been mentioned with regard to its ability to catalyze the formation of quinones

Figure 3. Two mechanisms of phenol oxidase catalyzed protein cross-links formation. A) Mechanism of Brown (1950). B) Mechanism of Pryor (1940).

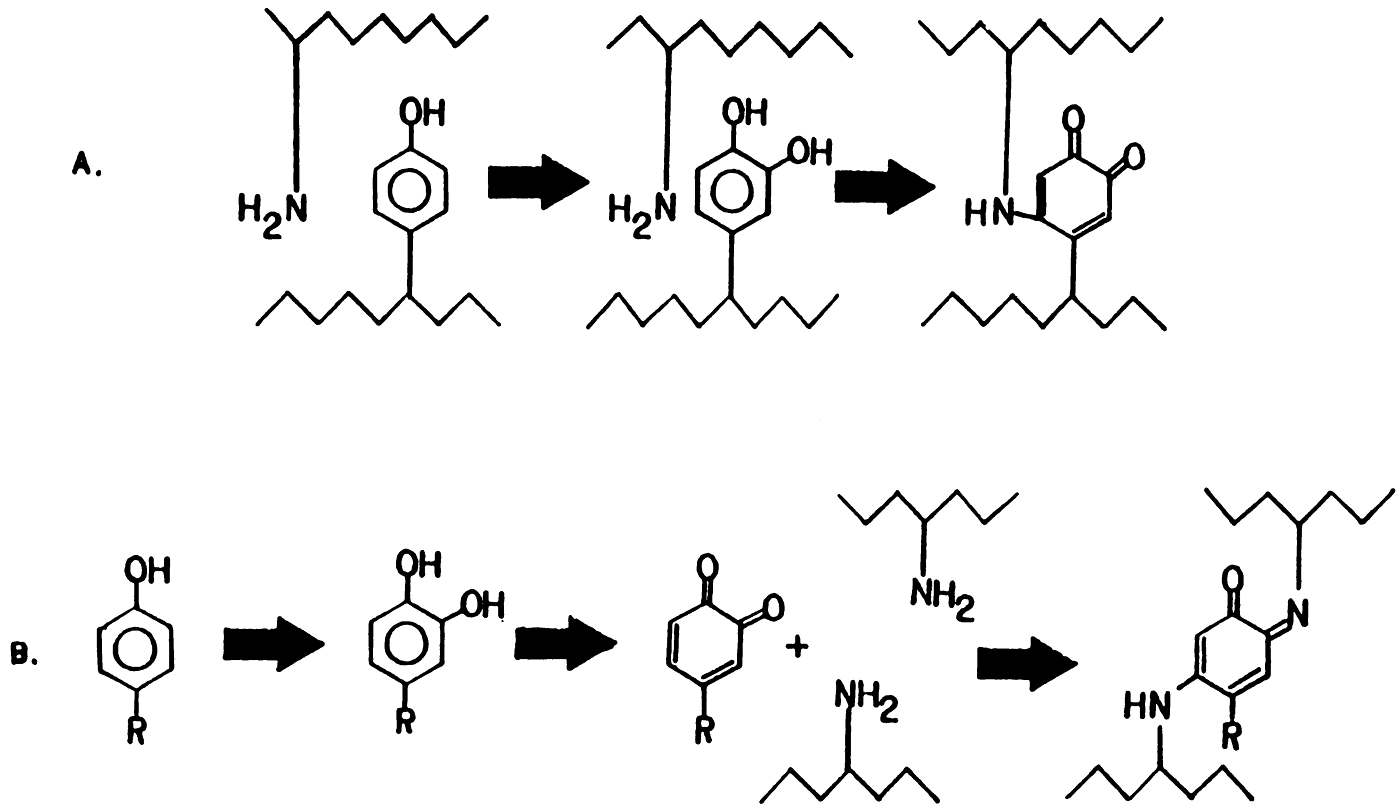


Figure 3

from dihydroxyphenols and tyrosine (Patel _et_ _al_., 1974; Mason, 1957), and thus also catalyze the formation of quinone-linked protein polymers (Stahmann _et_ _al_., 1977). This alternative scheme is based upon the ability of peroxidase to catalyze the formation of dityrosine (Gross and Sizer, 1953). Recent studies by Foerder and Shapiro (1977) and Garcia-Castineiras _et_ _al_. (1978) have demonstrated the presence of dityrosine residues in cross-linked proteins. Aeschbach _et_ _al_. (1976) have recently shown that peroxidase can catalyze the formation of dityrosine from proteins rich in tyrosine residues. Foerder and Shapiro (1977) have shown that the release of peroxidase from sea urchin egg membranes is intimately associated with the production of dityrosine residues and the simultaneous hardening of the eggshell. Thus, it is clear that peroxidase might be involved in the process of protein hardening either in the production of quinones or in the formation of direct dityrosyl links. Resolution of the question of whether phenol oxidase or peroxidase is involved in the formation of the hardened eggshell in _S_. _mansoni_ will depend upon the isolation and characterization of the enzyme or enzymes involved in this process.

Lipid peroxidation has also been implicated in the process of protein cross-link formation (Roubal and Tappel, 1966). However, the mechanism by which free radicals form protein cross-links in a lipid peroxidizing system has not been extensively investigated. Thus, lipid peroxidation has an uncertain potential for being involved in the process of protein hardening.

d. Evidence for quinone-tanning in trematodes

The evidence for quinone-tanning in trematodes is based primarily on histochemical studies (Nollen, 1971; Erasmus, 1975;

Smyth and Clegg, 1959; Clegg and Smyth, 1968; Ramalingan, 1973) with only limited support from biochemical studies in this area (Mansour, 1958; Gianutsos and Bennett, 1977; Bennett and Gianutsos, 1978). While these studies provide a limited amount of information from which the mechanism of eggshell formation might be inferred, the inherent lack of specificity of most histochemical stains does not provide sufficient information to definitively rule out any one mechanism of protein sclerotization.

In *F. hepatica*, histochemical evidence has suggested the presence of phenol oxidase in the vitelline cells of *F. hepatica* (Smyth, 1954) and in freshly laid eggshells (Ramalingam, 1973). However, this histochemical evidence is based simply upon the oxidation of catechol to a red color and thus does not differentiate between phenol oxidase and other enzymes (e.g., peroxidase) which are capable of oxidizing catechol. On the other hand, Mansour (1958) was able to isolate a crude, membrane-bound enzyme in *F. hepatica* homogenates which appeared to possess many of the characteristics of phenol oxidase including some degree of selectivity in its affinity for different phenolic substrates, a characteristic which is not typical of peroxidases (Mason, 1957). In addition, catalase was not found to alter the kinetics of catechol oxidation, thus ruling out the possibility of a contaminating peroxidase. Thus, there appears to be fairly good evidence based on histochemical and biochemical studies which implicates phenol oxidase in the process of eggshell formation in *F. hepatica*. Unfortunately, Mansour (1958) did not recognize the significance of his findings with regard to role of phenol oxide in egg production and hence did not attempt to correlate his *in vitro* data with *in vivo* studies on eggshell formation. Thus, it might still be argued that another enzyme such as peroxidase is involved in the process of eggshell formation (Ramalingham, 1973). Furthermore, although phenol oxidase has been identified in *F. hepatica*, free phenolic substrates for this enzyme have been difficult to identify. Smyth and Clegg (1959) were unable to histochemically identify any phenolic substances in *F. hepatica* vitelline glands except for tyrosyl peptides. In the absence of detectable phenolic substrates these investigators proposed a mechanism for eggshell formation in trematodes based on tanning of protein bound phenols (Figure 3a). However, the observation of Gianutsos and Bennett (1977) that the egg producing regions of *Fasciola* have elevated concentrations of DA, suggested a potential role for this compound in the classical process of quinone tanning described by Pryor (1940). With regard to the role of basic amino acids, Smyth (1954, 1956) and Stephenson (1947) have noted that the staining characteristic of basic amino acid residues gradually disappears as the eggshell becomes hardened in the uterus. Thus, there appears to be a fair amount of evidence which is suggestive of a role for quinone-tanning in the process of eggshell formation in *F. hepatica*. However, Ramalingam (1973) and Wilson (1967) have presented evidence which suggests that dityrosine may be present in hydrolysates of *F. hepatica* eggshells. These findings have created a controversy concerning the role of phenol oxidase and the possible role of peroxidase in eggshell formation in *F. hepatica*.

In *S. mansoni* it has been reported that phenol oxidase may be present in the vitelline cells of the female (Clegg and Smyth, 1968). However, this data has not been published in detailed form. The only histochemical evidence for phenolic substances localized in the vitelline cells of female _S. mansoni_ is based upon a positive diazo test (Johri and Smyth, 1956). Unfortunately, the test is positive for a number of other substances including the aromatic amines (Pearse, 1968) which may be present in the vitelline cells in the form of the basic amino acid histidine. Gianutsos and Bennett (1977) observed that DA was present in higher concentrations in the female than in the male. It was thus suggested that this difference might reflect the importance of this substance to the process of egg production. On the other hand, Erasmus (1975) obtained autoradiographic evidence which suggested that the eggshell globules in the vitelline cells of this trematode avidly incorporated tyrosine into proteins. This investigator suggested that a tyrosine rich peptide could serve as a substrate for _S. mansoni_ phenol oxidase. However, in the absence of detailed biochemical studies, one can conclude very little about the mechanism of eggshell formation in _S. mansoni_ except to say that, based on similarities to the evidence obtained from _F. hepatica_, it might involve the process of quinone-tanning (Clegg and Smyth, 1968). There is insufficient evidence available from studies on other trematodes to permit a discussion of the mechanism of eggshell formation in these species (Nollen, 1971).

C. Proposed Research

Studies by previous investigators (Boros and Warren, 1970; Lichtenberg and Raslavicious, 1967) have established that the presence of an intact eggshell is critical to the development of the egg granuloma which is responsible for most of the pathology associated with schistosomiasis (Lichtenberg, 1955). Eggshell formation can be inhibited reversibly by a number of drugs which are known to inhibit the enzyme phenol oxidase (Machado _et al._, 1970; Bennett and Gianutsos, 1978). It is the purpose of this research to 1) establish the mechanism by which the eggshell is formed, 2) determine the role of the enzyme phenol oxidase in this process, and 3) to evaluate the effects of phenol oxidase inhibitors on the development of schistosomal pathology.

MATERIALS AND METHODS

A. Schistosomes

The schistosomes used in these experiments are derived from a parent stock of *Schistosoma mansoni* (St. Lucian strain) maintained in outbred laboratory mice at the University of Lowell Research Foundation. The mice used in these experiments were outbred (ICR) mice (Spartan Research Animals, Haslett, Mich.) and were infected with 150-200 cercariae by tail immersion or i.p. injection. Paired mature schistosomes (40-60 days post-infection) were surgically removed from the mesenteric veins of infected mice and placed in a solution containing either Eagles Basal medium (BME) (Gibco, Long Island, N.Y.), Earle's salts, .05% sodium pentobarbital (Sigma Chemical Co., St. Louis, Mo.) and .02 M Tris buffer, pH 7.4 or phosphate buffered saline, pH 7.4. Sodium pentobarbital (.05%) was included in these media because paired male and female schistosomes cannot be separated without a muscular relaxant being present in the solution. Male and female *S. mansoni* were separated by gently removing the female with microforceps.

B. Activation of Phenol Oxidase in S. mansoni

Phenol oxidase was activated by the incubation of whole female schistosomes in the same culture medium as described above with 10% heat-inactivated fetal bovine serum and .05% sodium pentobarbital added. Sodium pentobarbital was included in the incubation medium in order to facilitate handling of the female schistosomes. In the absence of pentobarbital the schistosomes tended to cling to the surface of the incubation dish making subsequent removal difficult. Since the presence of pentobarbital also tended to marginally increase the extent of phenol oxidase activation (10-20%), pentobarbital was left in the incubation medium during the period of enzyme activation. From now on this culture medium without pentobarbital will be referred to as BMEFC. In studies on the effects of protein synthesis inhibitors on induction, the inhibitors and radiolabeled amino acids were added to the BMEFC plus pentobarbital prior to the transfer of the schistosomes. In these experiments, the paired schistosomes were transferred directly from the host into BMEFC before being separated. Males were removed from the induction medium immediately following separation from the female.

Some of the drugs and enzymes used in these studies and their sources are: cycloheximide; trypsin, type II; phospholipase A2; protease from *Streptomyces griseus*, type V; Lubrol Wx; Tween 80 (Sigma Chemical Co.); Triton X-100 (Research Products International, Elk Grove, Ill.); antimony potassium tartrate (a gift of Dr. Ernest Bueding); ^{3}H-[2,6]-L-tyrosine, 40-60 Ci/mmole (New England Nuclear, Boston, Mass.); and ^{3}H-[4,5]-L-leucine 50-60 Ci/mmole (Amersham/Searle, Arlington, Heights, Ill.).

C. Phenol Oxidase Assays

1. Standard phenol oxidase assay

Phenol oxidase activity was determined in female *S. mansoni* using a modification of the method described by Mansour (1958) for *Fasciola hepatica*. Forty female schistosomes were placed in a homogenizing vessel containing 2 ml of 0.1 M phosphate buffer pH 6.8 and homogenized with a teflon pestle. The homogenate was centrifuged at 3,000 x g for 5 min. The supernatant was discarded and the pellet was resuspended in 2 ml of phosphate buffer pH 6.8. All of the above procedures were performed at 4°C. The resuspended pellet was placed in a Clark-type oxygen electrode (Rank Brothers, Cambridge, England) maintained at 37°C. Three and a half minutes later substrate was added through a small port at the top of the electrode chamber. Oxygen consumption was measured during the first 5 minutes following addition of the substrate. The amount of oxygen consumed was based on the amount of current generated by the reduction of O_2 at a constant voltage of 0.6 v. The electrode was calibrated as described by Lessler and Brierley (1969) using air-saturated distilled water as a reference (.410 μmoles O_2/ml at 37°C). The data are expressed in terms of μmoles O_2/min-mg protein and represent the initial velocity recorded during the first two minutes of reaction. All substrates used in this assay were obtained from Sigma Chemical Co.

2. <u>Phenol oxidase assay for protein and peptide substrates</u>

Phenol oxidase was activated in eighty mature female schistosomes as previously described. The schistosomes were homogenized in 4 mls of 5% TX-100 buffered with 0.1 M sodium phosphate pH 7.0. Two mls of the homogenate were centrifuged at 3,000 x g. The pellet was resuspended in 2 mls of phosphate buffer and placed in oxygen electrode system maintained at 37°C. L-DOPA was then added to a final concentration of 30 μM. Three minutes following the addition of L-DOPA, 20 μl of a 0.1 M solution of L-tyrosine methylester (TME) was added to the system as substrate. Phenol oxidase activity was measured in terms of the amount of O_2 consumed during a 2 minute period immediately following the addition of the substrate. All reagents in this assay were freshly prepared and maintained at room temperature. Homogenization was performed at 4°C. Centrifugation was performed at room temperature.

The second portion of the 4 ml homogenate described above was treated as in the above protocol, with the exception that the pellet was resuspended in phosphate buffer containing a protein or peptide substrate plus 30 μM L-DOPA. In these studies the activity of the protein and peptide substrates were determined by subtracting the rate of oxidation obtained in the first 3 minutes of assay from the baseline rate of oxidation obtained from the paired sample immediately preceding it. In some studies, TME was added after the 3 min assay period to confirm that the rates of TME oxidation in the two samples were approximately equal. The protein and peptide substrates employed in these studies and their concentrations were, tri-L-tyrosine methyl ester (Sigma Chemical Co.), 2 mg/ml; a tyrosine:lysine polymer (1:1) (Miles Biochemicals, Elkhart, Ind.), a saturated solution in 0.1 M phosphate buffer; chymotrypsinogen A (Sigma Chemical Co.), 1 mg/ml and endogenous proteins from female S. mansoni, 1 mg/ml. The schistosome proteins were prepared as follows: 100 mature, female schistosomes were homogenized in 2 mls of 1% Triton X-100 buffered with 0.1 M sodium phosphate pH 7.0. The homogenate was dialyzed for 24 hrs against 4 liters of phosphate buffer. The buffer was changed twice during the course of dialysis. The dialyzed proteins were then removed and a 2 ml aliquot assayed as a substrate for phenol oxidase. Residual phenol oxidase activity in the homogenate was assayed as described at the beginning of this section.

3. Mushroom phenol oxidase assay

In these studies mushroom phenol oxidase (Sigma Chemical Co.) was substituted for schistosome phenol oxidase. The mushroom enzyme, containing 2750 units/mg dry weight was diluted to a concentration of 30 μg/ml. Twenty μl of this solution was then added to phosphate buffer in the oxygen electrode which already contained all substrates and cofactors. This dilution of the enzyme yielded a rate of TME oxidation which approximated that produced by a 3,000 x g pellet obtained from a homogenate of 80 female schistosomes, which had been activated by 8 hrs incubation in BMEFC (total activity ≃ 2 units). One μM L-DOPA was used as a cofactor in these experiments since 10 μM L-DOPA produced a significant rate of oxygen consumption. One unit of phenol oxidase activity is defined as a change in $O.D._{280}$ of .001/min at pH 6.5, 25°C in a 3.0 ml reaction volume using L-tyrosine as a substrate.

D. Inhibitor Studies

Phenol oxidase inhibitors were obtained from commercial sources as follows: allylthiourea, diethyldithiocarbamate (DDC), disulfiram, 1-phenyl-3-(2-thiazolyl)-2-thiourea (PTTU; UK 14624) (Aldrich Chemical Co., Milwaukee, Wis.); penicillamine, bathocupioure sulfonate, L-cysteine, rotenone, EDTA, phenylthiourea, α,α'-dipyridyl, tranylcypromaine, thiourea, 5-hydroxytryptamine (Sigma Chemical Co.); potassium cyanide (J.T. Baker Chem. Co., Phillipsburg, N.J.). The effects of these inhibitors on phenol oxidase activity was determined by resuspending the 3,000 x g pellet in 0.1 M phosphate buffer containing the desired concentration of the inhibitor to be used. The inhibitor was then incubated with the resuspended pellet for three and a half-minutes prior to addition of the substrate (2 mM DA). Rate constants of inhibition were determined by the method of Dixon (1951). Additional rate constants of inhibition were determined for DDC and allylthiourea by the method of Lineweaver Burk (1938).

E. Histochemistry

1. Catechol method

Phenol oxidase was localized histochemically in whole female worms by a modification of the method of Smyth (1954). Whole female schistosomes were fixed in 95% ethanol for a period of 2 hrs. The worms were subsequently incubated for a period of 15 min in an aqueous solution of 0.2% catechol. The worms were then mounted (wet mount) on a slide and examined under a light microscope for the presence of dark-staining material.

2. Fluorescent method

Whole female *S. mansoni* were placed in Hank's balanced salt solution (HBSS) containing 2 mM TME. The worms were allowed to incubate for a period of 20 minutes, after which they were mounted on a glass slide in a few drops of HBSS and the relative fluorescence intensity of the vitelline cells was determined using a Leitz microspectrofluorimeter. The optics of the microspectrofluorimeter were essentially the same as described by Jonsson (1969). The light source was a high intensity Leitz xenon lamp. Light emitted from this source was passed through a heat filter, a band pass filter (BG-38) which suppressed all emissions below 300 nm and above 650 and a band-pass filter (UG-5) which suppressed all emissions below 220 nm and above 400 nm. The sample on the slide was visualized with epi-illumination.

Light emitted from the sample passed through the objective of the microscope and a 500 nm band-pass filter which suppressed U.V. and visible light reflected off the slide from the xenon lamp. This light was then focussed on a photomultiplier tube and readings of fluorescence intensity were obtained by monitoring the output from the photomultiplier tube. Readings of fluorescence intensity were made at 100X magnification and were obtained from a minimum of 6 different regions of each worm. The average of these values was treated as a single data point representative of the fluorescence over the entire schistosome.

In studies with phenol oxidase inhibitors, the worms were preincubated in HBSS, containing inhibitor, for 10 min prior to being placed in the substrate solution which contained both substrate and inhibitor.

F. L-DOPA and DA Determinations

L-DOPA and DA levels were determined in fresh, mature (50-60 days post-infection), male and female schistosomes by mass fragmentography. Samples of 10 male schistosomes with a wet weight of approximately 4-5 mg or 20 female schistosomes with a wet weight of 1.5-2 mg were homogenized in 100 µl of acetone - 0.1N HCl (2:1 v/v) containing 100 pmol of deuterated (d_3)-L-DOPA and deuterated (d_3)-DA and were centrifuged at 8,000 x g on a Beckman microfuge (Beckman Instruments, Irvine, California) for 6 min. The supernatant was transferred to 1 dram vials which had been coated with dimethyldichlorosilane. The samples were then evaporated to dryness at 55°C under nitrogen. The samples were chemically modified by the addition of 50 µl of a 4:1 mixture of pentafluoropropionic anhydride (PFPA) and pentafluoropropanol (PFP)

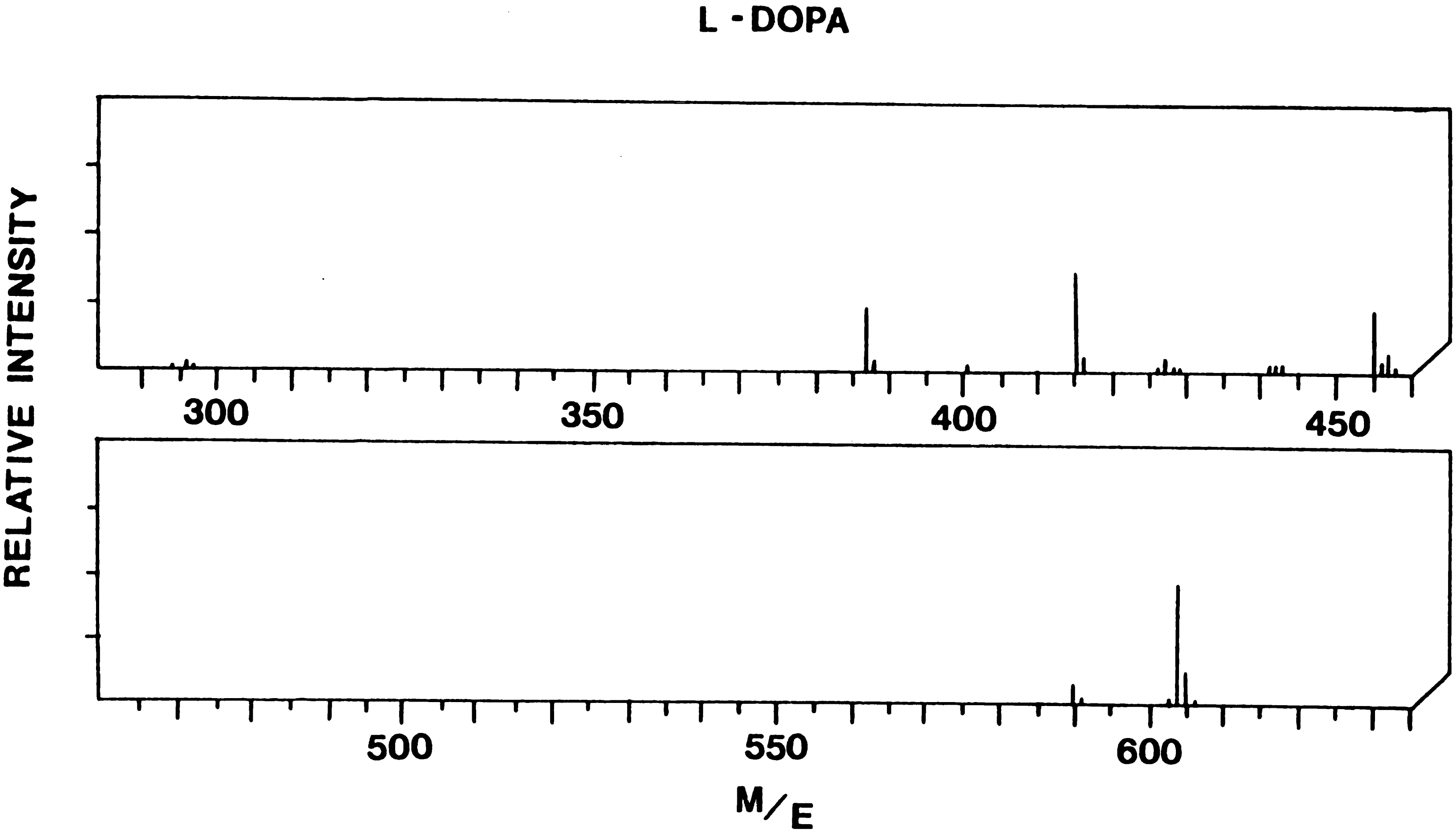
(Regis Chemical Co., Morton Groves, Ill.). The vials were sealed and reacted for 30 min. at 75°C. The reagents were subsequently evaporated under nitrogen at room temperature. PFPA (30 μl) was added and allowed to react for an additional 10 min at 75°C. The PFPA was again evaporated and the residue dissolved in 20 μl of a 10% solution of PFPA in ethyl acetate. This reaction results in the formation of volatile derivatives of L-DOPA and DA which are suitable for separation on a gas chromatograph and analysis in the mass spectrometer. Amounts of 1-4 μl were injected for analysis.

Blank-corrected standard curves for the quantitation of endogenous L-DOPA and dopamine were prepared from a series of standard solutions containing 100 pmol of deuterated L-DOPA and deuterated dopamine and varying amounts of DOPA (0-10 pmole) and dopamine (0-10 pmol). The standards were reacted as described above.

Quantitation of L-DOPA and DA in the samples was performed by mass fragmentography using a Finnigan 3200E gas chromatography/mass spectrometry system. A 1.6 m x 2 mm (i.d.) silanized glass column packed with 3% SP-2250 was used for separation. Temperatures were: column 150°C (isothermal), injector 250°C, separator 250°C. The carrier gas (Helium) was maintained at a flow rate of 15 ml/min. Fragmentation was accomplished by electron impact at 70 ev and 500 μamp. Ions selected for monitoring from the mass spectra of the derivatives were m/e = 428 and 281 for DA (431 and 284 for deuterated DA) and m/e = 415 and 604 for DOPA (418 and 607 for deuterated L-DOPA) (Figures 4 and 5).

The ion at m/e 604 results from cleavage of the bond between the α carbon and nitrogen. The ion at m/e = 415 results from cleavage of the

Figure 4. Electron impact mass spectrum of protonated L-DOPA. Each bar represents the size of the current generated by molecular fragments of the indicated mass:charge ratio. The current is directly proportional to the number of ions striking the detector.



L-DOPA

Figure 4

Figure 5. Electron impact mass spectrum of d_3-DOPA. Each bar represents the size of the current generated by molecular fragments of the indicated mass:charge ratio. The current is directly proportional to the number of ions striking the detector.

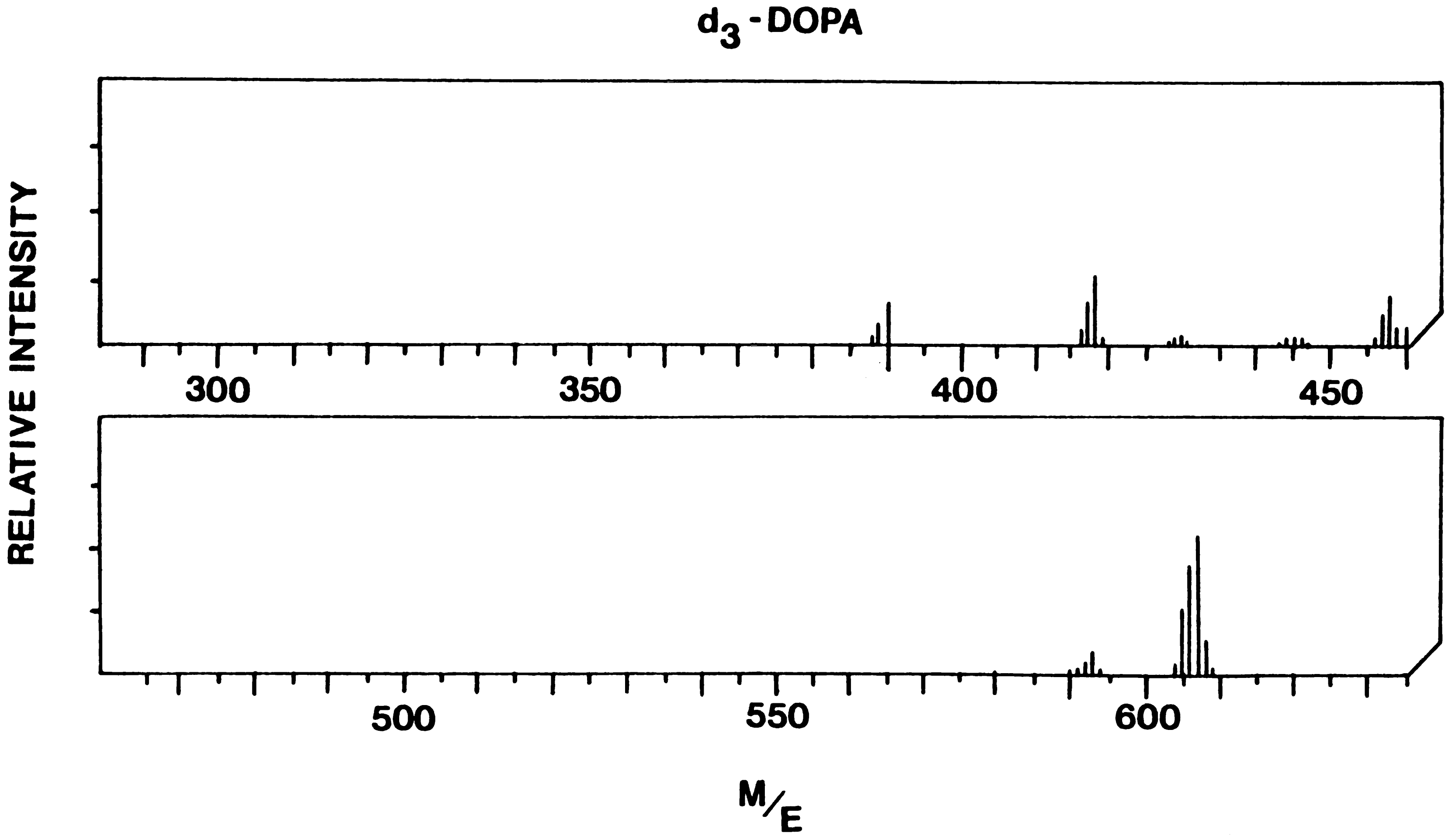


Figure 5

bond between the α and β carbons. The fragmentations yielding the ion pairs utilized in DA quantitation have been previously described (Kilts et al., 1977).

The L-DOPA and DA concentrations of the samples were simultaneously determined by plotting the ion current ratios generated by monitoring the ions characteristic for DA/d_3-DA and DOPA/d_3-L-DOPA against the varying amounts of dopamine and L-DOPA used to generate the appropriate standard curve.

G. Tyrosine Determinations

Samples of 10 male schistosomes with a wet weight of approximately 4-5 mg or 20 female schistosomes with a wet weight of 1.5-2 mg were homogenized in 150 µl of 0.1 N HCl and centrifuged at 8,000 x g for 4 min. The supernatant was assayed for tyrosine by the method of Waalkes and Udenfriend (1957). Fifty µl of supernatant from female worms or 25 µl of supernatant from male worms was diluted to 200 µl with 0.1 N HCl. To this sample was added 100 µl of 0.1% 1-nitroso-2-naphthol (Sigma) in 95% ethanol and 100 µl of a solution containing 24.5 ml of a 1:5 dilution of nitric acid in distilled water and 0.5 ml of 2.5% $NaNO_2$. The samples were then incubated for 30 min at 55°C, cooled to room temperature, and 1 ml of ethylene dichloride was added. The sample tubes were vortexed vigorously and the aqueous layer removed for fluorimetric analysis after centrifugation. Measurements of fluorescence intensity were performed on an Aminco-Bowman fluorimeter with the excitation wavelength set at 460 nm and emission wavelength set at 570 nm. In order to test for specificity of the assay, samples were chromatographed on preabsorbent silica gel thin layer plates in n-propanol-water (7:3 v/v). The developed area was divided into ten units. Each unit was subsequently scraped, eluted with 0.1 N HCl and assayed for tyrosine as described above in the presence of internal standards.

H. Identification of Crosslink Formation In Vitro

Proteins from female schistosomes were labeled by incubating 50 paired schistosomes for 48 hr in a modified RPMI 1640 (Gibco) containing 10 μCi/ml of ^{3}H-[2,6]-L-tyrosine, 40-60 Ci/mmol (New England Nuclear). The labeled worms were separated and the females homogenized in 16 mls of 0.1 M sodium phosphate buffer, pH 7.0. The specific activity of the label was 6140 dpm/μg protein, 34 μg protein/ml phosphate buffer. The reactions of these proteins with schistosome phenol oxidase were monitored as follows. Three-hundred twenty female schistosomes were homogenized at 4°C in 4 ml of ^{3}H-labeled proteins from female *S. mansoni*. Schistosome phenol oxidase was prepared from 80 female schistosomes as previously described, using 0.1 M phosphate buffer containing 5% Triton X-100 as the homogenizing solution. Samples which were to be incubated in the presence of phenol oxidase were added to test tubes containing the phenol oxidase pellet. Cold (4°C) phenol oxidase was added to those samples not treated with phenol oxidase, following the period of incubation. One ml of a 0.1 M phosphate buffer (pH 7.4), saturated with L-tyrosine was added to those ^{3}H-homogenates which were to be incubated in the presence of tyrosine. All samples were incubated at 37°C for a period of 20 minutes in an open system. Following the incubation period, the samples were placed in ice and spun at 3,000 x g for 15 minutes at 4°C. An aliquot of the supernatant was removed for scintillation counting. The pellet was resuspended in 200 µl of 2% SDS containing 1% β-mercaptoethanol. The pellet was incubated in this solution for 2 hrs at 37°C, spun for 15 minutes at 3,000 x g and 100 µl removed for gel electrophoresis. Twenty µl was used for liquid scintillation counting. The pellet was washed 2 times with 200 µl of 0.1 M phosphate buffer and subsequently digested in 250 µl of NCS (Amersham/Searle) at 50°C for 1 hour. The digest was neutralized with 60 µl of glacial acetic acid, decolorized with 50 µl of 30% hydrogen perioxide and diluted with 5 mls of toluene: triton (2:1) scintillation cocktail.

I. Uptake Studies

The kinetics of the uptake of ^{3}H-[2,6]-L-tyrosine (New England Nuclear) and ^{3}H-[2,5,6]-L-DOPA (Amersham) was evaluated as follows. Twenty female schistosomes were incubated at either 25°C or 37°C in the presence of either 5 µCi/ml ^{3}H-L-tyrosine (40-60 Ci/mmol) or 2.5 µCi/ml ^{3}H-L-DOPA (50-60 Ci/mmol), respectively. Incubations were performed in HBSS buffered with .02 M Tris containing varied concentrations of cold L-DOPA or L-tyrosine. Uptake was terminated after 30 sec while the uptake was still linear. Uptake was stopped by washing with cold HBSS. The worms were washed 5 times with 5 mls each of cold HBSS to remove excess radiolabel. To determine the amount of radiolabel accumulated, the schistosomes were digested in 250 µl of tissue solubilizer (NCS, Amersham) at 50°C. The digested sample was diluted with 5 ml of toluene:Triton X-100 (2:1) containing 12 g PPO and 0.3 g dimethyl POPOP/liter. Sixty µl of glacial acetic acid was added to reduce chemiluminescence and 30 µl of hydrogen peroxide was added to decolorize the sample prior to liquid scintillation counting. The data was expressed in terms of pmoles of L-tyrosine or L-DOPA incorporated/mg worm wet weight.

J. Tyrosine Utilization

1. Distribution of L-tyrosine into PCA-precipitable and non-precipitable fractions of S. mansoni homogenates

Fifty paired male and female *S. mansoni* were incubated in an appropriate sterile tissue culture medium containing 20 mCi/mmol of ^{3}H-[2,6]-L-tyrosine or ^{3}H-[4,5]-L-leucine at a concentration of 0.1 mM and 0.2 mM, respectively. At the end of the incubation period, the schistosomes were isolated by filtration on a Millipore filtration apparatus using a Whatman filter (Whatman Corp., Clifton, N.J.) and washed 5 times with 10 mls of cold tissue culture medium containing .05% sodium pentobarbital. Sodium pentobarbital was included in these media because paired male and female schistosomes cannot be separated without a muscular relaxant being present in the solution. The separated schistosomes were homogenized in 0.4 N PCA and an aliquot of the homogenate was removed for liquid scintillation counting. The homogenate was spun at 8,000 x g for 4 min and an aliquot of the resulting supernatant was removed for liquid scintillation counting. The pellet was subsequently washed 4 times with 0.4 N PCA. The final pellet was resuspended in distilled water and aliquots were removed for liquid scintillation counting and protein determinations.

2. Tyrosine metabolism

Fifty paired male and female *S. mansoni* were incubated in ^{3}H-[2,6]-L-tyrosine as described above. The worms were washed 5 times with cold tissue culture medium as described above and homogenized in (2:1) acetone:0.1 N HCl. The homogenate was spun at 8,000 x g for 4 min. An aliquot of the supernatant was spotted on a 20x20 cm cellulose MN-300 thin-layer plate (Whatman) and subjected to two dimensional chromatography as follows: first dimension was n-butanol:acetic acid:water (3:1:1) and the second dimension was n-butanol:ethanol:1 N acetic acid (Johnson and Boukma, 1970) (Figure 6). The samples were chromatographed twice in the first dimension so as to improve the separation between tyrosine and norepinephrine (NE). Plates were allowed to dry completely before the beginning of each chromatographic separation. Although very similar in characteristics, the above solvent systems provided the best separation possible of a number of different solvent systems on cellulose plates. Although other investigators (Aures _et al_., 1968) have claimed to achieve better separation of the catecholamines using basic solvents, similar results could not be obtained in these experiments due to the well-known, rapid, oxidation of the catecholamines. The following cold carriers were spotted on the thin layer plate: L-tyrosine (5 μg), L-DOPA, DA, NE, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine (3-MT) and normetanephrine (NM), 2 μg each. Upon completion of the chromatographic run, the plates were dried at room temperature and spots subsequently visualized with paraformaldehyde as described by Aures _et al_. (1968) and potassium ferricyanide (Osbornne _et al_., 1975). The spots were subsequently scraped into liquid scintillation vials, eluted with ethyl acetate:acetic acid:H_2O (3:1:1, v/v). Sixty μl of a 15% solution of freshly prepared ascorbic acid was added to each vial

Figure 6. Migration of tyrosine and selected metabolites in 2-dimensional cellulose thin-layer chromatography. Plates were developed twice with solvent 1 (n-butanol:acetic acid:water, 3:1:1) and once with solvent 2 (n-butanol:ethanol:1 N acetic acid, 35:10:10).

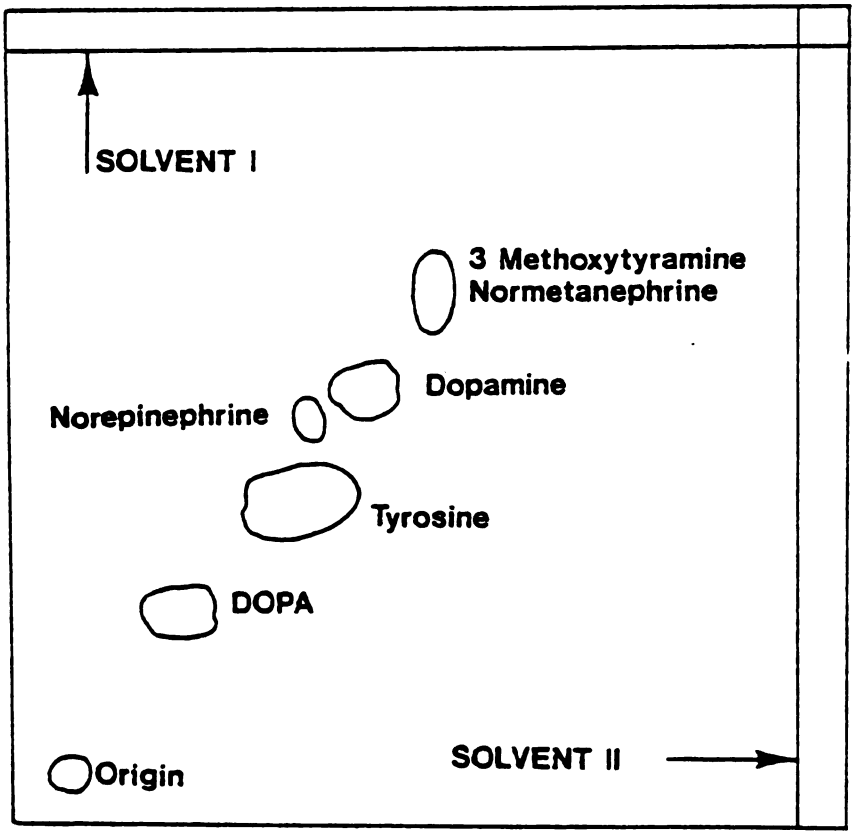

SOLVENT I
3 Methoxytyramine
Normetanephrine
Dopamine
Norepinephrine
Tyrosine
DOPA
Origin
SOLVENT II

Figure 6

prior to scintillation counting, so as to reduce chemiluminescence. The liquid scintillation cocktail was the same as previously described. The data are expressed in terms of the percent of total recoverable tritium.

The metabolism of unlabeled L-tyrosine to DA and NE was monitored by the catechol-o-methyltransferase radioenzymatic assay as described by Moore and Phillipson (1975).

K. In Vitro Incubation

All *in vitro* incubations were performed in sterile tissue culture media. Paired schistosomes were transferred directly from the mesenteric veins of the mouse to culture flasks containing sterile media under sterile conditions. The media employed in these studies were as follows. 1) Eagles Basal Medium containing Earle's salts (Gibco) buffered with .05 M sodium bicarbonate maintained at a pH of 7.4 under 5% CO_2 in air. This medium also contained 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml Amphotericin B. 2) RPMI 1640 (Gibco) (powdered form) buffered with .02 M Hepes buffer, pH 7.4. This medium also contained 10% heat-inactivated fetal bovine serum, 50 μM β-mercaptoethanol and antibiotics as above. These media shall be referred to as BMEFC and modified RPMI 1640, respectively. Incubations were performed in sealed flasks with a minimum of 5 mls of medium per schistosome pair. The media were changed every 3 days during long term cultures. Cultures which showed visual evidence of contamination were discarded.

L. Purification of Schistosome Eggshells

Schistosome eggs were obtained from the livers and intestines of mice infected with *S. mansoni*. These organs were homogenized in a 4-fold volume of cold saline in a Waring blender for 30 seconds. The homogenate was then passed through a series of wire mesh sieves with the following mesh sizes listed in order of use: 40 mesh, 80 mesh, 140 mesh and 325 mesh. The eggs were washed through each stage of filtration with excess cold saline. The eggs were trapped on the surface of the last sieve and were washed into a large petri dish with excess cold saline. The eggs were then passed through the 140 mesh sieve and washed on the 325 mesh sieve 3 more times. The final egg preparation contained no appreciable cellular debris (Figure 7). The eggs were then allowed to settle in a large test tube, the saline was aspirated and the eggs were resuspended in 10 mls of distilled water. The eggs were then disrupted by ultrasonication with a Heat Systems-Ultrasonic Model W185F sonicator with a Model H-1 microtip at maximum power for a period of 10 minutes. No intact eggs could be found in the sonicated preparation at the end of this time period. The eggshells were collected by centrifugation for 5 min at 600 x g. The eggshell pellet was washed and centrifuged 5 times with 5 mls each of distilled water. The final pellet contained apparently pure eggshells (Figure 8). The minimum number of eggs employed in these experiments was 10,000.

M. Fluorescence Spectroscopy

The fluorescence spectra of the *in vitro* and *in vivo* products of the phenol oxidase reaction were analyzed on an Aminco-Bowman

Figure 7. Purified schistosome eggs. Eggs were purified by successive washes through 140 mesh and 325 mesh sieves. Magnification is 35X.

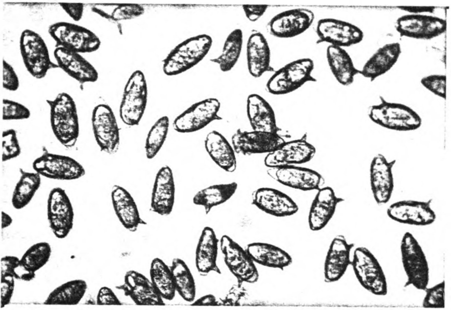


Figure 7

Figure 8. Purified schistosome eggshells. Eggshells were prepared by ultrasonication and washing. Magnification is 140X.

Figure 8

Spectrophotofluorimeter with a high intensity xenon lamp source. The spectra reported herein are uncorrected.

N. Fluorescence Characterization of the Endproducts of the Phenol Oxidase Reaction in S. mansoni Eggshells

The fluorescence characteristics of *S. mansoni* eggshells was determined by suspending purified eggshells in glycerol. The fluorescence spectrum was characterized as described above. The fluorescence characteristics of eggshell hydrolysates was analyzed as follows. Purified eggshells were hydrolyzed in 6 N HCl at 110°C for 48 hours under N_2 and resuspended in distilled water or 0.5 N NaOH. The fluorescence spectrum of the hydrolysate resuspended in 0.5 N NaOH was subsequently analyzed as described above. The portion of the hydrolysate resuspended in distilled water was spotted on a silica gel thin layer plate (Whatman) and chromatographed in one dimension with n-butanol:acetic acid:H_2O (4:1:1, v/v). The plates were then dried, scraped in 1 cm sections, eluted with 0.5 N NaOH and the fluorescence spectrum characterized. The products of the reaction of amino acids and peptides with mushroom phenol oxidase were hydrolyzed, chromatographed and analyzed as described for the schistosome eggshell.

O. In Vivo Inhibition of Eggshell Formation

The drugs used in these experiments were either administered chronically in the diet or by intraperitoneal injection 1 hr prior to sacrifice. The inhibition of eggshell formation was determined by examining the uterine ducts of female schistosomes for the presence of egg material. The data were expressed in terms of the total number of female schistosomes not possessing intact egg capsules divided by the total number of female schistosomes containing egg material. The drugs used for injection were prepared by dissolution in saline or suspension in 0.5% methyl cellulose. Acidic and basic compounds were adjusted to pH 7.0 with phosphate buffer prior to injection. Drugs used in these studies and not previously mentioned include ascorbic acid (U.S.P. Grade) (Mallinckrodt, West Germany), dl-α-tocopherol acid succinate (Sigma), phenylhydrazine hydrochloride (Aldrich), sodium sulfite (J.T. Baker), thiambutosine and clofazimine (gift of Dr. F. Krodolfer, Ciba Geigy).

P. ATP Assay

ATP levels in female S. mansoni were measured fluorimetrically with luciferase as described by Bergmeyer (1974). Twenty female schistosomes were homogenized in 100 µl of 0.4 N PCA. The homogenate was then neutralized by the addition of 40 µl of 1 N KOH. The sample was then centrifuged and the supernatant used for the ATP determination. Luciferase was prepared by dissolving 50 mg of firefly extract (Sigma Chemical Co.) in 5 ml of H_2O. Ten to forty ml of the schistosome supernatant was then added to 0.1 ml of luciferase in 2.1 ml of a 25 mM glycylgycine buffer pH 7.5 containing 0.1 M $MgSO_4$. The samples was then immediately placed in a liquid scintillation counter and counted for 1 min using a full tritium window. ATP standards were dissolved in distilled and assayed either alone or in the presence of schistosome extract.

Q. Proteins

Proteins were determined according to the method of Lowry et al. (1951) or by the method of Peterson (1977). The latter method was used when substances which interfered with the Lowry method (e.g., detergents) were present in the sample. The method of Peterson involves precipitation of the protein sample with 7.2% TCA prior to Lowry determination. This step removes most interfering buffers and detergents prior to Lowry determination. In the actual assay, between 5 and 100 µg of protein are dissolved in 1.0 ml of distilled water and 1.0 ml of reagent "A" which contains 2.5% sodium carbonate, .025% copper sulfate, 0.5% potassium tartrate, 2.5% SDS and 0.2 N NaOH. After allowing to stand for 10 min at room temperature, 0.5 ml of 0.3 N Folin reagent is added to the sample which is then allowed to sit for 30 min prior to being read at 750 nm on a spectrophotometer

R. Liquid Scintillation Counting

Tritium-labeled samples were counted on a Beckman LS100 scintillation counter. The scintillation cocktail contained 12 g PPO and 0.3 g dimethyl POPOP dissolved in either 3 liters of toluene or 2 liters of toluene and 1 liter of Triton X-100. On occasion, the 1 liter of Triton X-100 was replaced with 1 liter of 95% ethanol. The samples were counted in a full tritium window (95% of all tritium emissions) and gain was optimized to maximize counting efficiency. Samples were counted until the total counts reached 5,000 cpm to obtain accuracy of ± 5%. When necessary, disintegrations per minute were calculated from a quench curve generated by the addition of chloroform to a sample containing a known amount of ^{3}H-labeled water and using the external standard channels ratio method to determine the degree of quench. Individual quench curves were generated for each scintillation counter and each scintillation cocktail used.

S. Gel Electrophoresis

Proteins from the phenol oxidase studies were subjected to constant voltage SDS gel electrophoresis on 10% acrylamide gels as described by Weber and Osborn (1960). Polyacrylamide gels containing tritium labeled proteins were sliced in 1 mm sections with a BioRad gel slicer. The gels were solubilized in NCS (Amersham) and water (10:1) at 55°C as described by Grower and Bransome (1970). Chemiluminescence was quenched with 50 µl of a freshly prepared solution of 15% ascorbic acid. The scintillation cocktail contained 12 g PPO and 0.3 g dimethyl POPOP in 3 liters of toluene. Coomassie blue stained gels were analyzed for protein content by scanning on a Gilford Model 240 spectrophotometer at 550 nm.

T. Pathological Studies

Mice were infected with 150 cercariae of S. mansoni by tail immersion. Thirty days later, the animals were placed on a diet consisting of milled rodent chow either with or without 0.3% disulfiram (Aldrich Chemical Co.). Three groups with 10 animals in each group were used in this experiment. One group received control chow (no drug) and the remaining two groups received chow mixed with drug. In the two groups receiving drug treatment, one group was maintained on the diet for the duration of the experiment while the other group was returned to a control diet after 53 days of treatment. At the end of the experiment, 92 days following the start of drug administration, the remaining mice from each group were killed and the livers, spleens and intestines were removed. These tissues were then rinsed in saline, fixed in 10% formalin, sectioned and stained with hematoxylin and eosin (H & E), periodic acid-Shiff (PAS) and/or toluidine blue.

The pathology of the infection was subjectively evaluated by a trained pathologist who had only limited knowledge of the experimental conditions. The relative amount of egg deposition in the livers of treated and untreated mice was estimated by counting the numbers of eggs and egg granulomas within a 40 mm^2 area of a liver slice, 3 liver slices per mouse. Each 50 μM section was obtained from different regions of the mouse liver. The egg counts were performed on at least 3 mice from each group.

The average burden of worm pairs in this study was 26.5±2.6 (S.E.M.) as determined from worm counts in the surviving animals from the drug treated groups.

In an additional experiment, mice were treated as above, except that the period of drug treatment lasted 23 days and the mice in these experiments were maintained on their original diet for the entire period of the experiment. The average worm burden in these animals was 16.5±1.9 pairs.

U. Statistical Methods

Data are expressed as the mean ± S.E.M. Means were compared by the Student's t-test or analysis of variance as described by Snedecor and Cochran (1967). Multiple comparisons were made with the Student-

Newman-Keuls test (Sokal and Rohlf, 1969). Significance was established at $p<.05$. Linear regression analysis of kinetic data was performed by the method of least squares as described by Snedecor and Cochran (1967).

RESULTS

A. Induction of Phenol Oxidase Activity

The enzyme classified as phenol oxidase (E.C. 1.10.3.1, *o*-diphenol: O_2 oxidoreductase) has classically been described as a latent enzyme (Menon and Haberman, 1970; Fox and Burnett, 1959; Bodine *et al*., 1944). Thus, it has often been found to be difficult if not impossible, to detect phenol oxidase activity without first perturbing the enzyme preparation in order to unmask its activity. A similar situation appears to exist in female *S. mansoni*.

Using 2 mM DA as a substrate, it was not possible to detect phenol oxidase activity in homogenates of female schistosomes assayed for activity immediately following removal of the schistosome from its mouse host. Thus, it became necessary to evaluate a number of different methods for the activation of phenol oxidase in *S. mansoni*. Some of the methods which have commonly been used to activate phenol oxidase in other vertebrate and invertebrate preparations have included treatment with either detergents, proteases, phospholipases or repeated freezing and thawing (Menon and Haberman, 1970; Preston and Taylor, 1970; Quevedo *et al*., 1975). In these experiments, 3,000 x g pellets of female *S. mansoni* homogenates were incubated in the presence of 25,000 units of trypsin, 16 units of protease or 240 units of phospholipase A2 for 5 min at 37°C and then assayed for phenol oxidase activity. In detergent-treated preparations, the female schistosomes were homogenized in 1% Triton X-100 and centrifuged at 3,000 x g. The pellet was then assayed for phenol oxidase activity. In freezing and thawing experiments, whole female schistosomes were frozen at -40°C and thawed at 37°C, 3 times in succession. The worms were then homogenized, centrifuged and assayed for phenol oxidase activity. All of these treatments failed to induce enzyme activity in whole or homogenized female S. mansoni which were treated shortly after removal from the host (Table 1). However, when whole female worms were incubated at room temperature in BMEFC containing .05% sodium pentobarbital, phenol oxidase activity became readily detectable (Figure 9). Enzyme activity, measured as described in the Methods section, increased rapidly with time up to a peak of .41±.02 μmoles O_2/min-mg protein after 8 hrs of in vitro incubation.

There have been few reports in the literature wherein phenol oxidase has been reported to be activated by in vitro incubation in a tissue culture medium (Horowitz and Shen, 1952). In the experiments of Horowitz and Shen (1952), the incubation-dependent activation of phenol oxidase was shown to be sensitive to the effects of temperature, the type of incubation medium and ongoing protein synthesis. Similar experiments have been performed in order to determine if the incubation-dependent increase in phenol oxidase activity in S. mansoni was similar to the above mentioned system. The effects of temperature were examined in BMEFC where the incubation-induced increase in phenol oxidase activity was 40% greater at 22°C than at 37°C (Table 2). These findings are similar to those of the previously mentioned investigators who

TABLE 1

The Effectiveness of Various Treatments Which Have Been Used to Induce Phenol Oxidase Activity

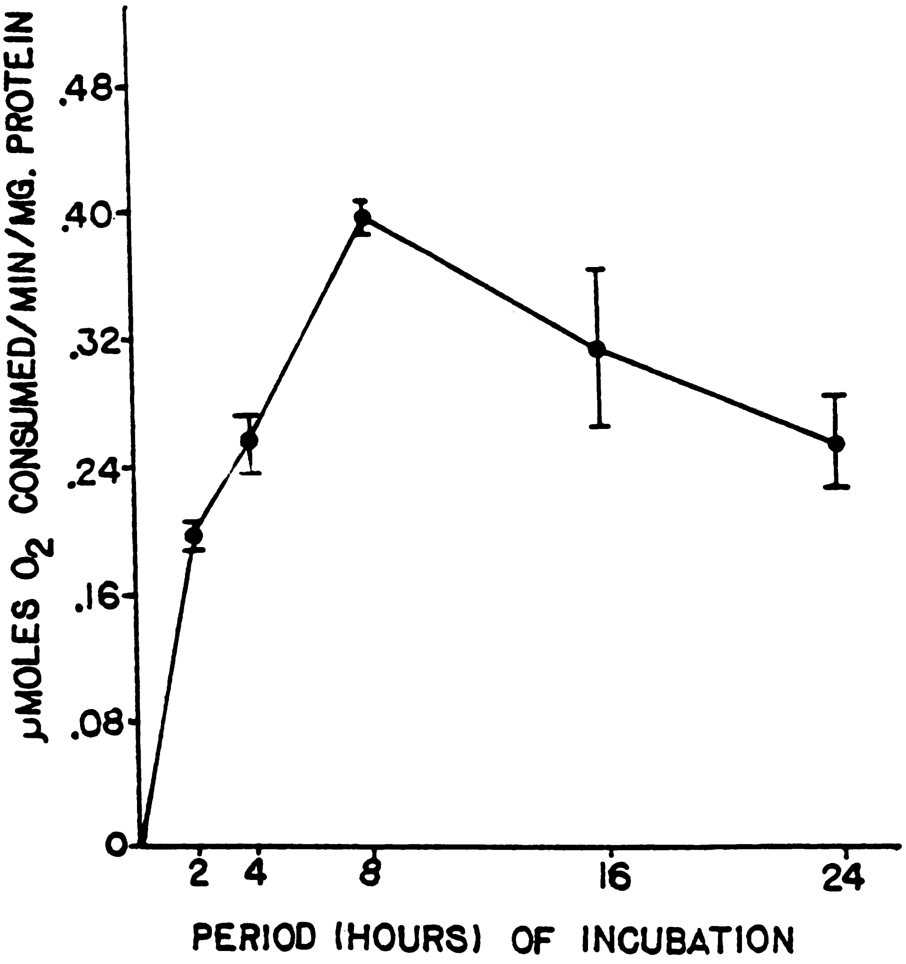
Treatment	Mammalian[a] Phenol Oxidase	Schistosome Phenol Oxidase
Freezing and Thawing	18.2	0
Detergents (TX-100)	22.6	0
Phospholipase A	17.4	0

Treatment	Cockroach[b] Phenol Oxidase	Schistosome Phenol Oxidase
Trypsin	.27	0
α-chymotrypsin	.21	0

[a] Menon and Habermon (1970). Data are expressed as the increase in nmoles tyrosine consumed/min. Baseline is 7.6 nmoles tyrosine/min.

[b] Preston and Taylor (1970). Data are expressed as the increase in O.D. 470/10 min using L-DOPA as a substrate. Baseline is 0.

Figure 9. Activation of *S. mansoni* phenol oxidase by *in vitro* incubation. Incubations were performed in BMEFC containing .05% sodium pentobarbital. Activity was assayed using 2 mM DA as substrate. Each point represents the mean ± S.E.M. of at least 3 determinations.



μMOLES O_2 CONSUMED/MIN/MG. PROTEIN
.48
.40
.32
.24
.16
.08
0
2
4
8
16
24
PERIOD (HOURS) OF INCUBATION

Figure 9

TABLE 2

The Effects of Temperature and Type of Incubation Medium on the Induction of Phenol Oxidase Activity

Culture Medium[c]	Buffer (pH 7.4)	Time of Induction	Temperature	Phenol Oxidase Activity[a,b]
BMEFC	.02 M Tris	4 hrs	25°C 37°C	100% 61±6%
RPMI 1640 + 10% calf serum	.025 M Hepes	4 hrs	25°C	114±8%
Eagle's medium	.02 M Tris	4 hrs	25°C	142±3%
Earle's Balanced Salts	.02 M Tris	4 hrs	25°C	103±6%
Hank's Balanced Salts	.02 M Tris	4 hrs	25°C	69±6%
Saline	0.1 M Sodium Phosphate	4 hrs	25°C	44±4%

[a] Mean ± S.E.M. for at least 3 determinations.

[b] Data expressed as percent of control, control being .162±.011 moles O_2/min-mg protein using 2 mM DA as the substrate.

[c] All media also contain .05% sodium pentobarbital.

have demonstrated that the lower the temperature of the induction medium, the greater the inducible levels of phenol oxidase activity. This unusual temperature dependence for the incubation dependent activation of phenol oxidase has been attributed to a greater rate of self-inactivation of phenol oxidase at 37°C (Ohnishi, 1959). (Self-inactivation is a well known attribute of all phenol oxidase enzymes [Nelson and Dawson, 1944].)

With regard to the effects of the tissue culture medium, incubation in Eagle's medium without calf serum produced a 40% greater activation of phenol oxidase than did the reference medium (BMEFC), while incubation in Hank's salts produced 30% less phenol oxidase activity than BMEFC. The mere maintenance of tonicity was not adequate to support the process of phenol oxidase activation. Incubation in the presence of phosphate buffered saline, pH 7.4, resulted in a nearly 60% decrease in the activation of phenol oxidase activity. This decrease in phenol oxidase activity was associated with a nearly 60% decrease in the total protein content of the female schistosome (Table 3). This observation suggested that the activation of phenol oxidase in _S. mansoni_ might be dependent upon protein synthesis.

In order to determine if the activation of phenol oxidase in _S. mansoni_ was dependent on protein synthesis, female _S. mansoni_ were placed in BMEFC containing .05% pentobarbital, 2 μCi of tritium labeled L-tyrosine, and 0.1 mM cycloheximide during the period of induction. There was a 16% reduction in total schistosome proteins which was associated with an approximately 50% reduction in ^{3}H-L-tyrosine incorporation into PCA-precipitable proteins (Table 3). This

TABLE 3

The Effects of Protein Synthesis Inhibitors on Schistosome Protein Synthesis, ATP Levels and Phenol Oxidase Induction[a,b]

Treatment	^{3}H-Leucine incorp. into PCA-precipitable proteins	^{3}H-Tyrosine incorp. into PCA-precipitable proteins	PCA-precipitable proteins	[ATP]	Phenol[c] Oxidase Activity
	cpm/mg worm	cpm/mg worm	μg/mg worm	pmoles/ mg worm	% of Control
Control	3987±424	2544±311	118± 7	4.79±.48	100
10^{-4}M cycloheximide	---	1257±135[d]	99± 5	----	83±.11
10^{-4}M Antimony potassium tartrate	376± 13[d]	284± 34[d]	90±17	1.33±.26[d]	85±.08
0.1 M phosphate buffered saline	71± 10[d]	----	44± 7[d]	----	44±.04[d]

[a] Worms were incubated for 4 hrs either in BMEFC containing .05% pentobarbital and a protein synthesis inhibitor or 0.1 M phosphate buffered saline (pH 7.4) containing .05% pentobarbital.

[b] Data represents the mean ± S.E.M. of at least 3 determinations in each group.

[c] Control is .162±.011 μmoles O_2/min-mg protein.

[d] Significantly different from control, $p<.05$.

reduction in protein synthesis was associated with a 17% reduction in phenol oxidase activity. Thus, it appeared that the reduction in phenol oxidase activity was more closely associated with the reduction in total protein content of the schistosome than the reduction in the synthesis of new proteins.

In order to control for the possibility that the reduction in synthesis of new proteins was a non-specific artifact due to the large concentrations of cycloheximide, an attempt was made to inhibit protein synthesis with antimony potassium tartrate (SbK). SbK has been shown to inhibit S. mansoni phosphofructokinase, a critical enzyme in the schistosome glycolytic pathway (Mansour and Bueding, 1954). Inasmuch as glycolysis is the sole source of energy for this parasite (Bueding, 1950), inhibition of schistosome phosphofructokinase should result in the inhibition of all energy dependent processes within the schistosome, including protein synthesis. Whole female S. mansoni were incubated in BMEFC containing .05% pentobarbital, 0.2 mM SbK and tritium-labeled L-leucine or L-tyrosine for a period of 4 hours. ATP concentrations dropped by 70% and this drop in ATP corresponded with a 90% inhibition of the incorporation of ^{3}H-amino acids into PCA-precipitable proteins (Table 3). This decreased incorporation of amino acids into protein could not be attributed to the inhibition of the uptake of amino acids since the uptake of one of the amino acids used in this study (tyrosine) was shown to be a diffusional process (Figure 10). However, despite the large decrease in protein synthesis and reduction of ATP concentrations, the activation of phenol oxidase was not significantly altered by SbK.

Figure 10. *In vitro* uptake of L-tyrosine by female *S. mansoni*. Worms were incubated at 37°C in Hank's salts containing varying concentrations of *unlabeled* L-tyrosine and 5 µCi/ml of ^{3}H-L-tyrosine (40-60 Ci/mmol). ( ) - worms incubated in Hank's salts containing 10^{-4}M SbK. ( ) - worms incubated in Hank's salts. Each point represents the mean ± S.E.M. of at least 5 determinations.

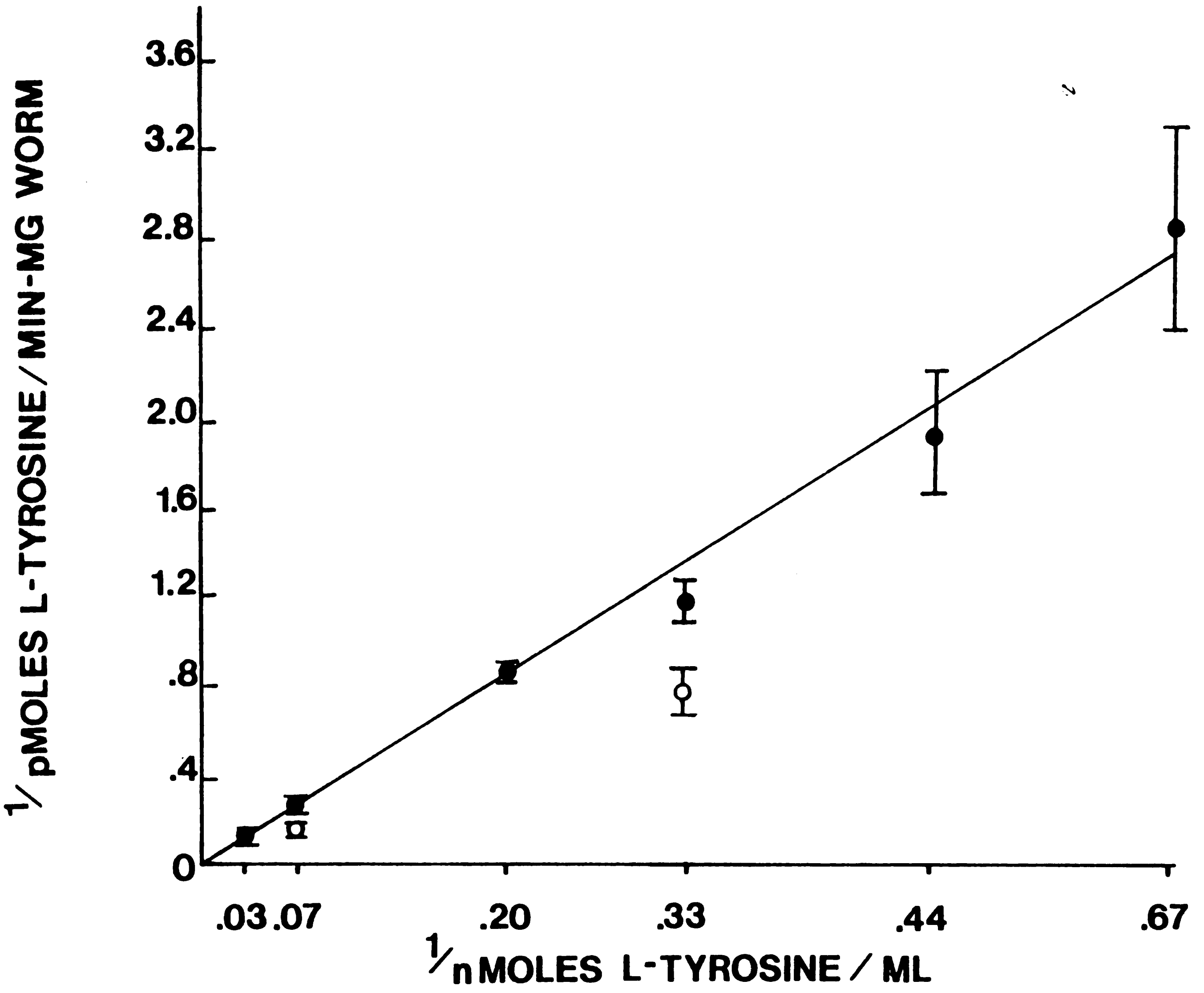

1/pMOLES L-TYROSINE/MIN-MG WORM
3.6
3.2
2.8
2.4
2.0
1.6
1.2
.8
.4
0
.03
.07
.20
.33
.44
.67
1/nMOLES L-TYROSINE / ML

Figure 10

The results of these studies indicate that the incubation-dependent activation of phenol oxidase is not dependent upon protein synthesis or on any ATP-dependent process. In addition, the mechanism does not appear to involve proteolysis or lipolysis since these treatments do not activate phenol oxidase in female *S. mansoni* prior to *in vitro* incubation (Table 1).

B. Isolation and Histochemical Localization of Phenol Oxidase

1. Isolation of the enzyme

Previous studies by Mansour (1958) on the trematode *F. hepatica* have shown that most of the phenol oxidase from this trematode is recovered in the 600 x g pellet of whole worm homogenates, thus indicating that most of this enzyme is localized in a crude nuclear and plasma membrane fraction of this trematode (DeDuve *et al.*, 1955; Mahler and Cordes, 1971). This observation is consistent with the results of histochemical studies which have shown that *Fasciola* phenol oxidase is localized in the membrane globules of vitelline cells (Smyth, 1954). In order to determine if phenol oxidase from female *S. mansoni* was similarly recovered in the crude nuclear and plasma membrane fractions of schistosome homogenates after differential centrifugation, activated female *S. mansoni* were homogenized in 0.1 M phosphate buffer, pH 6.8 and were centrifuged at 1,000 x g, 3,000 x g and 5,000 x g. The pellets were assayed for both succinate-stimulated oxygen consumption and DA-stimulated oxygen consumption since female schistosomes are known to contain abundant quantities of mitochondria (Erasmus, 1973). Succinate stimulation of oxidative activity in the presence of

1 mM malate, glutamate and ADP was used as a marker for the presence of mitochondria (Coles, 1973). In these experiments, 1 mM succinate was added 5 minutes prior to the addition of DA in order to determine if mitochondrial respiration had an effect on phenol oxidase activity. The results in Table 4 showed that 96% of the phenol oxidase activity in a 5,000 x g pellet could be recovered in 1,000 x g pellet. However, considerable amounts of succinate stimulated respiration were also noted in all of the pellet fractions. Nonetheless, the presence of this activity did not interfere with the assay of phenol oxidase activity, since the addition of succinate 5 minutes prior to the addition of DA failed to alter the rate of DA stimulated oxygen consumption. 5,000 x g pellets obtained from 3-fold larger quantities of male _S_. _mansoni_ did not contain any phenol oxidase activity (data not shown).

In further studies on the isolation and purification of phenol oxidase from female _S_. _mansoni_, a 3,000 x g pellet was used because it minimized the time of centrifugation (15 min at 1,000 x g vs. 5 min at 3,000 x g) without noticeable loss of phenol oxidase activity.

Solubilization of membrane bound phenol oxidase from mouse melanoma has been noted to occur following treatment with proteolytic enzymes (Miyazaki and Seiji, 1971), phospholipase (Seiji and Yoshida, 1968), detergents (Iwata and Takeuchi, 1977; Burnett _et al_., 1967) and combinations of proteolytic enzymes and detergents (Quevedo _et al_., 1975). In order to determine if phenol oxidase from _S_. _mansoni_ could be solubilized, female schistosomes which had been incubated in BMEFC

TABLE 4

Oxygen Consumption in Pellets of Female Schistosome Homogenates Following Successive Administration of 1 mM Succinate and 2 mM DA

	Oxygen Consumption[a]		
Substrate	1,000 x g pellet	3,000 x g pellet	5,000 x g pellet
1 mM succinate,			
glutamate, malate	.062	.018	.054
ADP	.014		.031
2 mM DA, 1 mM[b]			
glutamate, malate,	.112	.103	.101
ADP, succinate	.090		.117
2 mM DA	----	----	.112
			.101
			.101

[a] Data expressed as μmoles O_2/min-mg protein.

[b] Oxygen consumption stimulated by succinate was subtracted from the total oxygen consumption following the administration of dopamine to give a value which represents the increase in oxygen consumption stimulated by the administration of 2 mM DA.

for 4 hrs were either homogenized in detergents, centrifuged and the supernatant and pellet assayed for phenol oxidase, or the phenol oxidase pellet derived from a phosphate buffer homogenate was preincubated with proteolytic or lipolytic enzymes for 5 minutes at 37°C prior to recentrifugation and assay. Treatment with proteolytic enzymes or phospholipase decreased the enzyme activity in the pellet without increasing this activity in the supernatant (Table 5). Nonionic detergents had little or no effect on phenol oxidase activity in the 3,000 x g pellet. However, since nonionic detergents can selectively solubilize a number of membrane-bound proteins (Helenius and Simons, 1975) it is possible that incubation of the phenol oxidase pellet in the presence of a nonionic detergent could aid in the partial purification of the enzyme by reducing the total protein content of the pellet. Thus, activated female S. mansoni were homogenized and centrifuged in the presence of 5% Triton X-100 (TX-100) or 0.1 M phosphate buffer and the 3,000 x g pellets were assayed for both phenol oxidase activity and protein content. Homogenization with 5% TX-100 resulted in only a slight increase in the purity of the preparation. There was a 23% drop in total protein content of the pellet with no appreciable loss of phenol oxidase activity (Tables 5 and 6). However, treatment with TX-100 resulted in a significant stabilization of phenol oxidase activity. Incubation of the untreated pellet fraction at 37°C for 30 minutes resulted in a 70% drop in phenol oxidase activity (n=2). Incubation of the TX-100 treated pellet for 30 min at 37°C resulted in only a 6.8% drop in phenol oxidase activity (n=3). At this time, it is not known whether this increase in heat stability was due to

TABLE 5

The Effects of Enzymes and Detergents on Phenol Oxidase Activity[a]

Treatment	Phenol Oxidase Activity[b,c] in Pellet		Phenol Oxidase Activity in Supernatant
Control	100%	(48)	not detectable (n.d.)
Trypsin 12,500 units/ml	89.6	(2)	n.d.
Protease 8 units/ml	54.8± 4.2%[d]	(3)	n.d.
Phospholipase A_2 120 units/ml	88.3± 8.1%	(3)	n.d.
Freezing and Thawing	116.3± 3.6%[d]	(6)	n.d.
2% Triton X-100	107.3±11.3%	(4)	n.d.
2% Tween 80	97.5± 4.9%	(7)	n.d.
2% Lubrol WX	104.8± 4.7%	(3)	n.d.
0.2% SDS	n.d.	(2)	n.d.

[a] All phenol oxidase preparations were activated by incubation for 4 hrs in BMEFC containing .05% pentobarbital.

[b] Data expressed in terms of percent of control, control being .162±.011 μmoles O_2/min-mg protein.

[c] Number of experiments is indicated in parentheses.

[d] Significantly different from control $p<.05$.

TABLE 6

Protein Levels in Pellet of Female Schistosomes Homogenized in Either 5% Triton X-100 or 0.1 M Phosphate Buffer[a]

Type of Pellet	Total proteins in aqueous fraction of resuspended pellet (μg)	Total proteins in pellet fraction of resuspended pellet (μg)
Triton X-100	248± 13	64.0±1.7[b]
Phosphate buffer	224± 9	83.2±7.2

[a] Data represents mean ± S.E.M. of at least 6 determinations.

[b] Significantly different from phosphate buffer, $p<.05$.

direct interaction of the detergent with phenol oxidase or detergent mediated inhibition of proteolytic enzymes.

2. Histochemical localization

The results of the preceding section have indicated that *S. mansoni* phenol oxidase can be found in a crude membrane and mitochondrial fraction of female schistosome homogenates as was described previously in *F. hepatica* (Mansour, 1958). Thus, it is important to determine if *S. mansoni* phenol oxidase, like *F. hepatica* phenol oxidase, can be histochemically localized within the eggshell globules of the vitelline cells. Unfortunately, the histochemical technique used by previous investigators to localize phenol oxidase within the eggshell precursor globules of *F. hepatica* did not permit localization of activity to specific subcellular structures in *S. mansoni* due to an apparently diffuse reaction to the stain (Figure 11). A fluorescent histochemical assay for phenol oxidase was subsequently developed, based on the observation that schistosome eggshells fluoresce a characteristic yellow color when viewed under u.v. light using a 500 nm barrier filter to suppress other wavelengths of light emitted by the U.V. source.

Incubation of female schistosomes in HBSS in the presence of 2 mM TME resulted in the appearance of yellow fluorescence in the vitelline cells (Figure 12). This fluorescence was localized within small globules located within the vitelline cells. These globules are similar in morphology and distribution to the eggshell precursor globules, previously described by Smyth (1954), Stephenson (1947) and Smyth and Clegg (1959) in *F. hepatica* and Gonnert (1955) in *S. mansoni*.

Figure 11. Histochemical localization of phenol oxidase activity in female S. mansoni by catechol method. Note that the reaction is diffuse and does not permit identification of specific cellular structures. Magnification is 35X.

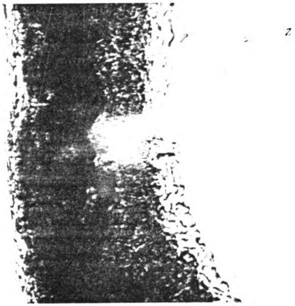


Figure 11

Figure 12. Histochemical localization of phenol oxidase activity in female S. mansoni by fluorescent method. Fluorescence was induced by incubation in HBSS containing 2 mM TME. Magnification is 140X.

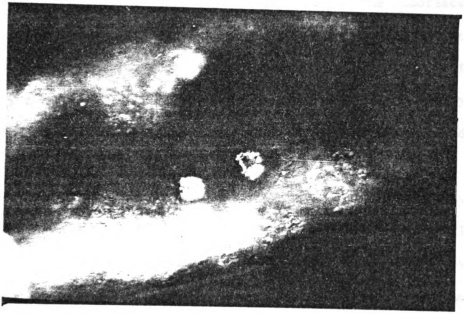


Figure 12

Incubation of female S. mansoni in HBSS alone did not result in the appearance of a fluorescent color (Table 7). To determine if the tyrosine methyl ester-induced fluorescence of the vitelline cells was being mediated by phenol oxidase, female worms were preincubated for 10 minutes in HBSS containing 0.1 mM DDC prior to the addition of TME. This compound is a well known copper chelator and has classically been described as an inhibitor of phenol oxidase activity (Lerner, 1949, 1953; Pomerantz, 1963). Quantitative analysis of the fluorescence intensity of the vitelline cells by microspectrofluorimetry (see Methods section for details) demonstrated that preincubation of the female worms in DDC significantly reduced ($p<.01$) the intensity of the fluorescence of the vitelline cells compared to worms incubated in TME without DDC.

Other phenolic substrates such as catechol, tyramine, L-DOPA methyl ester and DA were not as effective as TME in inducing fluorescence. None of these compounds induced fluorescence in the vitelline cells at 2 mM, although L-DOPA methyl ester and catechol did induce fluorescence in these cells at 10 mM.

Further evidence was obtained from studies on the fluorescence characteristics of material extracted from the female worm following incubation in the presence of TME or TME plus DDC as just described. Following incubation in 2 mM TME female worms were homogenized in 0.1 M phosphate buffer, pH 7.0 and then extracted successively with neutral butanol and butanol acidified with 2 N HCl. The acidified butanol extract demonstrated the presence of fluorescent material with a characteristic excitation maximum of 315 nm and an emission maximum

TABLE 7

Effects of Various Treatments on Relative Intensity of Fluorescent Light from Vitelline Glands of Adult Female *S. mansoni*

Treatment	Relative Intensity of Fluorescent Light at 537 nm[a]
1. 30 min incubation in HBSS	.071 ± .007
2. 30 min incubation in HBSS + 2.5 mM TME	.958 ± .090
3. 30 min incubation in HBSS + 2.5 mM TME + 0.1 mM DDC	.183 ± .024
4. 30 min incubation in HBSS + 0.5 mM TME	.106 ± .009
5. 5 hrs incubation in BMEFC followed by 30 min incubation in HBSS	.056 ± .002
6. 5 hrs incubation in BMEFC followed by 30 min incubation in HBSS + 0.5 mM tyrosine	.186 ± .017

[a] Data expressed as mean ± S.E.M. of at least 4 determinations.

of 420 nm (Figure 13, curve B). The excitation/emission maxima obtained in this study were identical to the excitation/emission maxima of the product formed from the reaction of TME with purified mushroom phenol oxidase (Figure 13, dotted curve). Incubation of the female schistosome in the presence of 0.1 mM DDC or in the absence of TME resulted in complete inhibition of this fluorescence (Figure 13, curve A). It has previously been shown (Section I) that incubation of female S. mansoni for extended periods of time in BMEFC at room temperature results in the activation of schistosome phenol oxidase. To determine if the fluorescent assay for phenol oxidase demonstrates similar characteristics, female S. mansoni were preincubated in BMEFC for 4 hours prior to the addition of 2 mM TME. The relative fluorescence intensity increased in both the butanol extracted schistosomes (Figure 13, curve C) and in the intact schistosome (Table 7). In the latter experiment, the concentration of TME was reduced to 0.5 mM in order to permit visualization of the increased fluorescence intensity. The fluorescence intensity in the intact worm appeared to saturate with 2 mM TME since induction of phenol oxidase activity by incubation in BMEFC failed to increase fluorescence intensity when this concentration of substrate was employed.

C. Characterization of S. mansoni Phenol Oxidase

Phenol oxidase has previously been characterized in a wide variety of vertebrate and invertebrate species (see reviews by Nelson and Dawson, 1944 and Brunet, 1963). In general, phenol oxidase has been reported to be a copper protein (Karlson and Liebau, 1961) and appears to catalyze the conversion of L-tyrosine to L-DOPA and the conversion

Figure 13. Fluorescence spectrum of products extracted from female *S. mansoni* following incubation in 2 mM TME. Spectra were recorded from acidified butanol extracts. Excitation wavelength set to 310 nm for emission scan. Emission wavelength set to 420 nm for excitation scan. Curve A, butanol extract of DDC pretreated worm; curve B, butanol extract of fresh worm; curve C, butanol extract of worm activated by 4 hrs *in vitro* incubation in BMEFC. (-----) Butanol extract of products formed from the reaction of mushroom or schistosome phenol oxidase with TME *in vitro*.

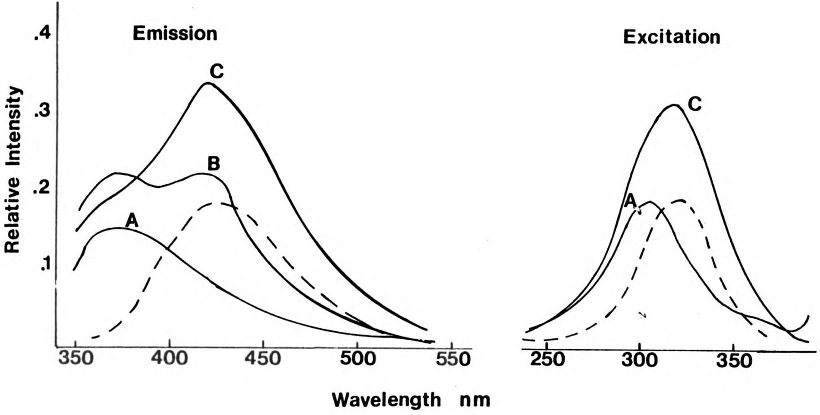

Emission
Excitation
Relative Intensity
.4
.3
.2
.1
C
B
A
C
A
350
400
450
500
550
250
300
350
Wavelength nm

Figure 13

Figure 13. Fluorescence spectrum of products extracted from female _S. mansoni_ following incubation in 2 mM TME. Spectra were recorded from acidified butanol extracts. Excitation wavelength set to 310 nm for emission scan. Emission wavelength set to 420 nm for excitation scan. Curve A, butanol extract of DDC pretreated worm; curve B, butanol extract of fresh worm; curve C, butanol extract of worm activated by 4 hrs _in vitro_ incubation in BMEFC. (-----) Butanol extract of products formed from the reaction of mushroom or schistosome phenol oxidase with TME _in vitro_.

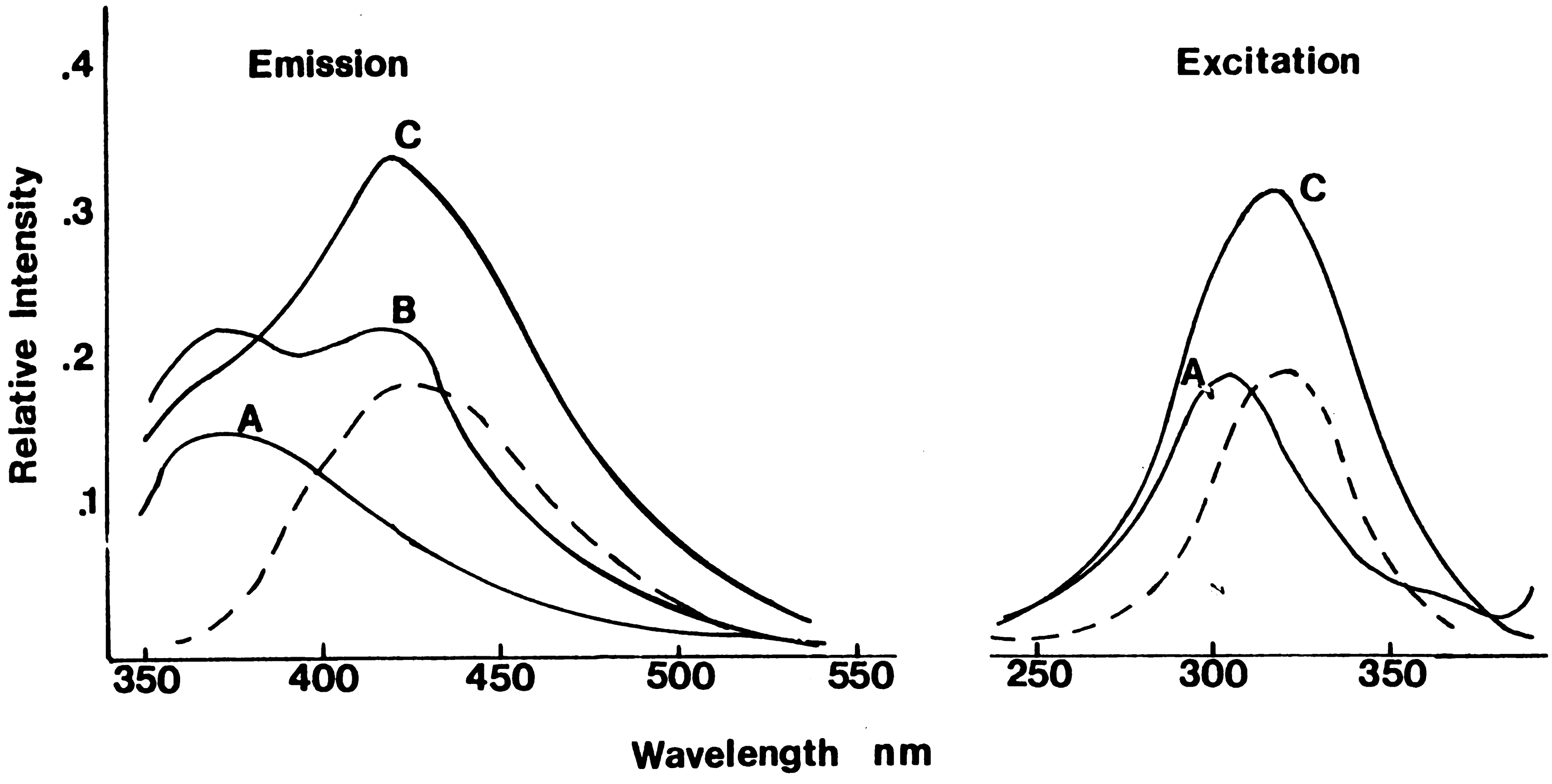

Emission
Excitation
Relative Intensity
.4
.3
.2
.1
C
B
A
C
A
350
400
450
500
550
250
300
350
Wavelength nm

Figure 13

of L-DOPA to DOPA-quinone (Lerner *et al.*, 1949). Much of the work on the characterization of phenol oxidase has been performed on extensively purified enzymes (Karlson and Liebau, 1961; Aerts and Vercauteren, 1965). However, as was illustrated in Section B, extensive purification of phenol oxidase from *S. mansoni* has not been feasible. Therefore, prior to the characterization of this enzyme it was necessary to perform numerous experiments designed to control for the possibility that other oxidative enzymes may contaminate the phenol oxidase preparation in *S. mansoni* or may in some other way interfere with the assay of phenol oxidase activity (for details of the assay, see the Methods section).

1. Specificity of the assay

A number of oxidative enzymes have been identified in *S. mansoni* which have the potential to interfere with the phenol oxidase assay by virtue of their ability to oxidize dopamine. These enzymes include cytochrome oxidase (Coles, 1972; Coles and Hill, 1972), monoamine oxidase (Nimmo-Smith and Raison, 1968) and dopamine-β-hydroxylase (Bennett and Gianutsos, 1978). In addition, Byram and Senft (1978) have suggested the possibility that peroxidase might also be present in the schistosome. In order to determine if any one of these enzymes was interfering with the phenol oxidase assay the phenol oxidase pellet was preincubated in the presence of selective inhibitors of the above-mentioned oxidative enzymes prior to the addition of phenol oxidase substrate. The inhibitors used in these studies included catalase (25,000 units), 1 μM rotenone, 10 μM, 1-phenyl-2-thiazolyl-2-thiourea (PTTU, UK14624), 100 μM α,α'-dipyridyl and 3 μM tranylcypromine (TCP).

One μM rotenone was found to inhibit succinate-stimulated oxygen consumption in the schistosome by 50% (n=4). Ten μM PTTU reduced schistosome NE levels from .28±.02 ng/mg worm to non-detectable levels in the catechol-o-methyl transferase radioenzymatic assay (n=3). α,α'-Dipyridyl has previously been characterized as a potent inhibitor of iron containing enzymes such as tyrosine hydroxylase (Taylor *et al*., 1969) and peroxidase (Mason, 1957). Tranylcypromine has been shown to be a potent inhibitor of schistosome monoamine oxidase, $K_i = 0.4$ μM (Nimmo-Smith and Raison, 1968). None of the inhibitors used in these studies blocked the increase in oxygen consumption produced by the addition of DA to the phenol oxidase pellet (Figure 14). However, when schistosome phenol oxidase was preincubated in the presence of compounds which are classical inhibitors of phenol oxidase, e.g., the copper chelating agents DDC, KCN and thiourea (Lerner, 1953), these compounds were found to be potent inhibitors of the oxygen consumption stimulated by the addition of 2 mM DA in the assay (Table 8). Other compounds known to chelate copper, including EDTA, bathocuproine sulfonate and penicillamine, were not found to be inhibitors of schistosome phenol oxidase. However, similar results have been obtained by other investigators working with phenol oxidase preparations of considerably greater purity (Pomerantz, 1963). The above results indicated that the assay for schistosome phenol oxidase was not significantly affected by other oxidative enzymes capable of utilizing DA as a substrate.

2. Characteristics of the enzyme

Having established the validity of the assay, it was then possible to examine some of the characteristics of schistosome phenol

Figure 14. Specificity of the reaction between crude S. mansoni phenol oxidase and DA. Inhibitors of interfering enzymes were added to the phenol oxidase preparation 3.5 min prior to addition of substrate. Each bar represents the mean ± S.E.M. of at least 3 determinations. Control is the rate of oxygen consumption by DA in the absence of inhibitors (.162±.011 μmoles O_2/min-mg protein).

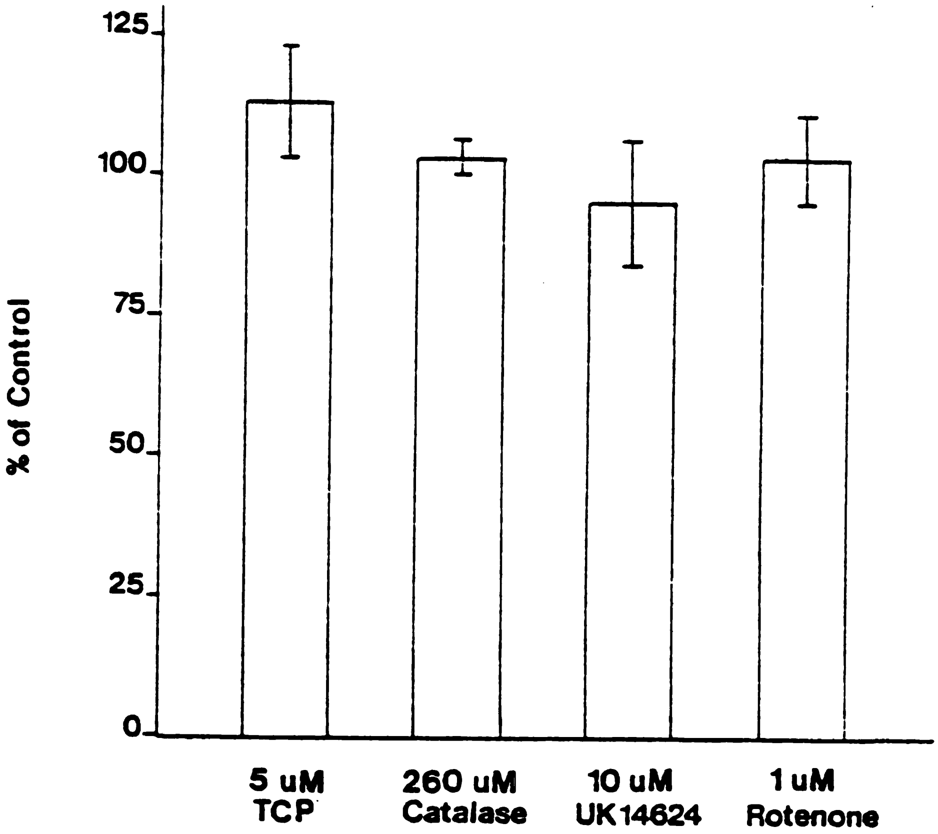

% of Control
125
100
75
50
25
0
5 uM TCP
260 uM Catalase
10 uM UK14624
1 uM Rotenone

Figure 14

TABLE 8

Effect of Inhibitors on Phenol Oxidase Activity in Female *S. mansoni*[a]

Compound[b]	K_i[c]
Sodium diethyldithiocarbamate	0.3 mM
Allylthiourea	.02 mM
Phenylthiourea	.04 mM
Potassium cyanide	.07 mM
L-Cysteine	13 mM
Disulfiram	2 mM

[a] Females were incubated for 4 hr in Earle's BME and assayed as described in Methods.

[b] The following compounds were found to have no significant effect at the noted concentrations. Penicillamine (0.1 mM), EDTA (0.1 mM), bathocuproine sulfonate (0.1 mM), antimony potassium tartrate (0.2 mM), 5-hydroxytryptamine (0.1 mM), thiourea (0.1 mM).

[c] K_i's were determined by Dixon plot.

oxidase. The enzyme was active over a fairly broad range of pH with an apparent maximum at pH 7.0 (Figure 15). Substrate specificity studies indicated that the enzyme preferred diphenolic over monophenolic substrates (Table 9). On the other hand, kinetic analysis of selected substrates indicated that the enzyme has its greatest affinity for phenolic substrates with an alanine side chain (Table 10). Thus, the enzyme oxidizes DA at a greater maximal rate than L-tyrosine, even though its affinity for DA is lower than its affinity for L-tyrosine. However, the rate of L-tyrosine oxidation can be stimulated by preincubation of the enzyme with 30 µM L-DOPA. This concentration of L-DOPA does not cause any appreciable consumption of oxygen by itself, but results in an 89% increase in the rate of tyrosine oxidation (Figure 16). On the other hand, 50 µM DA does not significantly stimulate tyrosine oxidation. Similar results have been obtained in studies on the stimulation of tyrosine hydroxylation by dihydroxyphenols in mammalian phenol oxidase (Hearing _et al._, 1978).

Based on its sensitivity to copper chelating substances, it would appear that the schistosome enzyme is a copper-containing enzyme. Kinetic analysis (Lineweaver-Burk plots) of the mode of inhibition of schistosome phenol oxidase by the copper chelating agents DDC and allylthiourea reveals that both the hydroxylation of tyrosine (Figure 17) and the oxidation of DA (Figure 18) are inhibited by these compounds in a non-competitive manner which is consistent with their purported mechanism of action. Although the addition of 20 µM $CuSO_4$ reversed the inhibition of DA oxidation induced by 10 µM DDC, it was

Figure 15. pH optimum of S. mansoni phenol oxidase. Each point represents the mean of 3 determinations.

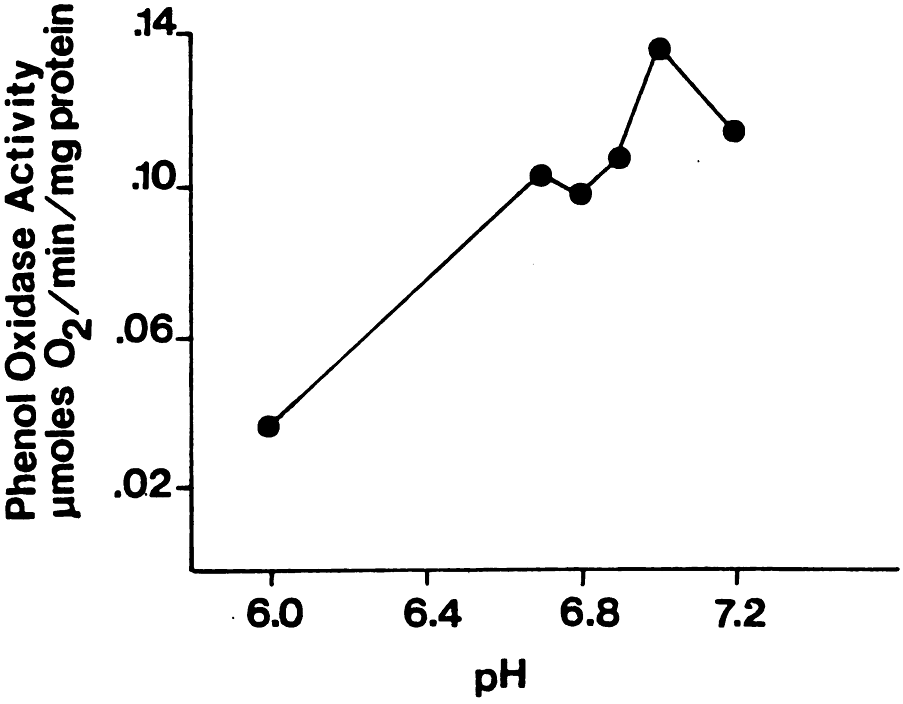

Phenol Oxidase Activity
µmoles O_2/min/mg protein
.14
.10
.06
.02
6.0
6.4
6.8
7.2
pH

Figure 15

TABLE 9

Effects of Substrates on Phenol Oxidase Activity in Homogenates of Female S. mansoni[a]

Substrate (2 mM)	μMoles O_2 Consumed/minute-mg protein[b]
L-DOPA methyl ester	.283±.021
Dopamine	.162±.018
Catechol	.138±.015
Tyrosine methyl ester	.144±.020
Epinephrine	.072±.008
Norepinephrine	.062±.004
Tyramine	.053±.004
Phenyl ethyl amine	.022±.003

[a] Female worms were incubated for 4 hours in Eagle's media and then homogenized as described in Methods.

[b] Data expressed as mean ± S.E.M. for at least 3 determinations.

TABLE 10

Michaelis Constants and Maximal Velocities for the Substrates Dopamine, L-DOPA Methyl Ester and L-Tyrosine Methyl Ester[a]

Compound	K_m (moles/liter)	V_{max} (μmoles O_2/min/mg protein)
Dopamine	1.8 mM	0.250
L-DOPA methyl ester	0.45 mM	0.338
L-Tyrosine methyl ester	0.42 mM	0.148

[a] Data obtained from double reciprocal plots of substrate concentration vs. velocity.

Figure 16. Stimulation of L-tyrosine oxidation by L-DOPA and DA. L-DOPA and DA were added to the phenol oxidase preparation 3.5 min prior to the addition of 1 mM TME. Asterisk indicates significantly different from control, $p<.05$. Each bar represents the mean ± S.E.M. for 5 determinations. Control is .144±.02 μmole O_2/min-mg protein.

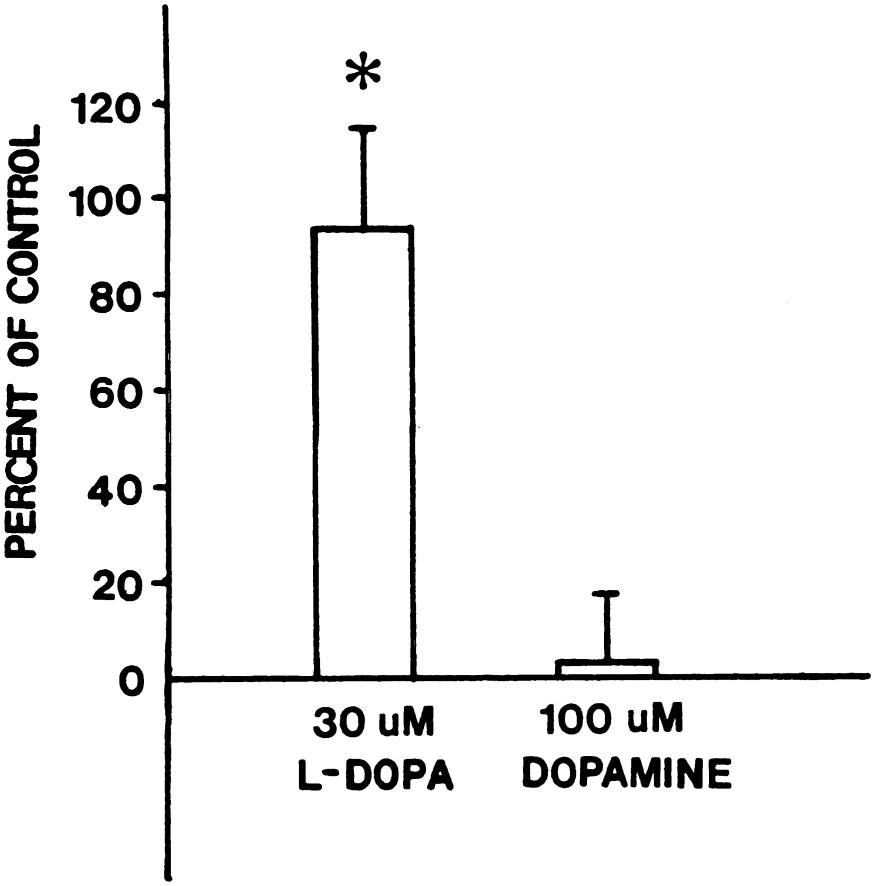

PERCENT OF CONTROL
120
100
80
60
40
20
0
*
30 uM
L-DOPA
100 uM
DOPAMINE

Figure 16

Figure 17. Noncompetitive inhibition of phenol oxidase by DDC. Substrate is 2 mM DA. Each point represents the mean ± S.E.M. of 4 determinations.

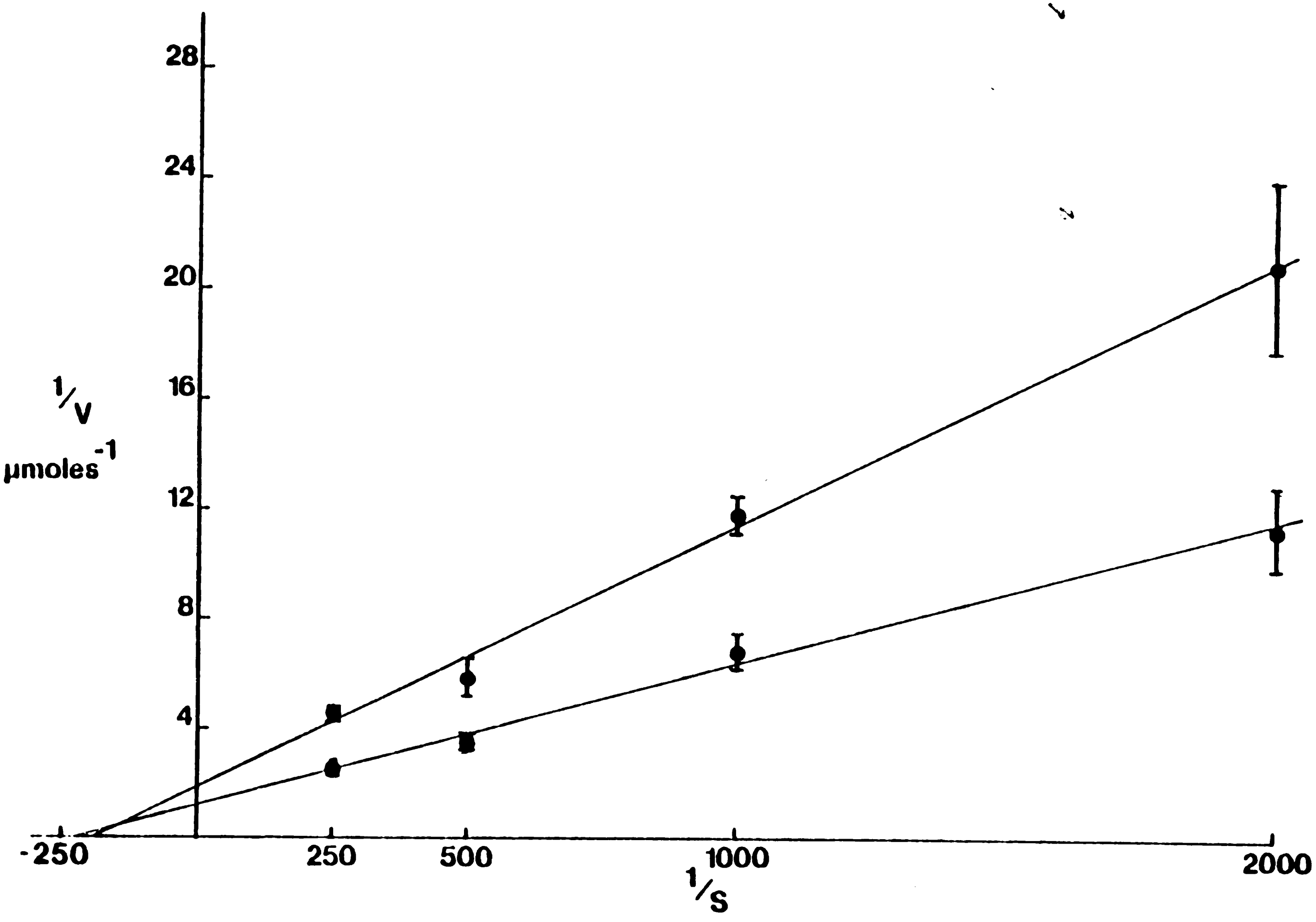


Figure 17

Figure 18. Noncompetitive inhibition of phenol oxidase by allylthiourea. Substrate is 1 mM TME. Each point represents the mean ± S.E.M. of 4 determinations.

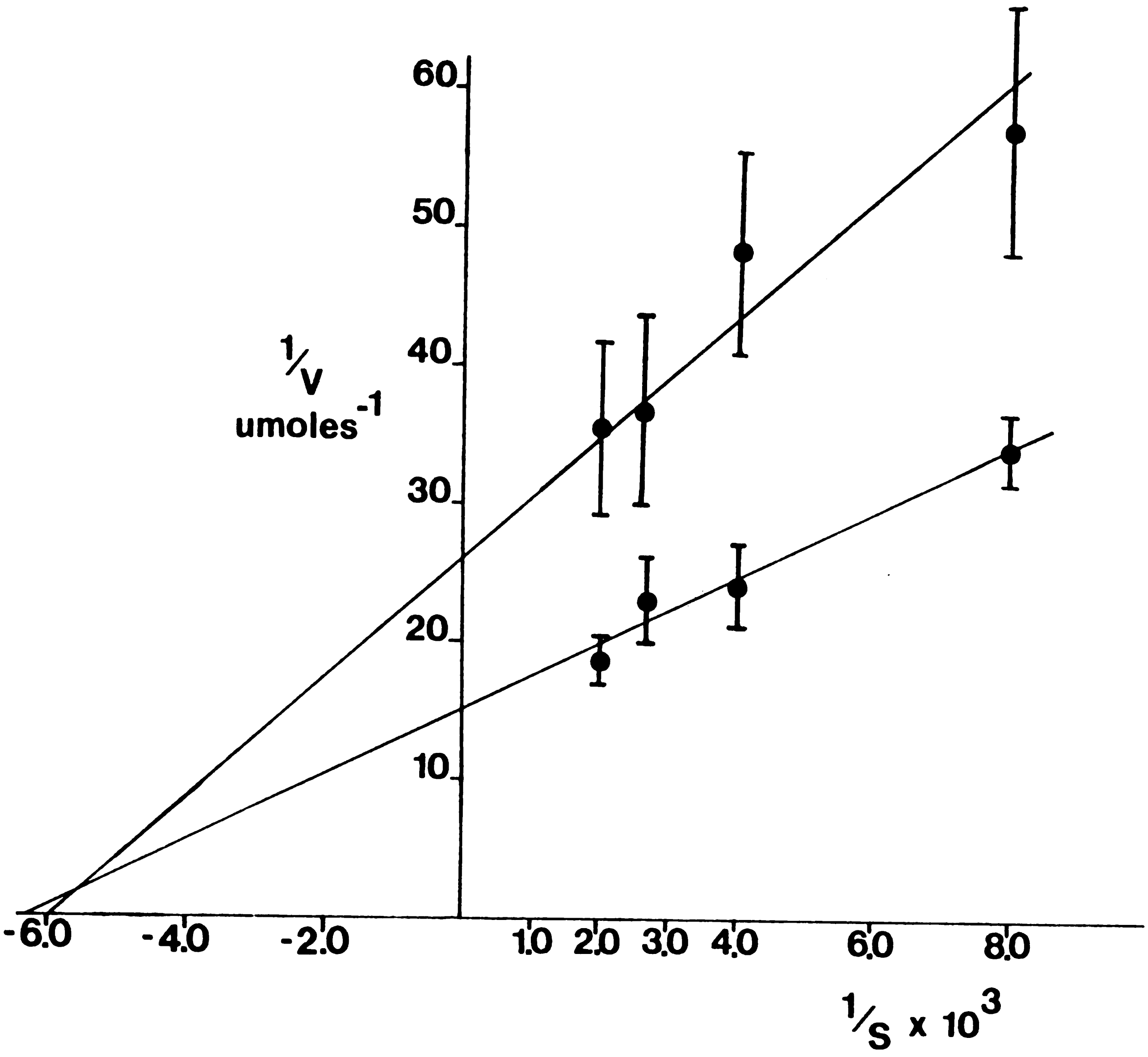


Figure 18

subsequently found that this phenomenon was not enzyme dependent since 10 μM $CuSO_4$ alone was capable of catalyzing the oxidation of DA.

Studies on the phenol oxidase reaction revealed that the product of the reaction between schistosome phenol oxidase and L-DOPA methyl ester fluoresces with a characteristic excitation and emission maximum in neutral buffer of 310 nm and 420 nm, respectively. This same product was formed by the reaction of purified mushroom phenol oxidase with L-DOPA methyl ester and is consistent with previous literature reports on this topic (Haas *et al*., 1951; Mason and Peterson, 1965).

D. Identification of the In Vivo Substrate for S. mansoni Phenol Oxidase

1. Soluble substrates

In the biochemical characterization of *S. mansoni* phenol oxidase (Section II), a number of compounds were noted to be potential substrates for this enzyme including L-tyrosine, L-DOPA and DA. These three compounds were the most likely candidates for the *in vivo* substrate since two of these compounds, L-tyrosine and DA had previously been demonstrated to be present in the schistosome (Senft *et al*., 1969; Gianutsos and Bennett, 1978). L-DOPA was also a likely candidate by virtue of its being an intermediate in the conversion of L-tyrosine to DA in the classical scheme of catecholamine metabolism (Cooper *et al*., 1978).

In order to determine which of these substrates was most likely to serve as the *in vivo* substrate for schistosome phenol oxidase, the concentrations of L-tyrosine, L-DOPA and DA were determined in the female schistosome and compared with the levels obtained from the male schistosome. Elevated concentrations of any one of these substrates in the female schistosome would be suggestive of a unique metabolic function for this compound in the female, possibly related to its role in eggshell formation.

a. L-Tyrosine concentrations: Female levels of tyrosine were found to be relatively constant over all ages tested (Figure 19). The average concentration of tyrosine was 252±7 ng tyrosine/mg wet weight of female schistosome. Tyrosine levels in the male schistosome declined from a concentration of 179±13 ng/mg 30 days post-infection to a plateau level of 92.9±2.1 ng/mg 38 days post-infection. In all cases, the concentration of tyrosine in the female was significantly greater than that seen in the male ($p<.01$). To insure that these differences represented true differences in tyrosine concentration, samples of male and female *S. mansoni* were assayed in the presence of internal standards or were subjected to thin layer chromatography prior to the assay as described in Methods. These assays failed to demonstrate the presence of any substances which could interfere with the tyrosine determinations. Values obtained for L-tyrosine concentrations in these studies are approximately 3-fold less than the values obtained by Senft *et al.* (1972). The reason for this discrepancy is apparently due to the use of dry weights by these authors while our studies are based on wet weights. If one assumes a wet/dry weight ratio of 4.48 (Isserhoff *et al.*, 1976), one can recalculate the values obtained by Senft *et al.* (1972) to yield a concentration of 180 ng/mg wet weight. This value is intermediate between the values determined for male and female *S. mansoni* in our studies.

Figure 19. L-Tyrosine concentrations in male and female *S. mansoni* as a function of age. (———) Male *S. mansoni*, (-----) female *S. mansoni*. Each point represents the mean ± S.E.M. of at least 4 determinations for male *S. mansoni* and 7 determinations for female *S. mansoni*.

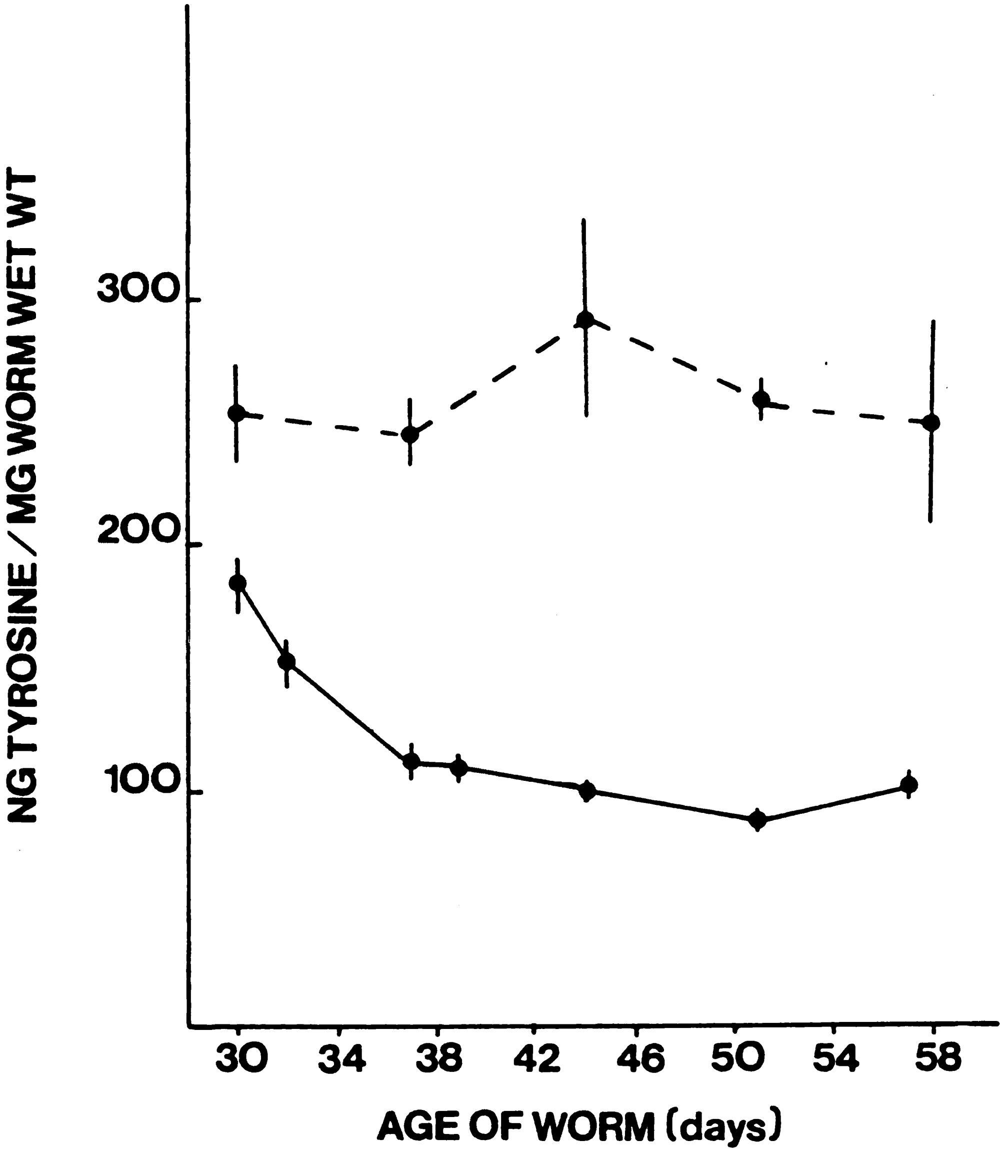

NG TYROSINE / MG WORM WET WT
300
200
100
30
34
38
42
46
50
54
58
AGE OF WORM (days)

Figure 19

b. L-DOPA and DA: The concentrations of these substrates in mature male and female S. mansoni (50 days post-infection) were determined by mass fragmentography or COMT radioenzymatic assay (Table 11). There was no significant difference between the DA levels determined by either mass fragmentography or COMT radioenzymatic assay. The concentration of DA in the female as determined by mass fragmentography (.790±.057 ng/mg worm) was approximately 3 times higher than the concentration in the male schistosome (.260±.037 ng/mg worm; $p<.01$). The concentration of L-DOPA in the female schistosome (.954±.129 ng/mg worm) was approximately 5 times higher than in the male schistosome (.196±.029 ng/mg worm)($p<.01$). These male-female differences in L-DOPA and DA concentrations are similar to the male-female differences in L-tyrosine concentration in mature schistosomes. Despite this high concentration of L-DOPA in the female schistosome, it was apparent that L-DOPA was not actively accumulated by the female schistosome (Figure 20), which suggests that most of the L-DOPA is derived metabolically from the hydroxylation of L-tyrosine.

These results suggest that L-tyrosine may be the *in vivo* substrate for schistosome phenol oxidase. However, Erasmus (1975) has presented evidence which suggests that exogenous L-tyrosine is preferentially incorporated into eggshell globule proteins. Thus, it may be that protein-bound tyrosine is the actual *in vivo* substrate for this enzyme, by virtue of its being present in high concentration and in close proximity to schistosome phenol oxidase, which has been histochemically localized in this thesis in the same eggshell globule as the tyrosine-rich proteins.

TABLE 11

L-DOPA and DA Concentrations in Male and Female S. mansoni as Determined by Mass Fragmentography (MF) and Catechol-o-methyl Transferase (COMT) Radioenzymatic Assay[a,b]

	Male		Female	
	COMT	MF	COMT	MF
L-DOPA	not determined	.196±.029	not determined	.954±1.29
DA	.234±.022	.260±.037	.736±.986	.790±.057

[a] Mean ± S.E.M. for at least 8 determinations.

[b] Data expressed in terms of ng/mg schistosome wet weight.

Figure 20. *In vitro* uptake of L-DOPA by female *S. mansoni*. Worms were incubated in Hank's salts containing varying concentrations of unlabeled L-DOPA and 2.5 μCi/ml of ^{3}H-L-DOPA (50-60 Ci/mmol). Each point represents the mean ± S.E.M. of 5 determinations.

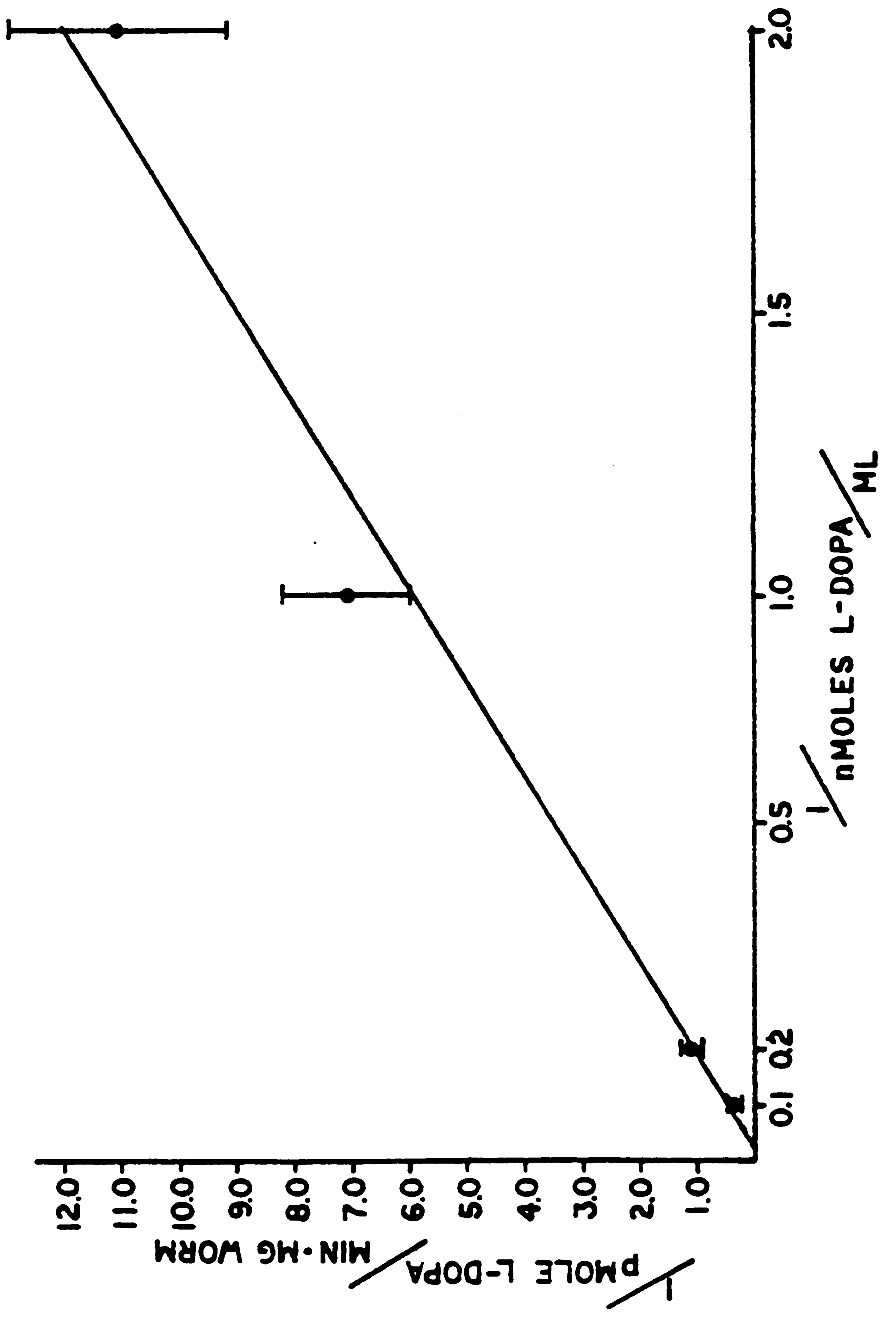

1/pMOLE L-DOPA/MIN·MG WORM
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
0.1
0.2
0.5
1.0
1.5
2.0
1/nMOLES L-DOPA/ML

Figure 20

2. Protein and peptide substrates

In order to determine if protein bound tyrosine could serve as a substrate for schistosome phenol oxidase, enzyme activity was determined in the presence of several protein and peptide substrates *in vitro*. A modified phenol oxidase assay was employed in these studies as described in the Methods section. Only one peptide, tri-L-tyrosine methyl ester (2 mg/ml) was found to produce a significant rate of O_2 consumption (Table 12). However, the rate of oxygen consumption produced by this substrate was extremely low (10.6±.7 nmoles O_2/min) compared to the rate of oxygen consumption produced by L-tyrosine alone (384±36 nmoles O_2/min). All other protein and peptide substrates failed to show significant rates of O_2 consumption in the presence of a relatively high concentration of schistosome phenol oxidase (1 unit/ml). On the other hand, mushroom phenol oxidase, when diluted to levels of activity which were equivalent to those obtained for schistosome phenol oxidase (i.e., 1 unit/ml) was found to oxidize a tyrosine:lysine polymer (1:1) which the schistosome enzyme was incapable of oxidizing. However, the rate of peptide oxidation by mushroom phenol oxidase (12.7±0.1 nmoles O_2/min) was only slightly higher than the rate of oxidation of tri-L-tyrosine methyl ester by schistosome phenol oxidase. Neither schistosome phenol oxidase nor mushroom phenol oxidase were capable of oxidizing chymotrypsinogen or homogenates of female schistosomes at protein concentrations of 1 mg/ml. However, chymotrypsinogen has previously been shown to be a substrate for mushroom phenol oxidase, albeit a weak substrate (Sizer, 1946, 1953). Therefore, it might be argued that the dilution of

TABLE 12

Proteins and Peptides as Substrates for _S_. _mansoni_ and Mushroom Phenol Oxidase

Enzyme	Substrate	Concentration	Oxygen Consumption[a,b]
_S_. _mansoni_[c] phenol oxidase	TME	1 mM	384±36
	Tyrosine:lysine polymer (1:1)	Sat'd Solution	not detected (N.D.)
	Tri-l-tyrosine methyl ester	2 mg/ml	10.6±.7
	Chymotrypsinogen	1 mg/ml	N.D.
	Female _S_. _mansoni_ proteins	1 mg/ml	N.D.
Mushroom phenol oxidase	TME	1 mM	350±25
	Tyrosine:lysine polymer (1:1)	Sat'd Solution	12.7±.1
	Tri-l-tyrosine methyl ester	2 mg/ml	Not tested
	Chymotrypsinogen	1 mg/ml	N.D.
	Female _S_. _mansoni_ proteins	1 mg/ml	N.D.

[a] Data expressed as mean ± S.E.M. for at least 3 determinations.

[b] Oxygen consumption expressed in terms of nmoles O_2/min.

[c] Phenol oxidase pellet produced by incubating 80 worms in BMEFC for 8 hrs.

schistosome proteins in this in vitro system has effectively prevented them from being oxidized at significant rates. However, it was not feasible to use higher concentrations of female schistosome proteins or phenol oxidase due to the constraints of volume (minimum volume = 1.5 mls) and the numbers of worms required to produce either 1 mg of protein or 1 unit of phenol oxidase (≈80 worms). Alternatively, it may be possible to answer the questions concerning the role of protein bound tyrosine in the process of phenol oxidase catalyzed eggshell formation by examining the relative abundance of tyrosine in the male as opposed to the female schistosome.

3. Relative abundance of tyrosine in female proteins

In all cases where it appears likely that protein bound tyrosine serves as a substrate for phenol oxidase in the process of protein sclerotization (Hackman, 1953; Brown, 1952) the substrate proteins have been found to be rich in aromatic residues. In order to determine if proteins from female schistosomes possessed a relatively high amount of tyrosine, male and female schistosomes were incubated in vitro in BMEFC in the presence of ^{3}H-L-tyrosine or ^{3}H-L-leucine. ^{3}H-L-leucine was used as a control for L-tyrosine since L-leucine possesses no reactive functional groups other than those involved in the peptide linkage and as such should not be involved in the formation of unusual protein structures such as the hardened eggshell protein. The results of these studies are summarized in Figures 21 and 22. L-tyrosine was incorporated into PCA-precipitated proteins obtained from homogenates of female S. mansoni to a lesser extent than L-leucine overall time points tested (Figure 22). More importantly, there were

Figure 21. *In vitro* incorporation of ^{3}H-amino acids into male and female *S. mansoni* PCA-precipitable proteins. Asterisk indicates significantly different from tyrosine, $p<.05$. Each point represents the mean ± S.E.M. of 6 determinations.

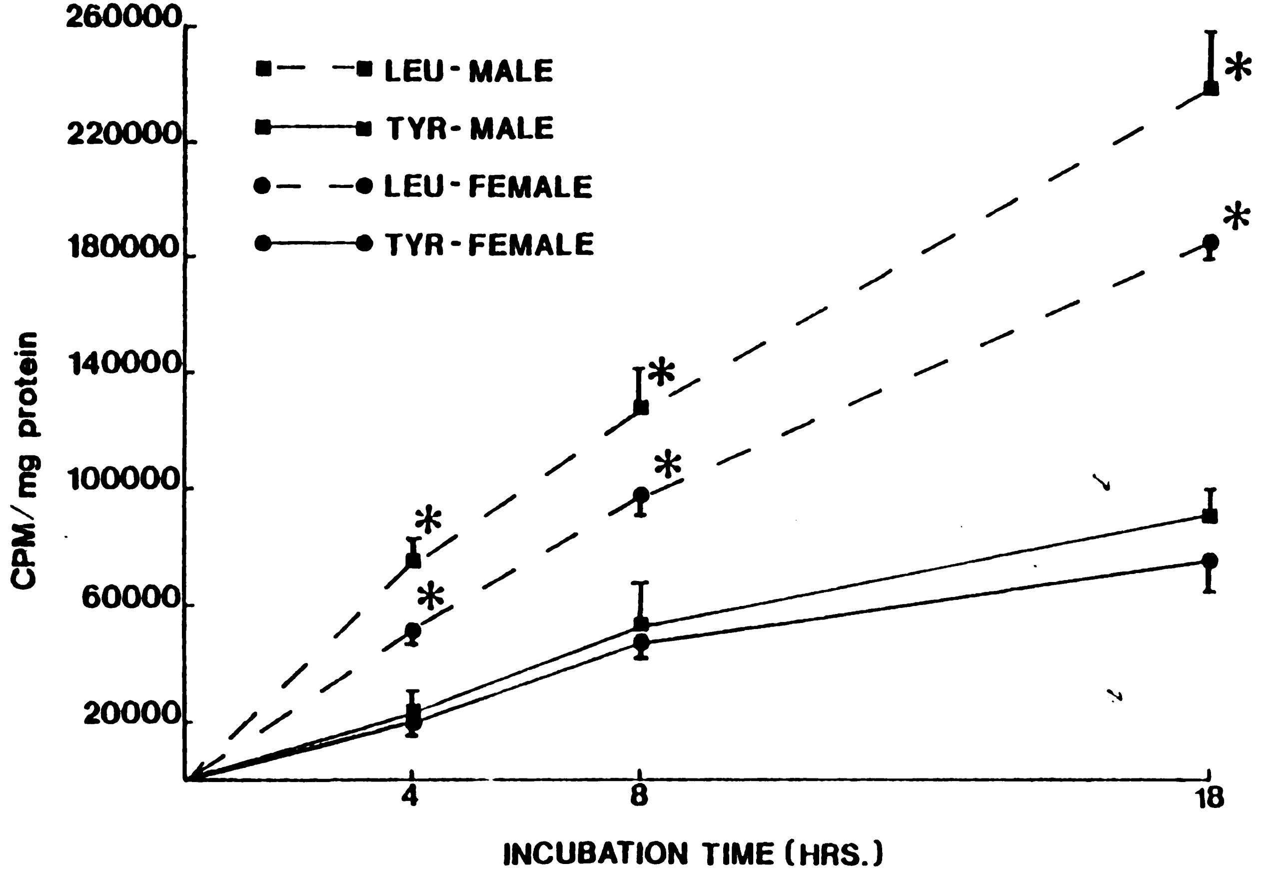

LEU - MALE
TYR - MALE
LEU - FEMALE
TYR - FEMALE
CPM/ mg protein
260000
220000
180000
140000
100000
60000
20000
4
8
18
INCUBATION TIME (HRS.)
*

Figure 21

Figure 22. *In vitro* male/female ratios for the incorporation of ^{3}H-amino acids into *S. mansoni* PCA-precipitable proteins. Data was paired for male and female *S. mansoni* obtained from the same incubation flask. Each bar represents the mean ± S.E.M. of 6 determinations.

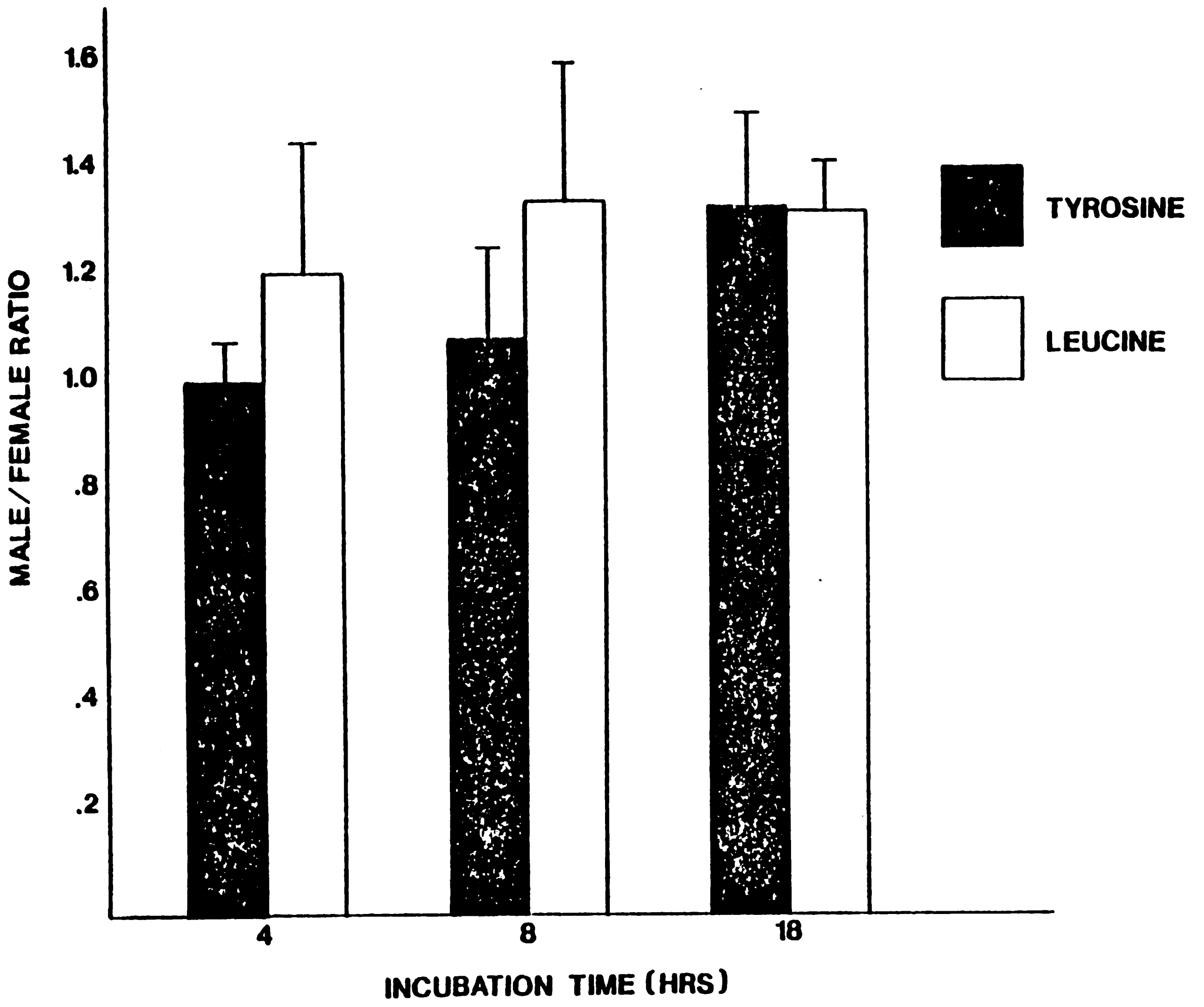

MALE/FEMALE RATIO
1.6
1.4
1.2
1.0
.8
.6
.4
.2
4
8
18
INCUBATION TIME (HRS)
TYROSINE
LEUCINE

Figure 22

no significant differences between the male/female ratios for the amounts of ^{3}H-L-tyrosine and ^{3}H-L-leucine incorporated into these PCA-precipitated proteins (i.e., the relative abundance of these amino acids in female schistosome proteins is similar to the relative abundance of these amino acids in the male schistosome)(Figure 23). However, since female S. mansoni do not produce eggs in vitro in BMEFC, this failure to find evidence of tyrosine rich proteins in female schistosomes might be due to inhibition of the protein synthesis mechanism which is responsible for the generation of eggshell proteins. In order to test this hypothsis, paired S. mansoni were incubated in modified RPMI 1640. In this medium, paired S. mansoni begin producing eggs on the second day of incubation at the rate of 31.4±8.1 eggs per day. In these studies, ^{3}H-L-tyrosine was added to the incubation medium for an 18 hour period beginning on day 2 of incubation. The results are summarized in Tables 13 and 14. These studies showed a significant ($p<.05$) increase in the amount of ^{3}H-L-tyrosine and ^{3}H-L-leucine incorporated into both male and female schistosome proteins when the incubations were performed in modified RPMI 1640 as opposed to BMEFC (Table 13). However, it was also noted that the relative abundance of both ^{3}H-L-leucine and ^{3}H-L-tyrosine was significantly ($p<.01$) higher in the proteins obtained from female worms incubated in modified RPMI 1640 as opposed to BMEFC (Table 14). These results indicated that although both male and female S. mansoni increased the rate of incorporation of exogenous, labeled, amino acids into protein in modified RPMI 1640, there was a greater but non-selective increase in the incorporation of these amino acids into proteins from female schistosomes.

Figure 23. *In vitro* incorporation of ^{3}H-amino acids into male and female *S. mansoni* PCA supernatants. Asterisks indicate significantly different from male, $p<.05$. Each point represents the mean ± S.E.M. of 6 determinations.

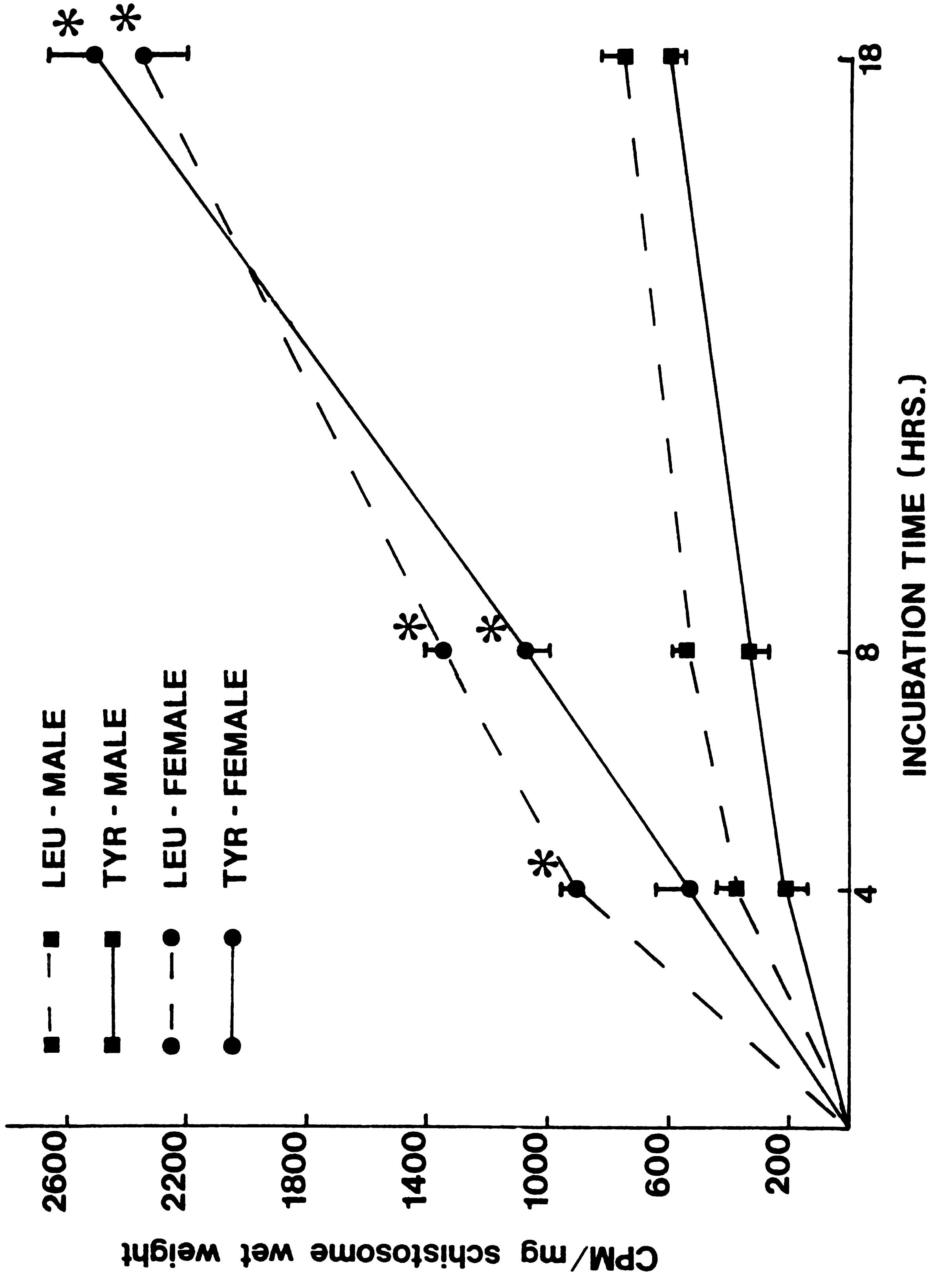

LEU - MALE
TYR - MALE
LEU - FEMALE
TYR - FEMALE
CPM/mg schistosome wet weight
2600
2200
1800
1400
1000
600
200
INCUBATION TIME (HRS.)
4
8
18
*

Figure 23

TABLE 13

Incorporation of ^{3}H-L-tyrosine and ^{3}H-L-leucine Into Male and Female S. mansoni PCA-precipitable Proteins Following 18 hrs In Vitro Incubation in Egg Producing (mod. RPMI 1640) and Non-egg Producing (BMEFC) Media

Culture Medium[b]	CPM x 10^3 Incorporated/mg Protein[a]			
	Male		Female	
	Tyrosine	Leucine	Tyrosine	Leucine
BMEFC	91.2± 7.8	237.5±18.3	75.5±10.9	186.4± 5.54
mod. RPMI[c] 1640	135.1±10.0	423.4±43.3	269.3±25.8	703.0±99.8

[a] Mean ± S.E.M. for 6 determinations.

[b] Specific activity of labeled amino acids in medium was 20 mCi/mmol.

[c] All values significantly greater than corresponding data in BMEFC (p<.05).

TABLE 14

Male/Female Ratios for the Incorporation of ^{3}H-L-Tyrosine and ^{3}H-L-leucine Into PCA Pellets and Supernatants of S. mansoni Pairs Incubated 18 hrs in BMEFC or mod. RPMI 1640

Culture Medium	Male/Female Ratios[a]			
	Tyrosine		Leucine	
	Pellet	Supernatant	Pellet	Supernatant
BMEFC[b]	1.33 ±.17	.226±.011[e]	1.32 ±.09	.325±.016
mod. RPMI[b,c] 1640	.684±.093[d]	.464±.040[d]	.637±.057[d]	.554±.063[d]

[a] Mean ± S.E.M. of 6 determinations.

[b] Sepcific activity of radiolabel was 20 mCi/mmol.

[c] Radiolabel was added 24 hrs after the start of incubation.

[d] Significantly different from BMEFC, $p<.05$.

[e] Significantly different from leucine supernatant, $p<.05$.

4. Metabolism of tyrosine as it relates to eggshell formation

Incubation of paired *S. mansoni in vitro* in BMEFC containing ^{3}H-L-leucine and ^{3}H-L-tyrosine as described previously results in a gradual increase in the amount of tritium in the supernatant with time (Figure 23). In contrast to protein bound tritium, the soluble tritium derived from either ^{3}H-L-tyrosine or ^{3}H-L-leucine is present in significantly ($p<.05$) greater concentration in the female than in the male at 8 and 18 hrs. While there are no significant differences between the amounts of soluble tritium incorporated into the male or female schistosome from either ^{3}H-L-tyrosine or ^{3}H-L-leucine, the male/female ratios reveal that the relative amount of tritium derived from L-tyrosine is significantly greater in the female schistosome after 18 hrs *in vitro* incubation (Figure 24). However, these differences are not apparent in the female schistosome following 18 hrs *in vitro* incubation in RPMI 1640 (Table 14). The observation that significant increases in the accumulation of tritium derived from L-tyrosine occur only after prolonged incubation in a non-egg-producing medium suggests that these differences may be related to differences in the metabolism of L-tyrosine in the two media.

In order to test this hypothesis, paired *S. mansoni* were incubated in either BMEFC or RPMI 1640 as previously described. After incubation, the females were separated from the males and homogenized in acidified acetone (2:1 v/v, acetone:0.1 N HCl). The supernatant fraction was spotted on a cellulose thin-layer plate and chromatographed as described in Methods. The results of these experiments are summarized in Table 15. Approximately 35% of the recoverable tritium was recovered as tyrosine in worms incubated in both BMEFC and RPMI

Figure 24. Male/female ratios for the _in vitro_ incorporation of ^{3}H-amino acids into PCA supernatants. Asterisk indicates significantly different from leucine, $p<.05$. Each bar represents the mean ± S.E.M. of 6 determinations.

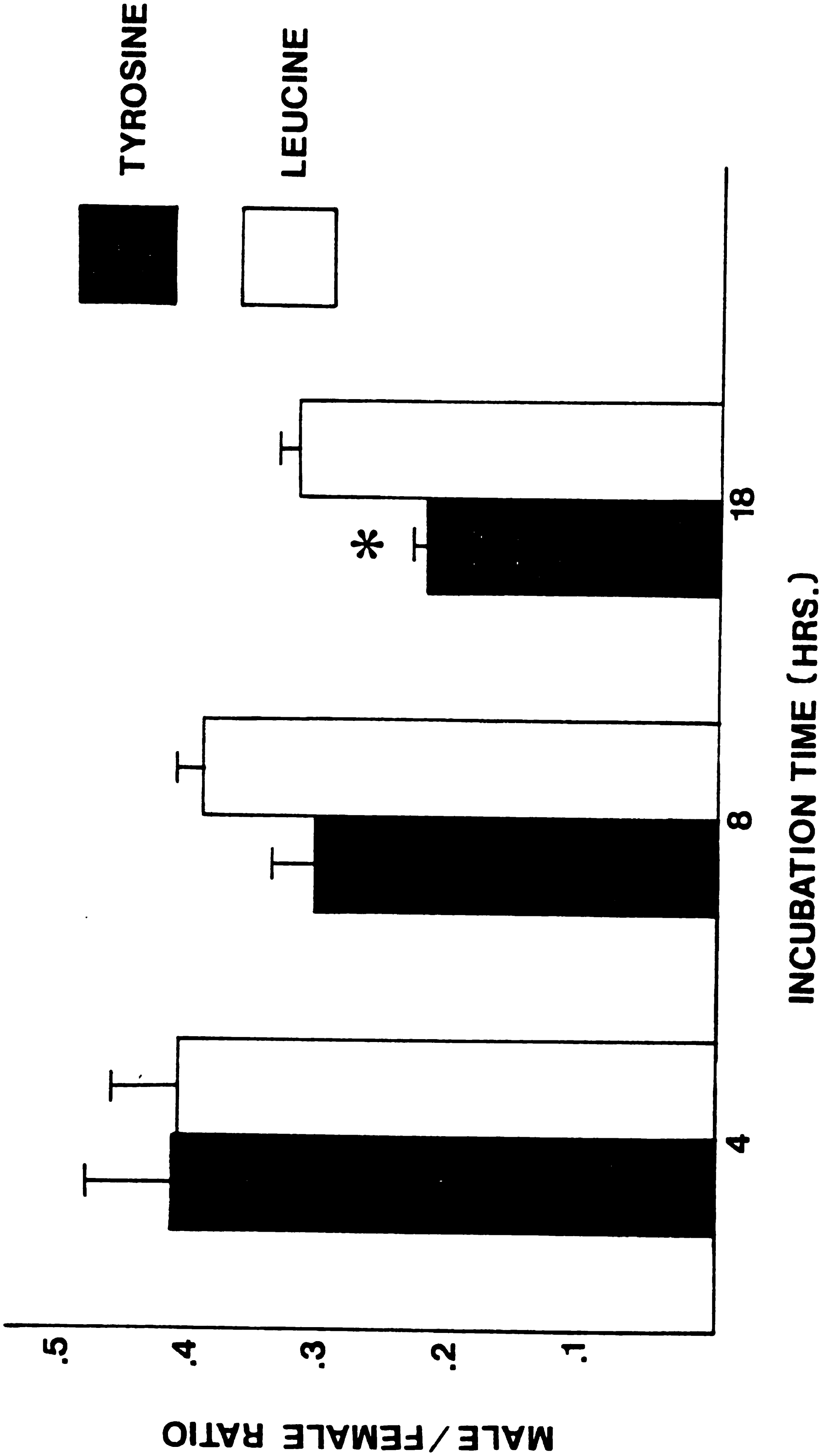

TYROSINE
LEUCINE
MALE / FEMALE RATIO
.5
.4
.3
.2
.1
4
8
18
*
INCUBATION TIME (HRS.)

Figure 24

TABLE 15

Metabolism of ^{3}H-L-Tyrosine in Female S. mansoni During 18 hrs In Vitro Incubation in BMEFC or mod. RPMI 1640

Culture Medium	% of Metabolite Recovered[a]				
	Tyrosine	DOPA	DA	NE	3MT,NM
BMEFC	32.0±1.8	5.6±1.7	2.2±0.7	1.4±0.2	1.3±0.3
mod. RPMI 1640	38.8±4.3	2.1±0.3	n.d.[b]	n.d.	n.d.

[a] Mean ± S.E.M. for 4 determinations.

[b] Not detectable.

1640. The only significant differences between the metabolism of tyrosine in BMEFC and modified RPMI 1640 were related to the metabolism of tyrosine to the catecholamines. Levels of tritiated catecholamines went from 2.2 and 1.3% for DA and the methoxylated metabolites (3MT and NM), respectively, in BMEFC to nondetectable levels in female schistosomes incubated in RPMI 1640. The remainder of the radioactive material (approximately 60%) was recovered on or near the origin and could not be identified. This material probably represents L-tyrosine present in proteins and peptides or other high molecular weight metabolites of L-tyrosine. These results suggest that L-tyrosine in RPMI 1640 may serve as a preferential substrate for another enzyme (e.g. phenol oxidase) which is not part of the classical scheme of tyrosine metabolism to the catecholamines which has been demonstrated in nervous tissues (Cooper _et al_., 1978).

5. _Soluble L-tyrosine and in vitro protein crosslink formation_

It is an essential tenet of this thesis that the reaction of phenol oxidase with its substrate L-tyrosine produces a highly reactive product which is capable of cross-linking proteins from the female schistosome during the process of eggshell formation. In order to determine if the female schistosome contains proteins which are capable of being polymerized by _o_-quinones, ^{3}H-labeled proteins from female _S. mansoni_ were incubated _in vitro_ in the presence or absence of L-tyrosine and phenol oxidase as described in the Methods section. The formation of insoluble protein polymers was monitored by measuring the increase in the amount of ^{3}H-labeled protein deposited in a 3,000 x g pellet obtained from homogenates of female schistosomes which had been incubated with phenol oxidase and L-tyrosine. The results of these studies are summarized in Table 16. There was a slight, but significant ($p<.05$) increase in the amount of ^{3}H-protein incorporated into the 3,000 x g pellet following incubation in the presence of a half-saturated solution of L-tyrosine and schistosome phenol oxidase. In the absence of L-tyrosine or phenol oxidase, there was no significant increase in the amount of ^{3}H-protein in the 3,000 x g pellet when compared to the amount of ^{3}H-protein in pellets obtained from S. mansoni homogenates incubated in the absence of both L-tyrosine and phenol oxidase. Extraction of the insoluble pellet with 2% SDS and 1% β-mercaptoethanol did not result in statistically significant increases in the amount of ^{3}H in SDS extracts of the pellets obtained from homogenates of female schistosomes. SDS polyacrylamide gel electrophoresis failed to reveal the presence of any apparent protein polymers when stained with Coomassie blue (Figure 25). However, when these polyacrylamide gels were sliced and counted to determine the extent of ^{3}H present, it became apparent that the SDS extract of the 3,000 x g pellet obtained from homogenates incubated in the presence of both L-tyrosine and phenol oxidase contained a significantly greater ($p<.05$) amount of tritium label within the first 1-2 mm of these gels when compared to pellets obtained from homogenates incubated in the absence of L-tyrosine and phenol oxidase (Figure 26). However, this increase was not statistically different from the other two controls (L-tyrosine alone or phenol oxidase alone), which in turn were not statistically different from the untreated homogenates. These data provided evidence to support the contention that the increase in ^{3}H

TABLE 16

Formation of Insoluble Protein Polymers Following *In Vitro* Incubation of ^{3}H-female *S. mansoni* Proteins with L-Tyrosine Plus *S. mansoni* Phenol oxidase [a,b]

Treatment	Insoluble Pellet	Supernatant	Total	% in Pellet
Phenol oxidase + L-tyrosine + female *S. manosni* proteins	44.8±1.78	215.8±23.5	267.1±25.6	16.27±.64[c]
L-tyrosine + female *S. mansoni* proteins	31.2±2.7	243.0± 9.4	300.9±11.0	12.41±.61
Phenol oxidase + female *S. mansoni* proteins	37.4±1.9	262.6±33.5	327.3±39.9	11.60±.76
Female *S. mansoni* proteins	41.3±1.5	297.8± 8.6	362.0±56.1	11.29±.42

[a] Mean ± S.E.M. for 3 determinations.

[b] Data expressed in terms of DPM x 10^3.

[c] Significantly different from controls, $p<.05$.

Figure 25. Distribution of female S. mansoni protein in SDS-polyacrylamide gel following incubation with S. mansoni phenol oxidase. A) Proteins incubated in the presence of phenol oxidase and excess l-tyrosine. B) Proteins incubated in the presence of phenol oxidase alone. C) Proteins incubated in the presence of excess l-tyrosine alone. D) Proteins incubated in the absence of l-tyrosine and phenol oxidase.

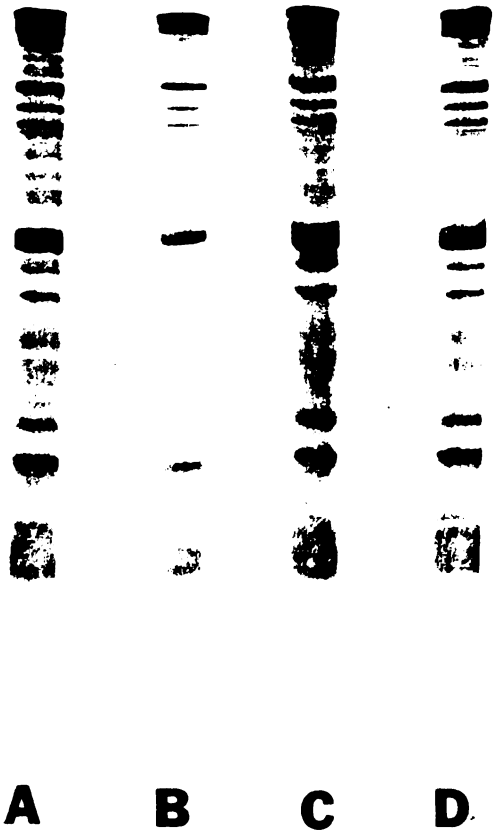


Figure 25

Figure 26. Distribution of ^{3}H-proteins from female *S. mansoni* in a 10% polyacrylamide gel following incubation with *S. mansoni* phenol oxidase. Each point is the mean of 3 determinations. (——) Proteins incubated in the presence of excess l-tyrosine and *S. mansoni* phenol oxidase. (----) Proteins incubated in the absence of l-tyrosine and phenol oxidase. Asterisk indicates significantly different from control.

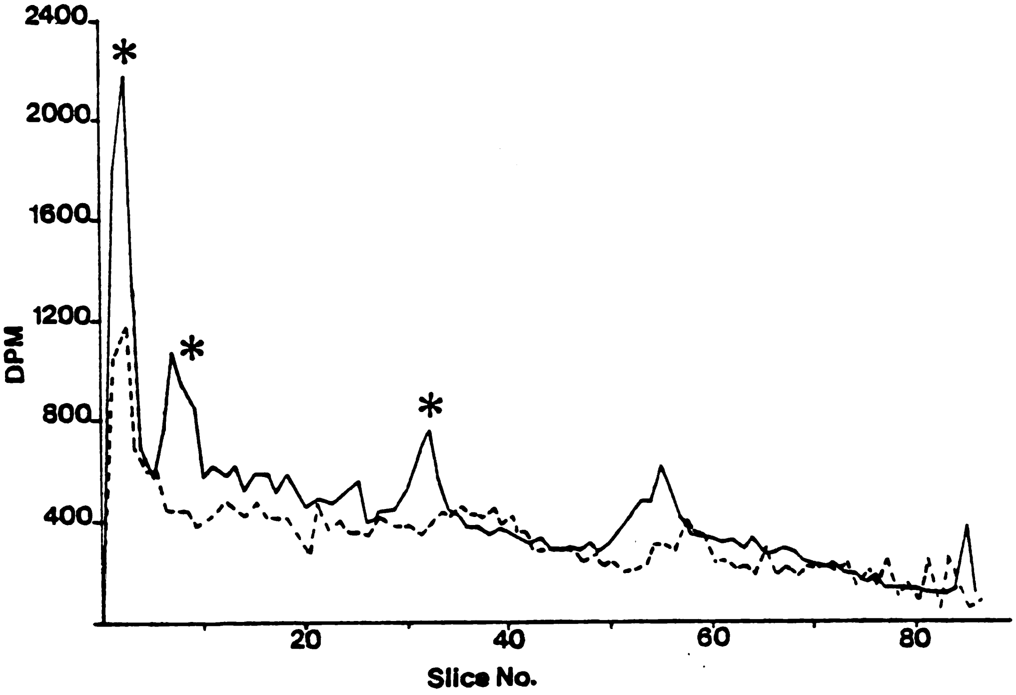


Figure 26

seen in the insoluble pellet after incubating with excess L-tyrosine and phenol oxidase represented the formation of high molecular weight polymers.

E. Identification of the Link Between In Vitro Phenol Oxidase Inhibition and In Vivo Eggshell Formation

In the preceding sections it has been shown that female *S. mansoni* contain an enzyme which can be characterized as phenol oxidase, and for which there is an excess of available substrate. However, the identification of these two most essential components of quinone-tanning in female *S. mansoni* does not establish that this enzyme is the major catalyst of eggshell formation *in vivo*.

Evidence to implicate phenol oxidase in eggshell formation was obtained from studies on the fluorescent products obtained from *S. mansoni* eggshell hydrolysates. In order to determine if the products of the reaction between phenol oxidase and tyrosine could be identified in the schistosome eggshell, purified *S. mansoni* eggshells were prepared as described in Methods. The eggshells were then hydrolyzed in acid and the hydrolysate dried under nitrogen. When the hydrolysate was redissolved in dilute acid (.01 N HCl), no strong fluorescent peaks could be identified. However, when the hydrolysate was redissolved in 1 N NaOH, a number of fluorescent peaks became readily apparent (Figure 27). There were two major excitation/emission peaks which could be identified in the eggshell hydrolysate. The first peak had excitation/emission maxima of 310/420 and the second peak 360/460 (Figure 27). In order to determine if these products corresponded to any known product of the reaction between tyrosine and phenol oxidase, the products of this latter reaction were hydrolyzed, dried and

Figure 27. Fluorescence spectrum of acid hydrolyzed S. mansoni eggshells. Spectra were obtained in 1 N NaOH. Excitation wavelength set to 310 nm for emission scan and emission wavelength set to 410 nm for excitation scan; curves A and C. Excitation wavelength set to 360 nm for emission scan and emission wavelength set to 470 nm for excitation scan, curves B and D. Curves A and D: Acid hydrolysis products from reaction of L-DOPA with phenol oxidase. Curves B and C: Eggshell hydrolysate. Relative intensity of excitation curve D and emission curve C are increased 3X.

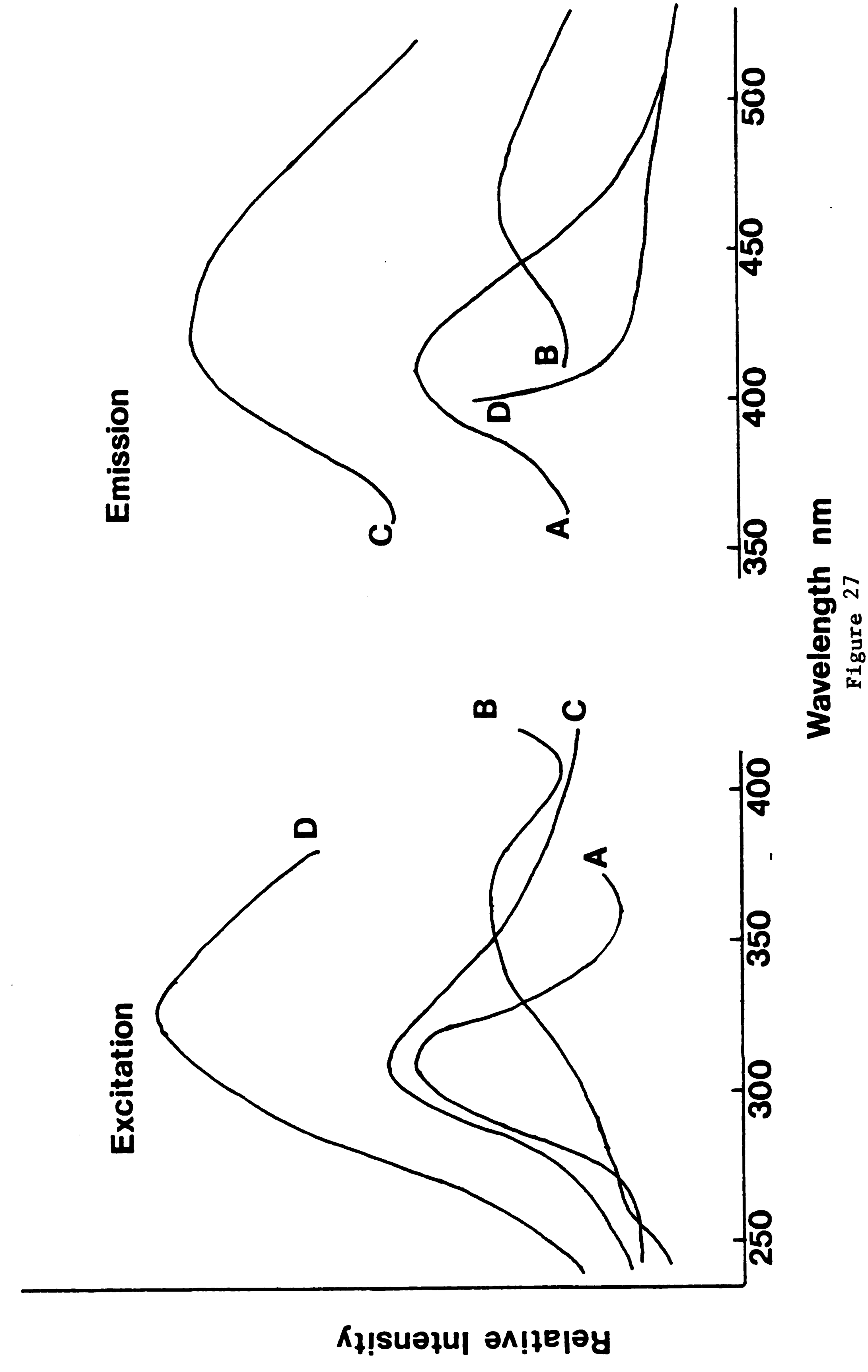

Excitation
Emission
A
B
C
D
A
B
C
D
250
300
350
400
350
400
450
500
Wavelength nm
Relative Intensity

Figure 27

redissolved as described for the eggshells. The products formed from this reaction had a fluorescence spectrum very similar to the 310/420 peak of the eggshell. However, almost all of the products formed by the reaction of L-DOPA with phenol oxidase in the presence of nucleophilic amino acids absorb in the U.V. spectrum at 310 nm except the thiol containing compounds (e.g., cysteine) which absorb at 360 nm. Although the hydrolyzed eggshell has an absorption maximum at 360 nm, both the excitation and emission peaks are very broad, suggesting that the peak seen is composed of multiple fluorescent entities. Therefore, the eggshell hydrolysate was chromatographed on a silica gel thin layer plate and the fluorescence spectrum obtained from slices of this gel were compared with the fluorescence spectrum of the products formed from the reaction of L-DOPA with mushroom phenol oxidase in the presence of various amino acids containing nucleophilic side chains. These latter products were hydrolyzed in acid and were treated the same as the eggshells.

The products formed from the reaction of a tyrosine-lysine (1:1) polymer with a quinone derived from the reaction of L-DOPA with phenol oxidase yielded 2 fluorescent products with apparent excitation/emission maxima which were similar to those found in the eggshell. One product which migrated with a R_f of between .07 and .13 yielded excitation/emission maxima of 325/420 which closely corresponded to a compound in the eggshell which appeared to fluoresce at 330/425. The second product migrated between .93 and 1.00 and gave a fluorescent product with excitation/emission maxima at 335/395. These maxima corresponded to an identical peak in the eggshell hydrolysate. No other fluorescent peaks were identified which possessed similar R_f values and fluorescence spectra.

The results of these studies suggested that the products formed from the reaction of protein bound lysine with a DOPA or tyrosine derived quinone can be identified in *S. mansoni* eggshells, thus providing additional evidence to support a concept of schistosome eggshell formation in which phenol oxidase reacts with tyrosine to form a quinone which cross-reacts with multiple proteins in the eggshell to form a single large and extensively cross-linked protein.

In order to determine if there was a good correlation between inhibition of phenol oxidase activity and inhibition of eggshell formation, infected mice were injected with varying quantities of compounds which have been demonstrated to be inhibitors of phenol oxidase *in vitro* (Section 3, Table 8). Administration of these compounds had a dramatic effect on egg production. Schistosome eggs in the uterus of female *S. mansoni* obtained from mice treated with phenol oxidase inhibitors lacked the well-defined eggshell characteristic of *S. mansoni* eggs (Figure 28). Instead there was a small amount of globular material present in the uterus which fluoresced in a manner similar to the intact eggshell (Figure 29). However, since sulfhydryl groups such as those present in DDC and allylthiourea (C=S) are capable of reacting with quinones (Hirsch, 1955) it is possible that these compounds prevented formation of the intact eggshell by preferentially reacting with the quinones formed from phenol oxidase rather than inhibiting the phenol oxidase catalyzed formation of quinone. In order to control for this possibility, infected mice were

Figure 28. Appearance of normal _S_. _mansoni_ egg in U.V. light. Photographed with dark-field condensor. Color of the egg is yellow. Magnification is 140X.

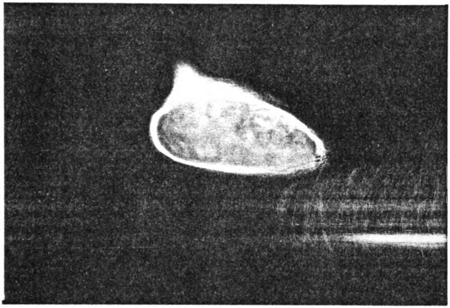


Figure 28

Figure 29. Appearance of *S. mansoni* egg in U.V. light 1 hr following the administration of 40 mg/kg diethyldithiocarbamate. Photographed with dark-field condensor. Color of material is yellow. Magnification is 140X.

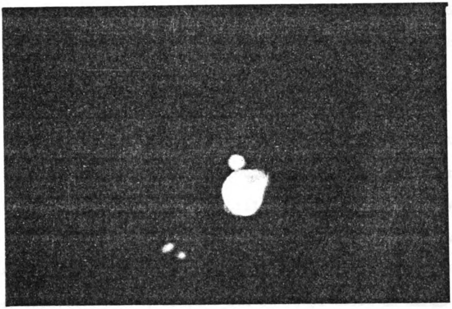


Figure 29

administered large doses of compounds which were known to be structural analogs of schistosome phenol oxidase inhibitors (e.g., thiambutosine, clofazimine, PTTU), but which were not demonstrated to be effective inhibitors of schistosome eggshell formation _in vitro_. These inactive analogues failed to produce inhibition of eggshell formation at doses which produced 100% inhibition of eggshell formation using known inhibitors of schistosome phenol oxidase (Table 17). Thus, it appeared that inhibition of eggshell formation was closely correlated with inhibition of phenol oxidase activity. However, since DDC and allylthiourea, the two phenol oxidase inhibitors used in these studies, are chelating agents, the possibility existed that the inhibition of eggshell formation by these compounds might be related to the inhibition of other metal ion containing enzymes such as peroxidase. In order to control for this possibility, infected mice received a single dose of 100 mg/kg of compounds which either had been demonstrated to inhibit peroxidases involved in eggshell formation (sodium sulfite, phenylhydrazine; Foerder and Shapiro, 1977) or which were known to inhibit iron containing enzymes in general (α,α'-dipyridyl). These drugs failed to inhibit eggshell formation (Table 17). It was noted, however, that these compounds appeared to produce more structural abnormalities (creasing, deformation, elongation of spike) in the eggshell than were apparent in the eggs of untreated schistosomes. It was not certain if these structural changes represented specific changes due to the action of these drugs on peroxidase or a nonspecific artifact of drug administration. However, it was clear that schistosome eggshell formation was more susceptible to the actions of phenol oxidase inhibitors than peroxidase inhibitors.

TABLE 17

The Effects of Inhibitors of Protein Cross-link Formation on *S. mansoni* Eggshell Formation *In Vivo*[a,c]

Compound	Dose mg/kg	# of Exp.	Percentage Abnormal Eggs	% Inhibition of Phenol Oxidase *In Vitro* at 10^{-4}M
DDC	100	(2)	100	69%
	40	(8)	79	
	20	(7)	20	
Allylthiourea	100	(2)	100	74%
	40	(3)	83	
	20	(3)	29	
Phenylthiourea	20	(1)	0[b]	69%
PTTU	100	(2)	0	5%
clofazimine	100	(2)	0	Not Tested
thiambutosine	100	(2)	0	3%
penicillamine	100	(2)	0	-15%

[a] All drugs were administered i.p. in 1% methyl cellulose 1 hr prior to sacrifice.

[b] Animal died.

[c] The following drugs did not inhibit eggshell formation at the concentrations indicated and were not found to inhibit phenol oxidase *in vitro* at 10^{-4}M: α,α-dipyridyl (100 ng/kg), phenhydrazine hydrochloride (100 mg/kg), sodium sulfite (100 mg/kg), dl-α-tocopherol acid succinate (400 m/gkg), ascorbic acid (100 mg/kg).

On the other hand, the inhibition of eggshell formation by phenol oxidase inhibitors did not appear to be complete (Figure 28). There was a small amount of residual globular material which fluoresced in a manner similar to the intact eggshell and which persisted following the administration of as much as 200 mg/kg DDC. These globules like eggshells, were brown when visualized with a light microscope and as will be discussed later, were not found to be antigenic. These characteristics of the globules are very similar to the characteristic of purified eggshells (Boros and Warren, 1970) and thus the globules were thought to represent eggshell material. Since doses of DDC well in excess of the dose required to produce 100% inhibition of eggshell formation failed to completely block the formation of eggshell globules, it was thought that some other process such as the autooxidative or peroxidative generation of quinones, or protein cross-linking mediated by free radical formation, might be involved in the generation of this residual eggshell material. In order to determine if one of these mechanisms was responsible for the continued formation of eggshell material in the presence of DDC, a number of antioxidants including ascorbic acid (100 mg/kg), dl-α-tocophenol acid succinate (400 mg/kg) and PTTU (100 mg/kg) were injected into infected mice 1 hr prior to the administration of 200 mg/kg DDC. These antioxidants, in addition to preventing the autooxidative formation of quinones (Hirsch, 1955) also inhibit free radical chain reactions (Tappel, 1972) which may be involved in the formation of protein cross-links (Roubal and Tappel, 1966). None of these compounds caused the complete cessation of eggshell globule formation following DDC administration. Nevertheless, these drugs appear to be capable of inhibiting eggshell formation to a limited extent. When given alone in the doses mentioned above, only ascorbic acid inhibited eggshell formation, and this inhibition occurred in only 1 worm in 20. However, administration of these drugs in doses of 100 mg/kg (dl-α-tocophenol acid succinate) or 50 mg/kg (ascorbic acid, PTTU) resulted in an increase in the percentage of abnormal eggs in the uterus following the administration of submaximal doses of DDC (Table 18). It should be noted that these results were based on only 2 experiments, 2 mice/experiment with a minimum of 10 worms/experiment. In addition to the antioxidants, α,α'-dipyridyl was also tested for its ability to inhibit eggshell formation. Like the antioxidants, this compound at 100 mg/kg did not inhibit eggshell formation by itself, but did potentiate the DDC-induced inhibition of eggshell formation at a dose of 50 mg/kg (Table 18). One hundred mg/kg dipyridyl alone did not inhibit the formation of eggshell globules (Table 17).

F. Chemotherapy of Schistosomiasis Using Phenol Oxidase Inhibitors

It is apparent from the results of the preceding section that the process of eggshell formation is most susceptible to the inhibitory actions of lipophilic copper chelating agents such as DDC and allylthiourea. Thus, in order to evaluate the effectiveness of alleviating the pathology of schistosomiasis by inhibition of eggshell formation, heavily infected mice were fed disulfiram chronically in the diet (0.3%). The effectiveness of disulfiram in alleviating schistosomal pathology was evaluated in terms of its ability to prolong the survival of heavily infected mice and reverse the microscopic pathology associated with egg deposition. It should be noted that disulfiram *per se*

TABLE 18

The Effects of Some Inhibitors of Protein Cross-link Formation on the DDC Induced Inhibition of Eggshell Formation *In Vivo*[a]

Compound	Dose mg/kg	# of Exp.	Inhibition of Eggshell Formation[b]		
			Control	DDC 20 mg/kg	DDC 40 mg/kg
Control	---	(2)	---	7/29	19/23
dl-α-tocopherol acid succinate	100	(2)	0/30	12/24	35/38
PTTU	50	(2)	0/27	9/31	26/28
α,α'-dipyridyl	50	(2)	0/34	9/27	34/36
ascorbic acid	50	(2)	0/29	9/21	19/21

[a] Vehicle or vehicle plus inhibitor was determined i.p. in 1% methyl cellulose 1 hr prior to the administration of DDC. Animals were sacrificed and worms examined 1 hr following the administration of DDC.

[b] Data expressed in terms of the number of abnormal eggs divided by the total number of worms containing egg material in the uterus or uterine canal.

is a relatively poor inhibitor of schistosome phenol oxidase (Table 8). However, disulfiram is rapidly metabolized to DDC (Linderholm and Berg, 1951) which, as has previously been mentioned, is a potent inhibitor of phenol oxidase *in vitro* and eggshell formation *in vivo*.

The results of these studies are illustrated in Figure 30. Some early deaths occurred in the drug treated group but these were few in number and ceased by the 48th day after infection. The early deaths in the drug treated and control animals were probably secondary to the severity of the infection. The gross pathology normally associated with the deposition of schistosome eggs in the liver (distended portal veins, swollen and granular appearance of liver and spleen) did not become apparent until after the 40th day of infection in the untreated mice.

After this initial period of increased mortality, no more deaths occurred in the drug-treated animals until the conclusion of the experiment. Deaths in the control group occurred at a more rapid rate, reaching a low of 20% surviving (4/20) by the 96th day after infection. Note that the animals which were withdrawn from the drug diet showed a sudden increase in mortality beginning 12 days following the cessation of drug treatment. Thus, it appears that in the absence of continued drug treatment, the beneficial effects of disulfiram treatment are reversed.

Light microscopic examination of the livers, spleens, intestines and lungs from both treated and untreated mice revealed substantial differences in pathology between these groups. Examination of the livers from untreated mice (Figure 31) revealed the presence of characteristic granulomatous lesions surrounding *S. mansoni* eggs or

Figure 30. Effects of disulfiram on mortality of mice infected with *S. mansoni*. (——) Mice on control diet; (---) mice receiving 0.3% disulfiram on diet; (– – –) mice withdrawn from 0.3% disulfiram diet. Arrow indicates time at which mice were withdrawn from the drug diet.

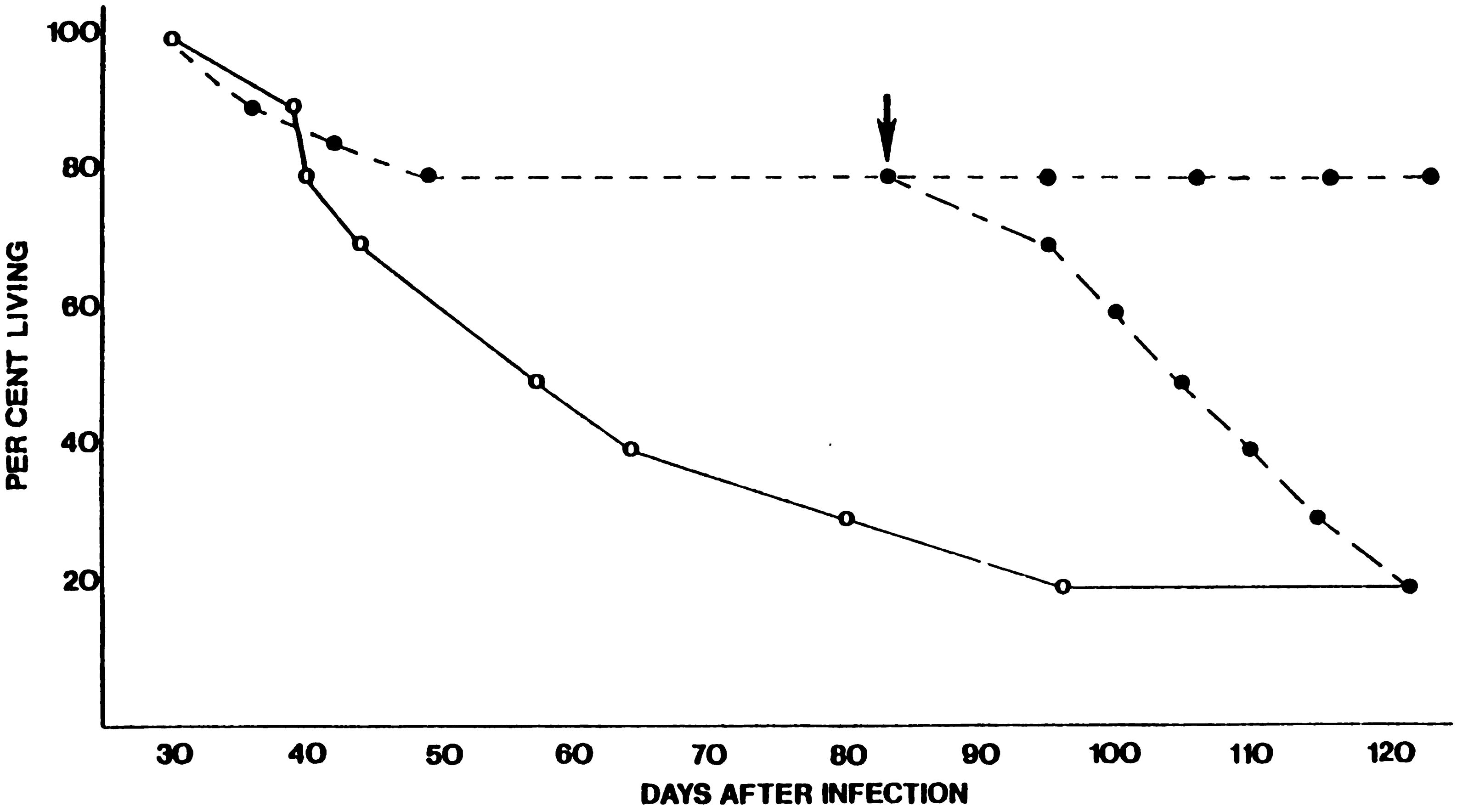

PER CENT LIVING
100
80
60
40
20
30
40
50
60
70
80
90
100
110
120
DAYS AFTER INFECTION

Figure 30

Figure 31. Portal triad from liver of mouse infected with S. mansoni 122 days post-infection. PV = portal vein. Tissue stained with hematoxylin and eosin. Magnification is 100X.

Figure 31

egg remnants. These lesions typically contained lymphocytes, macrophages, eosinophils and proliferating fibroblasts. In addition, considerable bile duct hyperplasia was noted along with extensive infiltration of periportal areas with lymphocytes, eosinophils and macrophages. Livers from animals receiving the disulfiram treatment showed relatively few granulomas (Table 19). Those granulomas which were present were associated with the presence of an occasional intact egg. The presence of these eggs and granulomas may be due to the development of tolerance to the effects of disulfiram. The dose of disulfiram used in these studies is only slightly above the threshold dose (0.2% in the diet) necessary to produce complete inhibition of eggshell formation (Bennett and Gianutsos, 1978). The liver of a disulfiram treated mouse sacrificed on Day 42 was not noted to contain any eggs, although the liver of an infected mouse was noted to contain numerous eggs. However, some eggs and egg granulomas were present in the livers of mice given the same dose of disulfiram over a period of 23 days (30-53 days post-infection) (Table 19).

In addition to the drastic reduction in the numbers of granulomas, the treated livers also contain small globules of schistosome egg material which are not seen in livers from untreated mice (Figure 32). This material is similar to that seen in the uterine canal of female S. mansoni obtained from the disulfiram treated mice (Figure 33). Note that the eggshell globules in the uterine canal are formed separately from the vitelline. These globules are fluorescent (Figure 29) and appear to be similar to the abnormal egg material described by Kelley and Lichtenberg (1970) in terms of its morphology and immunogenicity

TABLE 19

Number of Eggs per mm^2 of Liver in Mice Chronically Treated with Disulfiram (0.3% in Diet)

Length of Drug Treatment[a]	Control[b]	Treated
Long term study	7.22±0.26	1.34±0.33
Short term study	1.47±0.24	0.03±0.02

[a] Long term study - 92 days of drug treatment; short term study - 23 days.

[b] Data are expressed as mean No. of eggs/mm^2 ± S.E.M. for at least 3 determinations.

Figure 32. *S. mansoni* eggshell globules in liver of disulfiram treated mouse 122 days post-infection. Tissue stained with hematoxylin and eosin. Magnification is 100X.

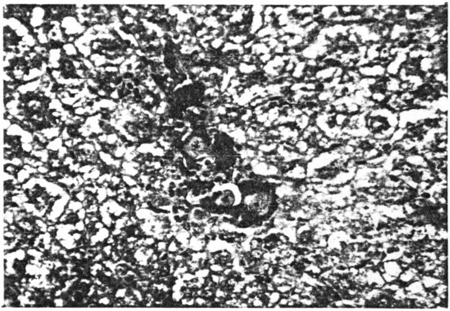


Figure 32

Figure 33. Eggshell globules in uterus of female *S. mansoni* obtained from disulfiram treated mouse. Photographed with dark-field condensor using incandescent light. Arrow indicates eggshell globules. Magnification is 140X.

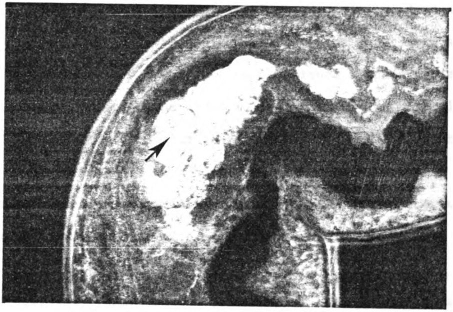


Figure 33

(i.e., they do not appear to elicit an immune response). It was difficult to demonstrate the lack of immune responsiveness to the presence of the eggshell globules. The globules are often found in the vicinity of egg granulomas or in periportal regions in which an inflammatory response is already present. Thus, it was difficult to dissociate ongoing inflammatory reactions to other schistosomal material from the reaction to the globule alone. However, these globules can occasionally be found in the liver parenchyma, isolated from most other schistosomal material. In such cases, no demonstrable inflammatory response was noted to occur during the short term study. In the long term study, macrophages and occasional lymphocytes and/or eosinophils could sometimes be found in the vicinity of these globules. These inflammatory cells were most likely associated with the presence of large deposits of schistosome pigment which tend to concentrate around the globules. Similar reactions were noted to occur in the presence of large quantities of pigment, and in the absence of the globules of egg material.

The livers of treated mice, like those of untreated mice, contained copious amounts of schistosome pigment. More importantly, there is a prominent infiltration of periportal areas with inflammatory cells, primarily lymphocytes, eosinophils and macrophages (Figure 34). Similar results were obtained in the short term study where egg production was inhibited by more than 98%. The meaning or cause of this reaction is not certain. It appears that vitelline cells and other egg-related materials are released independently of the eggshell globules (Figure 34). However, with the exception of the eggshell globules, it was not possible to detect the presence of any material

Figure 34. Portal triad from liver of disulfiram treated mouse infected with S. *mansoni* 122 days post-infection. Tissue stained with hematoxylin and eosin. Magnification is 100X.

Figure 34

in these periportal regions which could be construed to be schistosomal in origin, using both toluidene blue and PAS stains in addition to H & E.

Livers from mice not infected with S. mansoni, but receiving drug, were normal as were the spleen and small intestine.

Animals which had been placed on a drug diet and then reverted back to a control diet showed the typical granulomatous lesions of untreated mice with the exception that the globules previously found only in treated mice (Figure 32) were also present in these mice.

Microscopic examination of frozen sections of spleens from treated mice revealed them to be similar to those from untreated mice (marked hyperplasia with increased megakaryocytes) except for the notable absence of eggs and/or egg granulomas. The small intestine and colon of treated animals showed a response similar to that seen in the liver; few eggs or egg granulomas, and minimal fibrosis. One notable difference between these tissues and the liver was the virtual absence of schistosome egg globules in these tissues. Those globules which were present did not elicit any demonstrable inflammatory response.

These results suggest that treatment with phenol oxidase inhibitors can result in the alleviation of most, but not all of the pathology associated with schistosomiasis mansoni.

DISCUSSION

A. Induction of Phenol Oxidase

One of the major problems associated with the isolation and characterization of phenol oxidase concerns this enzyme's lack of activity in its natural state. In animals, phenol oxidase has been found to be either "soluble" in which case the enzyme appears to be a proenzyme (Preston and Taylor, 1970; Ohnishi *et al*., 1970; Ashida and Ohnishi, 1967), or it is found to be particulate, in which case the activity of the enzyme appears to be inhibited by the membrane to which it is bound (Menon and Haberman, 1970; Quevedo *et al*., 1975). In the former case, the enzyme can usually be activated by treatment with proteolytic enzymes such as trypsin or α-chymotrypsin (Preston and Taylor, 1970; Ohnishi *et al*., 1970). In the latter case, the enzyme can be activated by lipolytic, proteolytic or mechanical methods which serve to disrupt the membrane and release the enzyme in its soluble form (Quevedo *et al*., 1975; Menon and Haberman, 1970; Seiji and Yoshida, 1968). Unfortunately, *S. mansoni* phenol oxidase does not appear to fit into either one of these categories. Treatment of whole or homogenized female *S. mansoni* with proteolytic, lipolytic or mechanical procedures which disrupt membrane did not result in an increase in phenol oxidase activity (Table 1), despite biochemical and histochemical evidence (Table 4, Figure 11) which suggested that *S. mansoni* phenol oxidase was a membrane bound enzyme. Furthermore, treatment of a partially induced preparation of *S. mansoni* phenol oxidase with various proteolytic and lipolytic enzymes inhibited, rather than stimulated phenol oxidase activity without resulting in significant solubilization of the enzyme (Table 5). Only freezing and thawing produced a significant increase in phenol oxidase activity, but this increase (16%) was very slight when compared to the control preparation. These findings suggested that an alternative mechanism is responsible for the activation of *S. mansoni* phenol oxidase. Indeed, it was found that *S. mansoni* phenol oxidase was readily activated by incubation in tissue culture media or balanced salt solutions containing .05% sodium pentobarbital at room temperature. The mechanism by which *in vitro* incubation results in increased levels of phenol oxidase activity in the female schistosome is uncertain. It was originally thought that the iduction of the enzyme might be dependent upon protein synthesis (Seed *et al.*, 1978). Protein synthesis was shown to occur under the conditions of phenol oxidase activation in BMEFC plus .05% pentobarbital at 25°C (Table 3). Both ^{3}H-L-tyrosine and ^{3}H-L-leucine were incorporated into PCA-precipitable fractions of female *S. mansoni* during the period of activation. Furthermore, it was apparent that much of the protein synthesis which occurred in the female schistosome was directed towards egg production. Moore and Sandeground (1956) have estimated that one paired schistosome will produce one egg every 5 min or approximately 300 eggs/day. Senft (1969) has estimated that the vitelline cells, which contain important subcellular structures associated with the formation of the eggshell, are produced at a rate of 12,000/day.

Erasmus (1975) has shown that radiolabeled amino acids are preferentially incorporated into eggshell globule proteins in the vitelline cells of female S. mansoni. Thus, it was apparent that the synthesis of eggshell globule proteins--in which phenol oxidase can be histochemically localized (Figure 12)--constituted a major fraction of ongoing protein synthesis during egg production. However, removal of the schistosome from its host results in a cessation of egg production which lasts at least 24 hrs. Thus, it was thought that the increase in phenol oxidase activity might represent the brief but continued synthesis of the protein precursors of egg production, including phenol oxidase, following blockade of egg production. However, incubation of this parasite in the presence of 10^{-4}M SbK resulted in a 90% decrease in the incorporation of radiolabeled amino acids into proteins with no apparent decrease in the activation of phenol oxidase.

Having eliminated the possibility that either protein synthesis or proteolytic activation of a latent enzyme are involved in the activation of S. mansoni phenol oxidase, we are left to consider a couple of possible alternatives which include either activation of a prephenol oxidase similar to that which has been described by Ohnishi (1959) in the housefly Musca vinica and Bodine et al. (1938) in the grasshopper Melanoplus differentialis, or the derepression of a latent enzyme through the loss of an inhibitory agent which is insensitive to the actions of proteolytic enzymes. However, the mechanism of activation of S. mansoni phenol oxidase is somewhat different than that which has been described for M. vinica and M. differentialis. In both of these cases, the activation of the enzyme occurs in homogenates or extracts of the tissue in question. In *S. mansoni*, the activation of the enzyme appears to be dependent on the maintenance of specific ionic and nutrient conditions which are not necessarily correlated with optimization of *in vitro* culture conditions. BMEFC and RPMI 1640 are both excellent media for the culture of *S. mansoni* (Clegg and Smyth, 1968; Section 4, this thesis), but they induce significantly less phenol oxidase than BME (Table 2). The mechanism behind this unusual dependence of activation on the type of culture medium is not known.

Although it has not been possible to identify the mechanism by which phenol oxidase is induced in *S. mansoni*, it has been possible to eliminate a number of mechanisms which have previously been demonstrated to be responsible for the activation of phenol oxidase in other species including proteolytic activation and/or solubilization of the enzyme.

B. Isolation and Characterization of the Enzyme

The evidence presented in this thesis suggests that the oxygen consumed by homogenates of induced female *S. mansoni* in the presence of DA is due to the presence of an enzyme previously characterized in other organisms (including trematodes) and which is known as phenol oxidase or tyrosinase (Mason, 1955; Mansour, 1958; Pomerantz, 1963; Nakamura and Sho, 1964). However, since it was not possible to solubilize and purify this enzyme (Table 4), it might be argued that the dopamine induced consumption of oxygen seen in homogenates of female schistosomes was due to the activity of other parasite enzymes that are known to utilize oxygen, *i.e.*, monoamine oxidase, cytochrome oxidase or dopamine-β-hydroxylase. However, there is considerable evidence which argues against this position. All of these enzymes appear to be present in both the male and female schistosome. Nimmo-Smith and Raison (1968) and Coles (1972, 1973) have demonstrated that both monoamine oxidase and cytochrome oxidase are present in the male and female schistosome. Although dopamine-β-hydroxylase has not been isolated from male and female _S_. _mansoni_, both sexes have been demonstrated to contain DA and NE (Gianutsos and Bennett, 1977). The conversion of DA to NE can be selectively blocked in both male and female _S_. _mansoni_ by the administration of low doses of disulfiram _in vivo_ (Bennett and Gianutsos, 1978). Furthermore, it was shown here that the dopamine-β-hydroxylase inhibitor PTTU can also reduce NE levels _in vitro_. These findings indicated that male and female _S_. _mansoni_ both contain dopamine-β-hydroxylase. However, DA-stimulated oxygen consumption in the 3,000 x g pellet of female _S_. _mansoni_ was an exclusive characteristic of the female schistosome. Phenol oxidase pellets prepared from 3-fold larger quantities of male _S_. _mansoni_ failed to show any demonstrable oxygen consumption in the presence of 2 mM DA. In addition, it was shown that DA-stimulated oxygen consumption in female _S_. _mansoni_ was very sensitive to the actions of classical phenol oxidase inhibitors (Table 8) and very insensitive to the actions of compounds which are known to be inhibitors of the above-mentioned enzymes (Figure 14). These findings are not only consistent with the notion that the measurement of DA oxidation in the 3,000 x g pellet of female _S_. _mansoni_ is a valid measure of phenol oxidase activity, but are also consistent with the notion that phenol oxidase is important to _S_. _mansoni_ solely in terms of its involvement in the process of reproduction, _i_. _e_., it can only be found in the female schistosome, and is localized within the reproductive structures of this worm.

Characterization of the enzyme revealed that it was similar in many respects to the mammalian enzyme obtained from hamster (Pomerantz, 1963) or mouse (Lerner *et al.*, 1949) melanoma. Substrate specificity studies (Table 9) indicated that the schistosome enzyme prefers phenolic substrates with an alanine side chain, but reacts more rapidly with diphenolic than monophenolic substrates (Table 10). In addition, the oxidation of L-tyrosine methyl ester was found to be stimulated by preincubation with cofactor quantities of L-DOPA (3×10^{-5}M), but not with DA. These findings are very similar to the findings of Lerner *et al.* (1949, 1951) with respect to the enzyme from mouse melanoma and are consistent with the classification of this enzyme as phenol oxidase. In addition, the pH optima of the mammalian and schistosome enzymes are very similar and both are markedly inhibited by the metal ion chelating agent DDC. The finding that other copper chelating agents such as bathocuproine sulfonate and penicillamine are relatively poor inhibitors of schistosome phenol oxidase is puzzling, but may be related to differences in the affinity of these chelating agents for metallic ligands (Martel and Calvin, 1952; Sillén and Martel, 1964). These findings are consistent with previous reports concerning the effectiveness of copper chelating agents on the inhibition of this enzyme (Pomerantz, 1963). Thus, all the available evidence concerning the characteristics of this enzyme is consistent with the conclusion that the enzyme is a phenol oxidase and that it is similar to the enzyme which can be obtained from mammalian sources.

On the other hand, the phenol oxidase obtained from the closely related trematode *F. hepatica* (Mansour, 1958) appears to be markedly different in terms of its substrate specificity. For this enzyme, compounds with ethylamine side chains as opposed to alanine side chains, were preferred substrates (Mansour, 1958). Furthermore, the monophenolic substrate L-tyrosine ethyl ester was a much more effective substrate than L-DOPA. These differences in substrate specificity suggested that the mechanisms by which phenol oxidase catalyzes eggshell formation in *F. hepatica* and *S. mansoni* might be markedly different. However, while great care has been taken to ensure the validity of the assay for *S. mansoni* phenol oxidase, similar precautions were not taken by Mansour (1958) in his characterization of phenol oxidase in *F. hepatica*. For example, Mansour's observation that NE was a substrate for *F. hepatica* phenol oxidase could be due to the presence of a monoamine oxidase in the crude phenol oxidase pellet. It is interesting to note in this respect that only three of Mansour's substrates showed the rapid self-inactivation which is characteristic of the phenol oxidase reaction. These substrates were DA, catechol and tyrosine ethyl ester. Thus, it may be that the phenol oxidase from *F. hepatica* is not as different from *S. mansoni* phenol oxidase as has been suggested.

In order to more accurately characterize the role of *S. mansoni* phenol oxidase in the process of eggshell formation, an attempt was made to determine the nature of the *in vivo* substrate for this enzyme. Although L-DOPA was found to be the best *in vitro* substrate, it was not clear that it was the best *in vivo* substrate. L-tyrosine, L-DOPA and DA, all substrates for *S. mansoni* phenol oxidase, are all present in the female schistosome (Figure 18, Table 11). Furthermore, since all three substrates are present in substantially larger quantities in the female as opposed to the male schistosome, it cannot be argued that the presence of a higher concentration of substrate in the female schistosome is indicative a role for any one of these compounds in the process of egg production. However, it is apparent from these studies that only L-tyrosine is present in the female schistosome in concentrations which will saturate S. mansoni phenol oxidase. L-DOPA and DA concentrations in female S. mansoni, which are in the neighborhood of $5x10^{-6}M$, are 100-500 times less than the Km of this enzyme for these substrates. L-Tyrosine concentrations, which are on the order of $1.5x10^{-3}M$, are 3-4 times greater than the Km of this enzyme for the substrate. In addition, it was noted that the concentration of L-tyrosine remains elevated in the female schistosome during a period in the maturation of the female schistosome which is associated with the onset of egg production (day 30-38, Figure 18). During this same time period, which is characterized by the rapid growth of both the male and female schistosome, male levels of L-tyrosine were noted to be significantly decreased. Thus, the sustained elevation of L-tyrosine levels in female S. mansoni during this period of maturation is consistent with the notion that this substrate is important to the process of egg production. It is not likely that L-DOPA or DA are concentrated within the vitelline glands to serve as a substrate for phenol oxidase. Histochemical localization of the catecholamines by the Falck-Aillarp method has shown that most of the catecholamines localize within the nervous system of the male schistosome (Bennett and Bueding, 1971).

Although the autofluorescence of the vitelline cells obscured much of the catecholamine fluorescence in the nervous system of the female schistosome, catecholamine-like fluorescence could be observed in the female schistosome which was similar to that seen in the male schistosome (Bennett, 1973). On the other hand, biochemical studies revealed that the uptake of L-DOPA, like the uptake of L-tyrosine, was strictly a diffusional process (Figure 20). Furthermore, fluorescent histochemical studies (Section 2) have indicated that L-DOPA (methyl ester) and DA are poor substrates for phenol oxidase in the intact worm. While TME was capable of inducing the onset of DDC sensitive fluorescence at a concentration of 2 mM, L-DOPA methyl ester and DA were required to be present in the incubation medium at 5-fold higher concentrations in order to be able to induce this same fluorescence. These data are consistent with the notion that L-DOPA and DA are not actively accumulated in the cellular structures associated with reproduction and are therefore not likely to serve as substrates for an enzyme which is intimately associated with the process of egg production.

Alternatively, it might be argued that at the time of activation of schistosome phenol oxidase, *i.e.*, at the moment that sclerotization of the eggshell begins, *S. mansoni* phenol oxidase is exposed to a much greater concentration of protein bound L-tyrosine than soluble L-tyrosine. Erasmus (1975) has suggested that L-tyrosine is preferentially incorporated into female eggshell globule proteins and this phenol oxidase would be exposed to a high concentration of protein bound substrate during sclerotization due to its localization within the same membranes as these tyrosine rich proteins (Figure 12). The validity of this hypothesis is predicated on the notion that female _S. mansoni_ eggshell proteins are rich in tyrosine residues and that these protein bound tyrosine residues can serve as substrates for _S. mansoni_ phenol oxidase. However, the results presented in this thesis indicate that none of these basic criteria can be satisfied. Peptides rich in L-tyrosine were demonstrated to be either very poor substrates or not substrates at all. The only peptide which could be demonstrated to be a substrate for schistosome phenol oxidase was tri-L-tyrosine methyl ester at a concentration of 2 mg/ml. However, not only was the concentration of this peptide far in excess of what could be expected for the parent compound, tri-L-tyrosine, but the rate of oxidation was less than 5% of the rate of oxidation of 1 mM L-tyrosine methyl ester. Neither female schistosome proteins or chymotrypsinogen (1 mg/ml) could be demonstrated to be substrates for _S. mansoni_ phenol oxidase. In fact, when ^{3}H-labeled proteins from female _S. mansoni_ were incubated in the presence of triton-stabilized phenol oxidase for extended periods of time, only those preparations which also contained free L-tyrosine were found to form insoluble protein polymers (Table 16). These results indicate that _in vitro_, schistosome phenol oxidase does not react with protein-bound tyrosine. Nevertheless, this reaction with protein-bound tyrosine might still occur _in vivo_. However, if this is to be the case, then female schistosome proteins must satisfy the second criterion necessary for the demonstration of protein bound phenolic substrates for phenol oxidase, _i.e._, the identification of tyrosine rich proteins. In all cases where there is good reason to believe that there is a protein-bound substrate for phenol oxidase, the organism in question has been demonstrated to contain proteins rich in aromatic residues (Smyth, 1954; Brown, 1952; Smyth and Clegg, 1959). However, polyacrylamide gel electrophoresis of female S. mansoni proteins labeled with ^{3}H-L-tyrosine did not demonstrate the presence of any female schistosome proteins which possessed a high tyrosine content (Figure 20). In addition, tyrosine was incorporated into female schistosome proteins to a lesser extent than leucine in both egg-producing and nonegg producing media (Figure 21, Table 13). Furthermore, the male/female ratios for incorporation of ^{3}H-tyrosine and ^{3}H-leucine into schistosome proteins in either egg-producing or non-egg-producing media were not significantly different despite a rather marked increase in the incorporation of these amino acids into proteins during egg production (Table 14). This ratio reflects the ability of the female schistosome to incorporate ^{3}H-tyrosine into proteins to a greater extent than the male schistosome, which does not appear to have a high metabolic demand for tyrosine rich proteins. Indeed, this ratio becomes less than 1.0 in an egg-producing female. However, since an identical change occurs in the male/female ratio for ^{3}H-leucine, it is apparent that this increased incorporation of tyrosine into female schistosome proteins is non-specific and applies to other amino acids as well. Thus, it is apparent that proteins from female S. mansoni cannot be demonstrated to meet the two basic requirements necessary to establish protein bound tyrosine as an important component of the process of eggshell formation.

On the other hand, it was found that there was a significant difference in a non-egg-producing medium between the male/female ratios for incorporation of tritium from L-tyrosine into PCA-supernatants and the incorporation of tritium from L-leucine into PCA-supernatants. Conversely, no such differences were noted in an egg-producing medium (Table 14). These differences were apparently related to a decreased metabolism of ^{3}H-L-tyrosine to its catecholamine metabolites in the egg producing medium (Table 15). One explanation of this phenomenon is that the catecholamine pathway of tyrosine metabolism is being diverted for utilization of L-tyrosine as a substrate for phenol oxidase in the egg-producing medium. However, it might also be argued that L-tyrosine is being preferentially utilized for the purposes of protein synthesis, which is increased approximately 3.5-fold over the non-egg-producing medium. In any case, based on the apparent preferential association of the catecholamines with the nervous system of the parasite, the relatively low concentrations of L-DOPA and DA, the absence of a biochemical uptake process for L-DOPA and the relatively poor ability of L-DOPA and DA to serve as substrates for phenol oxidase in the intact worm, and in the absence of any evidence to indicate that protein-bound L-tyrosine serves as a substrate, one is led to conclude that the *in vivo* substrate for schistosome phenol oxidase is soluble L-tyrosine. This information provides an important basis for further studies on the role of phenol oxidase in eggshell formation.

C. The Role of Phenol Oxidase in Eggshell Formation

The currently available information provides strong evidence for an important role of phenol oxidase in the process of eggshell formation, yet the link between phenol oxidase activity and eggshell formation in *S. mansoni* remains somewhat tenuous. Katz (1977), Bennett and

Gianutsos (1978) and Machado *et al*. (1971) have shown that the compounds dapsone, disulfiram (or its *in vivo* metabolite, DDC) and allylthiourea are all inhibitors of schistosome eggshell formation *in vivo*. In this study, the ability of DDC and allylthiourea to inhibit eggshell formation *in vivo* was found to be well correlated with their ability to inhibit phenol oxidase activity *in vitro* (Table 18). Although dapsone was not tested for its ability to inhibit *S. mansoni* phenol oxidase, the inhibition of phenol oxidase was consistent with its purported mechanism of action as an antileprotic drug (Lavrijsen *et al*., 1977; Prabhakaran *et al*., 1976). The observation that structural analogs of DDC and allylthiourea which are not inhibitors of schistosome phenol oxidase did not inhibit formation of the eggshell, rules out the possibility that the actions of DDC and allylthiourea are nonspecific. Furthermore, the finding that this enzyme can be histochemically localized in the eggshell precursor globules of the vitelline cells (Figure 12) demonstrated that this enzyme is localized within structures whose role in the process of egg production is consistent with the purported role of phenol oxidase in this process. In addition, it was observed that *S. mansoni* phenol oxidase could form protein polymers in the presence of female schistosome proteins *in vitro*. This capability is an essential aspect of its role in the process of eggshell formation and is consistent with previous studies on protein polymer formation by enzymes which generate quinones (Mason, 1955; Stahmann *et al*., 1972). Other evidence to support the concept of phenol oxidase catalyzed eggshell formation was obtained from studies on the schistosome eggshell. However, conclusions concerning the identity of the substances present in the eggshell must be qualified with the understanding that these substances have been subjected to acid hydrolysis and thus probably do not represent the original product from the eggshell, but represent acid stable derivatives of these products.

Following silica gel chromatography of these products, it was found that there were several fluorescent products in schistosome eggshell hydrolysates (Table 19). Two of these products were similar in terms of R_f value and fluorescence spectrum to the hydrolysis product formed from the reaction of mushroom phenol oxidase with L-DOPA in the presence of a tyrosine:lysine polymer or lysine (Table 19). The fluorescence spectra of compounds formed from the reaction of DOPA-quinone with other nucleophilic amino acids did not correspond with peaks of similar R_f values in the eggshell hydrolysates. These data suggested that there are fluorescent products in the eggshell which are formed from the reaction of lysine with DOPA-quinone. These findings are consistent with a recently published report (Byram and Senft, 1978) which indicated that S. mansoni eggshells contain large quantities of lysine. Although the data presented so far do not prove that phenol oxidase catalyzes the formation of the eggshell by forming quinone-mediated cross-links between lysine residues in adjacent proteins, they do provide additional evidence to support this concept.

However, not all of the data are consistent with this concept. The inhibition of eggshell formation which is produced by DDC is not complete. Administration of as much as 200 mg/kg of DDC did not result in the complete cessation of eggshell formation. There was some residual eggshell material which was formed in the presence of DDC which could not be suppressed by large doses of this phenol oxidase inhibitor. There are a number of possible explanations for this phenomenon which are both enzymatic and non-enzymatic.

Based on evidence which suggests that dityrosine is present in hydrolysates of *F. hepatica* eggshells (Wilson, 1967) certain investigators (Ramalingham, 1973) have argued that the enzyme responsible for cross-linking of eggshell proteins in *F. hepatica* might be peroxidase. Similarly, Byram and Senft (1978) have reported finding small quantities of dityrosine in *S. mansoni* eggshells. Since dityrosine is one of the major products formed from the reaction of peroxidase with tyrosine (Gross and Sizer, 1959), these results suggest that schistosome eggshell formation is also catalyzed by peroxidase. However, the evidence presented in this thesis argues against a role for peroxidase. Compounds such as phenylhydrazine hydrochloride and sodium sulfite which have been demonstrated to be potent inhibitors of peroxidase activity and eggshell formation in the only well documented case of peroxidase catalyzed eggshell formation (Foerder and Shapiro, 1977) were not found to inhibit *S. mansoni* eggshell formation *in vivo* (Table 17). Furthermore, the administration of 100 mg/kg α,α'-dipyridyl, a potent inhibitor of peroxidase (Mason, 1957) did not block the formation of residual eggshell material following the administration of 200 mg/kg DDC. Since peroxidase is also inhibited by DDC (Mason, 1957) it would be difficult to argue that the residual eggshell material is formed by a peroxidase reaction.

Alternatively, the formation of eggshell material in the presence of DDC might be due to the autooxidative formation of quinones. The finding that ascorbic acid and PTTU can enhance the DDC induced inhibition of eggshell formation (Table 19) is consistent with this notion since these compounds have previously been demonstrated to prevent DOPA autooxidation both *in vivo* and *in vitro* (Hirsch, 1955). However, these findings may also be explained in terms of the ability of these compounds to either form complexes with metal ions and thus act as a weak inhibitor of phenol oxidase, e.g., PTTU, or reduce quinones back to dihydroxyphenols regardless of the means of quinone generation, e.g., ascorbic acid. Nevertheless, neither of these compounds could block the formation of residual eggshell material following the administration of 200 mg/kg DDC. These findings suggested that either the residual eggshell material is produced by a different mechanism or that the antioxidants used in these experiments were not present *in vivo* in sufficiently high concentration to prevent quinone formation. If the site of quinone formation is within the eggshell membranes, then the accessibility of these water soluble antioxidants to the site of quinone formation would be seriously impaired.

Vitamin E succinate (dl-α-tocopherol acid succinate) is a lipophilic antioxidant, which is also capable of enhancing the DDC induced inhibition of eggshell formation. Although vitamin E is known for its ability to reduce quinones, it is better known as an agent which protects against lipid peroxidation by free radicals (Tappel, 1972). Lipid peroxidation is known to result in the formation of protein polymers (Roubal and Tappel, 1966) and furthermore is known to result in the generation of a yellow fluorescent material in peroxidized microsomes and mitochondria (Dillard and Tappel, 1971). Nevertheless, vitamin E succinate could not be demonstrated to reverse the formation of the residual, fluorescent, eggshell material following the administration of large doses of DDC. However, the accessibility of vitamin E succinate to the uterus of the female schistosome may have been sufficiently low so as to prevent this antioxidant from protecting against lipid peroxidation, especially in a single dose protocol such as the one described in these experiments. Those experiments which have shown vitamin E to protect against lipid peroxidation have usually involved the use of large quantitites of vitamin E administered chronically in the diet (Dillard and Tappel, 1971).

Although it has not been possible to determine the reason for the persistant formation of eggshell globules *in vivo* in the presence of DDC, it is apparent that quinones generated from phenol oxidase play a major role in the formation of the schistosome eggshell. This conslusion is based on the observations that 1) phenol oxidase can be isolated and identified in the female schistosome and not in the male schistosome, 2) it has been localized within the subcellular structure associated with formation of the eggshell, 3) substrate is available in large quantities, 4) proteins from female schistosomes are polymerized during *in vitro* incubation with *S. mansoni* phenol oxidase and substrate, 5) substances found in acid hydrolysates of eggshells are fluorometrically and chromatographically similar to acid hydrolysates of lysyl-quinone and 6) the inhibition of eggshell formation by phenol oxidase inhibitors is sensitive and specific.

D. Chemotherapy of Schistosomiasis Using Phenol Oxidase Inhibitors

Since it appears that eggshell formation is most sensitive to the actions of phenol oxidase inhibitors, it is logical that one of these compounds be used to study the effectiveness of inhibiting eggshell formation on blocking the development of schistosomal pathology. The administration of disulfiram 0.3% in the diet resulted in a significant suppression of egg deposition in host tissues. This decrease in egg deposition in the disulfiram treated animals was associated with a substantial increase in the survival times of these animals. These findings are in agreement with the findings of previous investigators (Boros and Warren, 1970) who have shown that the presence of an intact eggshell is essential to the development of the granuloma and that granuloma formation is responsible for the development of most of the pathology associated with schistosomiasis (Lichtenberg, 1955). This latter concept is further reinforced by the finding that both the treated and untreated livers have apparently similar levels of periportal inflammation (Figures 31 and 34). The presence of this periportal inflammation could not be attributed to the presence of intact eggs since the periportal response in the treated and untreated animals was similar in both the short-term and long-term studies where the inhibition of egg production was 98 and 81%, respectively, as determined by egg counts in the livers of treated vs. control mice. The probable source of this periportal inflammation in the treated animals was the vitelline cells which were released from the female schistosome, separate from the eggshell globules (Figure 33). While it was not possible to detect deposits of vitelline cell material in the periportal regions of treated mice due to the severity of the immune response, the size and intensity of this response is definitely suggestive of an antigenic stimulus. Furthermore, while the severity of this response is considerable, it is nontheless apparent that it does not affect the survival of the infected mouse (Figure 30). Thus, it appears that treatment of schistosomiasis with compounds which inhibit production of the schistosome eggshell is of potential chemotherapeutic value. Unfortunately the usefulness of this drug treatment is limited by its reversibility. The death rate of treated animals increases rapidly shortly after withdrawal from the treatment group. The reversibility of the disulfiram induced inhibition of eggshell formation requires that this drug be given chronically, thus making this drug treatment somewhat susceptible to the development of tolerance. Furthermore, disulfiram can be considered to be a relatively dangerous drug since it apparently causes rather toxic side effects when administered in combination with other drugs (Ritchie, 1970). Thus, it appears that this drug, while potentially useful in the alleviation of schistosomal pathology, is severely limited by its potential toxicity and the necessity for chronic administration.

SUMMARY AND CONCLUSIONS

Female *S. mansoni* were found to possess an enzyme which can be classified as a phenol oxidase (E.C. 1.10.3.1) and which is thought to play a vital role in the formation of the schistosome eggshell. Like most phenol oxidases the schistosome enzyme appeared to be latent. It could be activated by incubation *in vitro* at room temperature in a tissue culture medium or balanced salt solution. Unlike phenol oxidases from other organisms, the activation of *S. mansoni* phenol oxidase was not found to be dependent on the action of proteolytic enzymes. Incubation of female *S. mansoni* homogenates from fresh or partially ininduced schistosomes in the presence of proteolytic enzymes tended to decrease rather than increase phenol oxidase activity. It was also found that the incubation dependent activation of phenol oxidase was not blocked by an inhibitor of protein synthesis. The mechanism of activation remains obscure. Nevertheless, the finding that it is a latent enzyme is consistent with its classification as a phenol oxidase.

S. mansoni phenol oxidase was histochemically localized in subcellular structures in the female schistosome known as eggshell globules by use of a fluorescent assay. The validity of the assay was confirmed by the extraction of fluorescent products from treated worms which were identical to the products formed from the *in vitro* reaction of substrate (L-tyrosine methyl ester) with phenol oxidase. The localization of this enzyme in these golbules was consistent with its biochemical localization in a 1,000 x g pellet of female S. mansoni homogenate and was also consistent with its purported role in the formation of the eggshell. Attempts to purify this enzyme were not successful since it was not possible to solubilize the enzyme from the eggshell globule membranes using a number of proteolytic, lipolytic and mechanical methods without losing enzyme activity. However, it was noted that treatment of a crude 3,000 x g pellet of female S. mansoni homogenates with Triton X-100 resulted in significant stabilization of enzyme activity.

Biochemical characterization of this crude enzyme demonstrated it to be very similar to the enzyme obtained from mammalian sources and confirmed its classification as a phenol oxidase. Its pH optimum (7.0), sensitivity to copper chelating agents such as diethyldithiocarbamate and allylthiourea and its substrate specificity were all very similar to that described for the mammalian enzyme. In addition, the finding that L-DOPA could act as a cofactor in the oxidation of tyrosine confirmed unequivocally that the enzyme was phenol oxidase. S. mansoni phenol oxidase was found to be markedly different in its substrate specificity from a similar enzyme found in the trematode F. hepatica, but these differences may have been related to the presence of interfering enzymes in the crude preparation of F. hepatica phenol oxidase.

The substrate specificity of S. mansoni phenol oxidase in vitro did not appear to parallel its ability to oxidize substrates in vivo. TME was a better substrate in the fluorescent histochemical assay for phenol oxidase in the living worm then L-DOPA methyl ester and DA, while the converse was found in the *in vitro* system. This finding was consistent with the conclusion that L-tyrosine was the *in vivo* substrate for *S. mansoni* phenol oxidase. Further evidence in support of this conclusion was obtained from studies which showed that L-tyrosine was present in female schistosomes in concentrations which were approximately 3 times higher than the phenol oxidase Km for L-tyrosine while L-DOPA and DA concentrations were found to be 100 and 500 times less than the respective Km's for these substrates. In addition, it was found that L-DOPA was not actively accumulated by the female schistosome, thus ruling out the possibility that L-DOPA was concentrated within the schistosome to serve as a substrate for phenol oxidase. Further studies showed that protein-bound tyrosine was a very poor substrate for schistosome phenol oxidase *in vitro*. This observation coupled with the finding that female *S. mansoni* did not possess tyrosine rich proteins and did not incorporate tyrosine into protein to any greater extent than leucine led to the conclusions that soluble L-tyrosine rather than protein-bound L-tyrosine was the *in vivo* substrate for *S. mansoni* phenol oxidase.

In vivo studies on the specificity of the response to phenol oxidase inhibitors showed that inactive analogs of the phenol oxidase inhibitors and inhibitors of other enzymatic and nonenzymatic processes capable of crosslinking proteins were not capable of inhibiting schistosome eggshell formation. On the other hand, not only did the inhibition of eggshell formation closely correspond with inhibition of phenol oxidase, but *S. mansoni* phenol oxidase was also found to be capable of cross-linking proteins from female *S mansoni in vitro* in the presence of excess L-tyrosine. Analysis of the acid hydrolysates of schistosome eggshells showed that the fluorescent substances found in the eggshell were similar to those formed from the reaction of phenol oxidase generated quinones with protein-bound lysine. Although these observations support the concept of phenol oxidase catalyzed eggshell formation it was noted that small amounts of fluorescent material were formed in the uterus of DDC treated schistosomes regardless of the dose of DDC employed during *in vivo* studies on the inhibition of eggshell formation. It could not be conclusively demonstrated that either autooxidation or lipid peroxidation were responsible for the formation of this material. However, the observation that inhibitors of these processes could enhance the DDC induced inhibition of eggshell formation suggested the possibility these processes could contribute to the formation of the eggshell.

In conclusion, it is apparent that the quinones generated by *S. mansoni* phenol oxidase play a major role in the formation of the eggshell in this schistosome. The data presented are consistent with a mechanism of eggshell formation in *S. mansoni* in which phenol oxidase in the eggshell membrane is activated within the uterus, subsequently reacting with tyrosine to produce a DOPA-quinone. This DOPA-quinone reacts non-enzymatically with proteins in the eggshell membrane which are rich in lysine residues to produce an extensively cross-linked protein. This cross-linked protein constitutes the major framework of the eggshell, although the recent finding of Byram and Senft (1978) concerning the amino acid content of the eggshell suggests that ionic and other forces may also play a role in its hardening.

With regard to chemotherapy, treatment of schistosomiasis with a phenol oxidase inhibitor was shown to be able to alleviate most, but not all of the microscopic pathology associated with schistosomiasis. In addition, it blocked the mortality associated with a heavy burden of schistosomes in mice. However, it was also noted that the actions of this compound were rapidly reversed thus necessitating daily administration of the drug. The necessity for daily administration of the drug coupled with its known potential for dangerous and toxic side effects makes the use of this and similar compounds a highly unsatisfactory clinical approach to the problem of alleviating schistosomal pathology.

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BIBLIOGRAPHY

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