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The Regulatory Role of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase in Egg Production by Schistosoma Mansoni

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Elizabeth Ann VandeWaa

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THE REGULATORY ROLE OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE IN EGG PRODUCTION BY *SCHISTOSOMA MANSONI*

by

Elizabeth Ann VandeWaa

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ABSTRACT

The Regulatory Role of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase in Egg Production by Schistosoma mansoni

by

Elizabeth Ann VandeWaa

The schistosome egg is responsible for most of the pathology associated with schistosomiasis. Eggs deposited in host tissues elicit an immune response, resulting in granuloma formation with subsequent tissue fibrosis, which can ultimately produce death in the host. In studies on the regulation of schistosome egg production, it was found that mevinolin, a competitive inhibitor of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase was able to significantly reduce egg production by the parasite both in vitro and in vivo. The in vitro effect of the drug was reversible by mevalonate and farnesol, both products of the HMG CoA reductase-catalyzed reaction. Furthermore, these lipids were also able to stimulate oviposition in the schistosome.

The presence of HMG CoA reductase activity was identified in this parasite, in microsomal fractions prepared from worm homogenates. In the assay system used, mevinolin at 10 μM was found to reduce parasite enzyme activity by 75%.

HMG CoA reductase activity is known to be both suppressible as well as inducible by mevinolin. To measure if induction of the enzyme occurred in the schistosome, parasites exposed to low concentrations of the drug in vivo were assayed for activity. Here, a doubling of HMG CoA reductase activity was measured. When these parasites were incubated in drug-free medium for a

determination of egg production, schistosomes exposed to low doses of mevinolin *in vivo* produced five times more eggs *in vitro* than did control parasites.

Mevinolin disrupts egg production in the schistosome and affects HMG CoA reductase activity in this parasite, indicating that a product in the pathway catalyzed by this enzyme is essential for egg production by the worm. When products of HMG CoA were analyzed in the parasite following mevinolin exposure *in vitro*, it was found that the polyisoprenoid lipids were diminished in the parasite by 70%. Polyisoprenoid lipids function in glycoprotein synthesis to transfer carbohydrate to newly formed proteins. Since glycoproteins are major constituents of the schistosome egg, the effect of mevinolin on HMG CoA reductase may be reflected in a reduction in parasite egg production. Therefore, HMG CoA reductase may be playing a regulatory role in the production of eggs by *S. mansoni*.

To my husband John, whose love, encouragement and sense of humor helped me to maintain my perspective and continue to enrich my life.

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LIST OF ABBREVIATIONS

Asn	asparagine
DPG	diphosphatidylglycerol
EDTA	ethylenediaminetetraacetic acid
GDP	guanosine diphosphate
HC	hydrocarbon
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
MG-DG	mono-, diglyceride
MVA	mevalonate
NADPH	nicotinamide-adenine dinucleotide phosphate
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PI-1,2,3	polyisoprenoid 1, 2, 3
PL-1,2,3	polar lipid 1, 2, 3
PS	phosphatidylserine
RNA	ribonucleic acid
SD	standard deviation
SE	sterol ester
SEM	standard error of the mean
Ser	serine
Sph	sphingolipids
TG	triglyceride
TLC	thin-layer chromatography
UDP	uridine diphosphate

INTRODUCTION

The schistosome is a helminth parasite which currently afflicts more than 300 million people worldwide. Schistosoma mansoni, one of the three major schistosome species, is endemic to much of Africa, as well as to parts of South and Central America. The schistosomes as a group differ from other trematodes in that these parasites exist as separate sexes within the definitive host. Schistosoma mansoni dwells in the portal and mesenteric blood vessels of its host, wherein the larger male parasite folds its body around the slender female forming the gynecophoral canal in which the female lies during copulation (Figure 1). The pairing and copulation of the adult parasites in the host results in the production of up to 300 eggs per worm pair per day (Moore and Sandground, 1956). The formation of eggs by S. mansoni is not only essential to the maintenance of the life cycle of this parasite, but the eggs are also responsible for much of the pathology associated with bilharziasis (schistosomiasis).

A. The Parasite: Schistosoma mansoni

1. The life cycle of S. mansoni

The life cycle of the schistosome is depicted in Figure 2. The life cycle of this parasite is complex, involving an intermediate host and a definitive host, the latter is in a mammal in which the parasite reaches sexual maturity. Within the definitive host, the sexually mature female produces eggs which are deposited into the venules of the mesentery. The eggs secrete lytic enzymes

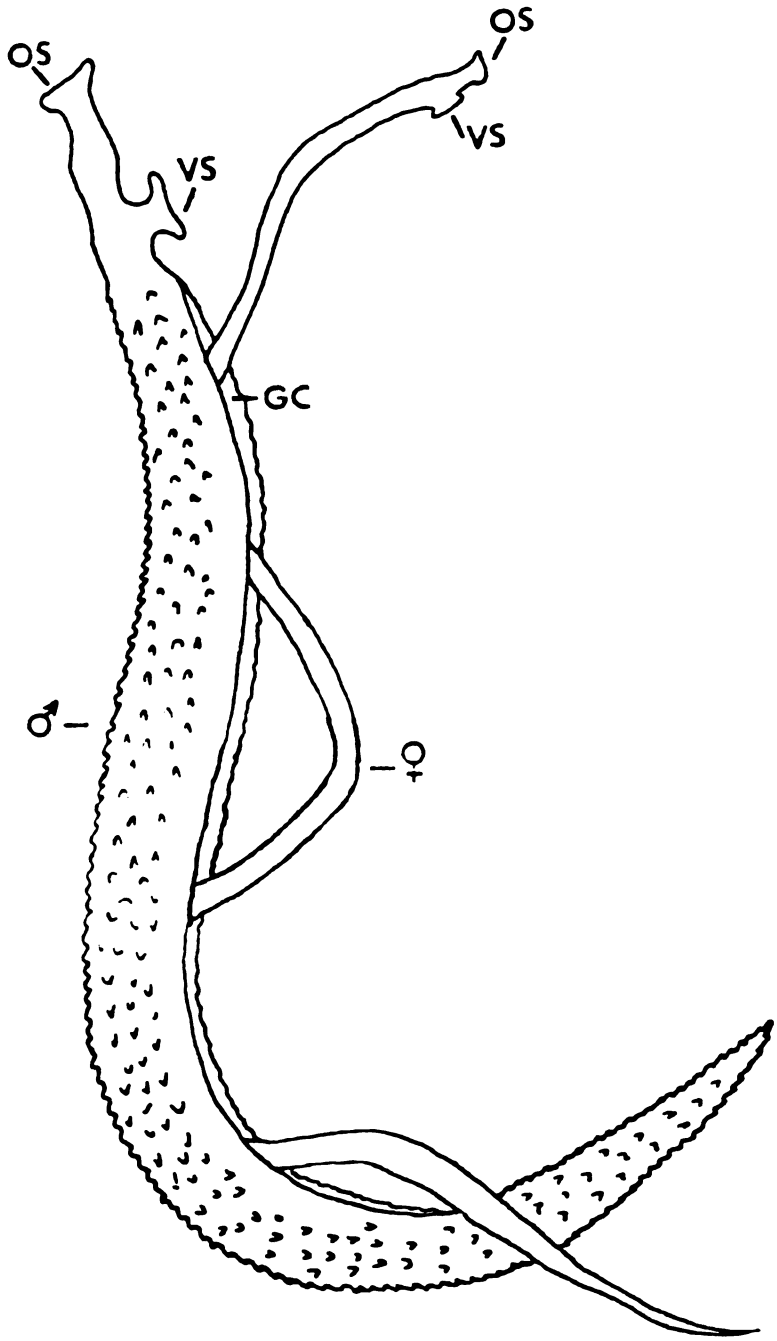


Figure 1. Drawing illustrating the external characteristics of male and female S. mansoni. OS, oral sucker; VS, ventral sucker; GC, gynecophoral canal.

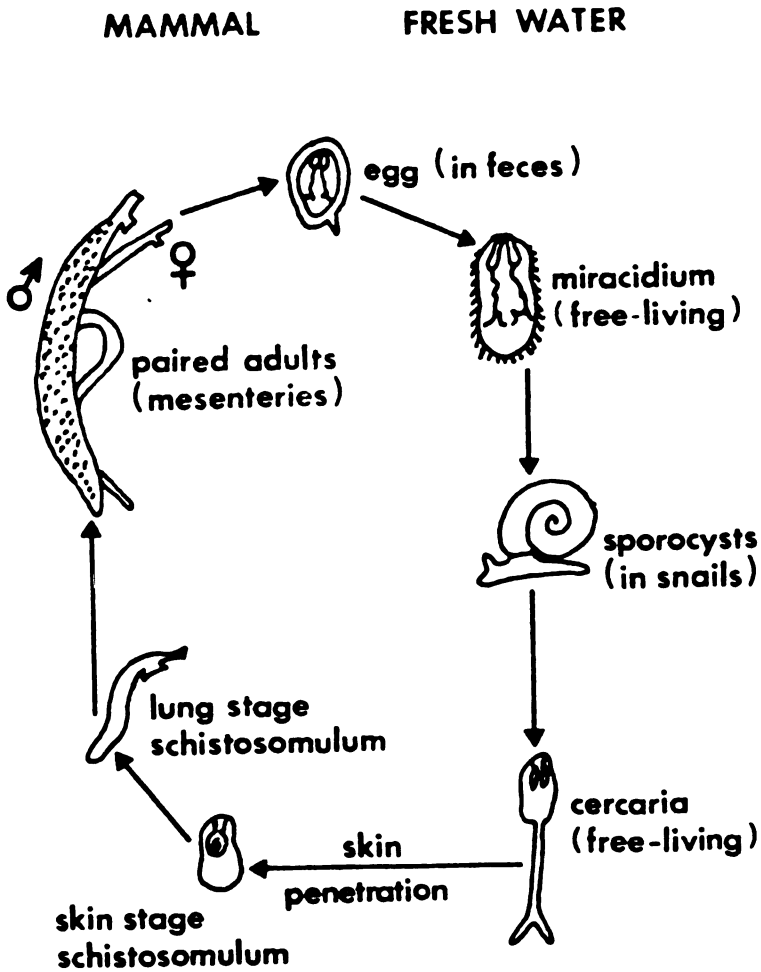


Figure 2. Life cycle of S. mansoni

which help them to rupture the venule and penetrate the intestine. Once in the lumen of the intestine, the eggs are carried out of the host via the feces.

The eggs contain a larval form of the parasite known as the miracidium. Upon contact with fresh water the eggs hatch, releasing the free-swimming miracidia. The miracidia then penetrate a snail which serves as the intermediate host in which the miracidia multiply, asexually, and differentiate into cercariae (Faust and Hoffman, 1934). The cercariae are released by the snail, and subsequently penetrate the skin of man (or other mammal), burrowing down to the peripheral capillary bed. Once in the bloodstream, the parasites are carried to the right heart and lungs, from which they migrate to the portal circulation. After a maturation period within the portal vessels, the adolescent worms migrate into the mesenteric venules where they pair, and oviposition begins. The adult parasites may live in the blood vessels of man for as long as 30 years actively producing eggs (Faust et al., 1934).

2. Egg Production in S. mansoni

The production of eggs by S. mansoni is considered an important process for two reasons. First of all, egg production is necessary to maintain the life cycle of the parasite, as discussed above. Secondly, the deposition of eggs in host tissues by S. mansoni is the primary cause of the pathology associated with schistosomiasis. Egg production by the parasite is a complex biological process, about which little is known. It has been established that the female schistosome contains an organelle called the ootype in which vitelline cells are combined with the ovum (egg). The vitelline cells release droplets which coalesce to form a thin membrane around the uterus, at which time egg shell formation occurs (Stephenson, 1947; Smyth and Clegg, 1959) (Figure 3). The egg shell, which consists primarily of cross-linked proteins, is highly resistant to immunolytic (Stenger et al., 1967) and chemical degradation (Stephenson, 1947). Furthermore, the

Figure 3. Anatomical features associated with egg production in female S. mansoni (adapted from Gonnert, 1955).

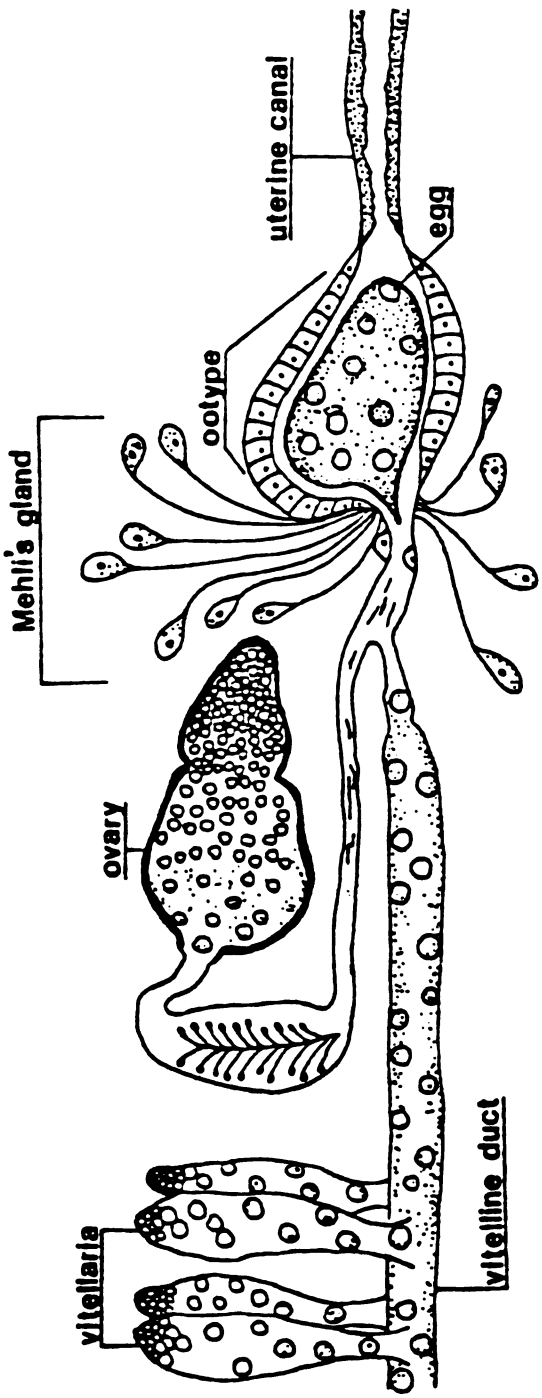

Mehli's gland
ootype
uterine canal
egg
ovary
vitellaria
vitelline duct

Figure 3

proteinaceous shell possesses no antigenic activity and thus plays little role in the induction of pathology associated with schistosomiasis (Boros and Warren, 1970; Lichtenberg and Raslavicious, 1967). Rather, the egg shell seems to function only to protect the developing miracidium from the host defense mechanisms, allowing the egg to safely pass to the external environment. In chronic schistosomiasis, more than fifty percent of the eggs produced by the worms may remain within the definitive host, being carried by the portal circulation to the liver, where they become trapped (Warren, 1978). Eggs which remain within the host are ultimately responsible for the pathology of schistosomiasis.

B. The Role of the Egg in the Pathology of Schistosomiasis

The pathology resulting from infections with S. mansoni may be divided into three general stages (Brown, 1973). The first of these is the pathology associated with the penetration of the cercariae through the skin and subsequent development of the adult worms. Pathological signs here consist mainly of mild cutaneous lesions and petechial hemorrhages as the developing parasites migrate through host tissues.

The second stage of schistosome-associated pathology manifests itself upon active egg production and extrusion. Here, eggs, via the secretion of collagenase-like enzymes, penetrate through the vascular endothelium, causing local foci of inflammation and immune cell infiltration. Penetration of the eggs into other tissues (such as the organs of the digestive tract) causes a similar response in these tissues.

The production and penetration of schistosome eggs leads directly to the third stage of the pathological response, that of proliferation and repair. As stated earlier, most of the eggs produced by the schistosome are retained by the host, wherein they may become trapped in host tissues, particularly in the liver.

Here, the presence of the egg elicits the formation of granulomas or non-necrotizing pseudotubercles. Granulomas consist of layers of epithelioid cells, fibroblasts and giant cells, surrounded by plasma cells and eosinophils. Formation of the granuloma is instigated by the secretion of a number of soluble antigens by the intact schistosome eggs (Pelley et al., 1976; Hamburger et al., 1976). These proteinaceous antigens are produced by the miracidium developing within the egg shell, and diffuse through the shell into the surrounding tissues, thereby triggering granuloma formation (Hang et al., 1974). The immunological response of the host to the egg antigens is vigorous, resulting in the formation of granulomas up to 100 times the volume of the egg itself (Winslow, 1967). The result of these large areas of cellular proliferation and infiltration in the liver is manifest as an occlusion of portal blood flow to these regions, which compromises liver function.

Several other clinical signs and symptoms are characteristic of schistosomiasis. These include hepatomegaly, splenomegaly, esophageal varices and portal hypertension (Warren, 1978). Again, these overt disease symptoms are due largely to the presence of schistosome eggs within the host and, more specifically, to the immunological response of the host to the eggs. Recognizing the major role which the egg plays in schistosomiasis, a number of investigators have attempted to suppress oviposition in this parasite. Clearly, the inhibition of egg production by the parasite presents an attractive chemotherapeutic target whereby schistosome-induced pathology can be reduced.

C. Chemotherapy of Schistosomiasis by Suppression of Egg Production

1. Past studies on the inhibition of schistosome egg production

Attempts to inhibit oviposition in S. mansoni have been aimed at reducing or eliminating schistosome-associated pathology known to be caused by the eggs. One such attempt focused on inhibiting schistosome phenol oxidase, the

enzyme which serves to cross-link the protein in the eggshell, causing eggshell hardening (Seed et al., 1979). Inhibition of parasite egg production by the suppression of phenol oxidase proved to be of little therapeutic value however, when it was discovered that the most active inhibitors of the enzyme were copper chelators which affected various mammalian copper-containing enzymes as well. Similar results were seen with various metabolic inhibitors (Lee and Michaels, 1968) which also adversely affected the host as well as the parasite.

Other attempts to interfere with schistosome egg production have focused on the endocrinology underlying oviposition. It is well accepted that stimulation of the development of the female reproductive tract of the schistosome is brought about through a close physical relationship with the male parasite (Sagawa et al., 1928; Severinghaus, 1928). It was thought therefore, that the male worm may be secreting a hormone-like substance which would regulate fecundity in the female. To assess this possibility, several antispermatogenic compounds (Jackson et al., 1968; Davies and Jackson, 1970) and steroid hormones (Morrison et al., 1986) were examined for their effects on schistosome egg production. In all of these studies, none of the compounds tested were efficacious in disrupting oviposition in the schistosome.

2. The effect of mevinolin on schistosome egg production

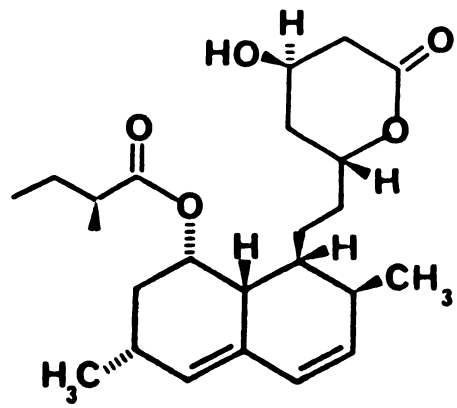
In the process of determining the effects of steroid hormones on schistosome egg production, a steroid synthesis inhibitor, mevinolin, was examined for its effects on parasite oviposition. Mevinolin is a potent inhibitor of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) (Alberts et al., 1980) which regulates the metabolic pathway for the production of cholesterol and several non-steroid lipids such as terpenes, dolichols and ubiquinones.

Chemically, mevinolin belongs to the class of substituted hexahydronaphthalene lactones (Figure 4). It is a small molecule, with a molecular weight of 404 daltons. Mevinolin was first isolated from the fungus Monascus ruber by Endo (1979) who called it monacolin K. Later, the same compound was isolated as a metabolite of the fungus Aspergillus terreus by Alberts and coworkers at Merck, Sharp and Dohme Research Labs (1980). The active form of mevinolin is the hydroxy acid (opened lactone) structure. The structure of the acid form of this agent contains a portion that resembles the HMG portion of HMG CoA, perhaps contributing to its ability to inhibit HMG CoA reductase (Figure 5). Although there exist several structural analogs of mevinolin, it is the most potent inhibitor of HMG CoA reductase in this series of compounds. Results with mevinolin in the schitsosome indicate that it is a highly efficacious inhibitor of egg production in S. mansoni both in vitro and in vivo.

D. HMG CoA Reductase

HMG CoA reductase (E.C. 1.1.1.34) is a transmembrane glycoprotein of the endoplasmic reticulum, consisting of a soluble and a membrane bound domain (Figure 6). HMG CoA reductase has a subunit molecular weight of 90 kilodaltons and, although it is a glycosylated protein, its carbohydrate moieties are not required for its enzymic activity (Liscum et al., 1983). This enzyme has a short half-life, turning over within 2-4 hours in vivo (Edwards and Gould, 1972).

The regulation of HMG CoA reductase is currently an area of intensive study because of the important role this enzyme plays in the synthesis of sterols in mammals. Specifically, HMG CoA reductase catalyzes the rate-limiting step in the synthesis of the steroid nucleus, catalyzing the conversion of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate (Figure 7). Mevalonate is then converted, in several steps, to farnesyl pyrophosphate which serves as a precursor



MEVINOLIN

Figure 4. Chemical structure of mevinolin.

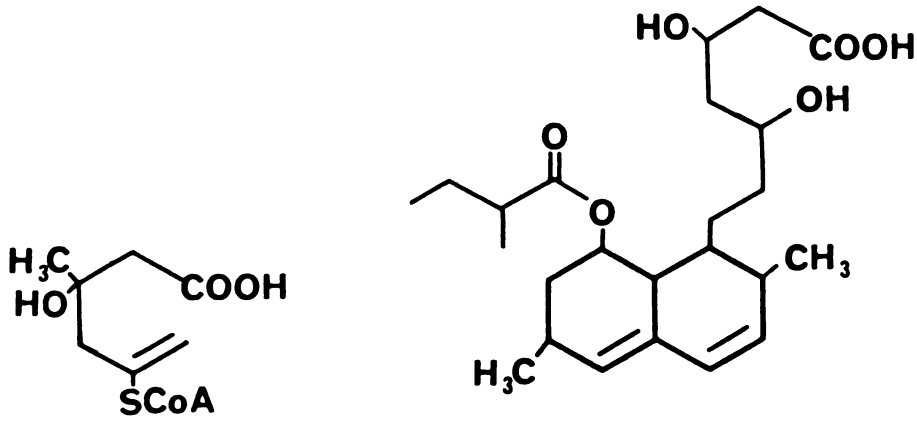


Figure 5. Structures of HMG CoA (left) and the acid form of mevinolin (right).

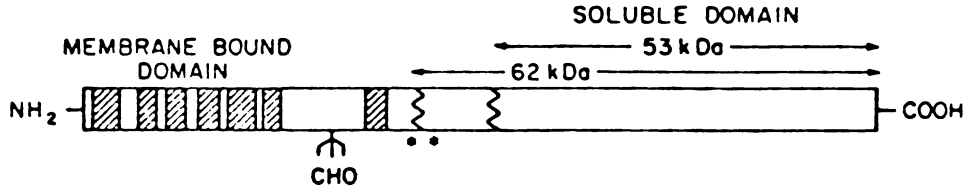
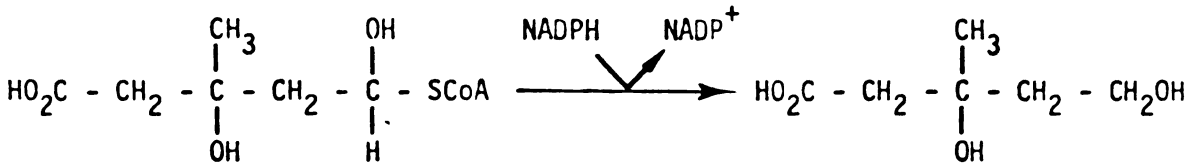


Figure 6. Domain map of HMG CoA reductase (adapted from Luskey, 1985).



3-Hydroxy-3-methylglutaryl SCoA Mevalonate

Enzyme: HMGCoA Reductase (EC 1.1.1.34)

Regulation of Enzyme: Cholesterol (and non-sterol products??) produced by this pathway influences the rate of transcription of the reductase gene (Luskey et al., 1983).

Figure 7. Catalytic role of HMG CoA reductase.

to a variety of compounds (Figure 8). In mammals, the major product of this pathway is cholesterol, however, the other products derived from mevalonate may be essential for a variety of cellular functions in various organisms.

1. Effects of mevinolin on HMG CoA reductase

The inhibition of HMG CoA reductase by mevinolin is competitive with respect to HMG CoA, and non-competitive with respect to NADPH, a necessary cofactor for the reaction (Endo et al., 1976; Endo, 1980). As stated earlier, the structure of mevinolin contains a portion which resembles the HMG moiety of HMG CoA (see Figure 5), this structural similarity is thought to contribute to the competitive nature of the inhibition of HMG CoA reductase by the drug. Furthermore, mevinolin, which has two methyl groups on its decaline ring moiety is a more potent inhibitor of the enzyme than are structural analogs of the drug with one or no methyl groups on the decaline ring (Endo et al., 1979). Additionally, HMG alone is insufficient to inhibit HMG CoA reductase (Brown et al., 1978), indicating again that the decaline ring is prominently involved in the inhibition of the enzyme. Therefore, it appears that both the HMG-like moiety as well as the decaline ring structure contribute to the inhibitory action of mevinolin on the reductase.

The precise mechanism involved in the inhibition of HMG CoA reductase by mevinolin is not entirely understood. The reaction catalyzed by the enzyme involves a two-step process. First, the enzyme interacts with HMG CoA to form a binary complex, this structure then forms a ternary complex with one molecule of NADPH (Tanzawa and Endo, 1979). It has been suggested (Rogers and Rudney, 1982) that mevinolin binds to the enzyme causing a conformational change in its tertiary structure. This structural change prevents the enzyme from forming a ternary complex with HMG CoA and NADPH and, as a result, the subsequent formation of mevalonate from HMG CoA is reduced.

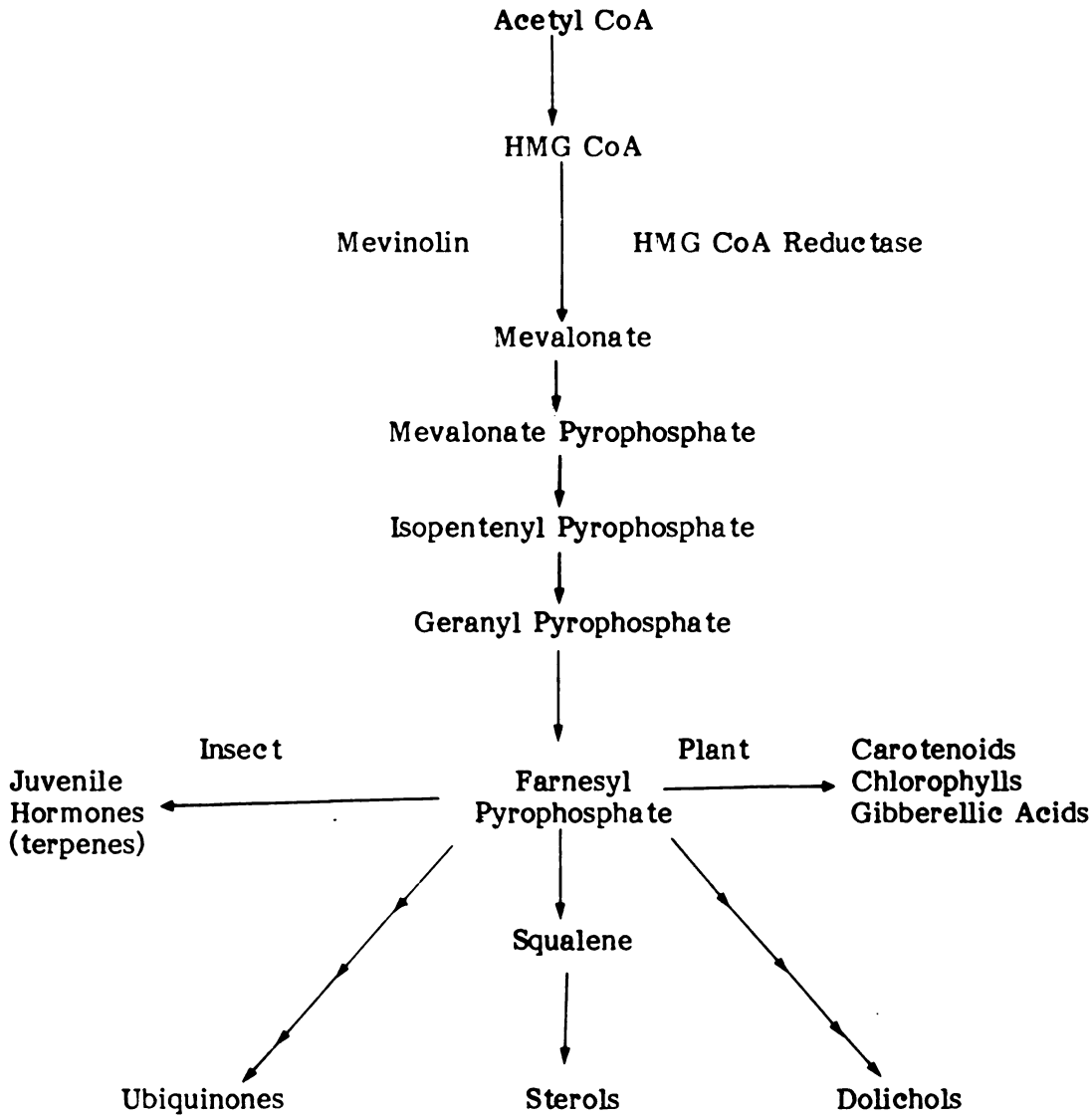


Figure 8. The branched pathway of mevalonate metabolism.

2. Induction of HMG CoA reductase by mevinolin

The discovery that HMG CoA reductase is an inducible enzyme was made by Goldstein and Brown (1977), who showed that when cells dependent on cholesterol were deprived of this lipid, HMG CoA reductase activity was increased. Since mevinolin deprives cells of mevalonate and its metabolites, including cholesterol, the ability of this drug to induce HMG CoA reductase was examined. Although mevinolin is a potent inhibitor of HMG CoA reductase, it has also been shown that this drug can induce enzyme activity in cells exposed to it. When human fibroblast cells in culture were exposed to compactin (a structural analog of mevinolin) a 15-fold increase in enzyme synthesis was seen within a 24-hour period (Brown et al., 1978). In in vivo studies, rats administerd 40 mg/kg compactin or mevinolin in their diet showed a significant induction of liver HMG CoA reductase (Endo et al., 1979; Tanaka et al., 1982).

The mechanism of induction of this enzyme is not yet clearly understood. In experiments by Sinensky and Logel (1983), the increased amount of enzyme detected following exposure of Chinese hamster ovary (CHO) cells to mevinolin was attributed to a decreased rate of enzyme degradation. Thus, mevinolin was found to extend the half-life of the enzyme in these studies. The vast majority of research into the induction of HMG CoA reductase, however, seems to indicate that the increased amount of the enzyme seen upon induction is due to increased enzyme synthesis, not decreased degradation in the presence of inducers. Specifically, rats fed mevinolin showed a significant induction in HMG CoA reductase activity which was correlated with an increase in messenger RNA (mRNA) for the enzyme (Clarke et al., 1983; Liscum et al., 1983).

Therefore, mevinolin both inhibits and induces HMG CoA reductase activity. It inhibits the enzyme by binding to it, altering its conformation as discussed earlier. The ability of mevinolin to induce HMG CoA reductase suggests

that the drug, by inhibiting the enzyme, blocks the synthesis of a product which may normally feed back to regulate it. The question of the regulation of HMG CoA reductase is considered further below.

3. Regulation of HMG CoA reductase

The HMG CoA reductase-catalyzed reaction is responsible for the production of lipids may be involved in several cellular functions. Because of the importance of these lipids to the cell, the regulation of HMG CoA reductase is a tightly controlled cellular process, dependent on the balance between the rates of synthesis and degradation of the enzyme.

The rates of synthesis and degradation of HMG CoA reductase are thought to be controlled by a multivalent feedback mechanism via products in the metabolic pathway catalyzed by the enzyme. In mammalian systems, it has been found that increased dietary cholesterol caused a decrease in the rate of enzyme synthesis (Erickson et al., 1975) subsequent to a decrease in mRNA for the enzyme (Chin et al., 1982). Cholesterol has also been found to cause the HMG CoA reductase which is present in the cell to be catalytically less active (Edwards et al., 1980; Arebalo et al., 1982). Furthermore, mevalonate, the immediate product of the HMG CoA reductase catalyzed reaction has been shown to have a similar inactivating effect on the enzyme in rats (Arebalo et al., 1982), although this effect is most likely due to the metabolism of mevalonate to cholesterol in these animals.

While the feedback control of cholesterol on HMG CoA reductase has been extensively studied, the regulation of the enzyme by other metabolic products in the enzyme pathway has also been examined. In these studies, mevinolin or compactin was used to determine what other products of the pathway could exert regulatory control on HMG CoA reductase. Carson and Lennarz (1979) noted that compactin caused abnormal gastrulation of embryos of

the sea urchin, Strongylocentrotus purpuratus. This effect was reversible by supplementing the embryos with dolichols, however, cholesterol supplementation did not prevent or reverse abnormal gastrulation in the presence of compactin.

In another study where nonsterol regulators of HMG CoA reductase were examined, CHO cells, whose growth was inhibited by compactin or mevinolin, were used (Brown and Goldstein, 1980). Here, the effect of these drugs on CHO cell growth was completely reversed by the addition of mevalonate to the cell culture, while the addition of squalene or cholesterol had no ability to prevent the growth inhibition. These studies, combined with the experiments on a variety of organisms from insect larvae (Monger et al., 1982) to plant seedlings (Bach and Lichtenthaler, 1982), in which cholesterol was unable to reverse the effects of mevinolin or compactin, suggest that different cell types vary in their regulatory mechanisms of HMG CoA reductase, relying on a variety of metabolites of mevalonate for this purpose.

In conclusion, it is known that HMG CoA reductase is regulated by a feedback mechanism, but the product of this enzyme's metabolic pathway which exerts this regulation is not necessarily cholesterol. With respect to the schistosome, this is a very important concept, for the schistosome lacks the ability to synthesize cholesterol de novo (Meyer et al., 1970). Thus, the effect of mevinolin on parasite egg production and the regulation of HMG CoA reductase in S. mansoni must center around a nonsterol lipid. The synthesis of this lipid, therefore, may be crucial not only to the regulation of parasite HMG CoA reductase, but may also be essential for the production of eggs by S. mansoni.

E. The Role of Nonsterol Lipid Metabolites of Mevalonate

Although the schistosome is incapable of cholesterol biosynthesis, mevinolin, a sterol synthesis inhibitor, was able to exert an effect on parasite egg

production. Thus, some other product formed in the HMG CoA reductase catalyzed pathway must be critical to oviposition in the parasite. While HMG CoA reductase catalyzes the rate-limiting step in the synthesis of cholesterol, studies by Mills and Adamany (1978), wherein inhibition of the enzyme resulted in decreased dolichol and subsequent glycoprotein synthesis in aortic smooth muscle cells, suggested that HMG CoA reductase may also catalyze the rate-limiting step in dolichol synthesis. Inhibition of dolichols, which are polyisoprenoid lipids involved in glycoprotein synthesis, by mevinolin would, therefore, affect the glycosylation of proteins. The effect of mevinolin or compactin on the synthesis of these lipids occurs at concentrations of these agents higher than those needed to completely block sterol synthesis (Filipovic and Menzel, 1981). In the schistosome, where sterol synthesis does not occur, the ability of mevinolin to interfere with polyisoprenoid lipids and glycoprotein synthesis can be studied without the concern that other parasite lipids (of cholesterol synthesis and metabolism) are also being affected by the drug.

1. Characteristics of the lipids involved in glycoprotein synthesis

The polyisoprenoid lipids are known to function as lipid intermediates in the synthesis of glycoproteins. Specifically, these lipids serve as carriers of multiple sugars, which they subsequently transfer to forming peptides. Chemically, these specialized polyisoprenoid lipids are referred to as dolichols, and exist as phosphorylated α-saturated polyprenols, varying in chain length from 80 to 110 carbons or more (Hemming, 1974). The basic unit of these large carbon chains is a five-carbon isoprene, several of which are linked together to form the hydrophobic dolichol molecule.

Dolichols are embedded in the surface of the endoplasmic reticulum, where glycosylation of proteins takes place. Polyisoprenoid lipids such as the

dolichols are known to be involved in the synthesis of several carbohydrate-containing molecules in many systems. They are involved in the synthesis of bacterial extracellular polysaccharides (Osborn, 1969), cell-wall mannan-protein complexes in yeast (Parodi, 1977), yeast cell walls (Hopp et al., 1978), and plant and animal glycoproteins (Waechter and Lennarz, 1976; Elbein, 1979). Several examples of these lipids may be seen in Figure 9.

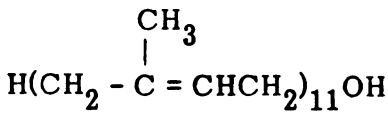
2. The role of polyisoprenoid lipids in the glycosylation of proteins

As stated above, polyisoprenoid lipids function as carriers of carbohydrates, transferring these sugars to peptides in the formation of glycoproteins. In this capacity, these lipids accept sugars from sugar-nucleotides until a large lipid-oligosaccharide is formed. The oligosaccharide portion is then transferred from the lipid to the polypeptide en bloc (Behrens, 1974; Elbein, 1979). In this manner, it is speculated, these lipids act as intermediates, aiding the transport of hydrophilic sugars into or through the membranous environment of the endoplasmic reticulum (ER) so that polymerization can occur (Elbein, 1979).

The scheme illustrating the role of these lipids in glycoprotein synthesis is depicted in Figure 10. In the early portion of the scheme (upper left), the phosphorylated lipid accepts two N-acetylglucosamine residues from the nucleotide. This is followed by the stepwise addition of nine mannose residues, with the subsequent addition of three glucose residues (Schachter and Roseman, 1980). At this point, the oligosaccharide is ready to be transferred to the protein in the ER. The oligosaccharide is generally linked to the amino group on the side chain of an asparagine (Asn) residue of the protein (Kornfeld and Kornfeld, 1976). These asparagine-linked oligosaccharides, referred to as N-linked oligosaccharides, are the most prevalent in glycoproteins. However, oligosaccharides linked to the protein via the hydroxyl group on the side chain of a serine, threonine, or

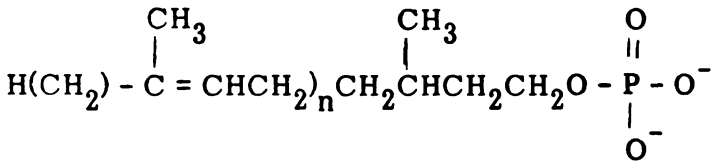
Involvement of polyisoprenoid alcohols in the synthesis of peptidoglycan (Hopp), Salmonella O-antigen (Osborn), and yeast mannans (Parodi).

General Structure:



Waechter and Lennarz demonstrated that animal tissues produce lipid-linked oligosaccharides as intermediates in glycoprotein synthesis.

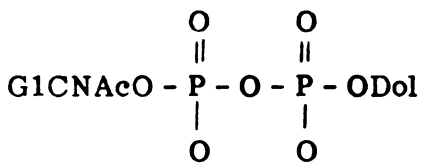
General Structure:



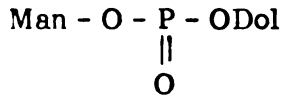
dolichylmonophosphate
n = 15-20

Monoglycosyl derivatives of dolichylmonophosphate exist (Waechter and Lennarz).

General Structure:



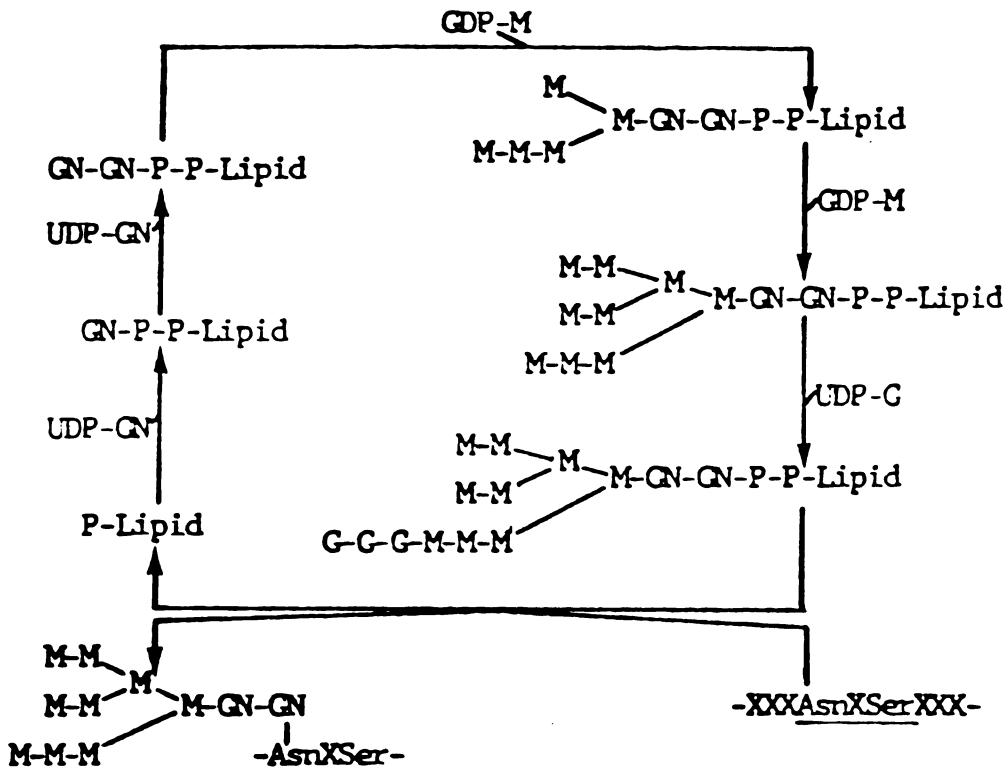
α-linked



β-linked

Figure 9. Biosynthesis of lipid-linked intermediates.

The Role of Lipids in Protein Glycosylation



M=Mannose
GN=N-acetylglucosamine
G=Glucose
X=Amino acid

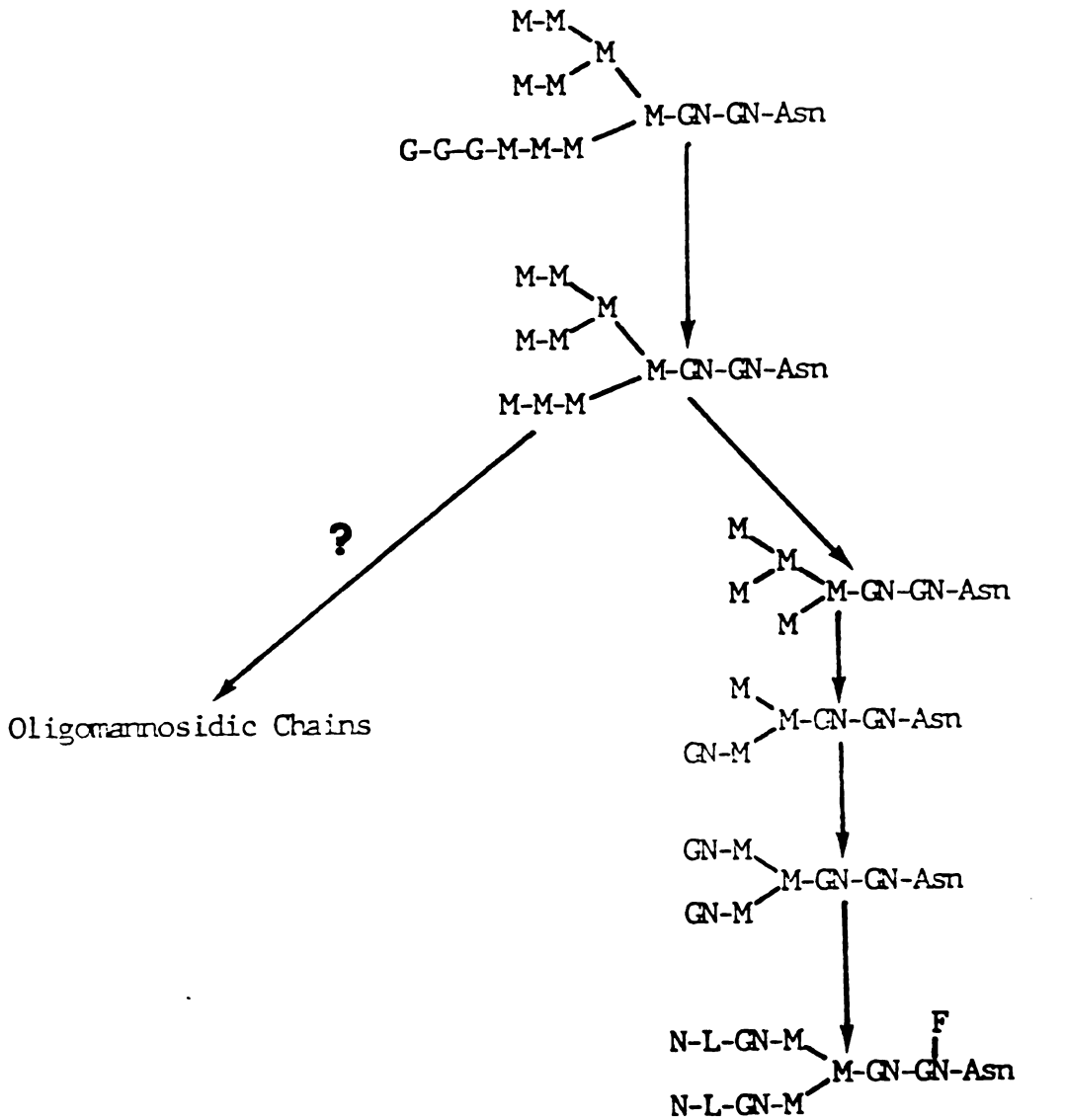
Figure 10. Role of lipids in the glycosylation of proteins.

hydroxylysine (O-linked oligosaccharides) may also be found in glycoproteins on occasion (Wagh and Bahl, 1981).

The glycosylation of a protein in the ER is the first step in the synthesis of complex glycoproteins. In order to modify the glycoprotein for its specific purpose, processing and remodeling of the oligosaccharide occurs. An example of the processing of the oligosaccharide is shown in Figure 11. The first step in this process, which occurs exclusively in the Golgi apparatus, is the trimming of the three terminal glucose residues. The oligosaccharide is then generally processed further to form an inner core structure, consisting of two N-acetylglucosamine and three mannose residues, still linked to asparagine. The terminal region of the oligosaccharide may now be modified by the addition of a variable number of different sugars, including N-acetylglucosamine, fucose, galactose and sialic acid (Hubbard and Ivatt, 1981), all added by their appropriate glycosyl transferases.

Once the glycoproteins are synthesized, they are packaged into secretory vesicles and released from the cell if they are secretory proteins. Glycoproteins may also be retained by the cell to serve in a structural capacity, or to act as enzymes. Figure 12 presents a summary of glycoprotein synthesis, along with the cellular sites for each step in this process.

In summary, polyisoprenoid lipids located in the ER play a vital role in the synthesis of glycoproteins; without these lipids, the formation of N-linked oligosaccharides would be impossible. Thus, these products of the HMG CoA reductase catalyzed pathway perform a key cellular function and, in certain cells they may be involved in the regulation of HMG CoA reductase as well.



M=Mannose

GN=N-Acetylglucosamine

G=Glucose

N=N-Acetylneuraminic Acid

L=Galactose

F=Fucose

Figure 11. Formation of complex glycoproteins.

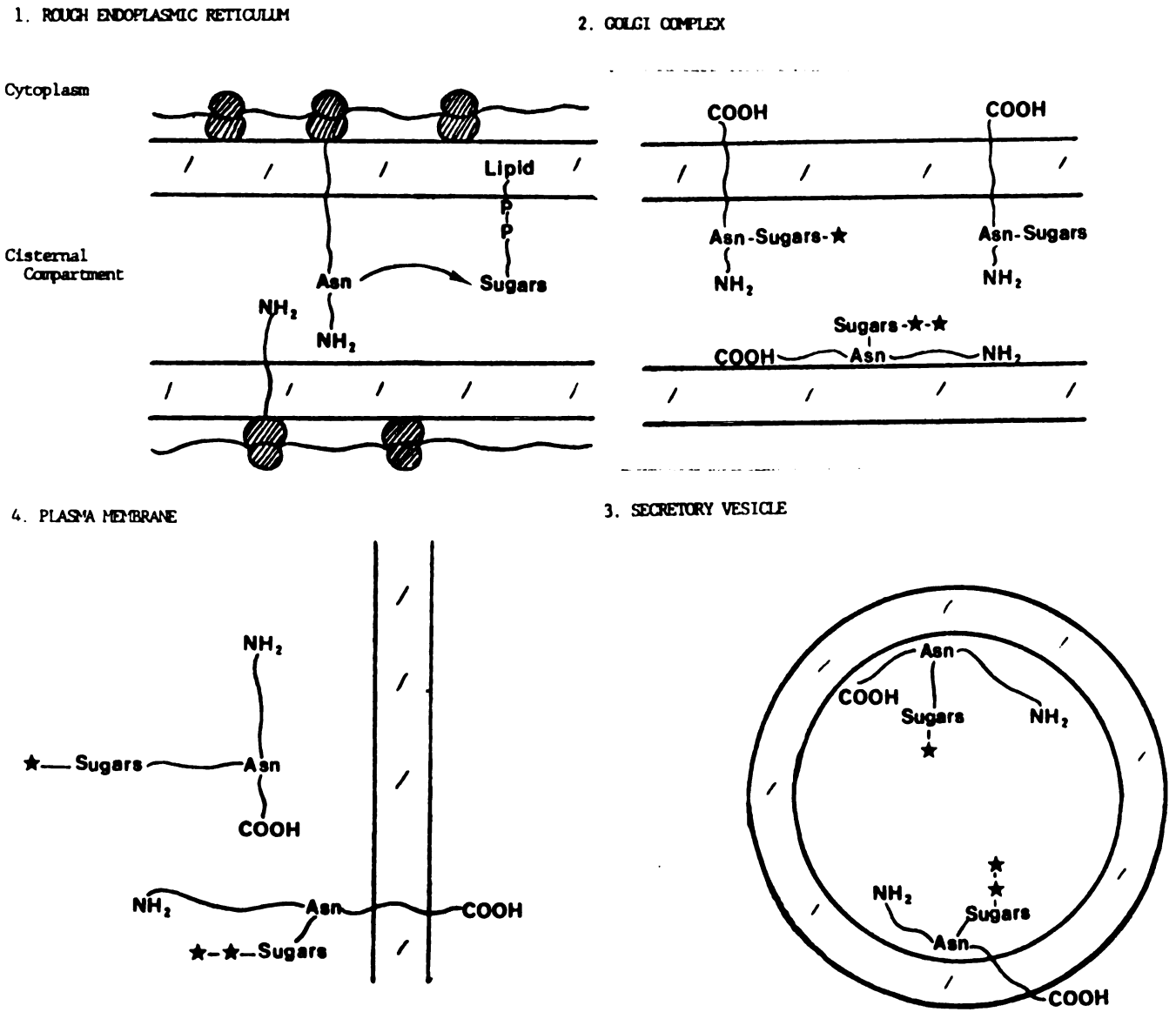


Figure 12. Cellular sites of glycoprotein synthesis.

F. Summary and Proposed Research

The production of eggs by S. mansoni is responsible for much of the pathology associated with schistosomiasis. In an attempt to inhibit egg production in the parasite, mevinolin, a sterol synthesis inhibitor, was examined. The purpose of this research is to investigate the effects of mevinolin on the parasite, and to assess the mechanism of action of this drug in S. mansoni. Since the schistosome cannot synthesize sterols de novo, the regulation of the enzyme which mevinolin inhibits, HMG CoA reductase, will be examined in the parasite. It is hoped that this research will contribute to the limited knowledge available regarding the biochemical regulation of egg production in S. mansoni, opening potential avenues for chemotherapy of schistosomiasis.

MATERIALS AND METHODS

A. Schistosomes

The schistosomes used in these experiments were Puerto Rican strain Schistosoma mansoni. Schistosome infections were maintained in white outbred (ICR) female laboratory mice (Harlan, Sprague-Dawley, Inc., IN), infected when body weight was approximately 15-20 g. The method of infection involved injection of 250-300 schistosome cercariae intraperitoneally. Sexual maturity of the parasites is reached by 40 days post-infection. From 45-55 days post-infection, adult parasites were surgically removed from the mesenteric veins and the hepatic portal vein of the host for use in in vitro studies. For in vivo studies with mevinolin, mice 35-50 days post-infection were used, depending on whether the protocol required sexually immature or sexually mature worms.

B. Incubation of Parasites In Vitro: Egg Production Assays

1. Exposure to mevinolin in vitro

Paired adult schistosomes were removed from the host and were placed in sterile medium containing RPMI 1640 (powdered form) buffered to pH 7.4 with 20 mM HEPES buffer. The media also contained 50% heat-inactivated horse serum plus 100 units/ml penicillin and 100 µg/ml streptomycin (media components from Gibco, Long island, NY), and 50 µM beta-mercaptoethanol. Schistosomes were rinsed several times in this media and were freed of any host-derived tissue prior to culture. Paired worms were then placed, 15 pairs to a flask, into sterile, capped Erlenmeyer flasks containing 50 ml of the medium

described above. Mevinolin (obtained from A.W. Alberts, Merck, Sharp and Dohme Research labs) was dissolved in dimethylsulfoxide (DMSO) and was added to the flasks in a volume not exceeding 50 µl. At least three flasks were run per drug concentration in each experiment. As a control, DMSO alone was added in the appropriate volume to several other flasks.

Incubation of parasites took place in a water bath heated to 37°C. During incubation, the flasks were agitated gently at 20 oscillations per minute. In the egg-laying assays, parasites were incubated in this manner for 72 hours.

2. Addition of lipids to the culture media

The effects of various lipid substrates on schistosome egg production were assessed by the direct addition of these substrates to the media. Specifically, mevalonate, farnesol, and dolichols were dissolved in DMSO, and were then added to the culture system described above in a volume not exceeding 50 µl. At least three flasks per lipid concentration were run for each experiment. Control flasks were assayed in the presence of DMSO alone.

3. Determination of egg production

Following incubation, each flask was shaken vigorously and three 5 ml media aliqouts were removed and placed in gridded petri plates. The number of eggs in the media sample was determined by counting the eggs under a dissecting microscope at 30X power.

C. Administration of Mevinolin to Schistosome-Infected Mice

Mice infected with S. mansoni were given mevinolin by gavage in various dosing regimens. In order to assess the ability of mevinolin to interfere with schistosome egg production in vivo, mice 35 days post-infection were dosed with 250 mg/kg mevinolin or its vehicle (25% glycerol-1% Cremophor EL) once daily

for ten days. In a separate experiment, where in vitro egg production after drug exposure was monitored, the dose was 50 mg/kg per mouse.

For the examination of parasite HMG CoA reductase activity, mice 42-45 days post-infection were dosed daily with mevinolin (50 mg/kg or 250 mg/kg) by gavage for three days.

Finally, in dosing experiments where gross and histopathology of liver tissue were examined, a subset of the livers were homogenized so that egg number in these livers could be determined. Briefly, this involved rinsing the excised livers several times in cold (4°C) saline solution, followed by homogenization in a four-fold volume of saline in a Waring blender for 30 sec. 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, from which they were washed into beakers and resuspended in 50 ml saline. Five ml aliquots of the eggs were counted to determine numbers of eggs in the livers.

D. Histological Preparations of Liver Tissue

Following ten days of drug administration, the animals were killed and livers from mice dosed with 250 mg/kg mevinolin or its vehicle were removed, rinsed in saline, and fixed in Bouin-Hollande fluid for 72 h. After fixing, the livers were sectioned and stained with hemotoxylin and eosin.

The liver pathology, due to the deposition of schistosome eggs in this tissue, was subjectively evaluated by a trained pathologist (Dr. Alan Cheever, N.I.H., Bethesda, MD) who had only limited knowledge of the experimental conditions. The relative amount of egg deposition in the livers of drug-treated and vehicle-treated mice was determined by measuring the granuloma volume within a 40

mm^2 area of liver slice. Additionally, the ratio of mature eggs to those containing immature miracidia was measured in the sections from each group. Three to six liver sections were obtained from different regions of each mouse liver. The granuloma counts were performed on at least 2 mice from each group per experiment.

The average burden of worm pairs in this study was 29.4±1.3 (SEM) as determined from worm counts in the mice at the time of sacrifice. None of the experiments in this group indicated a significant difference between worm burden in control versus mevinolin-treated mice.

E. Hydroxymethyl Glutaryl Coenzyme A Reductase Assays

1. Preparation of microsomal fractions

HMG CoA reductase activity was determined in paired S. mansoni using a modification of the method described by Alberts et al. (1980), which measures the conversion of radiolabeled HMG CoA to radiolabeled mevalonate (MVA). One hundred (average) paired worms were removed from infected mice, rinsed several times with RPMI 1640, and freed of any host-derived tissue. Worms were then placed in a homogenizing vessel containing 2 ml of buffer consisting of 50 mM potassium phosphate (pH 7.0), 0.2 M sucrose, 2 mM dithiothreitol and 50 mM EDTA. Homogenization involved ten strokes with a motor-driven Teflon pestle in a Potter-Elvehjem type glass homogenizer. The parasite homogenate was sonicated with three 25-sec pulses, and was then centrifuged at 8,000 x g for 30 min. The supernatant solution was removed to a separate tube and further centrifuged at 100,000 x g for 70 min. All of the above procedures were carried out at $4^{\circ}C$. The microsomal pellets resulting from high-speed centrifugation were frozen overnight at $-20^{\circ}C$. After thawing at room temperature, the microsomes were homogenized in solubilization buffer containing 50 mM potassium phosphate

(pH 7.0), 0.1 M sucrose, 2 mM dithiothreitol, 50 mM KCl and 30 mM EDTA. Following solubilization, the final volume of the microsomal fraction was adjusted with the above buffer so that 1 µl of the homogenate contained 10 µg protein (as determined by the Albro method).

2. Standard HMG CoA reductase assay

Reaction mixtures were made to assess HMG CoA reductase activity in the solubilized microsomes. Each reaction in the standard assay used 10 µl of the microsome (100 µg protein), along with 10 µl each of the following: 100 mM dithiothreitol, 1.8 M KCl, 1 mg/ml bovine serum albumin (BSA), 1.4 M potassium phosphate buffer (pH 6.8) and 35 mM EDTA. To this mixture, 5 µl of DL-3-(glutaryl-3-^{14}C)-hydroxy-3-methylglutaryl coenzyme A (47.2 mCi/mmol, New England Nuclear) was added. This mixture was incubated at 37°C for 10 min, after which 20 µl NADPH (10 µg/µl) was added to initiate the enzyme reaction. At the appropraite time, the reaction was stopped with the addition of 20 µl 5 M HCl to each tube. The mixtures were incubated an additional 15 min at 37°C following termination of the reaction.

At the end of the incubation period, the mixtures were centrifuged for 10 min at 1000 x g. The supernatant was then removed and placed on Kontes columns (dimensions: 0.5x5 cm) of Dowex-1, chloride form, 200-400 mesh (Sigma Chemical Co.). This resin functions to retain unreacted HMG CoA, while having very low affinity for the HMG CoA reductase-catalyzed product, mevalonate. The specificity of the resin was tested by thin-layer chromatography (TLC) of HMG CoA and mevalonate standards after Dowex-1 chromatography of these compounds (data not shown). To measure the amount of product formed in the assay, the Dowex columns were eluted with distilled water, and 3 ml eluate was collected into scintillation vials. To this, 17 ml aqueous counting scintillant (ACS, Amersham) was added, and radioactivity in the products was determined by liquid

scintillation spectrometry. The data obtained from these experiments are expressed as picomoles product (mevalonate plus other minor metabolites downstream from MVA) per mg protein.

3. Inhibitor studies

The effect of mevinolin on HMG CoA reductase activity in the schistosome was measured in two ways. First of all, mevinolin was added directly to the enzyme reaction mixture in a range of concentrations (0.1 μM to 100 μM). In these studies, mevinolin was dissolved in 100% ethanol, and the volume of drug added to the reaction mixture did not exceed 1% of the reaction mixture volume. The drug was added to the mixture containing the microsome, and a preincubation of 10 min at 37°C was carried out so that equilibrium could be established between mevinolin, microsome, and substrate. After preincubation, NADPH was added as before to initiate the enzyme reaction.

A second manner in which mevinolin's effect on the enzyme was assessed involved using schistosomes from mice that had been dosed for 3 days with mevinolin (50 mg/kg or 250 mg/kg) prior to making the microsome. These parasites were homogenized directly after removal from the mice or, in some cases, were incubated for 24 h *in vitro* prior to homogenization. The enzyme assays were then run on these microsomes as described above.

F. Radiolabeling of Parasite Lipids In Vitro

In order to determine which parasite lipids were sensitive to mevinolin, lipids were metabolically labeled using [U-^{14}C]-acetic acid or [DL-2-^{14}C]-mevalonic acid (Research Products International Corp.). In these studies, 200-300 paired schistosomes were incubated in 100-150 ml of the media described earlier for 24 h. At the onset of incubation, 50 μCi [U-^{14}C]-acetate (94 mCi/mmol) or 25 μCi [DL-2-^{14}C]-mevalonate (39.5 mCi/mmol) were added to the flasks.

Uptake of radiolabel by the parasites was terminated at 24 h by removing the schistosomes from the culture flasks and rinsing them several times in cold, sterile RPMI 1640.

G. Characterization of Radiolabeled Lipids

1. Extraction and isolation of labeled schistosome lipids

Following incubation and rinsing, schistosomes were placed in 60 ml chloroform:methanol ($CHCl_3$:CH_3OH, 2:1 v/v), and were extracted overnight at 4°C. Following extraction, the worms were filtered, and the collected extract was concentrated using a flash evaporator (Buchler Instruments). Non-lipid contaminants were removed from the extract by Sephadex chromatography on a Sephadex G-25 column (Wells and Hanahan, 1969). The resulting fraction was concentrated and then passed through a silicic acid column (100-300 mesh, Sigma Chemical Co.) which was used to separate the lipids into neutral and polar fractions (Rouser et al., 1967). This column was eluted with 100 ml each of chloroform, acetone and methanol which resulted in three fractions of increasingly polar lipids. Yields from the silicic column ranged from 81-100% as determined by liquid scintillation spectrometry of samples just prior to and immediately following silicic acid chromatography.

The three fractions (referred to as neutral, polyisoprenoid, and polar lipids) were further separated using column chromatography. The neutral lipids were passed through a 7% hydrated Florisil column (Sigma Chemical Co.) of 1.2x15 cm. A gradient of hexane and diethylether (EE) was used to elute hydrocarbons (HC), sterol esters (SE), triglycerides (TG), and diglycerides (DG). Methanol and EE (98:2 v/v) was used to elute monoglycerides (MG), and EE and acetic acid (96:4 v/v) was used to elute free fatty acids (FFA) (Carroll and Serdarevich, 1967).

The polyisoprenoid-type lipids (PI) were separated on a Florisil column (dimensions 1.2x15 cm). These lipids were eluted, using the method of Radin (1969), with chloroform, acetone:methanol (3:1 v/v), and methanol.

The polar lipids were further purified using a DEAE cellulose column (Sigma Chemical Co.), dimensions 1.5x20 cm. These lipids were eluted with a gradient of chloroform and methanol, according to the method of Rouser et al. (1969).

2. Purification of labeled lipid components

Lipids from each of the three lipid classes were further purified using preparative thin-layer chromatography (TLC). Neutral lipids were spotted using a Hamilton syringe in a volume of 5-10 µl onto silica gel-impregnated instant TLC (ITLC-SG) plates (Gelman Sciences, Inc.), which were then developed in hexane: diethylether (95:5 v/v). Polyisoprenoid lipids were similarly spotted onto silicic acid containing ITLC plates (ITLC-SA) which were subsequently developed in isopropanol:ammonium hydroxide (100:7 v/v). The polar lipids were spotted onto ITLC-SG plates and were also developed in isopropanol:ammonium hydroxide (100:7 v/v).

Radiolabeled lipids were detected on the TLC plates by scanning lanes of the developed plates for ^{14}C-lipids using a Tracerlab 4 pi chromatogram scanner. Unlabeled lipid standards (all lipid standards were from Sigma Chemical Co.) were chromatographed alongside of the radiolabeled samples. After development, the standards were detected by spraying the plate with a sulfuric acid-dichromate stain (55% H_2SO_4 containing 0.6% K_2CrO_3) followed by heating at 180°C for 10-15 min (Rouser et al., 1967). The location of the standards by this method was compared with the location of peaks on the radioscans of the samples, and tentative identification of various schistosome-derived lipids could be made.

Distribution of radiolabel into individual schistosome lipids was calculated based on the area under the curve of the peaks of the radioscans.

H. Determination of the Effect of Mevinolin on Lipid Intermediates, Lipid Oligosaccharides and Glycoproteins of S. mansoni

1. Preparation of reaction mixtures for the isolation of lipid intermediates, lipid oligosaccharides and glycoproteins

Parasites incubated for 24 h in the presence of 10 μM mevinolin or DMSO were minced with a razor blade, then disrupted with three 25-sec pulses using a sonicator (Ultrasonics, Inc.). Protein determinations were made on the worm homogenates using the method of Lowry et al. (1951), and 0.5 mg protein was used in the reaction mixture. The remainder of the reaction mixture consisted of 25 mM Tris-HCl buffer (pH 7.4), 10 mM $MnCl_2$, and 0.5 μCi (2.0 nmoles) GDP-[U-^{14}C]-mannose (New England Nuclear), to a total volume of 1250 μl. The reaction was begun with the addition of the GDP-[U-^{14}C]-mannose to the mixture which was maintained at 37^{o}C, with gentle shaking, for the duration of the incubation. At the appropriate time, aliquots of the reaction mixture were removed and examined for ^{14}C-mannose incorporation into lipid intermediates, lipid oligosaccharides, and glycoproteins.

2. Isolation of lipid intermediates and lipid oligosaccharides

The isolation of lipid intermediates and lipid oligosaccharides from S. mansoni was accomplished using a modification of the method of Rumjanek and Smithers (1978). Afer 30 or 60 minutes of incubation of the S. mansoni homogenate reaction mixture, 250 μl aliquots of the reaction mixture were removed and placed into tubes containing 3 ml $CHCl_3$:CH_3OH (2:1 v/v). The homogenates were allowed to extract for 1 h at room temperature. Following extraction, the tubes were spun in a bench-top centrifuge at 700 x g for 10 min. The organic phase was then removed and was placed into a second test tube. This

fraction was washed three times with 2.0 ml water, then twice with an equal volume (approximately 1.5-2.0 ml) of $CHCl_3$:CH_3OH:H_2O (3:48:47 v/v/v) to remove any non-lipid contaminants. The remaining organic phase was removed, dried completely under a stream of nitrogen, and was then resuspended in 10-20 µl $CHCl_3$:CH_3OH (2:1 v/v). This entire fraction was spotted onto an ITLC-SG plate, which was developed in $CHCl_3$:CH_3OH:NH_4OH (65:35:5 v/v/v). Following development, the plate was scanned for lipids carrying radioactive mannose residues. This fraction is hereafter referred to as the lipid intermediate fraction.

The pellet remaining after extraction of the lipid intermediate was re-extracted with 3.0 ml of $CHCl_3$:CH_3OH:H_2O (1:1:0.3 v/v/v) for 1 h at room temperature. Following extraction, the sample was centrifuged for 10 min at 700 x g, and the supernatant was concentrated to dryness under nitrogen, then resuspended in 10-20 µl $CHCl_3$:CH_3OH (2:1 v/v). Again, the entire sample was spotted onto an ITLC-SG plate which was developed in $CHCl_3$:CH_3OH:NH_4OH (65:35:5 v/v/v). The lipid detected by the TLC scanner in this fraction is referred to as the lipid oligosaccharide.

3. Kinetic studies

The effect of mevinolin (10 µM) on the rate of incorporation of ^{14}C-mannose into the lipid intermediate, the lipid oligosaccharide, and the remaining protein pellet was determined. The reaction mixtures used in these studies were identical to those described earlier. After initiation of the reaction with the addition of 0.5 µCi GDP-[U-^{14}C]-mannose, 250 µl aliquots of the mixture wre removed at 2.5, 5.0, 10.0, and 20.0 minutes and were placed in $CHCl_3$:CH_3OH (2:1 v/v) as described. The lipid intermediate and the lipid-oligosaccharide were extracted as before. In these studies, however, the lipids were dried under nitrogen, then resuspended in 100 µl $CHCl_3$:CH_3OH (2:1 v/v). The 100 µl samples were then placed in scintillation vials, and 9.9 ml ACS were added to each vial.

The radioactivity in the samples was then determined by liquid scintillation spectrometry.

Once the lipids were extracted, the residual pellet was resuspended in 0.5 ml NCS tissue solubilizer (Amersham). The protein samples were then heated at 70°C for 15 min in capped tubes. Following solubilization, the samples were placed in scintillation vials and 9.5 ml ACS were added to each vial prior to scintillation spectrometry. A summary diagram of the protocol for the isolation of the lipids and protein is presented in Figure 13.

I. Protein Determinations

Protein determinations of whole worm homogenates used in the lipid intermediate studies were determined by the method of Lowry et al. (1951). Protein contained in microsomal fractions was measured using the method of Albro (1975). Briefly, this method involved dissolving 5-100 µg of protein in 200 µl 0.625 N NaOH. After a 30-min room temperature incubation, 250 µl of reagent "D" (containing 11% sodium carbonate, 2.2% soduim tartrate, and 1.1% copper sulfate) is added to each sample tube along with 1.0 ml dilute (5%) folin reagent. The tubes are then heated at 55°C for 5 min, then cooled and read at 650 nm in a spectrophotometer.

J. Liquid Scintillation Counting

Carbon ^{14}-labeled samples were counted using a Beckman LS7000 scintillation counter. Samples were counted in a full C-14 window (95% of all C-14 emissions). Accuracy of counting was ±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 C^{14}, and using the H-number technique to determine the degree of quench.

Methodology for the Extraction and Separation of Lipid Intermediates from Schistosoma mansoni

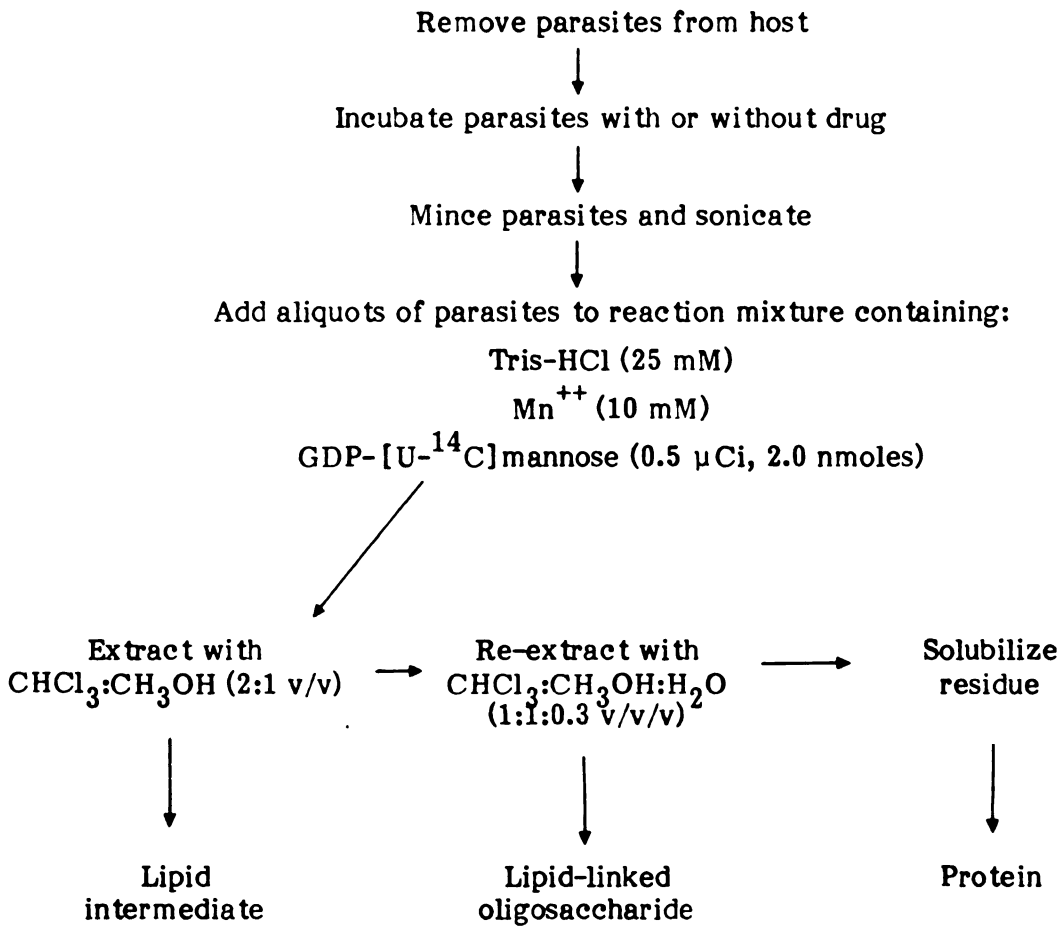


Figure 13. Flow diagram for the isolation of ^{14}C-mannose-labeled lipid intermediates, lipid oligosaccharides and glycoproteins of S. mansoni.

K. Statistical Methods

Data are expressed as the mean plus or minus the standard deviation (S.D.). Means were compared by the Student's t-test or by analysis of variance as described by Snedecor and Cochran (1967). Significance was established at $p \leq 0.05$.

RESULTS

A. Regulation of Schistosome Egg Production: In Vitro Studies with Mevinolin

1. Effect of mevinolin on in vitro egg production by S. mansoni

The effect of mevionlin on schistosome egg production in vitro is shown in Table 1. Mevinolin at concentrations greater than or equal to 10 μM caused a significant decrease in the number of eggs produced by the parasite. Specifically, mevinolin at 10 μM caused a 70% decrease in egg production by S. mansoni during a 72 h in vitro incubation. The reduction in egg number was not associated with a lesser degree of pairing or loss of adherence of the worms to the culture flask (both of which would be detrimental to egg production), since both treated and control worms appeared normal in these respects throughout the incubation period. Furthermore, it was found that mevinolin at concentrations ranging from 0.1 to 100 μM did not significantly alter schistosome longitudinal muscle tension or surface electrical activity as compared to control parasites (Morrison et al., 1986). Finally, although egg number was reduced in the mevinolin-treated parasites, egg morphology was not noticeably different between control and drug-treated worms upon microscopic examination. Thus, the in vitro effect of mevinolin was noted to be relatively specific for the amount of schistosome egg production.

2. Effect of metabolites of HMG CoA on schistosome egg production

Mevinolin is known to inhibit the formation of mevalonate (MVA) from HMG CoA by acting as a competitive inhibitor of the enzyme HMG CoA reductase (Alberts et al., 1980). This action of mevinolin starves cells for MVA and

Table 1

Effect of Mevinolin on *In Vitro* Egg Production by *S. mansoni*[a]

[Mevinolin] μM	No. Eggs/Female/72 h
0	112.3±37.2[b]
0.1	81.7±29.0
1.0	59.3±22.1[c]
10	32.8±18.6[c]
100	12.2± 8.4[c]

[a]Paired schistosomes were incubated for 72 h in the presence of mevinolin as described in the Methods.

[b]Data are expressed as mean number of eggs ± S.D.

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

downstream metabolites of MVA such as farnesol, an important intermediate in the synthesis of sterols and polyisoprenoid lipids. Since mevinolin is known to cause a dimunition of these lipids, the effects of MVA and farnesol on schistosome egg production were studied.

When parasites were incubated in the presence of MVA, a concentration-dependent effect was noted (Table 2). At low concentrations of MVA (25 μM) egg production was stimulated significantly above control. However, at high concentrations of MVA (200 μM), egg production was reduced to one-fifth of control levels.

The findings with farnesol were similar to those seen with MVA. Again, low concentrations of this lipid (20 μM or 40 μM) significantly stimulated parasite oviposition while higher concentrations of farnesol (80 μM or 200 μM) caused a four-fold reduction in egg production. Therefore, it appears that downstream metabolites of HMG CoA are capable of exerting an effect on schistosome egg production.

3. Reversal of mevinolin's effect in vitro by mevalonate and farnesol

Recognizing that MVA and farnesol could stimulate egg production in the schistosome, the ability of these lipids to reverse mevinolin's effect in vitro was studied. When parasites were incubated in the presence of both MVA and 10 μM mevinolin, a concentration-dependent effect was again seen (Figure 14). Incubations with 25 μM MVA and 10 μM mevinolin restored egg production to controls levels (0 MVA, 0 mevinolin), and significantly increased it above that in worms incubated in 10 μM mevinolin alone ($p < 0.01$). When 200 μM MVA and 10 μM mevinolin were added to the same flask, a marked suppression of egg production was seen.

Similar results were seen in experiments where schistosomes were incubated in the presence of farnesol and 10 μM mevinolin. Here, 80 μM farnesol

Table 2

Effects of Mevalonate and Farnesol on In Vitro Egg Production by S. mansoni[a]

[Mevalonate] μM	Eggs/Female/72 h	[Farnesol] μM	Eggs/Female/72 h
0	98.5±32.5[b]	0	76.0±13.2
10	121.5±25.0	20	148.3±24.9[c]
25	214.4±33.7[c]	40	140.3±10.4[c]
50	125.8±36.7	80	20.0± 1.8[c]
100	102.9±14.1	200	18.9± 4.6[c]
200	22.2±13.9		

[a]Paired schistosomes were incubated in the presence of mevalonate or farnesol as described in the Methods.

[b]Data are presented as mean number of eggs ± S.D.

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

Figure 14. Effect of mevalonate on in vitro egg production by S. mansoni exposed to mevinolin. Incubations were performed in the presence of various concentrations of mevalonate (MVA) plus 10 μM mevinolin. Each bar represents the mean ± S.D. of at least 6 determinations. Open bars represent control worms; closed bars represent worms incubated in 10 μM mevinolin.

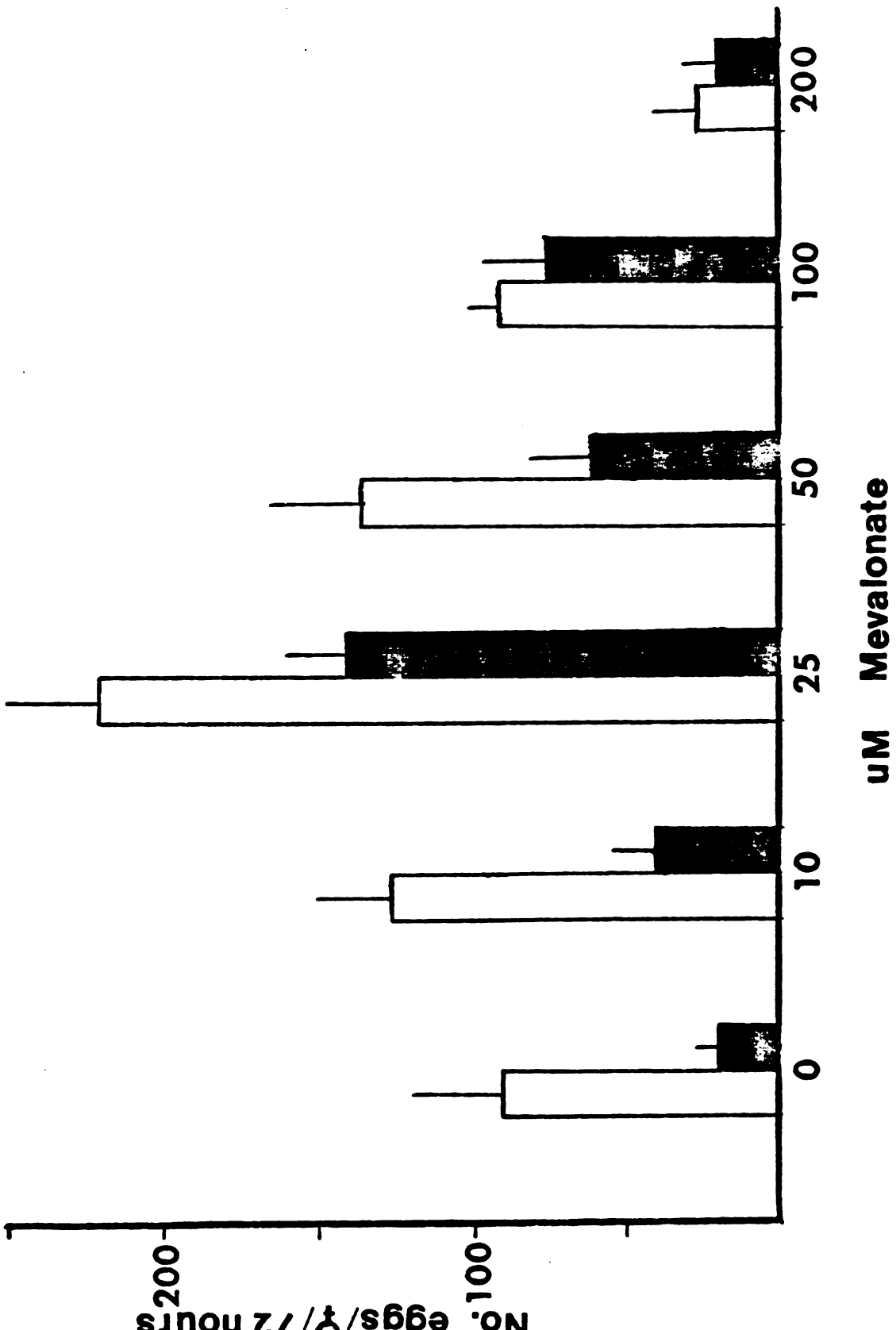


Figure 14

was able to restore egg production to control levels in the presence of 10 μM mevinolin (Figure 15). The egg production in these parasites was again significantly higher than that seen in worms incubated only in 10 μM mevinolin ($p < 0.01$). Therefore, the MVA and farnesol data indicate a true ability of these lipids to reverse mevinolin's effect on schistosome egg production.

In a third study, dolichols, a downstream metabolite of farnesol, were examined for their ability to stimulate egg production or reverse mevinolin's effect. In these studies, dolichols at concentrations shown to be effective in other in vitro systems (Carson and Lennarz, 1979) were unable to reverse the effect of mevinolin, and also had no ability to stimulate egg production in the parasite (Table 3). Since radiolabeled dolichols were unavailable, it is unknown whether the failure of dolichols to exert an effect was due to an inability of the parasite to use these lipids, or due to lack of penetration of these lipids into the parasite in culture.

4. Stimulation of egg production by S. mansoni following in vitro or in vivo exposure to mevinolin

The enzyme HMG CoA reductase has been shown to be highly inducible by low doses of mevinolin or its structural analog, compactin (Brown et al., 1978; Skalnik et al., 1985). To determine whether or not this induction phenomenon also occurred in the schistosome, assays were carried out which measured in vitro egg production by the parasite followng acute exposure to mevinolin in vitro or in vivo.

In the in vitro studies, paired schistosome were exposed to 10 μM mevinolin for 24 h, after which they were placed in drug-free media for an additional 72 h incubation. Following incubation, it was noted that parasites exposed to mevinolin acutely produced twice as many eggs as did the controls, and four times as many eggs as did parasites exposed to mevinolin for the entire incubation period (Table 4).

Figure 15. Effect of farnesol on in vitro egg production by S. mansoni exposed to mevinolin. Incubations were performed in the presence of various concentrations of farnesol plus 10 μM mevinolin. Each bar represents the mean ± S.D. of at least 6 determinations. Dotted bar: Farnesol plus 10 μM mevinolin. Solid bar: Farnesol alone. Asterisk indicates significant difference from appropriate control.

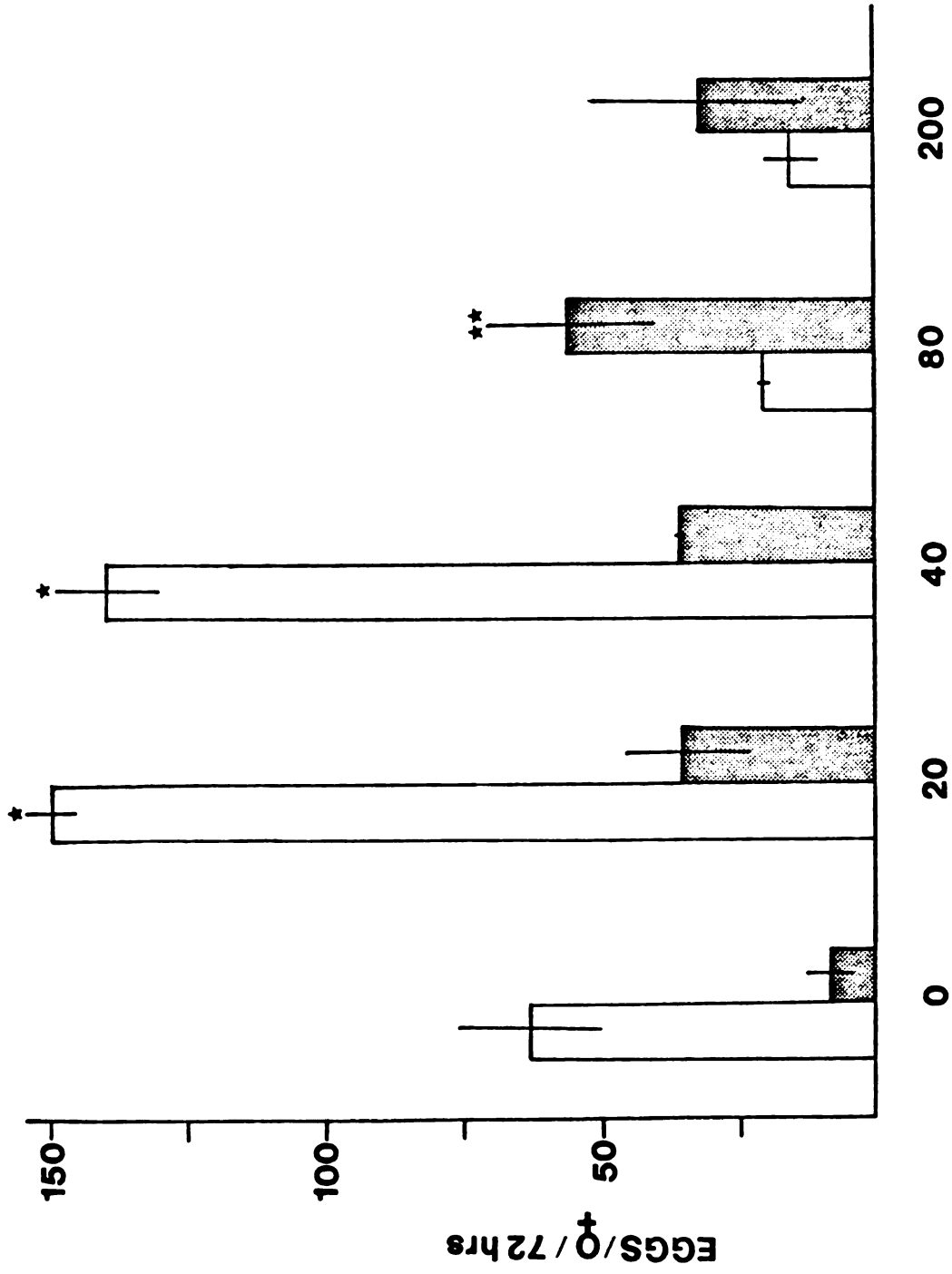


Figure 15

Table 3

Effect of Dolichols on Egg Production by S. mansoni in the Presence and Absence of Mevinolin[a]

[Dolichol] µg/ml	[Mevinolin] µM	No. Eggs/Female/72 h
0	0	51.7± 5.9[b]
0	10	9.3± 5.7
10	0	64.1± 9.3
10	10	17.4±15.7
20	0	58.6±11.3
20	10	15.8± 6.3

[a]Paired schistosomes were incubated in the presence or absence of dolichols or mevinolin as described in the Methods.

[b]Data are presented as mean number of eggs ± S.D.

Table 4

Effect of Mevinolin on In Vitro Schistosome Egg Production Following In Vitro Exposure to the Drug[a]

24 h In Vitro Treatment	Concentration of Mevinolin in Culture Media	No. Eggs/Female/72 h
Vehicle	0	63.0±28.1[b]
Mevinolin (10 μM)	0	117.8±32.3[c]
Mevinolin (10 μM)	10 μM	29.3±22.7[c]

[a]Paired schistosomes were incubated in the presence or absence of mevinolin after in vitro exposure to the drug or its vehicle.

[b]Data are presented as mean number of eggs ± S.D.

[c]Significantly different from control, $p < 0.05$.

In a second group of studies, mice 35 days post-infection were dosed daily with 50 mg/kg mevinolin orally until day 45, when the parasites were retrieved. Here, parasites removed from mice treated with the drug and placed in drug-free media demonstrated a significant increase in egg production over parasites from mice treated only with vehicle ($p < 0.01$, Table 5). Schistosomes from either *in vivo* group always produced fewer eggs when maintained in 10 μM mevinolin *in vitro*. Thus, it appears that schistosome HMG CoA reductase is inducible by mevinolin and this induction appears to be correlated with egg production *in vitro*.

B. Regulation of Schistosome Egg Production: In Vivo Studies with Mevinolin

The *in vivo* effects of mevinolin were studied by dosing *S. mansoni*-infected mice with the drug utilizing several dosing regimens. It was found that daily doses of 50 mg/kg or 100 mg/kg mevinolin did not significantly reduce worm burden or pathology (as assessed by granuloma content in the liver) compared to mice dosed with vehicle on a comparable regimen. When mice were dosed from days 35-45 post-infection with 250 mg/kg mevinolin however, a significant reduction in pathology was noted while worm burden was not significantly changed.

Pathological changes associated with mevinolin treatment at 250 mg/kg were assessed in two ways. First, gross liver pathology was compared between control and treated mice, and numbers of eggs deposited in each group of livers was determined. Secondly, livers from vehicle- and mevinolin-treated mice were sectioned and examined histopathologically.

Changes in gross pathology between control and mevinolin-treated mouse livers were readily apparent upon sacrifice of the animals. Livers from mevinolin-treated mice were smaller (20-30%, based on weight) and, upon gross

Table 5

Effect of Mevinolin on *In Vitro* Egg Production by *S. mansoni* Following *In Vivo* Exposure to the Drug[a]

In Vivo Treatment	Concentration of Mevinolin in Culture Media	No. Eggs/Female/72 h
Vehicle	0	60.4±32.6[b]
Vehicle	10 μM	10.7± 7.3[c]
Mevinolin (50 mg/kg)	0	321.8±90.4[c]
Mevinolin (50 mg/kg)	10 μM	6.3± 4.1[c]

[a]Parasites were incubated in the presence or absence of mevinolin following *in vivo* exposure to the drug or its vehicle.

[b]Data are presented as mean number of eggs ± S.D.

[c]Significantly different from control, $p < 0.01$.

examination, appeared to contain fewer granulomas (Figure 16). Homogenization of livers from each group (n=6 livers per group per experiment) yielded an average of 45.4% fewer eggs from livers of the mevinolin-treated mice. Thus, the reduction in gross pathology seemed to be correlated with reduced egg deposition in the livers of treated mice.

To better examine the pathological changes induced by mevinolin, histological sections were taken of livers from mice of both treated and control groups. Histopathologically, livers of mevinolin-treated animals were again strikingly different from controls. In one respect, the granulomas in livers of mevinolin-treated animals were much smaller than in vehicle-treated controls (Figures 17 and 18). This was probably attributable to the observation that in the drug-treated animals, generally speaking, only one egg was at the center of each granuloma. In the control animals, however, several eggs were involved in each area of granulomatous inflammation (Figures 17 and 18). Furthermore, in the drug-treated mice, histopathological examination by a trained pathologist revealed an average of 50% fewer mature miracidia in the deposited eggs than in granulomas of control animals. These results indicate an ability of mevinolin to interfere with schistosome egg production in vivo, an effect correlated with an amelioration of schitosome-associated pathology.

C. HMG CoA Reductase Assays

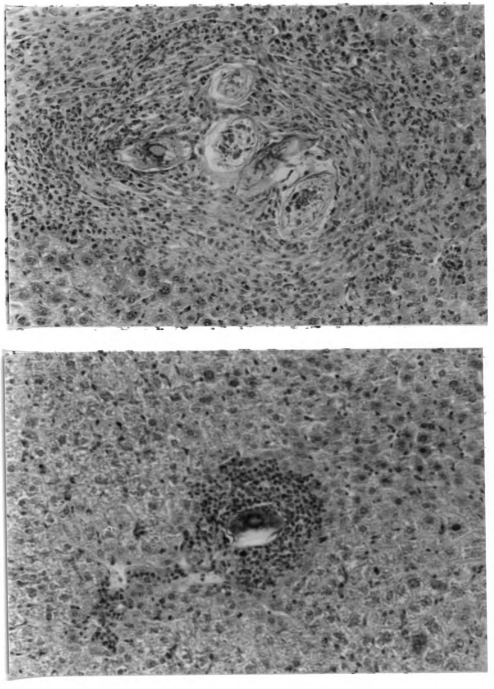
1. Characteristics of the HMG CoA reductase-catalyzed reaction in S. mansoni

Schistosome HMG CoA reductase activity has previously not been identified or characterized. However, using the method of Alberts et al. (1980) for measuring HMG CoA reductase activity in rat liver, some purification and preliminary characterization of schistosome HMG CoA reductase was accomplished. It should be pointed out that the studies on this enzyme in the

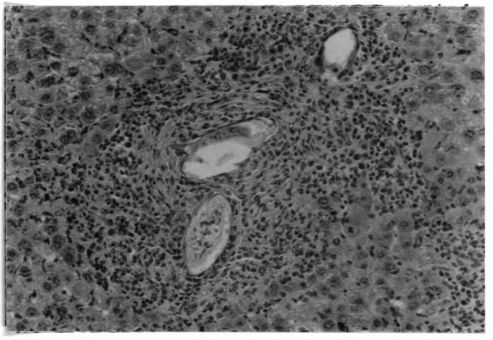
Figure 16. Gross pathology of livers removed from vehicle-treated and mevinolin-treated mice. Mice were dosed daily with 250 mg/kg mevinolin or its vehicle, days 35-45 post-infection, after which their livers were removed. Top two livers were removed from mevinolin-treated animals, bottom liver was removed from a vehicle-treated control mouse. Magnification is 2X.

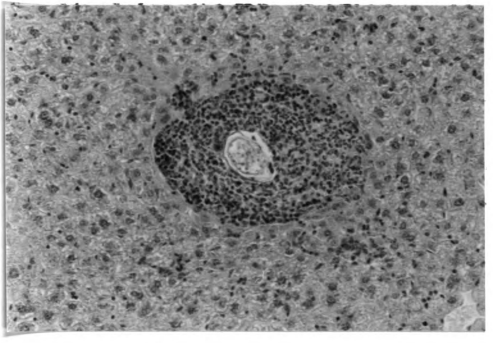
Figure 16

Figures 17 and 18. Histological preparations of livers from control and mevinolin-treated mice. Mice were dosed daily from days 35-45 post-infection with 250 mg/kg mevinolin. Tissue sections were made of livers from vehicle-treated infected mice (top photos) and drug-treated infected mice (bottom photos). Tissues are stained with hematoxylin and eosin, magnification is 100X.



Figures 17





Figures 18

schistosome were limited by the amount of activity recoverable from this parasite. The main objective, therefore, was to demonstrate the presence of this enzyme in S. mansoni and determine its response to mevinolin in vitro and in vivo. In rat liver, HMG CoA reductase activity has been isolated in microsomal fractions (Kleinsek et al., 1977). In the schitosome studies, parasite fractions including a "crude" pellet (resulting from 8,000 x g centrifugation of worm homogenate), a microsomal pellet (100,000 x g) and the supernatant remaining after ultracentrifugation of the microsome were assayed for HMG CoA reductase activity using the Alberts assay. The 8,000 x g pellet and the microsomal supernatant demonstrated no measurable HMG CoA reductase activity (data not shown); the enzyme activity was confined to the microsomal pellet.

Once the enzyme activity was localized to the microsome, enzyme assays were run testing three different amounts of microsome, 5 µl, 10 µl, and 20 µl (50, 100 and 200 µg of protein, respectively), for enzyme activity during a ten-minute incubation. Each microsomal fraction was incubated with 5 µCi ^{14}C-HMG CoA and the reaction mixture described in the Materials and Methods, and the generation of ^{14}C-labeled products of the reductase-catalyzed reaction was monitored. Radiolabeled products were eluted from Dowex-1 columns which retained the unreacted substrate, and fractions were counted with liquid scintillation spectrometry. Counts per minute were converted to picomoles of radiolabeled product generated per milligram of microsome protein. The data from these experiments are presented in Figure 19. In order to standardize the assay, 10 µl (100 µg protein) was chosen as the amount of microsome used in all further enzyme assays.

The time-dependence of the HMG CoA reductase activity in the microsome was next determined. Here, 10 µl worm microsome was reacted with ^{14}C-HMG CoA for 1, 5, 10, 20 or 40 minutes, after which the reaction was

Figure 19. The effect of various amounts of microsome on HMG CoA reductase activity in S. mansoni. Enzyme activity was recorded after a 10 min incubation. Each bar represents the S.D. for at least 6 determinations.

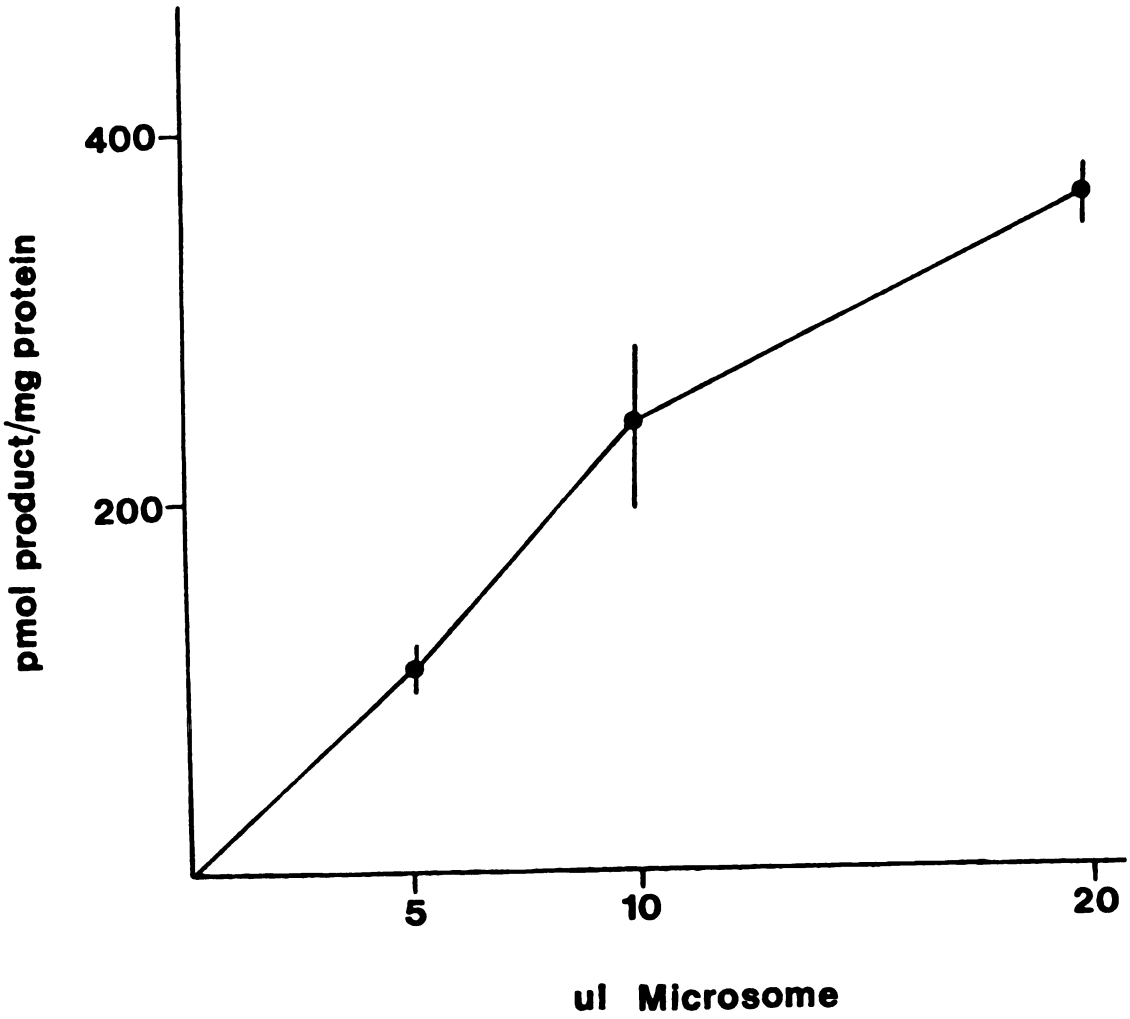


Figure 19

terminated with 5 M HCl. The results of these assays are shown in Figure 20. In these studies, the HMG CoA reductase-catalyzed generation of product was linear for at least 10 min, after which products continued to be formed, but at a slower rate.

D. Inhibition of Schistosome HMG CoA Reductase in the Enzyme Assay System

Mevinolin has been shown to affect schistosome egg production in concentrations ranging from 1 μM to 100 μM (see Table 1). To determine whether this range of concentrations was also effective to inhibit enzyme activity in the assay previously described, mevinolin, in concentrations of 0.1, 1.0, 10, and 100 μM, was included in the assay. The vehicle for mevinolin in these studies was 100% ethanol, as it was discovered that a DMSO vehicle completely abolished all enzyme activity whether or not mevinolin was present. Mevinolin in EtOH was added to the reaction mixture in a volume not greater than 1% of the total reaction mixture. All control assays (0 μM Mevinolin) contained the appropriate amount of EtOH.

The sensitivity of schistosome HMG CoA reductase to mevinolin is shown in Figure 21. When plotted on a semi-logarithmic scale, the IC_{50} for the reaction was found to be approximately 7.5 μM mevinolin. Concentrations from 1 μM to 100 μM mevinolin all significantly inhibited the formation of radiolabeled products relative to the control ($p < 0.05$).

E. In Vivo Effects of Mevinolin on HMG CoA Reductase Activity in S. mansoni

That HMG CoA reductase appears to be highly sensitive to mevinolin has been shown. The data thus far have also demonstrated the ability of mevinolin to either inhibit or stimulate schistosome egg production, depending on dose or duration of exposure (Tables 4 and 5). It was, therefore, attempted to correlate

Figure 20. Time-dependence of the HMG CoA reductase-catalyzed reaction in microsomes prepared from S. mansoni. Each bar represents the S.D. of at least 6 determinations.

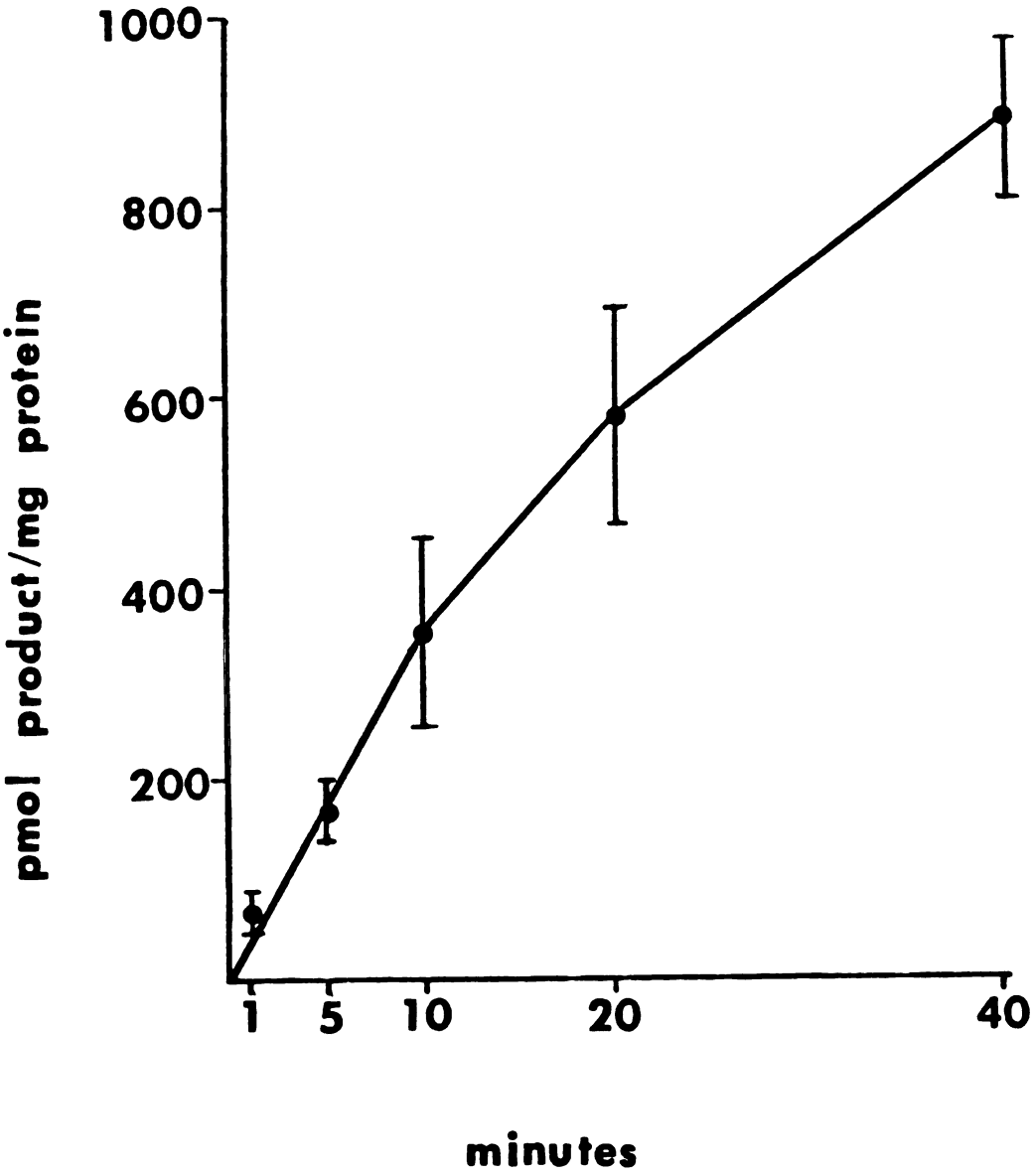

1000
800
600
400
200
pmol product/mg protein
1
5
10
20
40
minutes

Figure 20

Figure 21. Effect of mevinolin on HMG CoA reductase activity. Schistosome microsomes were incubated 30 min in the presence of various concentrations of mevinolin. Bars represent S.D. for at least 6 determinations.

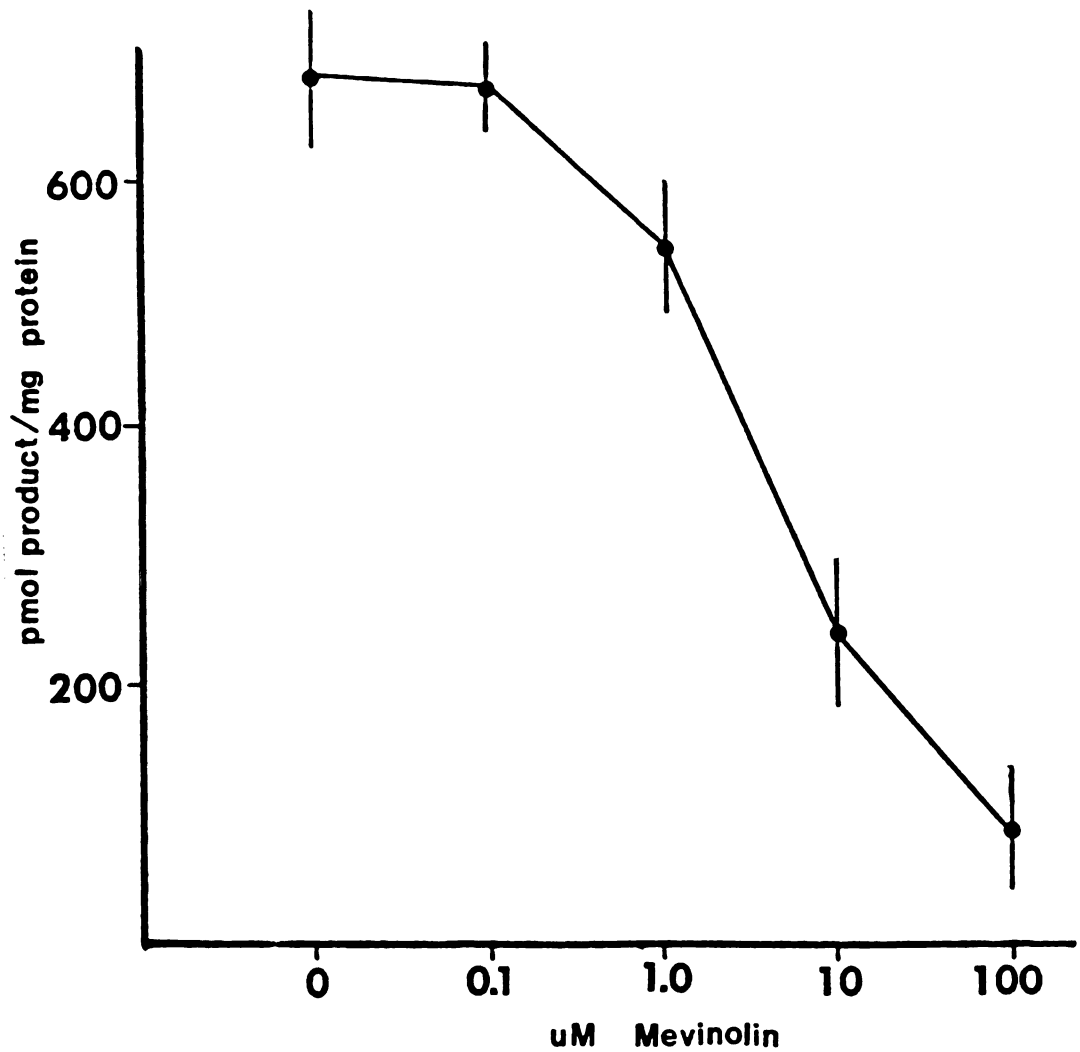

600
400
200
pmol product/mg protein
0
0.1
1.0
10
100
uM Mevinolin

Figure 21

the effects of mevinolin on egg production with effects on schistosome HMG CoA reductase activity.

In these studies, the enzyme assay was run as previously described, utilizing 10 µl (100 µg protein) worm microsome per assay tube during a 30-min incubation time. The parasites used to prepare each microsome were treated differently, however. For one group of parasites, mice were dosed from days 42-45 post-infection with 250 mg/kg mevinolin, after which the worms were removed from the mice, and microsomes prepared. In a second group, mice were dosed as above (250 mg/kg mevinolin for 3 days), but here, the parasites were aseptically removed from the mice and were incubated for 24 h in drug-free culture medium. In a third group, mice were dosed for 3 days with 50 mg/kg mevinolin. Finally, mice dosed with mevinolin's vehicle (25% glycerol-1% Cremophor EL) were considered to harbor control parasites. Following microsome preparation from each group of worms the enzyme assay was run. Enzyme activity was found to be significantly lower than control activity ($p < 0.05$) in microsomes prepared from worms exposed to 250 mg/kg mevinolin in vivo (Figure 22). However, if similarly-treated parasites were incubated in drug-free medium for 24 h, enzyme activity was stimulated above control. Furthermore, schistosomes exposed to 50 mg/kg mevinolin in vivo showed a siginficant ($p < 0.05$) induction of enzyme activity compared to that seen in control parasites. These data suggest that schistosome HMG CoA reductase activity can be induced or inhibited by mevinolin, and these changes in enzyme activity may affect schistosome egg production.

F. The Effects of Mevinolin on Lipids of S. mansoni

1. Normal distribution of ^{14}C-labeled lipids in the parasite

In order to assess what effects, if any, mevinolin was exerting on parasite lipids, the normal pattern of lipid distribution in the schistosome was

Figure 22. Effect of in vivo doses of mevionlin on schistosome HMG CoA reductase. Infected mice were dosed with 250 mg/kg mevinolin, 50 mg/kg mevinolin or its vehicle. Enzyme activity was either measured immediately after the dosing regimen (control, 250 mg/kg, 50 mg/kg), or following a 24 h incubation (250 mg/kg plus incub.). Lines represent S.D. of at least 6 determinations. Asterisk repesents significant difference from control.

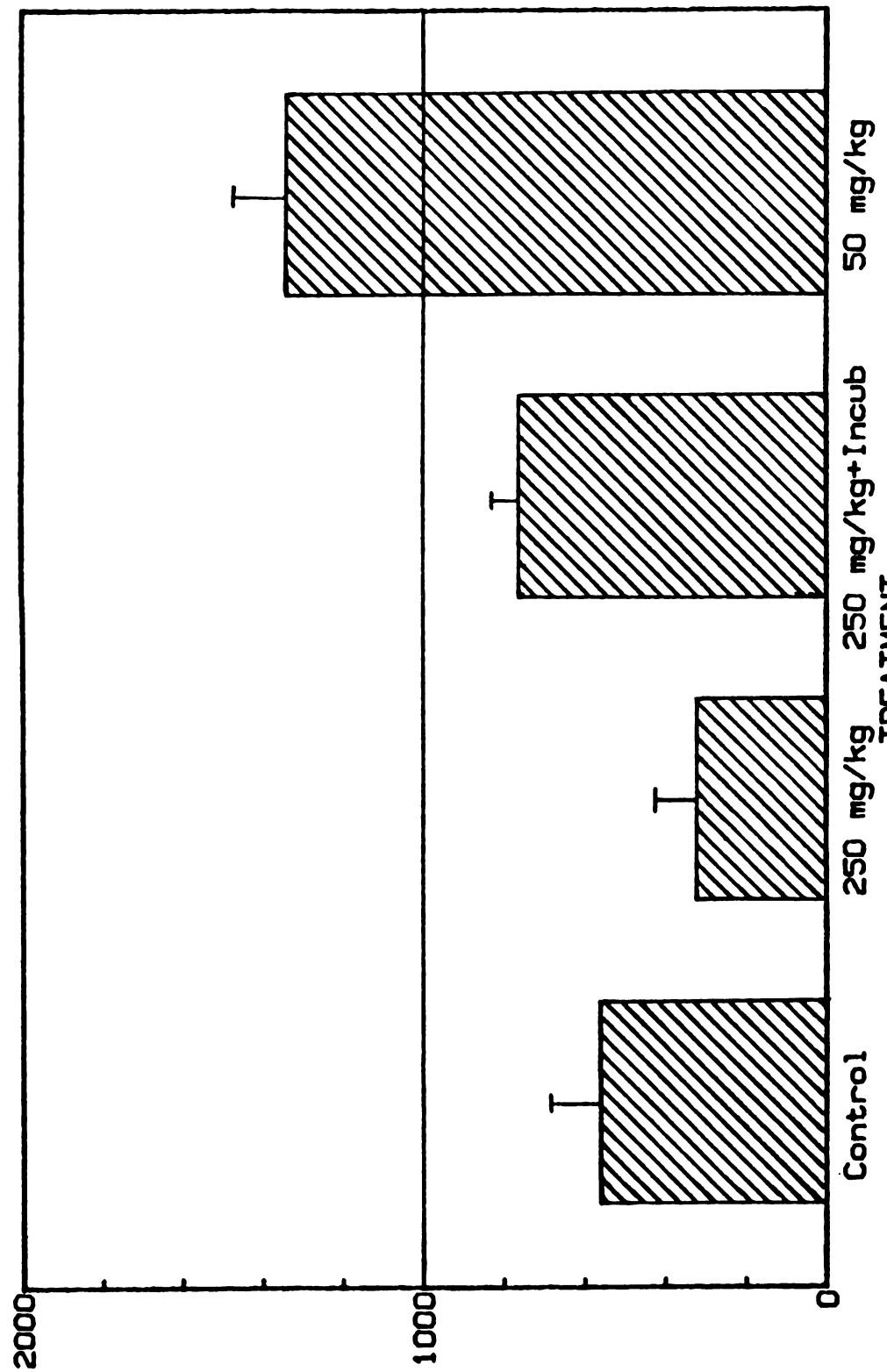

pmol product/mg. protein
2000
1000
0
Control
250 mg/kg
250 mg/kg+Incub
50 mg/kg
TREATMENT

Figure 22

examined. In these studies, parasite lipids were metabolically radiolabeled by incubating worms for at least 24 h in the presence of [U-^{14}C]acetate or [2-^{14}C]mevalonate. Following incubation, the radiolabeled lipids synthesized from these precursors were extracted and isolated. Radiolabeled lipids were purified by column and thin-layer chromatography (TLC) as described in the Materials and Methods. The TLC plates were scanned for radioactivity to determine the location and relative amounts of the radiolabeled lipids. A profile of the distribution of radiolabel into the lipids of S. mansoni is shown in Figure 23. As a control, media with radiolabel but no parasites were assayed to assure that the parasite, and not a contaminant in the culture, was responsible for the labeling pattern (bottom trace of Figure 23). For identification of the schistosome-synthesized labeled lipids, unlabeled authentic lipid standards were co-chromatographed with the parasite lipids; this allowed for tentative identification of some of the lipids synthesized by the parasite.

The lipids of S. mansoni were chromatographically separated into 3 classes: neutral lipids, polyisoprenoids, and polar lipids (Figure 24). Each class was then further purified, so that the individual components of the classes could be separated and identified. The distribution of radiolabel into the neutral lipids is shown in Table 6. Identification of the lipid components was based on co-chromatography with lipid standards. The bulk of the labeled acetate was incorporated into the triglycerides (72.4%), while the labeled mevalonate was incorporated into a sub-class of compounds which behaved chromatographically like hydrocarbon-alcohols. No labeled mevalonate was associated with sterol esters, triglycerides, or mono- and diglycerides.

The distribution of labeled acetate and mevalonate into the polyisoprenoids and the polar lipids was also determined by TLC. While identification of the polyisoprenoid lipids was not complete, these lipids were shown to contain

Figure 23. Radioscans of TLC plates spotted with extracts from ^{14}C-acetate labeled schistosomes, ^{14}C-mevalonate labeled schistosomes, and media containing ^{14}C-acetate but no parasites. Schistosomes or media were extracted with chloroform:methanol (2:1 v/v) and the extract was purified with Sephadex and silicic acid chromatography, then spotted and developed in the TLC system described in the Methods.

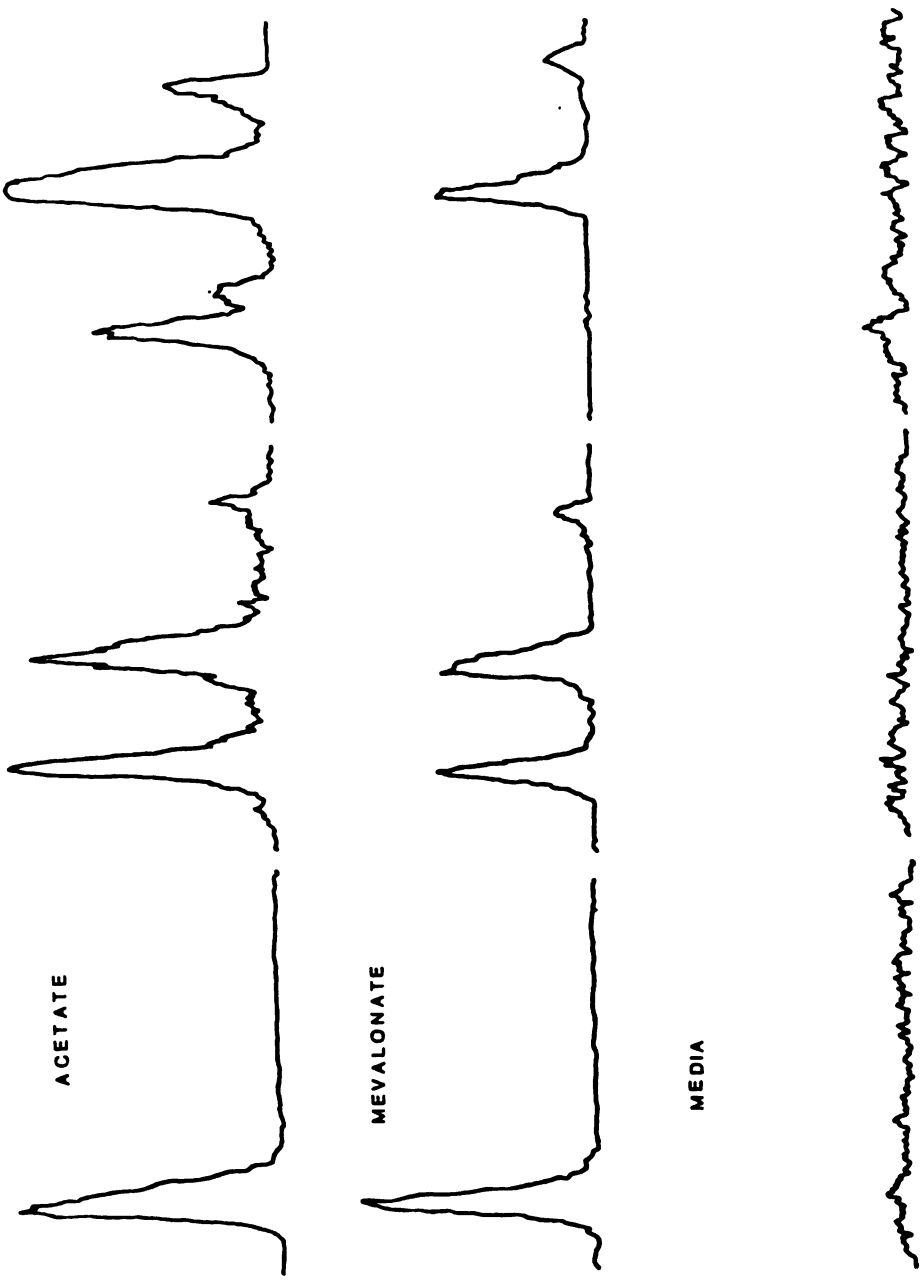


Figure 23

Figure 24. Radioscans of the lipid classes of S. mansoni. Parasites were incubated for 24 h in media containing ^{14}C-acetate. After incubation, lipids were extracted and each lipid class was purified by Sephadex and silicic acid column chromatography, then spotted on individual TLC plates and developed in solvent systems described in the Methods. Plates were then scanned for the presence of radioactive lipids.

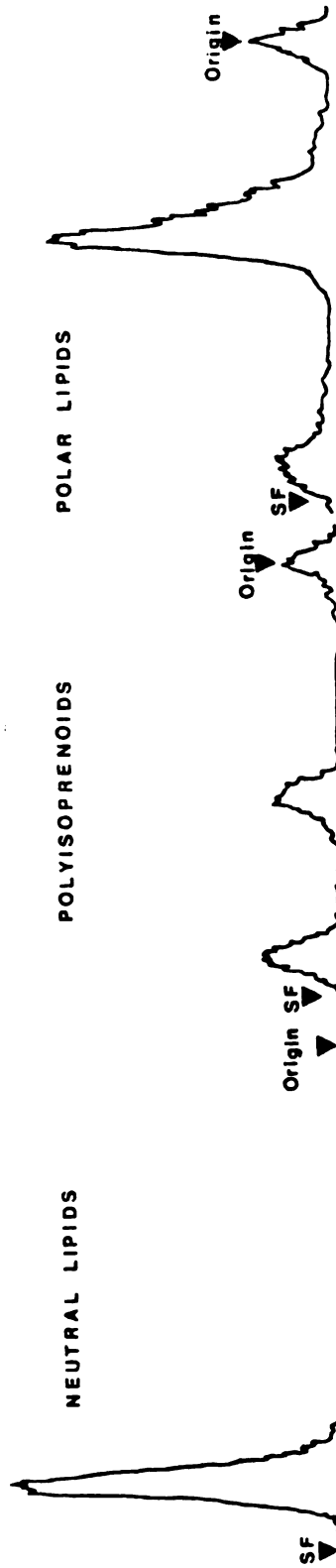

NEUTRAL LIPIDS
POLYISOPRENOIDS
POLAR LIPIDS
SF
Origin
SF
Origin
SF
Origin

Figure 24

Table 6

Percent Distribution of Label into Neutral Lipids of S. mansoni

Lipid	^{14}C-Acetate[a]	^{14}C-Mevalonate[b]
Sterol Esters	21.3	---
Triglycerides	72.4	---
Mono-, Diglycerides	6.3	---
HC-Long chain alcohols	---	100.0

[a]Mean incorporation of label from 8 experiments.

[b]Mean incorporation of label from 6 experiments.

carbohydrate using the method of Dubois et al. (1956). Both acetate and mevalonate were incorporated into the same three polyisoprenoid lipids (Table 7).

At least nine polar lipids were labeled by ^{14}C-acetate; these lipids were all shown to contain phosphorous by the method of Bartlett (1959), and were subsequently identified using lipid standards (Table 8). Radiolabeled mevalonate was incorporated into three polar lipids of S. mansoni. Two of these were present in relatively minor amounts, and did not co-chromatograph with any available standards. The major mevalonate-labeled polar lipid co-chromatographed with phosphatidyl choline, however, choline analysis of this lipid using the method of Wagner et al. (1961) and Beiss (1964), which utilizes the Dragendorff stain (bismuth nitrate and potassium iodide) to stain for choline, was negative for this lipid. Thus, while the mevalonate-labeled lipids were not identificable, the acetate-labeled lipids proved less difficult to identify.

2. The effect of mevinolin on the distribution of labeled lipids in S. mansoni

The incorporation of ^{14}C-acetate into parasite lipids was monitored in the presence of 10 µM mevinolin. In these studies, schistosomes were incubated for 24 h in 50 µCi [U-^{14}C]acetate plus 10 µM mevinolin or its DMSO vehicle. After incubation, the labeled lipids were divided into classes as described earlier. The effect of mevinolin was seen primarily on the polyisoprenoid lipids. Label incorporation into this class of lipids was reduced by approximately 70% in parasites exposed to mevinolin during incubation. The effects of mevinolin on each of the three polyisoprenoids can be ssen in Table 9. The neutral and polar lipid classes were not significantly affected by the drug.

A summary of the distribution of label into each of the lipids of S. mansoni under each of the incubation conditions described is shown in Table 10. While the polyisoprenoid lipids and the mevalonate-labeled polar lipids remain

Table 7

Percent Distribution of Label into Polyisoprenoid Lipids of S. mansoni

Lipid	^{14}C-Acetate[a]	^{14}C-Mevalonate[b]
Polyisoprenoid 1	48.3	23.5
Polyisoprenoid 2	37.7	61.0
Polyisoprenoid 3	14.0	15.5

[a]Mean incorporation of label from 8 experiments.

[b]Mean incorporation of label from 6 experiments.

Table 8

Percent Distribution of Label into Polar Lipids of S. mansoni

Lipid	^{14}C-Acetate[a]	^{14}C-Mevalonate[b]
Phosphatidylcholine	48.8	---
Lysophosphatidyl-choline	1.0	---
Sphingolipids	1.1	---
Phosphatidylethanol-amine	18.3	---
Lysophosphatidyl-ethanolamine	7.2	---
Phosphatidylserine	10.5	---
Phosphatidic Acid	2.7	---
Phosphatidylinositol	3.2	---
Diphosphatidyl-glycerol	7.2	---
Unidentified Polar Lipid 1	---	81.2
Unidentified Polar Lipid 2	---	2.5
Unidentified Polar Lipid 3	---	16.3

[a]Mean incorporation of label from 8 experiments.

[b]Mean incorporation of label from 6 experiments.

Table 9

Percent Distribution of Label into Lipids
of S. mansoni Exposed to Mevinolin

Lipid	A	B
Neutral Lipids	37.7±5.4	36.0±3.7
Polyisoprenoid 1	5.5±4.5	0.9±0.6*
Polyisoprenoid 2	4.3±3.3	1.6±0.8*
Polyisoprenoid 3	1.6±1.3	0.4±0.2*
Polar Lipids	50.9±7.5	61.1±3.9

A. Parasites were incubated in [U-^{14}C]acetate.

B. Parasites were incubated in [U-^{14}C]acetate plus 10^{-5}M mevinolin.

Asterisk indicates significance from control.

Table 10

Percent Distribution of ^{14}C-Label into Total Lipids of *S. mansoni* Under Various Incubation Conditions

Lipid*	^{14}C-Acetate[a]	^{14}C-Acetate + Mevinolin (10 μM)[b]	^{14}C-Mevalonate[c]
SE	7.2+1.0	3.1+0.2	---
TG	27.6+4.0	30.1+2.4	---
MG-DG	2.9+0.4	1.5+0.1	---
HC-Long chain alcohols	---	---	48.5+5.6
PI-1	5.5+4.5	0.8+0.4*	3.8+1.0
PI-2	4.3+3.3	1.8+0.6*	11.5+2.9
PI-3	1.6+1.3	0.5+0.2*	3.3+2.6
PC	23.4+5.5	25.1+1.5	---
LPC	0.5+0.2	0.5+0.2	---
Sph	0.6+0.1	0.1+0.0	---
PE	10.4+4.4	15.4+4.2	---
LPE	4.1+1.7	5.2+1.5	---
PS	5.5+2.2	4.9+1.8	---
PA	1.4+0.5	5.4+2.0	—
PI	1.6+0.4	0.3+0.1	---
DPG	3.5+0.8	5.3+0.3	---
PL-1	---	---	25.9+3.1
PL-2	---	---	0.8+0.7
PL-3	---	---	5.2+1.6

[a]Mean ± S.D. for 8 experiments.

[b]Mean ± S.D. for 5 experiments.

[c]Mean ± S.D. for 6 experiments.

* Key to Abbreviations: SE = Sterol esters; TG = Triglycerdies; MG-DG = Mono-and Diglycerides; HC = hydrocarbons; PI 1,2,3 = Polyisoprenoid 1, 2, 3; PC = Phosphatidylcholine; LPC = Lysophosphatidylcholine; Sph = Sphingolipids; PE = Phosphtaidylethanolamine; LPE = Lysophosphatidylethanolamine; PS = Phosphatidylserine; PA = Phosphatidic acid; PI = Phosphatidylinositol; DPG = Diphosphatidylglycerol; PL-1,2,3 = Polar lipid 1, 2, 3. Asterisk = significantly different from control.

unidentified, lipids which co-chromatographed with known standards and were identifiable by their chemical characteristics were tentatively named.

Finally, it was shown earlier that HMG CoA reductase activity in the schistosome is inducible (see Figure 22), and the induction of activity after in vitro exposure to mevinolin was correlated with increased egg production (Table 4). To measure if in vitro induction of the enzyme caused increased label incorporation into total lipids of the schistosome, parasites were exposed to 10 μM mevinolin for 24 h after which they were removed from the drug, and were placed in culture with [U-^{14}C]acetate for up to 72 h. The results of this experiment showed that by 24 h, the amount of label incorporated into total lipid was significantly lower ($p < 0.05$) in mevinolin-exposed worms as compared to control parasites (Figure 25). However, by 48 h, the drug-treated worms had recovered, and had begun to incorporate a significantly higher amount of labeled acetate into the total lipid. By 72 h, the time point at which egg production is usually measured, the amount of radioactive acetate incorporated into schistosome lipids remained significantly higher than in the control worms. Egg production at this time point was double in the mevinolin-treated worms as compared to control parasites (data not shown).

3. The effect of mevinolin on lipid intermediates (polyisoprenoid lipids)

The effect of mevinolin on lipid intermediates (i.e., the polyisoprenoid lipids) in S. mansoni was assessed by measuring the ability of these lipids to serve as carriers for sugars in the synthesis of glycoproteins before and after exposure to mevinolin. In order to do this, it had to be established that the schistosome contained lipids capable of carrying sugars. For these studies, homogenates were prepared from paired parasites, and GDP-[U-^{14}C]-mannose was incubated with the homogenate. After a 30-min incubation, the incubation mixture was extracted with chloroform:methanol (2:1 v/v) to remove the lipid intermediate

Figure 25. The incorporation of ^{14}C-acetate into total lipids of S. mansoni following 24 h exposure to 1 μM mevinolin (dashed line) or its vehicle (solid line). Fifteen paired parasites (n=6) were incubated in the presence of mevinolin (1 μM) or DMSO for 24 h, after which they were removed from the drug and placed in media containing 5 μCi ^{14}C-acetate. At various time points, the parasites were extracted with chloroform:methanol (2:1 v/v), and the extract was counted by liquid scintillation spectrometry. Bars represent S.D. of six determinations. Asterisk represents significant difference from control.

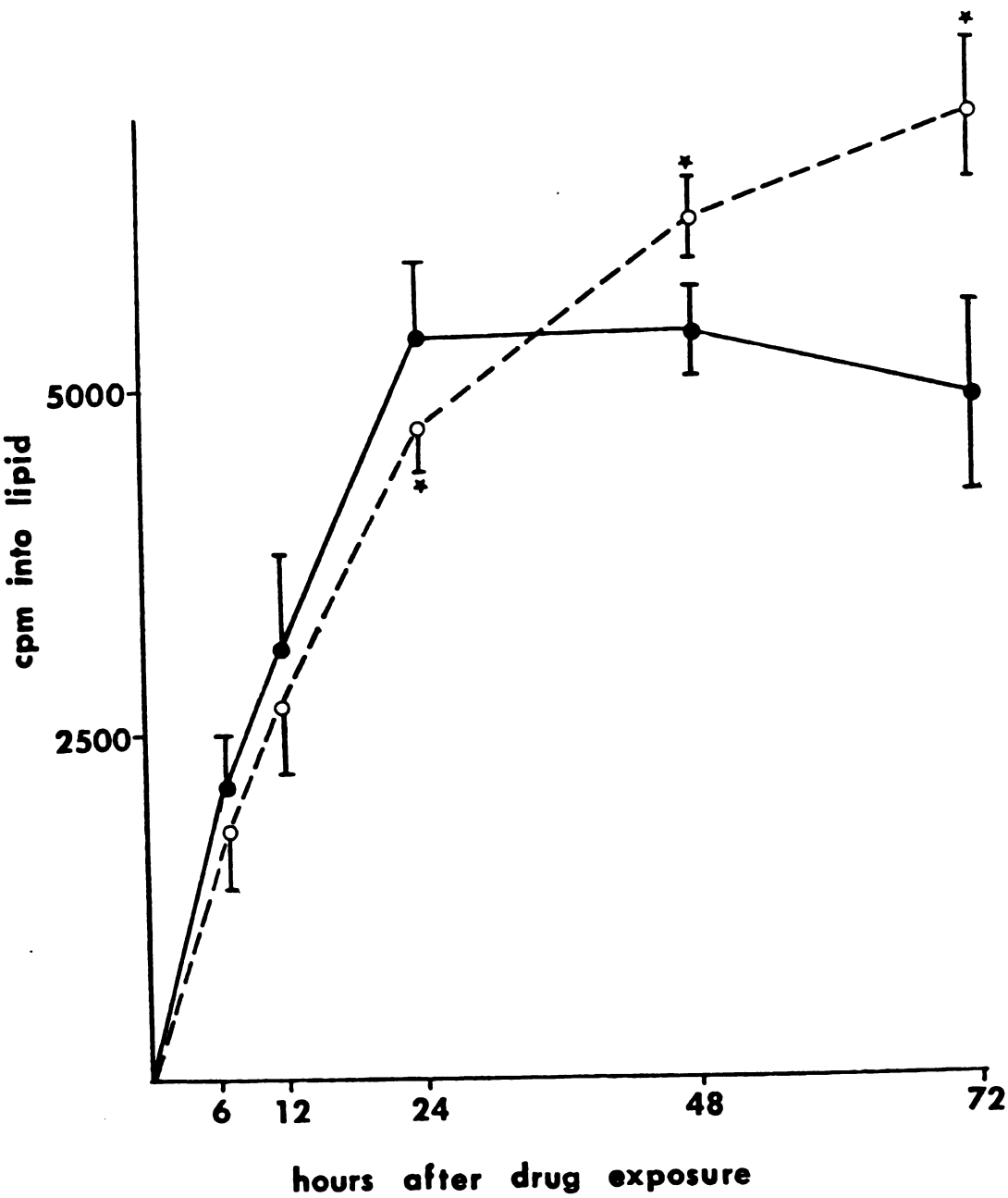

cpm into lipid
5000
2500
6
12
24
48
72
hours after drug exposure

Figure 25

(lipids carrying 1 mannose residue), then the mixture was re-extracted with chloroform:methanol:water (1:1:0.3 v/v/v) to remove lipid oligosaccharides (lipids carrying 2 or more mannose residues). The extracts were spotted onto TLC plates which were developed as described in the Materials and Methods. Upon scanning the plate for lipids carrying radioactive mannose residues, a single lipid intermediate (R_f 0.73) was found (Figure 26, upper trace). Similarly, a single lipid-oligosaccharide was detected when the 1:1:0.3 extract was scanned for radioactivity (R_f 0.41, Figure 26, lower trace).

Since these sugar-carrying lipids were detected in the schistosome, the sensitivity of these lipids to mevinolin was next determined. Here, homogenates were prepared from parasites incubated for 24 h in vitro in the presence of 10 μM mevinolin or its vehicle. The homogenates of these parasites were then incubated in the presence of GDP-[U-^{14}C]-mannose, and were extracted and spotted as above. Scanning of the TLC plates in this case revealed a marked reduction in the amount of labeled mannose incorporated into the lipids of the mevinolin-treated worms (Figure 27). Specifically, the mevinolin-treated parasites incorporated 66% less mannose into the 2:1-extractable lipid, and 76% less mannose into the 1:1:0.3-extractable lipid. These results indicate a greatly reduced amount of these lipids in the drug-treated schistosomes.

The rate of transfer of the mannose residues from the lipids to the forming protein in control and mevinolin-treated worms was next examined. Here, aliquots of the incubation mixtures for each group of parasites were removed and analyzed for radioactivity in each of the lipid fractions and in the remaining protein residue, which was solubilized and counted by liquid scintillation spectrometry. As the reaction progressed, the incorporation of ^{14}C-mannose was virtually abolished in the lipid-oligosaccharide fraction of the mevinolin-treated worms (Table 11). While label incorporation was also lower into the lipid

Figure 26. Radioscans of lipid intermediates of S. mansoni. The lipid intermediate (2:1) and the lipid oligosaccharide (1:1:0.3) were labeled with GDP-[U-^{14}C]-mannose. SF = solvent front.

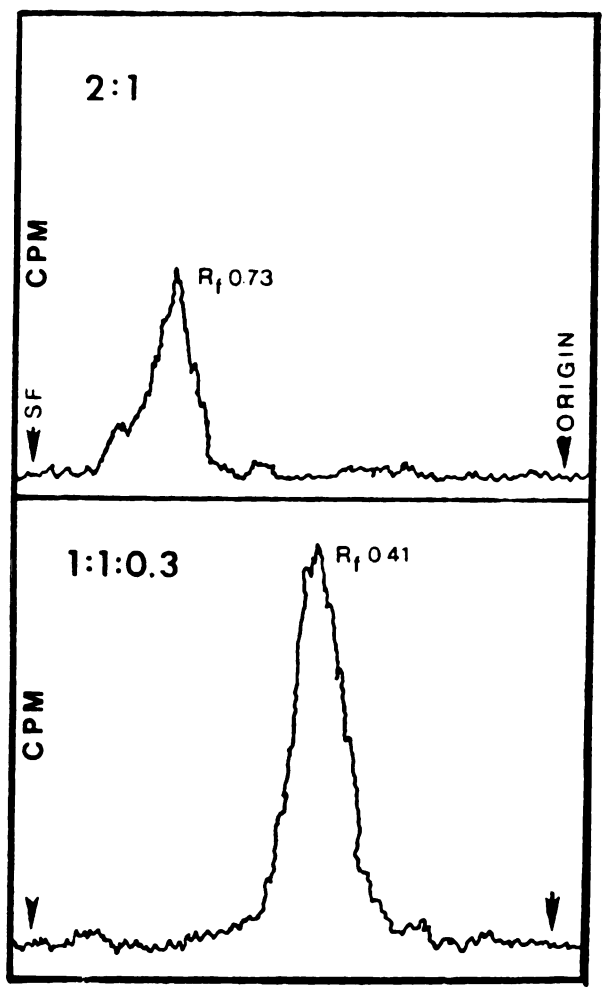


Figure 26

Figure 27. Radioscans of lipid intermediates of S. mansoni exposed to mevinolin versus control. TLC plates were scanned for lipids carrying ^{14}C-mannose residues in control (left upper and lower panels) and mevinolin-treated worms (right upper and lower panels).

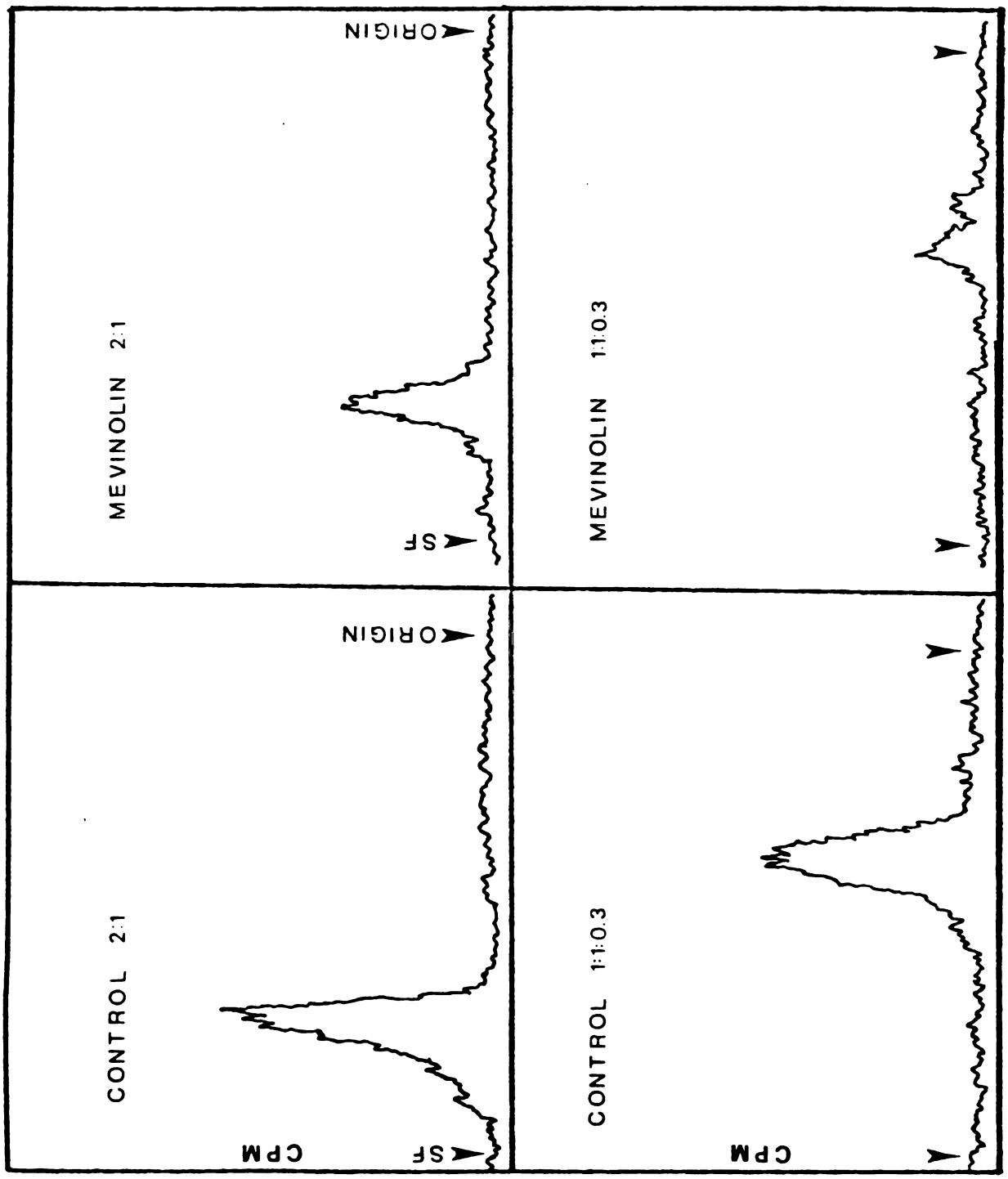

CONTROL 2:1
MEVINOLIN 2:1
CONTROL 1:1:0.3
MEVINOLIN 1:1:0.3
ORIGIN
SF
CPM

Figure 27

Table 11

Effect of Mevinolin on the Formation of Lipid Intermediates and Glycoproteins in S. mansoni Homogenates

Incubation time (min):	2.5	5.0	10.0	20.0
Sample	cpm/mg protein homogenate[a]			
2:1 Lipid Intermediate				
Control	178	978	990	1,024
Mevinolin	64	76	608	644
1:1:0.3 Lipid Oligosaccharide				
Control	3,956	5,034	13,784	24,732
Mevinolin	480	496	712	1,184
Protein				
Control	49,978	134,644	224,482	334,754
Mevinolin	37,554	80,322	174,436	202,512

[a]Data are expressed as mean cpm incorporated per mg protein homogenate for at least 6 determinations.

intermediate and protein residue of the drug-treated worms, the lipid-oligosaccharide seemed particularly sensitive to the effects of mevinolin.

These results again indicate that mevinolin is acting relatively specifically to deplete the schistosome of polyisoprenoid lipids. These lipids may be necessary for glycoprotein synthesis in the parasite and glycoproteins, in turn, are necessary for the synthesis of eggs. Therefore, mevinolin, by inhibiting schistosome HMG CoA reductase, effectively disrupts egg production in Schistosoma mansoni.

DISCUSSION

Knowledge of the complex biochemical processes underlying egg production by Schistosoma mansoni is highly relevant to any efforts toward the amelioration of pathology associated with schistosomiasis. Unfortunately, the understanding of the process of parasite egg production is quite limited. It is the purpose of this dissertation to further elucidate the events involved in schistosome egg production. For this purpose, mevinolin, a sterol synthesis inhibitor, has been used as a tool with which the biochemistry involved in parasite fecundity could be studied. Using this agent, aspects of the regulation of schistosome egg production have been uncovered which may aid in the development of chemotherapeutic drugs for schistosomiasis.

A. The In Vitro Effects of Mevinolin on S. mansoni

1. Effect of mevinolin on in vitro egg production by S. mansoni

The finding that mevinolin, an inhibitor of sterol biosynthesis, decreased egg production in S. mansoni in vitro (Table 1) was surprising in light of studies where steroids and other steroid inhibitors were shown to have no effect on the parasite (Morrison et al., 1986). From these studies and those in which it was shown that the schistosome is incapable of de novo sterol synthesis (Meyer et al., 1970), it was concluded that steroid hormones were probably not involved in parasite fecundity. However, the in vitro effect of mevinolin, which inhibits HMG CoA reductase, on the process of egg production indicates that some product in the metabolic pathway in which this enzyme is involved, plays a role in oviposition

in S. mansoni. Since the sterol synthetic pathway does not exist in this parasite, the metabolite of HMG CoA being affected by mevinolin is most likely to be a product between mevalonate and farnesyl pyrophosphate, or a polyprenol, such as ubiquinone or the dolichols, since the synthesis of all of these compounds is dependent on HMG CoA reductase (see Figure 8). This lipid seems to exert a significant influence on schistosome egg production since the effect of mevinolin was highly specific for this process, affecting no other parameters of parasite physiology during in vitro incubations. Thus, a mevinolin-sensitive metabolite of HMG CoA appears to play a prominent role in schistosome egg production.

2. Effects of metabolites of HMG CoA on egg production by S. mansoni in vitro

Mevinolin, by competitive inhibition of HMG CoA reductase, starves cells for the metabolic products of HMG CoA. Since mevinolin was able to inhibit egg production by the schistosome, some of the metabolic products of HMG CoA were studied for their ability to influence parasite oviposition. The first of these lipids tested was mevalonate, the immediate product of the HMG CoA reductase-catalyzed reaction. Mevalonate was added to schistosome cultures in concentrations ranging from 0 to 200 μM (Table 2) and an interesting effect of this metabolite on schistosome fecundity was noted. At lower concentrations (10 μM or 25 μM) mevalonate exerted a slight to a significant effect on egg production, increasing egg number over two-fold at 25 μM. However, at a higher concentration (200 μM), mevalonate significantly suppressed egg production in the parasite, reducing it by 75% from the control value. At this concentration of mevalonate, worm pairing and adherence to the glass substrate (the incubation flask) was no different than that of the control parasites. Thus, it appears that at high concentrations, mevalonate or a schistosome-derived metabolite of mevalonate, is able to exert a negative effect on parasite egg production, analogous to the effect seen with 10 μM mevinolin.

To assess whether metabolites of mevalonate could influence schistosome fecundity, farnesol and dolichols were added to the culture system. When farnesol was added to the schistosomes in culture, an effect similar to that seen with mevalonate was observed (Table 2). Specifically, lower concentrations of this lipid (20 μM or 40 μM) caused a two-fold stimulation in egg production. However, 80 μM or 200 μM farnesol significantly reduced the number of eggs produced during the 72 h incubation period. Here, the reduction in egg number was approximately 75% at either concentration of farnesol. Again, this reduction in egg production is comparable to that seen in parasites incubated in the presence of 10 μM mevinolin.

The effect of dolichols on S. mansoni egg production (Table 3) was measured using two concentrations of these lipids (10 μg/ml and 20 μg/ml) proven effective in other systems (Carson and Lennarz, 1979). In these studies on the schistosome, however, dolichols at either concentration were ineffective at stimulating schistosome egg production. The lack of an effect observed with the dolichols could be attributable to several factors. First of all, it could be that the schistosome is unable to use these lipids or cannot take them up in culture. Radiolabeled dolichols were unavailable, and therefore uptake of these lipids by the parasite was not monitored. Secondly, dolichols are very hydrophobic, while the culture medium for the parasites is aqueous. The addition of hydrophobic dolichols to this aqueous environment lessens the chance that these lipids would be in a form which the parsites could utilize. Thus, the lack of effect of the dolichols on schistosome egg production is not unexpected.

In summary, low concentrations of either mevalonate or farnesol were able to stimulate egg production in the schistosome. It is interesting that higher concentrations of either of these lipids reduced the number of eggs produced, but not in a graded, dose-dependent manner (i.e., 80 μM farnesol had the same effect

as 200 μM farnesol). This seems to suggest that a critical concentration of these lipids or their metabolites is necessary to stimulate and maintain egg production in the schistosome. In the case of both of these lipids, that concentration is approximately 25 μM. As stated earlier, higher concentrations of these lipids have an effect similar to 10 μM mevinolin in the schistosome. While mevinolin is a potent inhibitor of HMG CoA reductase (Alberts et al., 1980), mevalonate and its metabolites are also known to exert negative feedback control on HMG CoA reductase both in vivo (Arebalo et al., 1980; Beg and Brewer, 1981) and in vitro (Gibson et al., 1982; Parker et al., 1983). This may also be occurring in the schistosome, where lower concentrations of the products of the HMG CoA reductase-catalyzed reaction may be optimal for the production of eggs by the parasite. High concentrations of these lipids may, however, be inhibitory to egg production due to negative feedback regulation of these lipids on HMG CoA reductase. This study on schistosome egg production is therefore unique, in that it relates the regulation of this enzyme by nonsterol lipids in the parasite to a physiological process, the production of eggs by the schistosome.

3. Reversal of mevinolin's effect on schistosome egg production

Since mevalonate and farnesol were effective in stimulating egg production in S. mansoni, their ability to "rescue" the parasite from the effect of mevinolin was examined. When parasites were co-cultured with both mevalonate and 10 μM mevinolin, 25 μM mevalonate appeared to be the optimum concentration at which mevinolin's effect could be reversed (Figure 14). Here, egg production was restored to the control level, which was significantly higher than that seen in parasites incubated in 10 μM mevinolin alone. This same experiment was repeated in the presence of farnesol and dolichols. Farnesol at 80 μM was able to reverse mevinolin's effect, restoring egg number to control values (Figure 15). Dolichols were unable to cause a significant reversal of the effect of 10 μM

mevinolin on the parasite (Table 3). Considering the inability of these lipids to stimulate egg production, their lack of effect in the presence of mevinolin is not surprising.

The ability of metabolites of HMG CoA to reverse mevionlin's effect has been intensively studied using cells in culture. Here, growth inhibition of Swiss 3T3 cells (Habenicht et al., 1980) and cultured mouse spleen lymphocytes (Perkins et al., 1982) by mevinolin or compactin was shown to be reversible by mevalonate. However, the ability to reverse mevinolin's effect in a whole organism, such as the schistosome, allows one to relate the reversal of the drug's effect to a physiological process. For in the schistosome, the effect of mevalonate or farnesol on parasites incubated in the presence of mevinolin, involves restoration of the process of egg production. Therefore, the metabolites of HMG CoA may not only play a significant role in the regulation of HMG CoA reductase in the schistosome, but they may also be necessary for the production of eggs by this parasite.

4. Stimulation of egg production by S. mansoni following in vitro or in vivo exposure to mevinolin

HMG CoA reductase is known to be a highly inducible enzyme, stimulated after exposure to low doses of mevinolin or compactin (Brown et al., 1978; Skalnik et al., 1985). The effect of mevinolin on oviposition in S. mansoni, if mediated through HMG CoA reductase, should also prove to be inducible to an extent. To test this possibility, both in vitro and in vivo exposure of the parasite to mevinolin was carried out, followed by incubation in the typical egg-laying system so that egg production could be monitored. The results of in vitro exposure to mevinolin (Table 4) indicated that a slight induction of HMG CoA reductase activity concomitant with an increase in egg production may occur after in vitro exposure to 10 μM mevinolin. Here, parasites exposed to the drug for 24 h produced twice as many eggs as controls, whereas parasites maintained in

mevinolin for the entire incubation period showed markedly depressed egg production. This finding is suggestive of a dual effect of the drug namely, an induction of HMG CoA reductase after acute exposure to mevinolin, but an inhibition of the enzyme (even when induced) as long as the drug is present.

The induction effect of mevinolin on egg production was even more dramatic in parasites exposed to the drug in vivo (Table 5). Here, parasites exposed to 50 mg/kg mevinolin in vivo, when placed in culture, produced five times as many eggs as controls. Again, the presence of mevinolin (10 μM) in the culture was able to suppress this effect. Although induction of HMG CoA reductase activity has been found in several types of cells exposed to mevinolin or compactin, the studies with mevinolin on schistosome egg production are the first to show an effect on the enzyme with a subsequent physiological response in an intact organism. Thus, not only is mevinolin exerting an effect on schistosome egg production that can be described as inhibitory in vitro, but it also appears that this agent can induce egg production in the parasite under the appropriate conditions. The differences in the extent of the induction of this process following in vitro versus in vivo exposure to mevinolin is striking. This difference in the stimulation of egg production (a 2-fold increase in worms exposed to mevinolin in vitro versus a 5-fold increase in worms exposed to the drug in vivo) can best be explained by differences in the amounts of drug which the parasite encounters in each situation, and the duration of this exposure. In the in vivo situation, the parasite probably experiences a lower dose of the drug for a shorter period of time -- a situation which may be more conducive to induction of the enzyme than 24 h exposure to 10 μM mevinolin. Furthermore, the parasites exposed to mevinolin in vitro were maintained in culture for an additional 24 h, a condition more stressful to the parasite than maintenance within the host.

However, either set of experimental conditions resulted in a significant increase in egg production by the schistosome following exposure to mevinolin.

B. The In Vivo Effects of Mevinolin on S. mansoni Egg Production

Recognizing the in vitro effect of mevinolin on schistosome egg production, the in vivo effect of this drug was examined in mice infected with these parasites. The schistosome-infected mouse lends itself well to the study of the effects of mevinolin on the parasite, since the mouse is refractory to the cholesterol-lowering effects of the drug (Alberts et al., 1980). Thus, the metabolic or dietary status of the mouse host is not a consideration in examining the effects of mevinolin on the parasite in treated mice.

A significant effect on schistosome-induced pathology in the mouse was not seen until a daily dose of 250 mg/kg mevinolin was used. Since the LD_{50} of this drug in mice is greater than 1000 mg/kg (Endo, 1979), no toxic or lethal responses were seen in any of the animals during treatment with mevinolin. At a dose of 250 mg/kg however, schistosome-associated pathology appeared to be greatly altered in mevinolin-treated animals. Pathology was assessed in two ways, the first of these being the observation of gross liver pathology in drug-treated and control animals, with subsequent retrieval of eggs from these livers. In these studies, gross liver pathology was markedly reduced in the mevinolin-treated infected mice when compared to controls (Figure 16). This result was related to the isolation of 45% fewer eggs from livers of the drug-treated mice. While liver size and egg burden were significantly reduced in treated mice, a clearer picture of the effect of mevinolin on the liver pathology was seen in the second assessment of pathology, the examination of thin sections of liver tissue from both groups of animals.

When thin sections were made of livers from mevinolin- and vehicle-treated mice, there was a readily apparent difference between the two groups (Figures 17 and 18). Specifically, in the mevinolin-treated mice, the granuloma volume was obviously smaller than in livers from the control animals. This is most probably due to two factors. First of all, the granulomas in the drug-treated livers almost invariably contained only one egg, while the granulomas in the control livers involved at least 2-3 eggs per area of granulomatous infiltration. Generally speaking, the number of eggs deposited in a given area of the liver is directly related to the granuloma volume, thus the decrease in granulomatous tissue in the mevinolin-treated mice is probably partly due to the deposition of fewer eggs by the parasite in these animals. Secondly, it was found that the eggs in the livers of mevinolin-treated mice contained 50% fewer mature miracidia than did the eggs in livers of control animals. Since it is known that the miracidia secrete antigens which instigate granuloma formation (Hang et al., 1974), eggs containing immature miracidia would be less likely to cause a vigorous immune response. Therefore, mevinolin seemed to affect two aspects of egg production in vivo, both reducing egg number and affecting the production of the eggs which were formed, such that development of the miracidium within the egg was altered or slowed. It is interesting to note that although mevinolin disrupted schistosome egg production, parasite number or maturity was not significantly different in vehicle-versus drug-treated mice. This again suggests that the effect of mevinolin, even at high concentrations in vivo, is rather specific for schistosome fecundity. Furthermore, the ability of mevinolin to reduce schistosome-associated pathology in vivo by affecting egg production, again suggests that HMG CoA reductase is an important enzyme in the formation of eggs by this parasite.

C. Isolation of Schistosome HMG CoA Reductase

The effect of mevinolin on the schistosome both *in vitro* and *in vivo* is, as discussed above, quite specific for parasite egg production. Specifically, mevinolin did not cause a general depression of all parasite functions, rather, it seemed that the drug was targeted to a single biological process. Since mevinolin is known to inhibit HMG CoA reductase, and since it also inhibits schistosome egg production, an attempt was made to find this enzyme in the parasite in order to determine its characteristics and its role in schistosome egg production.

Mammalian HMG CoA reductase is a microsomally-bound enzyme (Rodwell *et al.*, 1973), thought to be associated with the membranes of the rough endoplasmic reticulum (ER) (Guder *et al.*, 1968; Shapiro and Rodwell, 1971), although the enzyme has also been isolated from smooth ER membranes (Goldfarb, 1972). Thus, in the search for this enzyme in the schistosome, microsomal fractions of parasites were prepared. The process of solubilization of the enzyme from the microsomal fractions has been an area of intense research, attempting to optimize enzyme activity from the microsomal pellet. While the microsomally-bound enzyme is highly labile to cold temperatures (Kleinsek and Porter, 1979), a modification of the freeze-thaw technique devised by Heller and Gould (1973) has been found to be an effective means of solubilizing the enzyme. Thus, schistosome microsomes were frozen overnight, then thawed at room temperature prior to use. Because the enzyme could be solubilized by this relatively mild treatment, it has been referred to as a peripheral ER protein. However, other studies using Triton X-100 to solubilize microsomes showed a 70% solubilization of membrane proteins while less than 5% of the HMG CoA reductase was solubilized (Ness *et al.*, 1981). Thus, the enzyme may have some properties normally associated with integral proteins of the ER. The purification of HMG CoA reductase from microsomes has been complicated by this seeming dual

nature of the enzyme, as well as by the lability of this enzyme to temperature, and by inactivating enzymes (particularly HMG CoA lyase) released during the homogenization of tissue. However, purification of the enzyme has been accomplished from rat liver (Ness et al., 1979; Rogers et al., 1980; Gil et al., 1981), and specific acivity of the pure enzyme was reported to be between 10,000 and 20,000 nmol mevalonate synthesized per minute per mg protein. Specific activity of HMG CoA reductase is determined using an enzyme assay (Alberts et al., 1980) which is based on the generation of radiolabeled mevalonate from ^{14}C-HMG CoA substrate by microsomal fractions containing the enzyme. While the recovery of pure enzyme from rat liver resulted in HMG CoA reductase with very high specific activity, the isolation procedures for the enzyme involved several extensive purification steps and large amounts of starting material. In the studies with the schistosome, where material was limited, only a crude purification of the enzyme was accomplished using the freeze-thaw technique to solubilize the enzyme as described above.

Like the mammalian enzyme, schistosome HMG CoA reductase activity was localized to the microsome. In the first study of this enzyme in the parasite, various amounts of the microsomal preparation were assayed for enzyme activity (Figure 19). Ten microliters of worm microsome (equal to approximately 100 µg protein) was used as a standard amount of enzyme in all subsequent assays when it was found that 10 µl microsome generated enough radiolabeled product to be quantified. Thus, in order to conserve parasite material, radiolabeled substrate, and to quantify the assay, 10 µl of parasite microsome were routinely used.

The time-dependence of the HMG CoA reductase-catalyzed reaction in the schistosome was next determined (Figure 20). In these studies, the activity of the enzyme in parasite microsomes was linear for 10 minutes, a finding in accordance with assays using rat liver, where the reaction was found to be linear for at least

5-10 minutes (Kleinsek et al., 1977; Alberts et al., 1980). Thus, excepting the purity of the enzyme preparation from the schistosome, which undoubtedly contributed to its lower specific activity in these preparations, schistosome HMG CoA reductase behaved like the mammalian enzyme in the localization of the enzyme and its rate of catalysis.

D. Inhibition of Schistosome HMG CoA Reductase by Mevinolin in the Enzyme Assay System

The presence of HMG CoA reductase activity in the schistosome, and its similarlity to the mammalian enzyme in some respects raised the question of whether the schistosome enzyme, like the mammalian enzyme, was inhibitable by mevinolin in the assay system. To measure the effect of mevinolin on the enzyme activity, the same concentrations of mevinolin was those utilized in the in vitro egg-laying studies (0.1 μM to 100 μM mevinolin) were used in the enzyme assay. It is interesting to note that 10 μM mevinolin, which caused a 70.8% decrease in schistosome egg production (Table 1), was able to inhibit HMG CoA reductase activity by 71.5% in the enzyme assay (Figure 21). The relationship between the mevinolin-induced effect on egg production at 10 μM and the inhibition of HMG CoA reductase activity by this concentration of the drug in the enzyme assay is excellent, even though 10 μM mevinolin is a high concentration of the drug, pharmacologically. However, although mevinolin has been shown to be a potent inhibitor of sterol biosynthesis, the HMG CoA reductase-catalyzed synthesis of other lipdis (such as dolichols) is more resistant to the drug. For instance, mevinolin or compactin was able to inhibit the conversion of ^{14}C-acetate into sterolds by 50% at concentrations of 1 nM in rat hepatocytes (Alberts et al., 1980), while the incorporation of ^{3}H-mannose into dolichol-linked oligosaccharides was inhibited by 50% only in the presence of 5 μM of these drugs, a concentration where sterol synthesis from ^{14}C-acetate was almost completely blocked (Filipovic

and Menzel, 1981). Since the schistosome cannot synthesize sterols, its HMG CoA reductase may be more resistant to mevinolin, thus explaining why higher concentrations of the drug were needed to exert an effect on schistosome enzyme activity in vitro. In this respect again, schistosome HMG CoA reductase may be similar to the mammalian enzyme.

E. In Vivo Effects of Mevinolin on Schistosome HMG CoA Reductase

A final study of schistosome HMG CoA reductase activity involved the determination of whether the parasite enzyme, like enzyme purified from other systems, was inducible by mevinolin. For the purpose of these studies, parasites were exposed to mevinolin in vivo, as this seemed to be an effective means of inhibiting egg production at high doses (Figure 15) while inducing egg production at low doses (Table 5). The goal of these experiments was to correlate mevinolin's effects on egg production with HMG CoA reductase in parasite microsomes.

When schistosomes were exposed to 250 mg/kg mevinolin in vivo for 3 days, their HMG CoA reductase activity was markedly suppressed (Figure 21). However, when parasites treated the same way in vivo were incubated for 24 h in drug-free media prior to the preparation of the microsomes, enzyme activity as measured in the enzyme assay was restored to above control levels. This result was indicative of an ability of the enzyme to rebound quickly following drug exposure, suggesting perhaps that the enzyme is inducible in the schistosome. The ability of mevinolin to induce parasite HMG CoA reductase was confirmed by using 50 mg/kg mevinolin in infected mice for 3 days. Here, parasites exposed to the drug showed a doubling of enzyme activity compared to that in controls (Figure 21). Although this level of induction is not as great as that seen in cultured cells exposed to compactin or mevinolin, where induction of activity ranged from 3.5- to 15-fold (Brown et al., 1978), the induction of this enzyme in

the schistosome, and the correlation between this induction and a physiological response, egg production, is unique. Specifically, induction of HMG CoA reductase activity in this parasite seems to be related direclty to an induction of egg production by the worm.

In conclusion, HMG CoA reductase activity in S. mansoni appears to have characteristics similar to those of enzyme preparations from diverse mammalian systems. These characteristics include similar rates of catalysis, subcellular location, sensitivity to the inhibitory effects of mevinolin, as well as the ability to be induced by this drug. An additional feature of this enzyme in the schistosome is the ability to ascribe to it not only a metabolic role, but also a physiological function in the parasite. Since the production of eggs by the worm closely parallels the activity of the enzyme, HMG CoA reductase may regulate this process via the production of lipid metabolites necessary for parasite fecundity.

F. The Effect of Mevinolin on the Lipids of S. mansoni

A nonsterol product of the HMG CoA reductase-catalyzed reaction is obviously involved in the production of eggs in S. mansoni. This lipid may serve to affect egg production by acting in two capacities; first, it may actually play a functional role in the synthesis of egg components. Secondly, this lipid may exert regulatory control over HMG CoA reductase, influencing the rate or number of eggs produced. Studies described earlier with mevalonate and farnesol (Table 2), suggest that this second role for a lipid metabolite of HMG CoA may exist in the schistosome. Whether this lipid could also be acting in a functional capacity in the synthesis of schistosome eggs could be best assessed by examining the lipids which the parasite synthesizes and determining which, if any, of these lipids are sensitive to mevinolin.

By far the most prevalent lipids synthesized by the schistosome from ^{14}C-acetate were the triglycerides and phosphatidyl choline (Table 10). This finding is consistent with that of Smith and coworkers (1970) who labeled parasite lipids with both ^{14}C-acetate and tritiated free fatty acids, and recovered the bulk of the label in the above two lipids. In the present studies, radiolabeled mevalonate was incorporated primarily into lipids which were unidentifiable, however, these lipids could be classified into groups referred to as hydrocarbons-long chain alcohols, polyisoprenoids, and polar lipids. While the lipids into which mevalonate was incorporated were of the most interest in the determination of lipid metabolites of HMG CoA, ^{14}C-mevalonate could not be used to determine which lipids were being affected by mevinolin, as it is the product of the reaction which the drug inhibits.

For the purpose of determining the effect of mevinolin or lipids in S. mansoni, parasites were incubated with ^{14}C-acetate in the presence of the drug. The result of these studies indicated a profound, selective effect of mevinolin on the synthesis of polyisoprenoid-type lipids (Table 9). These lipids were identified as polyisoprenoids based on their behavior on various chromatographic columns and thin-layer chromatography. The fractions from which these lipids were isolated generally may contain glycolipids, cerebrosides, or glycosylated isoprenoid-type lipids (Carroll and Serdarevich, 1967). The ^{14}C-acetate-labeled schistosome lipids in this fraction did not co-chromatograph with various glycolipids or cerebrosides in several chromatographic systems. Rather, these lipids ran slightly behind farnesol and slightly ahead of a mixed dolichol standard in the thin-layer systems tested. This suggests that these lipids may be isoprenoids of chain length between that of farnesol (which contains 15 carbons) and the long chain dolichols. Additionally, although minor amounts of ubiquinone have been detected in this

parasite (Folkers et al., 1983), none of the schistosome-labeled lipids co-chromatographed with a ubiquinone standard.

Mevinolin at 10 μM exerted a 70% decrease in labeled acetate incorporation into the polyisoprenoid lipids as a class. This, again, correlates nicely with the effects of this compound on in vitro egg production and HMG CoA reductase activity at this concentration. Thus, the effect of mevinolin, as expected based on the pathway of mevalonate metabolism (Figure 7), was confined to the synthesis of polyisoprenoid lipids, and, more specifically, the effect of this drug is probably limited to metabolites in the pathway involving mevalonate, farnesol, and the dolichols.

G. The Potential Role of Polyisoprenoid Lipids in the Synthesis of Schistosome Eggs

Polyisoprenoid lipids are known to function as lipid intermedites in the synthesis of glycoproteins, transferring carbohydrates to forming peptides in the endoplasmic reticulum (Waechler and Lennarz, 1976). In order to determine if the polyisoprenoids in the schistosome could function to carry sugars, a special procedure to isolate these lipids was used (Rumjanek and Smithers, 1978). The result of this procedure was the isolation of two polyisoprenoid lipids capable of carrying radiolabeled mannose residues (Figure 26). One of these lipids, the lipid intermediate, was found to carry one mannose residue, while the second lipid, the lipid-linked oligosaccharide, was found to carry two or more of these sugars.

Once it was established that schistosome polyisoprenoids could function to carry sugars, the effect of mevinolin on these lipids was determined. Parasites incubated in mevinolin contained significantly lower amounts of these lipids (Figure 27), as measured by a decrease in the ability of these lipids to incorporate radiolabeled sugars. Thus the effect of mevinolin on the parasite seems to involve a depletion of polyisoprenoid lipids which function to carry sugars.

The ramifications of a depletion of lipid intermediates in S. mansoni could be significant, since the parasite relies heavily on glycoprotein synthesis, particularly for their incorporation into eggs (Pelley et al., 1976). To assess the effect of mevinolin on the incorporation of ^{14}C-mannose into glycoproteins, a kinetic study was carried out in which the radioactivity incorporated into parasite lipid intermediates and glycoproteins was measured (Table 11). The results of this study indicate a particular sensitivity of the lipid oligosaccharide to the presence of mevinolin. One would expect that since this lipid functions to transfer the sugars to the protein, a proportional decrease in radioactivity incorporated into protein would be seen. This, however, was not the case, as radiolabeled mannose incorporation decreased by 39.5% into protein, while incorporation is reduced by 95.2% into the lipid-linked oligosaccharide. This discrepancy may be the result of a high level of O-linked glycosylation, which does not function through a lipid intermediate, in the schistosome, or may be due to the addition of more mannose residues to the glycoprotein following ER processing. Nonetheless, the suppression of the formation of the lipid intermediates and the subsequent decrease in mannose incorporation into glycoprotiens of the parasite exposed to mevinolin could seriously affect processes in the parasite which are dependent on glycoprotein synthesis. Since the parasite egg is highly proteinaceous, containing a significant amount of glycosylated proteins, it is very likely that mevinolin, by inhibiting parasite HMG CoA reductase, deprives the schistosome of lipid intermediates necessary for the synthesis of egg glycoproteins.

SUMMARY AND CONCLUSIONS

The effect of mevinolin, an inhibitor of HMG CoA reductase, was examined in the schistosome. Specifically, it was found that this drug inhibits schistosome egg production in vitro at concentrations of 10 μM or greater. Furthermore, this effect on egg production was shown to be reversible by the addition of mevalonate or farnesol, metabolites of HMG CoA to the parasite culture. These lipids were also shown to have stimulatory effects on egg production at low concentrations, while at higher concentrations, they inhibited it to a degree similar to that seen with 10 μM mevinolin. The effect of mevinolin on the schistosome in culture, coupled with the results on egg production obtained with the metabolites of HMG CoA, opened two further areas of research, that involving an investigation for HMG CoA reductase in the parasite, and a determination of a role for the lipid metabolites of HMG CoA in the schistosome.

Schistosome HMG CoA reductase was found to resemble the mammalian enzyme in several respects. For instance, the parasite enzyme found to be associated with the microsome, had a similar rate of catalysis as well as similar sensitivity to the inhibitory effect of mevinolin as the enzyme isolated from rat liver. Perhaps the most interesting similarity between the enzymes derived from both sources was the inducibility of enzyme activity following low dose or acute exposure to mevinolin. The mechanism for this induction remains unknown, but it is probably mediated by a product of the HMG CoA reductase metabolic pathway, which feeds back to exert regulatory control over the enzyme. The process of control of this enzyme may involve enhanced rates of transcription or of

translation of the message for the enzyme when it is induced. Currently, this remains an area of active research. In the schistosome, the induction of HMG CoA reductase activity paralleled an induction of egg production, thereby confirming a role for this enzyme in parasite oviposition.

The presence of HMG CoA reductase in S. mansoni suggests that products of the metabolic pathway in which the enzyme is involved must also be present in the parasite. These products, polyisoprenoid lipids, were found to be synthesized by the parasite from ^{14}C-acetate and ^{14}C-mevalonate, and this synthesis was highly sensitive to the presence of mevinolin. Mevinolin caused a selective reduction in the polyisoprenoid lipids of S. mansoni, leaving the other lipid classes of the parasite unaffected.

A function of polyisoprenoid lipids in the schistosome which could be related to the effect of mevinolin on egg production was their role in glycoprotein formation. Schistosome polyisoprenoids were found to be capable of carrying sugars and transferring these to protein. Parasites exposed to mevinolin did not have the ability to carry this process out efficiently, presumably due to a depletion of the polyisoprenoids in these schistosomes. As a result, the glycosylation of proteins in mevinolin-treated worms was markedly reduced. Due to the prevalence of glycoproteins in schistosome eggs, a reduction in protein glycosylation could greatly affect egg production, accounting for the ability of mevinolin to inhibit this process in vitro and in vivo.

The effect of mevinolin in vivo was striking, with doses of 250 mg/kg greatly reducing schistosome-induced pathology. While the low toxicity of this agent as well as its efficacy in reducing the pathology of schistosomiasis make it attractive chemotherapeutically, its use has limitations. For instance, in the human mevinolin would cause a significant effect on cholesterol metabolism -- indeed, this drug will probably find most use as a cholesterol-lowering agent.

Furthermore, since HMG CoA reductase is inducible by mevinolin, a chronic dosing regimen would be necessary to continuously suppress egg production (and not induce it) in the parasite. In areas where schistosomiasis is a health problem, geographical, econcomic and medical limitations do not make chronic administration of drugs feasible.

Therefore, the chemotherapeutic potential of mevinolin for schistosomiasis is very low. However, this drug has been useful to help elucidate a biochemical pathway which is involved in egg production in the schistosome, and this may aid the future development of effective drugs directed against this parasite.

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