TEGUMENTAL Ca-STIMULATED ADENOSINE TRIPHOSPHATASE ACTIVITY IN ADULT *SCHISTOSOMA MANSONI* WORMS

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A Ca-stimulated ATPase activity (pH 9.5) associated with the tegumental membrane enriched (TME) fraction of Schistosoma mansoni *adults was partially inhibited by NAP-taurine or by increasing concentrations of chlorpromazine; endogenous calmodulin was found associated with the TME fraction. A similar activity (pH 8.6) was histochemically visualized within the tegument of fixed worms on the cytoplasmic leaflet of both the double surface membrane and the basement membrane; this reaction was inhibited by 1 μM chloropromazine and it was also observed on the inner side of double membrane vesicles present in the TME fraction. No ATPase activity could be seen at alkaline pH with added Mg or Na/K ions. Without ATP, the addition of external Ca to the fixed worms induced the appearance of lead precipitates on the tegumental discoid bodies; this reaction was inhibited by molybdate and not by chlorpromazine. The intrategumentary regulation of calcium by the systems described and the possible use of phenothiazines against schistosomes are discussed.*

Key words: *Schistosoma mansoni* − tegumental membranes − discoid bodies − Ca-ATPase − Calmodulin − NAP-taurine inhibition − chlorpromazine inhibition

The tegumental membrane fraction of *Schistosoma mansoni* displays intense phospho-hydrolytic activity at alkaline pH against a variety of phosphate ester substrates (Cesari, 1974; Cesari et al., 1981). At least three different kinds of enzymes are responsible for this activity, phosphomonoesterase, phosphodiesterase and adenosine triphosphatase which are presumably associated with the surface membrane of the tegument (Cesari et al., 1983) where transport of solutes and nutrients occurs (Pappas & Read, 1975; Coles, 1984). Previous reports have suggested the presence in the tegument of a ouabain-insensitive Na-Mg-ATPase (Podesta & McDiarmid, 1982) and a classical ouabain-sensitive possibly electrogenic Na- and K-transport ATPase system working at neutral pH (Fetterer et al., 1981; Podesta & McDiarmid, 1982; Noel & Soares De Moura, 1986); an ATPase activity that was high in the absence of externally added ions in the range of pH 8-10 was also reported (Cesari et al., 1981). This latter activity was little ouabain-sensitive, not definitely stimulated by Na and/or K, independently stimulated by Ca or Mg while

inclusion of both ions showed no additive effect; the partial inhibitory effect of chelators like EDTA or EGTA suggested that Ca or Mg stimulated only a partial but important portion of the basal activity. The activity was also enhanced in the presence of exogenous calmodulin (Cesari et al., 1981), a Ca-binding protein known to stimulate Ca-dependent enzymes (Cheung, 1982). These properties distinguish it from other biochemically studied *S. mansoni* ATPases (Nechay et al., 1980; Podesta & McDiarmid, 1982; Noël & Soares De Moura, 1986). Histochemically, a nonspecific (Na-K-Mg)-dependent ATPase activity was visualized by Shaw (1987) in the tegument and subtegumental tissues of adult *S. mansoni* worms. A Ca-stimulated, ouabain-insensitive activity was also visualized within the tegument of adult male and female worms on the cytoplasmic side of the surface and the basal membrane of the tegument (Aldana et al., 1984; Shaw, 1987); however, in female worms the presence and distribution of tegumental Ca-ATPase seemed to depend upon the degree of sexual development, being observed only in sexually mature females (Shaw, 1987).

In the present work the apparently Ca-stimulated ATPase activity of the *S. mansoni*

tegument was further characterized biochemically and histochemically, particularly in isolated tegumental membranes. The possible function of this activity in the tegument and its possible role as a target for pharmacological attack are discussed.

MATERIALS AND METHODS

Chemicals — Adenosine 5'-triphosphate (ATP) disodium salt (vanadium-free), chlor-3-(dimethylamino-3'-propyl)-10 phenothiazine (chlorpromazine), 2-amino-2-methylpropane-1,3-diol (ammediol), ethylene glycol bis (aminoethyl-ether) tetra acetic acid (EGTA), ethylene diamine tetra acetic acid (EDTA), trizma base and bovine serum albumin fraction V were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Modified Eagle's Medium (MEM) was obtained from Flow Laboratories, VA, USA). Glutaraldehyde from Ladd Research Industries Inc., USA. N-(4-azide-2-nitrophenyl)-2-aminoethyl sulfonate (NAP-taurine) was obtained from Pierce Chemical Co., Rockford, IL, USA. Ammonium molybdate, lead nitrate, lead citrate, uranyl acetate, maleic acid and sucrose were from Merck, GFR. Calmodulin (CaM) from bovine brain was obtained from Fluka Ag Chemische Fabrik, Buchs, Switzerland. Reagent kit to determine CaM by radioimmunoassay was obtained from New England Nuclear, USA. Other reagents were of analytical grade.

Parasites and hosts — Adult male and female *Schistosoma mansoni* worms of the Venezuelan JL strain were collected from female NMRI/IVIC mice infected 7 weeks previously with 150 cercariae, as described by Smithers & Terry (1965). After perfusion, the worms were washed in cold MEM and stored briefly in the same medium before use for biochemical studies. Histochemical studies were performed with non-perfused, freshly obtained worms, as described below.

Preparation of schistosome tegument fraction — Live adult parasites were incubated for 5 min at 37 °C in 0.01 M phosphate buffered-saline (PBS) pH 7.4 to induce tegument disruption (Simpson et al., 1981). After the parasite had settled under unit gravity, the released tegumental material was sedimented from the fluid phase by centrifugation at 100000 g for 30 min in a Beckman Ultracentrifuge Model L5-65 (Palo Alto, CA, USA); the tegumentary membrane enriched (TME) pellet was dispersed in 8% (w/v) sucrose in 50 mM tris/HCl pH 7.4 and used directly for biochemical or histochemical assays. Parasites from which tegumental material was removed were homogenized and dispersed in the sucrose-tris solution.

Biochemical assays — The ATPase activity was determined at 37 °C by measuring the inorganic phosphate produced during the ATPase reaction, by the method of Carson (1976). The reaction volume was 0.2 ml, the protein concentration in the assay amounted to 1-5 μg/ml and the reaction medium consisted of 50 mM ammediol titrated with HCl to pH 9.5 and 1 mM $CaCl_2$. After 2-3 min preincubation of the TME protein, the reaction was started by adding ATP (1 mM, final) to the assay. After 5-10 min of reaction cold (4 °C) distilled water was added to the duplicate samples thereafter followed by the addition of phosphate determination reagents. The mixture was stirred and after 20-30 min at room temperature the absorbance was measured at 690 nm in a Gilford Model 250 Spectrophotometer (Gilford Instrument Lab., Oberlin, OH, USA). Corrections were made for phosphate production in the absence of ATP and in the absence of protein, and compared to a standard curve of 0.01 to 0.08 μmol K_2HPO_4 in water treated in the same way as the samples. Inhibitors (NAP-taurine, chlorpromazine) were included in the assay as stated in the text. Protein was estimated according to Lowry et al. (1951), using bovine serum albumin as standard.

Endogenous calmodulin (CaM) assay — Samples for CaM radioimmunoassay were prepared according to Wallace & Cheung (1979). An aliquot (0.5 mg approx.) of the TME fraction or of worm homogenate was resuspended in 0.2 ml of 50 mM tris (pH 7.4), 3 mM $MgCl_2$, 1 mM β-mercaptoethanol, 1 mM EGTA and 50 μl were used for each assay. ^{125}I-labelled CaM was used as the tracer and a specific sheep anti-CaM as the binder (New England Nuclear radioimmunoassay kit). The assay buffer was 125 mM borate (pH 8.4), 75 mM NaCl, 1 mM EGTA, 0.2% bovine serum albumin, 0.1% NaN_3. Standards of bovine brain CaM from 0.31 to 20 ng and adequate controls were run in parallel with samples. The tubes were incubated overnight with anti-CaM and 30 min with the second antibody and then were centrifuged at 5000 g for 15 min in a Eppendorf microcentri-

fuge. All operations were carried out at 4 °C. The supernatant were discarded and ^{125}I was counted in the pellets. The amount of CaM in the samples was determined by interpolation from the standard curve.

Histochemistry — Fresh whole adult parasites were transferred directly from the mouse portal vein (Morris & Threagold, 1968) to cold MEM for washing and thereafter into 2.5% glutaraldehyde-containing MEM for immediate fixation, and allowed to stand for 15 min at room temperature. The specimens were then rinsed again in cold MEM without the fixative. To detect the Ca-stimulated ATPase activity prefixed worms of both sexes were incubated with 0.1 mM $CaCl_2$, 8.3 mM ATP and 3.0 mM lead nitrate in 80 mM tris/maleate buffer pH 8.6 (final volume, 1.0 ml); the control sample was treated as above, except that it was not exposed to ATP and/or Ca. Other ion-stimulated ATPase activities were explored by substituting Ca for either Mg or Na/K ions in the enzymatic reaction mixture. Inhibitors (chlorpromazine, molybdate) were included in the assay as stated in the text. The enzymatic reactions were carried out for 15 min at room temperature and were arrested by briefly washing the worms with distilled water. Postfixation was done for 2 h at 4 °C in 2% osmium tetraoxide. Worms were finally dehydrated through alcohols and included in Epon; samples were cut with a Porter-Blum MT-2 ultramicrotome and stained with uranyl acetate and lead citrate. Stained or unstained sections were observed in a Jeol JEM 100 B electron microscope. A dozen worms from each sex and not less than 10 segments of different regions of the worm were examined in each experiment. At least three replicates per experiment were performed.

The histochemical techniques were also applied to the isolated TME pellets; these were prefixed in Eppendorf tubes by adding 100 µl of 2.5% glutaraldehyde containing MEM for 15 min without resuspension. After remotion of the fixative appropriate enzymatic and control reaction mixtures (final volume, 100 µl) were added to the pellets and the incubation allowed to proceed for 10 min at room temperature; the supernatants were removed, the pellets washed briefly with distilled water and finally processed for electron microscope analysis as above, avoiding any time resuspension.

RESULTS

Effect of inhibitors on the Ca-stimulated ATPase activity of the TME fraction — When the TME fraction was subjected to a freeze and thaw procedure (F/T) in the presence of 0.1 mM NAP-taurine, the Ca-stimulated ATPase activity decreased about 30-35% with respect to a F/T control; however, if NAP-taurine was added after F/T, the enzymatic activity was only affected by 7-10% as compared to the same control; the frozen and thawed sample differed little from the unfrozen control (Table I).

TABLE I

Effect of 0.1 mM NAP-taurine on the Ca-stimulated ATPase activity of the TME fraction of adult *Schistosoma mansoni*

Experimental	Activity* µmoles phosphate/min	%
Freezing (F)/ thawing (T)	2.68	100.0
F/T, then addition of NAP-taurine	2.42	90.3
F/T in the presence of NAP-taurine	1.83	68.3
Unfrozen control	2.56	–

* The activity values shown are the results of one representative experiment from three performed under the same conditions.

Chlorpromazine was able to inhibit the Ca-stimulated ATPase starting at a concentration of 1 µM. Stronger inhibitions were observed at phenothiazine concentrations higher than 10 µM (Fig. 1). At this drug concentration the inhibition of the Ca-stimulated ATPase (32.3%) was totally reversed by 2 µM exogenous CaM which was able to stimulate, by itself, about 28% the hydrolysis of ATP (Table II).

Endogenous CaM in adult worms — This protein was detected by radioimmunoassay in the TME fraction (3.265 µg/mg protein) and in the homogenate of partially detegumented adult male and female worms (0.147 µg/mg protein).

Histochemical localization of alkaline ATPase activities — Reaction products indicatives of Ca-stimulated ATPase activity were seen at pH 8.6 within the tegument of prefixed

TABLE II

Effect of calmodulin and/or chlorpromazine on the Ca-stimulated ATPase of the TME fraction of adult
Schistosoma mansoni

Chlorpromazine $(1 \times 10^{-5} M)$	Calmodulin $(0.2 \times 10^{-5} M)$	Activity* μmoles phosphate/min	%
−	−	1.30	100.0
+	−	0.88	67.7
+	+	1.45	111.5
−	+	1.66	127.7

* The activity values shown are the results of one representative experiment from three performed under the same conditions. The TME preparation was different from that used for experiment in Table I.
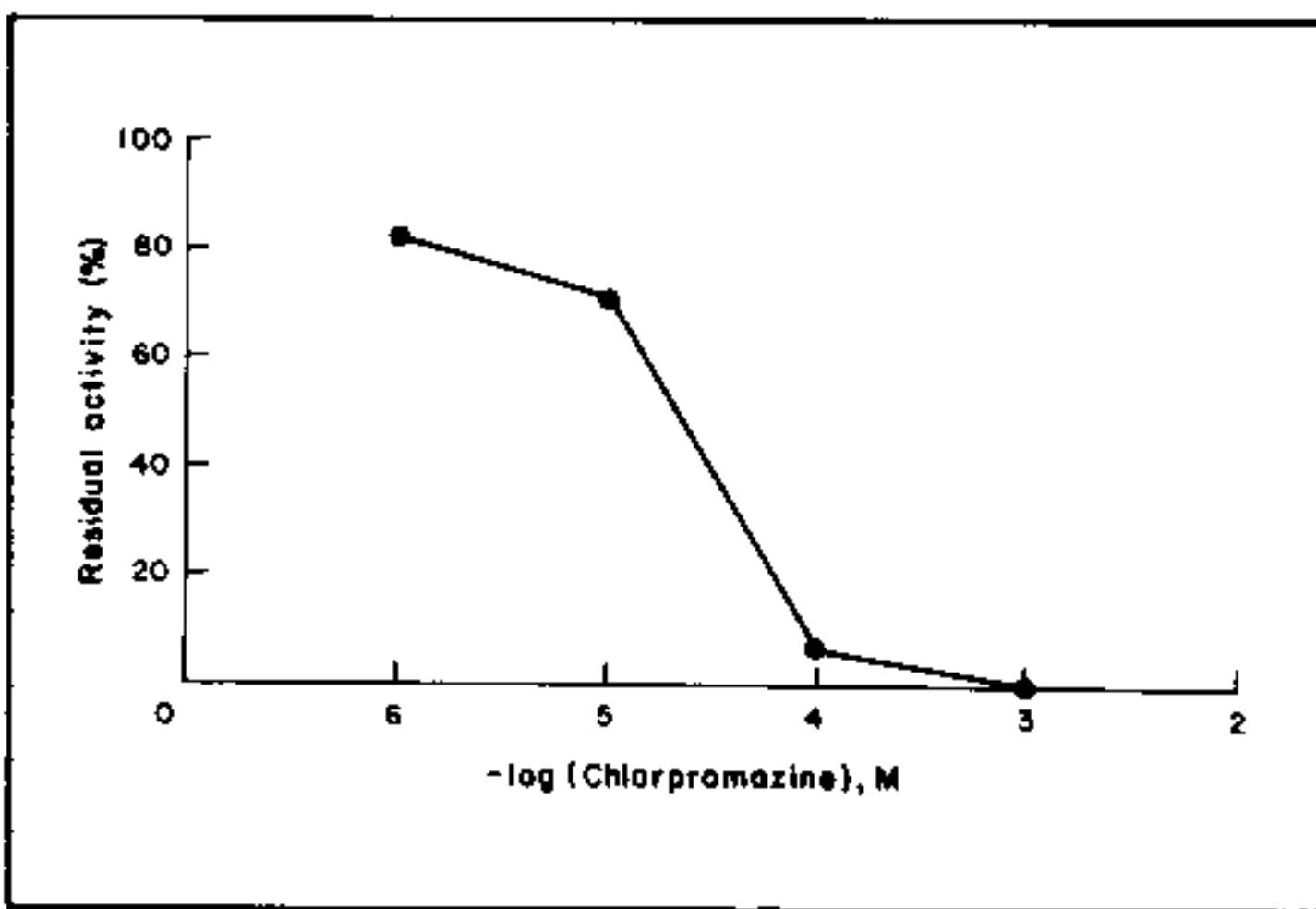

Fig. 1: effect of increasing concentrations of chlorpromazine on the Ca-stimulated ATPase activity of the adult *Schistosoma mansoni* TME fraction. Per cent of enzymatic activity relative to controls without the inhibitor.

adult male (Fig. 2) and female (Fig. 3) worms; the sites of reaction were observed as fine electron dense precipitates apparently in the apical and invaginated regions of the double surface membrane (DSM) and on the cytoplasmic side of the basement membrane (BM) and its intrategumentary digitations (Figs 2, 3). No activity was visualized in the tegument in the absence of both ATP and ions (Fig. 4) or in the presence of ATP and Mg or Na/K ions. With ATP and in the absence of ions no enzymatic reaction was seen. The Ca-stimulated ATPase reaction was not uniformly distributed within the tegument of male and female worms and appeared as discontinuous zones of activity (Fig. 5). When 1 μM chlorpromazine was included in the assay the Ca-stimulated ATPase reaction was not seen. However, in the absence of ATP, the incubation of prefixed worms with Ca induced occasionally the appearance of lead precipitates on the membrane of the discoid bodies (DB) (Fig. 6); this reaction was not

inhibited by chlorpromazine although it was interferred with by 0.1 mM molybdate the tegument appearing without precipitates as in the control shown in Fig. 4. No reaction was observed on the DB of worms incubated without ATP in the presence of Mg or Na/K ions.

Apparently, no dorso-ventral differences were observed for the tegumental Ca-stimulated ATPase reaction along the female worm body. However, the reaction appeared mostly in the dorsal regions of male worms (data not shown).

The isolated TME fraction showed Ca-stimulated ATPase activity in the form of lead phosphate precipitates when incubated in the presence of ATP plus Ca. The reaction was for the most part seen associated to the inner side of double membrane domains (Fig. 7). No precipitates were seen in controls without ATP or with Ca alone (Fig. 8).

DISCUSSION

The tegument possesses a high basal ATPase activity in the range of pH 8-10 which is further enhanced by addition of millimolar concentrations of Ca or Mg and is probably independent of the presence of Na and/or K ions for activity (Cesari et al., 1981).

NAP-taurine at 0.1 mM, known to be inhibitory on the Ca-dependent ATPase of human erythrocytes (Minocherhomjee & Roujogalis, 1982), could partially inhibit the Ca-stimulated activity of the TME fraction only when the compound was present during freezing and thawing (F/T) of the fraction; its posterior addition to the F/T process did not inhibit definitely the activity (Table I). The TME frac-
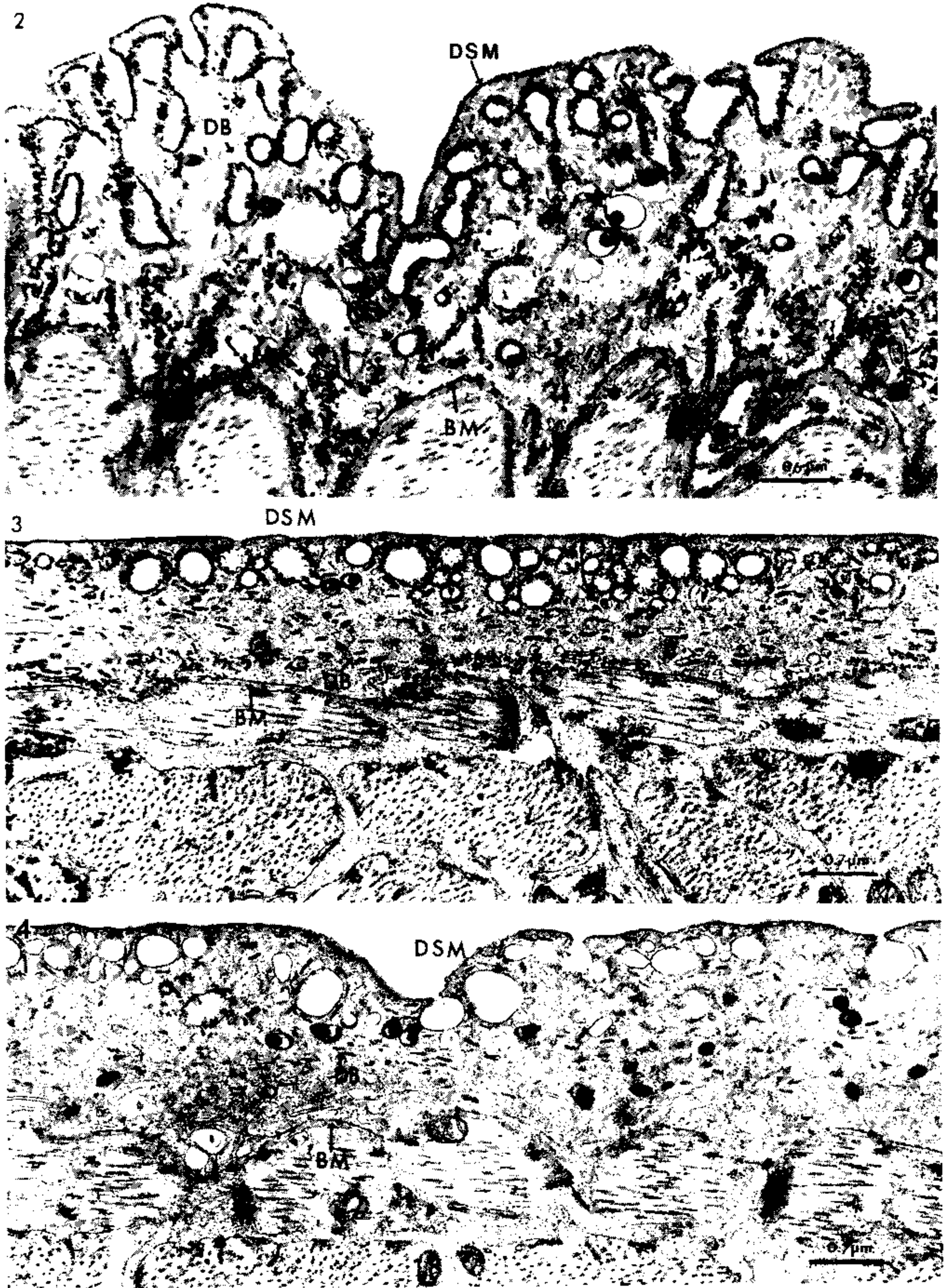
Fig. 2: male adult worm incubated with ATP and 0.1 mM $CaCl_2$; note reaction at the double surface membrane (DSM) and basal membrane (BM) but not on discoid bodies (DB) (Bar = 0.6 μm). Fig. 3: female adult worm incubated as above; note reaction as in Fig. 2 (Bar = 0.7 μm). Fig. 4: adult worm without both ATP and ions (Bar = 0.7 μm).

tion shows numerous and heterogeneous vesicles under the electron microscope, many of them from the DSM (Simpson et al., 1981;

Fig. 7) that are possibly open upon rapid freezing at − 70 $^\circ$C and reconstituted after thawing at 4 $^\circ$C; NAP-taurine present during
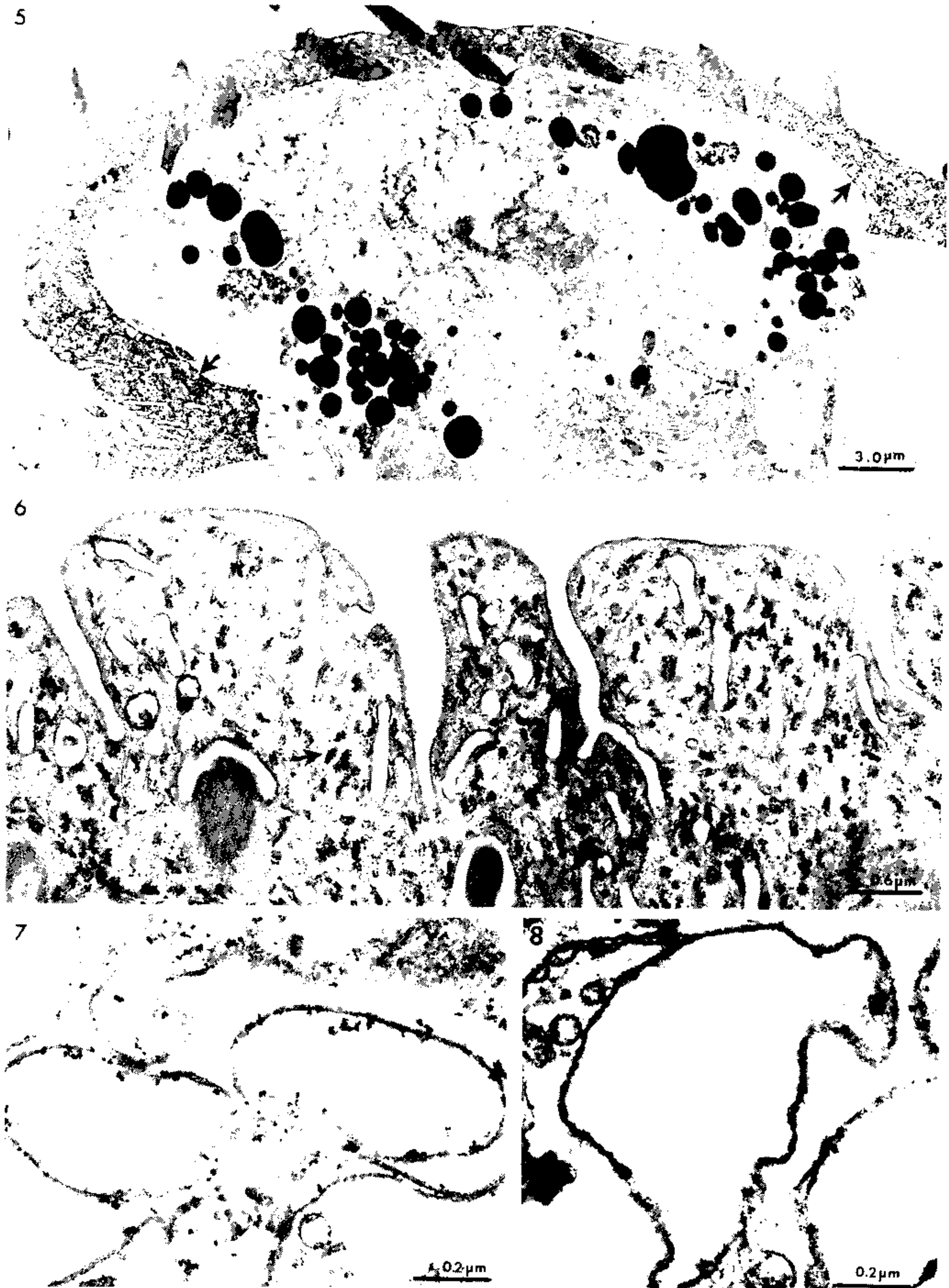
Fig. 5: male adult worm incubated with ATP and Ca; arrows show sites of intense activity in the tegument (Bar = 3 μm). Fig. 6: male adult worm incubated with Ca without ATP; arrows indicate precipitates on DB membrane (Bar = 0.6 μm). Fig. 7: TME fraction with ATP and Ca; note precipitates on the inner side of double membrane vesicles (Bar = 0.2 μm). Fig. 8: TME fraction without ATP and Ca (Bar = 0.2 μm).

but not after the F/T process could have been trapped within the vesicles to exert its inhibitory action on an asymmetrically located ATPase.

Chlorpromazine, an antipsichotic tricycle phenothiazine drug, exerts an inhibitory effect on Ca-stimulated ATPases probably due to its

known antagonizing effect on CaM (Weiss & Levin, 1978). This drug was able to inhibit the Ca-ATPase of the TME fraction starting at a concentration of 1 μM (Fig. 1). At 10 μM, it partially inhibited (32.3%) the activity; however, this inhibition was counteracted and totally reversed by 2 μM exogenous CaM which was able to stimulate (27.7%) the hydrolysis of ATP (Table II), in agreement with previous findings (Cesari et al., 1981). Although a K_i determination for the chlorpromazine effect was not carried out, this result suggests that in *S. mansoni,* endogenous CaM might be a target for chlorpromazine.

CaM was found in the TME fraction at a level of 0.3% of the total protein fraction and there was about twenty-two times higher CaM content in the TME fraction than in the homogenate of detegumented parasites. This result suggests the presence within the tegument of important Ca-dependent and CaM modulated systems. CaM may be of interest in Platyhelminthes. Branford White & Hipkiss (1985) have noticed that the integrity of the cestode *Hymenolepis diminuta* is related to its CaM content and suggests the potential cestocidal properties of the phenothiazine drug trifluoperazine; this suggestion might be also taken into consideration for schistosomes.

Since *S. mansoni* displays ATPase activity in a wide range of alkaline pHs (Nechay et al., 1980; Cesari et al., 1981), the histochemical assays were performed at pH 8.6 and not at pH 9.5 to avoid nonspecific lead precipitation. In agreement with the results obtained by Shaw (1987), in most segments and sections examined the Ca-stimulated ATPase activity appeared mostly located within the tegument of the parasite, associated with the cytoplasmic side of the apical and invaginated regions of the DSM and no reaction product was observed on the free surface of the worm; the activity was also observed on the cytoplasmic side of the BM and its intrategumentary digitations (Figs 2, 3). However, different to the results obtained by Shaw (1987), no conspicuous activity could be appreciated in the tegumental cytoplasm. Apparently, the activity was mostly located on the dorsal region of the male tegument whereas a more uniform reaction was observed within the tegument around the female worms. Shaw (1987) reported additional observations in the distribution of tegumental Ca-ATPase activity in female worms which could be related to their

degree of sexual development, with little or no tegumental activity being found in 70-day-old females from single sex infections. Ca-ATPase activity was also seen by this author in male and/or female tissues other than tegument.

The histochemical studies are in agreement with the biochemical results reported in Table I. Since plasma membranes in different biological systems are, in general, impermeable to NAP-taurine (Staros & Richards, 1974) the results in Table I would suggest that a high proportion of the ATPase-associated vesicles in the TME fraction retained their natural outside-in asymmetry upon isolation and after F/T. When the histochemical techniques were applied to the isolated TME fraction discrete lead phosphate precipitates indicatives of the enzymatic activity appeared associated mostly with the inner side of double membrane domains (Fig. 7); no precipitates were seen in the absence of substrate or in the presence of Ca alone (Fig. 8).

Control experiments done with whole parasites, without ATP but in the presence of 0.1 mM Ca showed sometimes lead phosphate precipitation on the DB's membrane (Fig. 6); no precipitation was observed around these bodies when Mg or Na/K were used instead of Ca. The Ca-induced reaction on the DB was not interferred with by 1 μM chlorpromazine. Phosphate release in these bodies was possibly accomplished by the action of a phosphatase over an endogenous substrate. On the other hand, molybdate, a phosphate chelator and apparently a strong inhibitor of the *S. mansoni* alkaline phosphatase activity (Nimmo-Smith & Standen, 1963) interferred with the Ca-induced histochemical reaction. Preliminary biochemical results suggest that molybdate does not inhibit the hydrolysis of ATP; on the contrary, in its presence there is an increased hydrolysis of this substrate. Since it has been also shown that the tegumental alkaline phosphatase does not hydrolyze ATP but its activity is inhibited by this nucleotide (Cesari et al., 1981) both results suggest that alkaline phosphatase and ATPase may compete for ATP. This is further suggested by the fact that lead phosphate precipitations were never seen in the DB when both Ca and ATP were present in the histochemical assay with the whole parasite (Figs 2, 3), due possibly to the inhibitory action of ATP on the phosphate generating system. Discoid bodies (about 45 x 150 nm) are numerous in the

cytoplasm of the distal tegument of schisto-
somes and are particular to platyhelminthes but
not known function has been so far assigned to
them. The generation of phosphate in these
organelles might be related to Ca sequestering
by them. In many eukaryotics, the cytosolic
free Ca concentrations are maintained within
strict limits by a series of regulatory mechanisms
mediated by plasma membrane Ca-pumping
ATPases, endoplasmic reticulum and organelles
that sequester Ca like mitochondria (Rasmus-
sen, 1975; Wuytack & Casteels, 1980; Akerman
& Nicholls, 1983). However, the role of the Ca-
stimulated ATPase described here is unknown; it
requires high pH for optimal activity and milli-
molar concentrations of Ca for its stimulation,
in contrast to the pumping Ca-ATPases from
mammalian sources which have lower pH
optimum (Sigler, 1982) and are usually stimu-
lated by micromolar concentrations of Ca
(Schatzmann, 1982). It will be necessary to
measure   accurately   the   intrategumentary
concentration of Ca, calcium transport and Ca
affinity to know if there is a Ca ATPase-
mediated pumping system. On the other hand,
in the distal tegument of the *S. mansoni* strain
used in the present work the mitochondria are
small with few and scarce cristae, and there is
no endoplasmic reticulum; instead, there are
numerous DB and a considerable amount of
CaM that might be contributing to regulate Ca
concentration and modulate the activity of this
cation and other Ca-dependent systems within
the tegument.

The Ca stimulated ATPase (Fig. 5) and Ca-
induced DB reaction appeared distributed in
patches along the tegument of adult male and
female worms as also reported by Shaw (1987).
This would suggest the simultaneous and
contiguous presence of active and inactive
regions in the syncytium; otherwise, the surface
membrane of the parasite may have been
intermittently   permeabilized   so   that   the
substrate penetrated unevenly.

The   histochemical   distribution   of   the
ATPase discussed in this study and in previous
ones (Aldana et al., 1984; Cesari, 1985; Shaw,
1987) differed from that described by Bogitsh
(1980) who visualized the products of alkaline
ATPase activity extracorporeally in the spaces
between invaginations and at the apical regions
outside the DSM. Different fixing and handling
procedures may be at the origin of this discrep-
ancy. An extracorporeal activity of the DSM-

associated Ca-stimulated ATPase may not be
physiological due to the fact that the spaces
between the invaginations are probably acidic
environments because of lactic acid release
(Rotmans et al., 1981).

Our results suggest the existence of an
important Ca/CaM-stimulated ATPase system
in the tegument of *S. mansoni* of so far unknown
function; on the other hand, endogenous CaM
was found in considerable amounts within the
tegument and together with the DB might be
involved in the maintenance of Ca-homeostasis
within the syncytium. These systems may be
suitable for further studies as possible targets
for pharmacological attack.

RESUMO

**Atividade da adenosina trifosfatase estimula-
da pelo Ca no tegumento de vermes adultos de
*Schistosoma mansoni*** — A atividade ATPase
(pH 9.5) estimulada por ions de Ca associados a
uma fração enriquecida de membranas do tegu-
mento (fração EMT) de vermes adultos de
Schistosoma mansoni, foi inibida por NAP-
taurina ou por concentrações crescentes de clor-
promacina.   Foi   encontrada   calmodulina
endogena associada principalmente a esta fra-
ção. Em vermes adultos fixados com glutaral-
deido se detectou histoquimicamente uma ativi-
dade ATPase similar (pH 8.6) na face citoplas-
mática da dupla membrana de superfície e da
membrana basal do tegumento. Esta reação foi
inibida por 1 μM de clorpromacina e foi tam-
bém observada na face interna de vesículas de
dupla membrana presentes na fração EMT. Não
se pôde detectar atividade ATPase em pH alcali-
no na presença de ions de Mg ou Na/K. A adi-
ção externa de Ca, sem ATP, aos vermes fixados
induz ao aparecimento de precipitados nos cor-
pos discóides do tegumento; esta reação foi ini-
bida pelo molibdato mas não pela clorpromaci-
na. Os resultados são discutidos em relação a
uma possível regulação intrategumentária de
Ca pelos sistemas descritos e o possível uso de
fenotiacinas contra os esquistossomas.

Palavras-chave: *Schistosoma mansoni* — membranas
tegumentárias — corpos discóides — Ca-ATPase —
calmodulina — inibição de NAP — taurina — inibição
de cloropromazine

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