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ACETYLCHOLINESTERASE OF *SCHISTOSOMA MANSONI* – AN ANTIGEN OF FUNCTIONAL IMPLICATIONS

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One of the major and puzzling aspects in the study of the host-parasite relationship in schistosomiasis is the ability of the schistosome to evade the immune defense mechanism of its host (Smithers, 1976; Phillip & Colley, 1978). It seems that this mechanism of resistance to complement-dependent killing is developed within a few hours of transformation of cercariae to schistosomula (Clegg & Smithers, 1972; Butterworth et al., 1979), and depends on intrinsic and yet undefined properties of the schistosomular surface membrane (Levi-Schaffer et al., 1982). We were interested, therefore, in schistosoma surface antigens that might serve for either diagnosis or protective immunization. We devised a procedure that allowed us to isolate the tegumental membranes of schistosomula. Somewhat unexpectedly, two enzymatic activities were found to be highly enriched in the isolated external membranes: alkaline phosphatase with about 100 fold enrichment and acetylcholinesterase which showed a 350 fold enrichment as compared to whole worm homogenate, indicating that these enzymes are probably associated with the outer membranes of the parasite (Levi-Schaffer et al., 1984b).

Acetylcholinesterase (AChE) was of particular interest in view of the physiological role that it plays in the biology of the parasite, due to its involvement in neurotransmission. AChE is generally required for the metabolic breakdown of acetylcholine at cholinergic synapsis. The enzyme AChE (E. C. 3.1.1.7) was first demonstrated in adults of *Schistosoma mansoni* by Bueding (1952), and was partially characterized by histochemical (Fripp, 1967) and kinetic studies (Gear & Fripp, 1973). Earlier studies demonstrated that AChE is involved in the neurotransmission mechanism of *S. mansoni* (Barker et al., 1966). More recently, Pax et al. (1984) has shown that eserine, an AChE inhibitor, produces significant relaxation of the longitudinal muscle of the parasite. It is therefore apparent that cholienergic receptor mechanisms are importantly associated with both circular and longitudinal muscle function. Indeed, AChE is the target for several anti-parasite drugs that have specific toxic activities, including hycanthone and lucantone (Hillman & Senft, 1975) and metrifonate (Bueding et al., 1972; Jewsbury et al., 1977).

Thus, having established that AChE is located on the outer surface of the parasite, and in view of its functional importance, we were attracted by the possibility of using it as an effective immunological target.

The simplest way to address this question was to investigate whether antibodies against AChE would be lethal against the parasite. In the absence of specific antibodies against AChE of Schistosoma, we employed antibodies elicited by AChE of *Electrophorus electricus* (electric eel), which were found to cross-react with *S. mansoni* AChE from different stages of the parasite life cycle (Fig. 1). Immunofluorescence microscopy showed specific staining of intact schistosomula as well as of adult worms, by this clearly positive cross-reaction was demonstrated (Fig. 2). More importantly, the interaction with these antibodies resulted in a marked complement-dependent cytotoxicity towards intact schistosomulae (Table I). These results demonstrated that AChE might constitute an antigen of significance in this parasite. We therefore started a systematic effort towards its purification and characterization.

The use of affinity chromatography techniques, similar to those used previously for purifing AChE from animal tissues, enabled us to obtain the enzyme in a very high degree of purity. The affinity matrices that are used for the purification of the enzyme are usually constructed by immobilizing an appropriate competitive inhibitor of the enzyme to Sepharose resins via hydrophobic spacer arm. The affinity ligand M-[E-(E-aminocaproyl-E-aminocaproyl)-m-aminophenyl] trimethylammonium bromide hydrobromide (mTA) was one of the inhibitors used for purification, and so was its

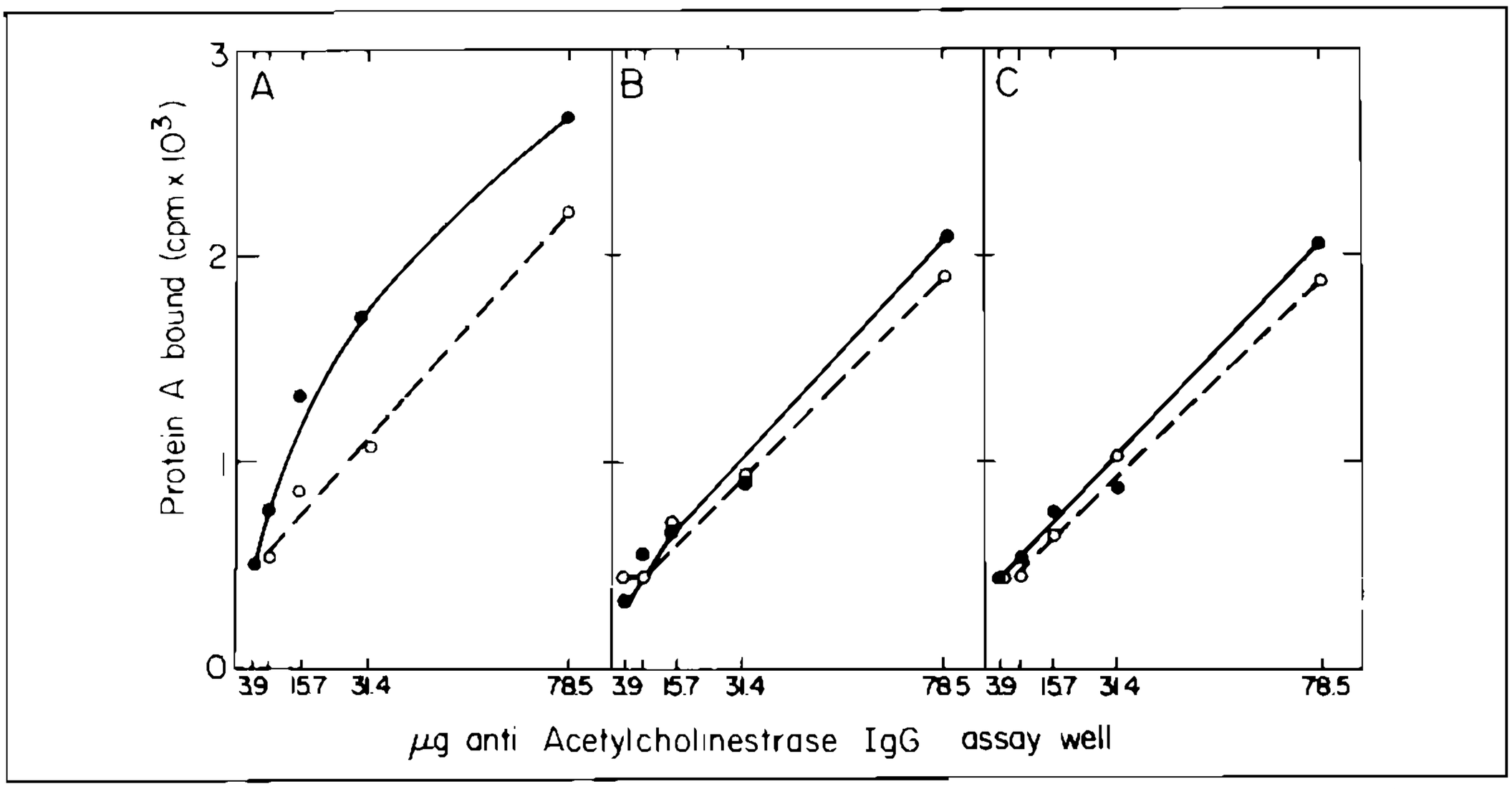


Fig. 1: Binding of anti-AChE to the *Schistosoma mansoni* enzyme in extract of the worms in various life stages. Binding of rabbit anti-AChE from *Electrophorus electricus* to worms crude sonicate (○) or to a 100 000 x g-supernatant fraction of an extract in 0.05 m Tris-HCl buffer, pH 7.5, 0.5m NaCl (●). The assay was monitored by RIA (A) Adult (B) Schistosomula, (C) Cercariae.

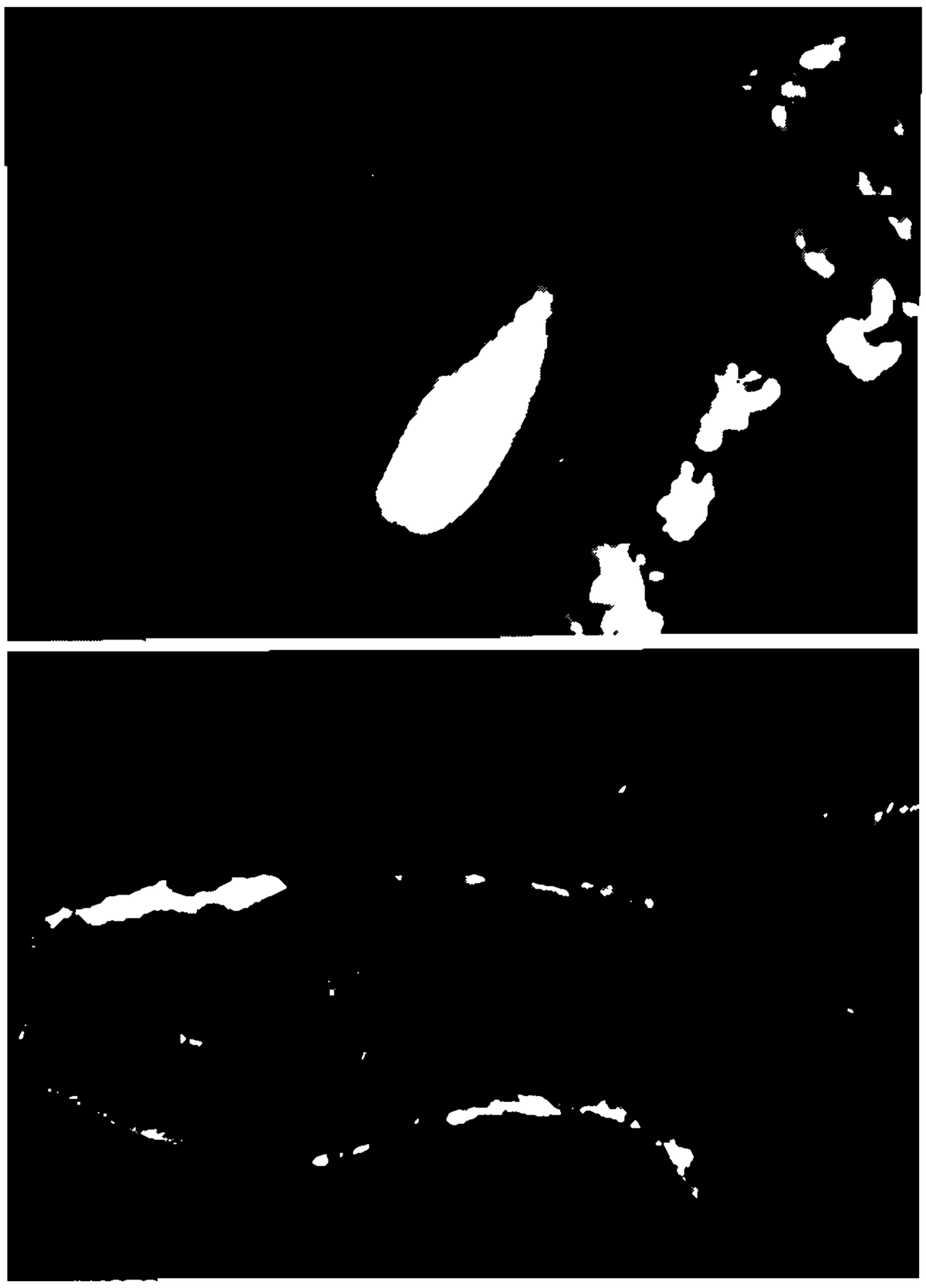


Fig. 2: Immunofluorescence micrographs of adult worms after binding of rabbit anti-AChE. Bound rabbit antibodies were visualized by staining with FITC-goat anti-rabbit IgC. The upper frame depicts the apical part of the worm, whereas the lower frame shows the region close to the ventral sucker. The worms were photographed at a magnification of x 250.

TABLE I

Susceptibility of 3-hr mechanical schistosomula to anti-AChE and complement

Specificity of antibodies	Percent net killing* whole antiserum	Obtained with IgC fraction** (mg/ml)
AChE	23.8 + 9.7	Batch I 51.5 + 5.52 (5) II 33.3 + 9.6 (3) III 39.3 + 18.4 (2)
AChR	1 + 2.2	8.2 + 6.8 (3)
Rabbit anticercariae	62.5 + 11.1	67.5 + 5.1 (5)
Normal rabbit	2.5 + 4.3	13.26 + 5.1 (4)

* These values are the differences between the absolute values of the percentage of total dead schistosomula in the presence of antibodies and complement and the percentage of dead schistosomula in the presence of antibodies and heat-inactivated complement. Each number represents an average of 4-8 experiments.

** IgG fractions obtained by $(NH_4)_2SO_4$ precipitation followed by DEAE-cellulose chromatography.

isomer the para trimethyl (pTA). Other inhibitors such as methylacridinium (MAC) and N-methyl-3-aminopyridinium (MAP) were tested during the purification of the enzyme from electric fish. The AChE was usually released by a competitive inhibitor solution such as decamethonium or tensilon. The efficiency of the affinity chromatography varied for different resins and different tissues (Chan et al., 1972; Vidal et al., 1981; Reavill & Plumer 1978; Dudai et al., 1971, 1972).

Sepharose conjugates of three inhibitors were evaluated for purification of *S. mansoni* AChE. These include mTA, pTA and 1-methyl-9-[Nβ-(E-amino-caproyl)-β-aminopropylamino)] acridinium bromide hydrobromide (MAC). Only the sepharose mTA resin was found suitable for purifying the *S. mansoni* AChE (Goldlust, 1984). The purified enzyme eluted from the column by decamethonium bromide with a specific activity of 11,000 units/mg of protein and 300 fold purification migrated as a single band of 500 Kd on non-denaturing polyacrylamide gel electrophoresis (PAGE). Analysis by SDS-PAGE, revealed two major polypeptide bands of 76 Kd and 30 Kd (Fig. 3). This last band of 30 Kd was the only one to be labelled by ^{3}H-DFP (which labels the active site of serine hydrolases) indicating that this subunit encompasses the active site of the enzyme (Goldlust et al., 1986). It is important to note that we have previously demonstrated that the cercarial enzyme extracted in the presence of non-ionic detergents, behaves as monodisperse 8 S form in sucrose gradients, and emerges as a sharp peak of 450,000 dalton in gel filtration (Tarrab-Hazdai et al., 1984a). We also demonstrated the existence of multiple molecular forms of this enzyme differing in their sedimentation coefficients. This phenomenon which has been observed in many other species (Massoulie & Bon, 1982), was pronounced mainly in adult worms of *S. mansoni* (Tarrab-Hazdai et al., 1984a), and less in cercariae and schistosomula.

With the pure enzyme we initiated a series of studies for a more thorough characterization of the molecule. The enzyme is a "true" acetylcholinesterase since its hydrolysis rate of acetylcholine was more than seven times higher than that of butyrylcholine. Furthermore, one of its characteristics was substrate inhibition. AChE was shown to be efficiently inhibited by eserine, a powerful inhibitor of cholinesterses, but was relatively resistant to the specific AChE inhibitor or BW 284C51. The enzyme was almost unaffected by the specific pseudocholinesterase inhibitor 1S0-OMPA (Goldlust et al., 1986), a final indication for its constituting a true acetylcholinesterase.

The availability of pure enzyme permitted the induction of specific antibodies against the *S. mansoni* AChE. These specific antibodies induced in rabbits, were able to bind at the surface of 3hr schistosomule, and to immunoprecitate a single band of serine hydrolase of 30-35 Kd from the intact cercarial extract. Most significantly, these antibodies were highly cytotoxic to the parasite in the presence of guinea pig complement effecting 75-95% killing (Fig. 4) These findings (unpublished data) serve as further corroboration in the funcional role played by AChE in *S. mansoni.* Monoclonal antibodies induced against the enzyme, are presently under investigation and characterization.

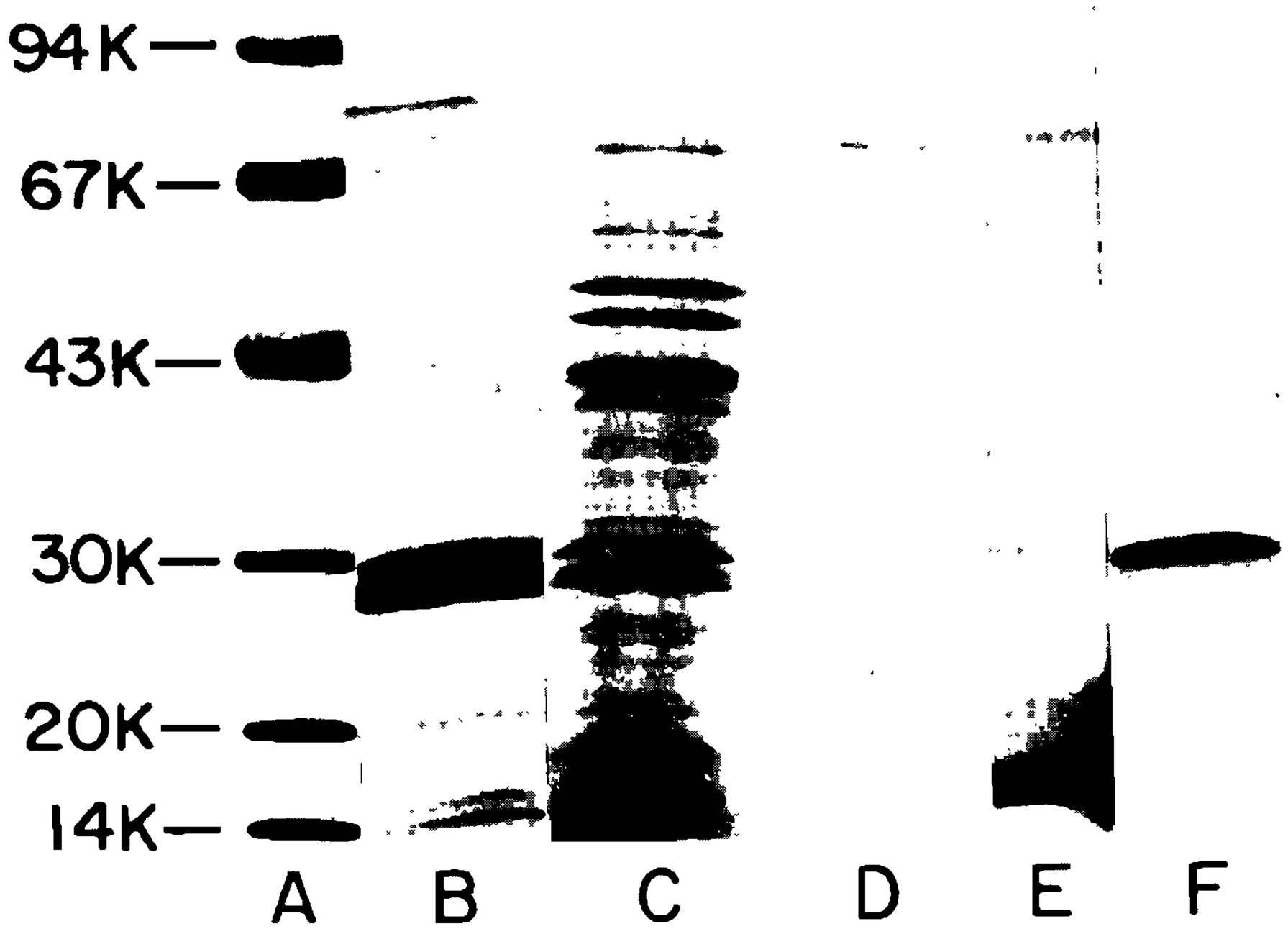


Fig. 3: SDS-PAGE of the Triton X-100 *Schistosoma mansoni* extract and of purified *S. mansoni AChE*. Samples were electrophoresed in Tris-glycine, pH 8.8, through a 7.5-15% polyacrylamide gradient gel in the presence of 2-mercaptoethanol. A. Markers: phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20 kD), and lactalbumin (14 kD). B. Autofluorography of ^{3}H-DFP-labeled 100,000g supernatant. C. Coomassie blue staining of 100,000g supernatant. D. Coomassie blue staining of purified *S. mansoni* AChE. Autoradiography of ^{125}I-labeled purified *S. mansoni* AChE. F. Autofluorography of ^{3}H-DFP-labeled purified *S. mansoni* AChE.

Another aspect of our work is concerned with studying the feasibility of using anti-cholinergic drugs as therapy. We chose to analyze the effect of a series of phosphonium salts, and phosphoranes on the catalytic activity of acetylcholinesterase and on the viability of the various life stages of *S. mansoni* (Levi-Schaffer et al., 1984a). Seventeen different derivatives were tested and all of them showed an inhibitory effect towards the parasite AChE activity. The most effective compound, p-xylylene bis(triphenyl phosphonium) dibromide displayed 100% inhibition even at concentration of 10^{-6}M. No significant differences were found in the sensitivity of the enzyme, obtained from the various stages of the parasite life cycle, to the effect of the drugs. Each compound was also tested for its toxicity towards 3hr old schistosomula and 7-9 week adult worms under *in vitro* culture conditons. In the case of the larvae, after 2 days in culture, only three compounds out of sixteen tested exhibited efficient killing of the schistosomula while the others had a very slight toxic effect or showed no toxicity at all. On the other hand, all the compounds showed a significant toxicity towards the adult worms (Table II) and the most effective one, allyltriphenyl phosphonium bromide, (compound No. 11), retained its toxic effect even at extremely high dilutions (10^{-8}M). This compound was effective *in-vivo*, and led to 50% reduction in worm burden in infected mice at concentration (20 mg/kg) which showed no toxicity to the mice. Although the results did not demonstrate a complete correlation between the inhibitory effect of the

drugs on the AChE activity of the schistosomes and their toxicity towards the worms, they are still of significance due to the fact that the more efficient in killing adult worms and schistosomula inhibit AChE activity 60%. It is not claimed herewith that phosphonium salts or phosphorans are the drugs of choice in therapy of schistosomiasis. However, the availability of functional enzyme of the parasite in a pure form could enable a specific approach to drug design in the future. In particular, the difference in the sensitivity of *S. mansoni* and *S. hematobium* to anti-cholinergic drugs such as metrifonate, may be explored by a detailed comparison of their AChE and their behaviour towards different drugs.

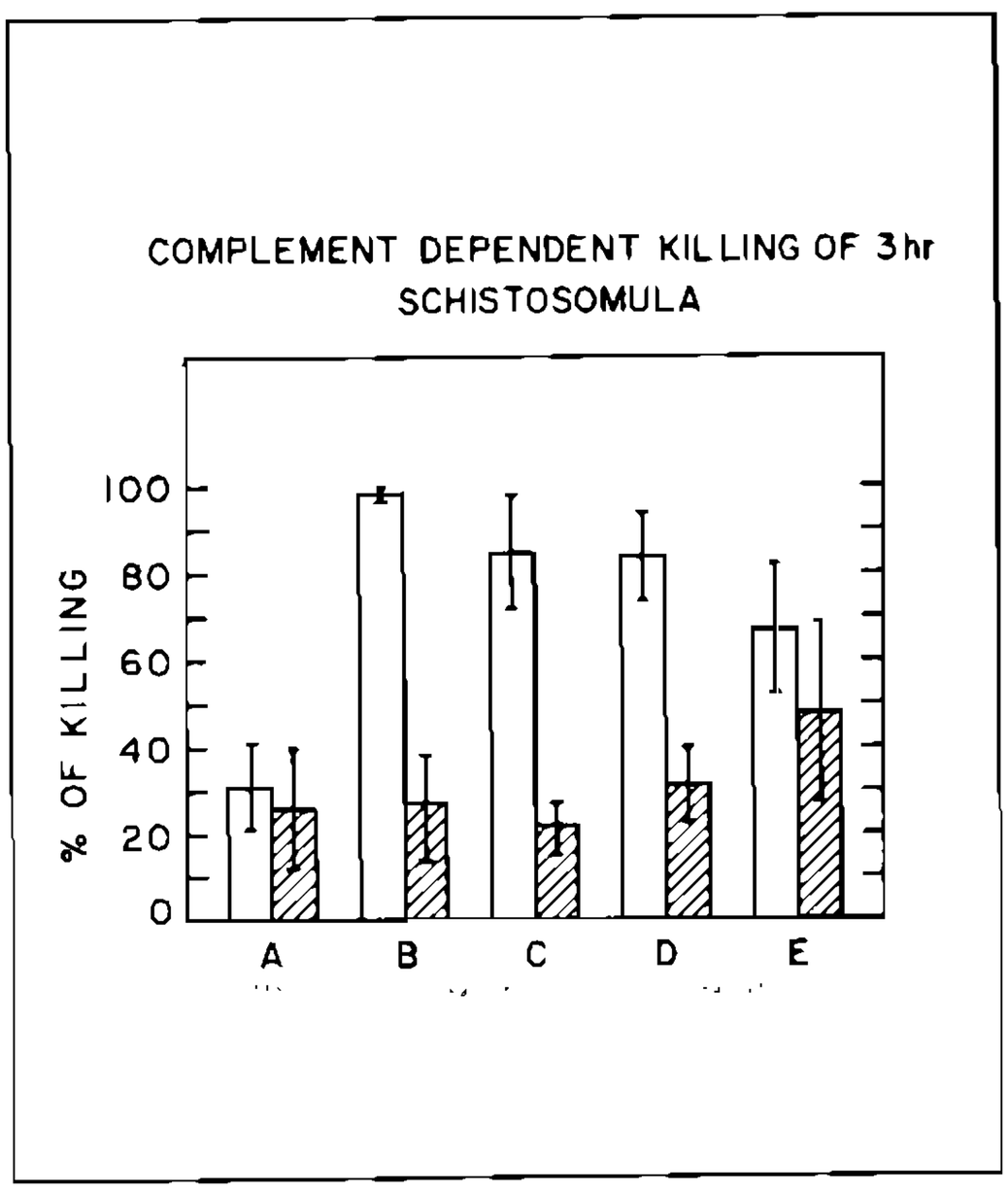


Fig. 4: complement dependent killing of 3 hr schistosomula by anti-Acetylcholinesterase antibodies. Mechanical transformed schistosomula were mixed with the antisera, after 20 min of incubation at 37 °C fresh guinea pig serum (□), or heat-inactivated guinea pig serum (▨) was added and then were incubated for 18 hr at 37 °C in 10% CO_2. The dead worms were counted under an inverted microscope.

A different issue is the intereaction between AChE and the membrane of the parasite. Recently, a novel mechanism for the hydrophobic attachment of proteins to surface membranes has been described, which is apparently shared by an increasing number of proteins of diverse functions and origins (Low et al., 1986). The hydrophobic anchor is the 1.2-diacylglycerol moiety of a single phosphatidylinositol (PI) molecule, which is covalently attached to one end of the polypeptide chain via an intervening oligoglycan. The initial evidence for this mode of attachment came from the observation that certain ectoenzymes could be released from the membranes to which they were attached by a phosphatidyl inositol-specific phospholipase (PIPLC) of bacterial origin (Low, 1987). Our results showed that AChE is susceptible of release from the *S. mansoni* tegumental membrane by PIPLC of *S. aureus*, suggesting that AChE of the parasite, as in higher organisms, is anchored to the membrane via covalently attached phosphatidylinositol. We also demonstrated that PIPLC removes large amounts of AChE from the surface of schistosomula in culture, with no impairment of the viability of the parasite. This AChE release by PIPLC increased with the age of the parasite in culture, reaching a maximal release with 24hr old schistosomula (Fig. 5), manifested also in a drastic increase in the overall levels of AChE in the parasite (Table III) (Espinoza et al., 1988). This interesting phenomenon is currently under investigation.

In conclusion, we have demonstrated that:

1) AChE of *S. mansoni* is a surface membrane protein.

2) The purified enzyme (by affinity chromatography) lends itself to physiochemical and enzymatic characterization.

3) Antibodies to both heterologous and homologus are cytotoxic towards the parasite.

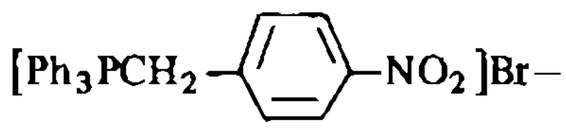
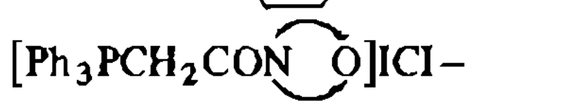
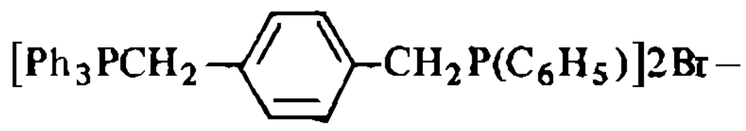
4) Anti-cholinergic drugs, such as phosphonium compounds are inhibitory to the AChE, and cytotoxic to both schistosomula and adult worms.

5) AChE is anchored to the membrane of schistosomes via phosphoinositol. Its release from the live parasite by PIPLC induces enzyme activity indicating the vital function of AChE in *S. mansoni*.

AChE of *S. mansoni* is thus demonstrated to be a functional antigen, and a suitable candidate for both diagnostic purposes, vaccine development and drug design.

TABLE II

Toxic effects of phosphonium compounds on *Schistosoma mansoni**

Compound No.	Formula	Adult worms 1×10^{-5} M drug	Schistosomula 1×10^{-4} M drug
1	$[Ph_3PCH_3]Br-$	45 ± 22	36.5 ± 12.9
2	$[Ph_3P(CH_2)_2CH_3]Br-$	55 ± 23	36.3 ± 1.5
3	$[Ph_3P(CH_2)_2]CH_3]Br-$	63 ± 8	35.2 ± 14.8
4	$[Ph_3P(CH_2)Br]Br-$	89 ± 15	93.8 ± 15.1
5	$[Ph_3P(CH_2)_2Br]Br-$	65 ± 29	27.5 ± 2.2
6	$[Ph_3P(CH_3)_3)Br]Br-$	55 ± 13	38.1 ± 11.9
7	$[Ph_3PCH_2CO_2C(CH_3)_33]Br-$	50 ± 15	59.0 ± 13.4
8		100	46.2 ± 18.1
9		0	20.5 ± 3.5
10	$[Ph_3P{=}CHCOCH_3$	0	36.5 ± 2.1
11	$[Ph_3PCH_2CH{=}CH_2]Br-$	100	98.7 ± 2.2
12	$[Ph_3PCH_2CH{=}CHCO_2CH_3]Br-$	88 ± 16	91.7 ± 11.3
13	$[Ph_3PCH-CH{=}CHCO_2CH_3$	52 ± 4	69.5 ± 27.3
14	$[Ph_3PCH_2-CH{=}CH\ CH_2Br]Br-$	100	27.6 ± 19.4
15	$[Ph_3P(CH_2)_3P(C_6H_5)_3]_2Br-$	0	39.5 ± 5.3
16		0	39.0 ± 4.1

* Results are an average of six different experiments performed in cultures of the parasite in defined synthetic medium.

TABLE III

Effect of PIPLC on AChE activity in cultured Schistosomula*

Exp. No.	Age of Schistosomula (h)	AChE activity – PIPLC		AChE activity + PIPLC	
		Sup	Pellet	Sup	Pellet
1	4	56,500	313,400	388,500	1,350,500
2	5	170,900	603,400	1,028,600	1,913,000
3	5	5,500	77,000	62,000	347,000
4	24	42,000	75,200	427,800	1,203,600

* PIPLC (8 μg/ml) was added to the cultured schistosomula (10,000 per experiment) and incubated for 90 (Exp 4) or 120 min (Exp 1, 2, 3), after which the cultured were centrifuged at 10,000 xg. AChE activity was measured in the supernatants as well as in the sonicated pellets. Results are expressed as total cpm of ^{3}H-ACh hydrolyzed.

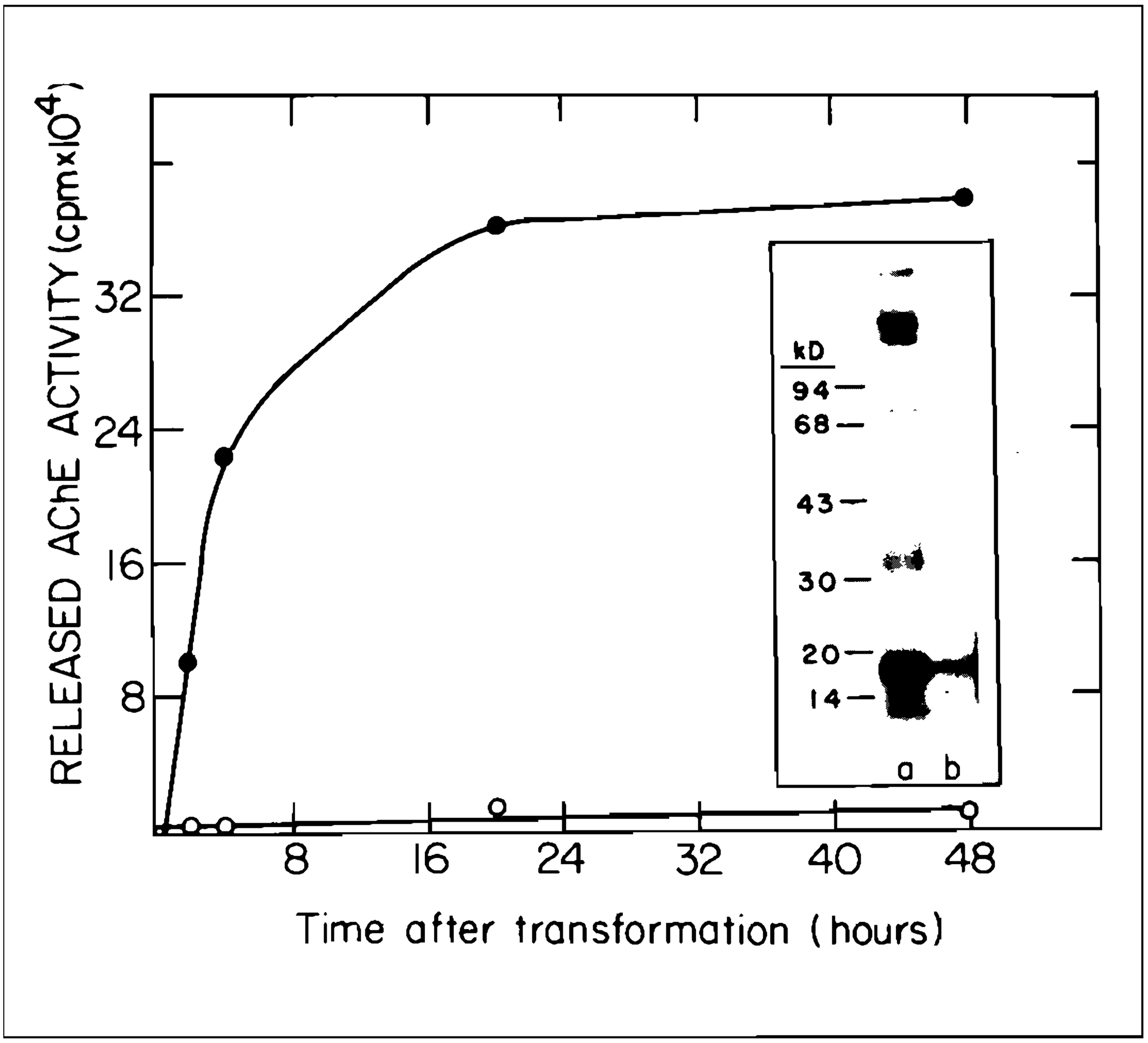


Fig. 5: effect of age of schistosomula on the release of AChE by PIPLC. Schistosomula obtained at different times after transformation were washed and incubated with 8 μg/ml PIPLC for 60 min. Released AChE activity was assayed in the supernatants of these cultures (●—●) as well as in controls without PIPLC (○—○). Insert represents the autoradiograms of SDS-PAGE of the PIPLC-released material (a) from intact 24h schistosomula labeled with ^{125}I by the lactoperoxidase method and of a corresponding control without PIPLC (b).

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