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PRELIMINARY STUDIES WITH A SCHISTOSOMA MANSONI SALINE EXTRACT INDUCING PROTECTION IN RABBITS AGAINST THE CHALLENGE INFECTION

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SUMMARY

Rabbits immunized with total extract of adult **S. mansoni** worms were found to be total or partially resistant to a challenge infection. In fact, in the immunized animal the percentages of protection, as compared with the control, were in two rabbits 100% and in the other 77.0% and 93.4%. This total extract obtained by incubation in 0.15M sodium chloride-phosphate buffer (pH 6.8), containing protein, carbohydrates and nucleic acid and/or by products of the latter component, showed four major fractions by Gel chromatography in G-100 Sephadex column. Sephadex column G-200, resolved fraction I into two or three subfractions and fraction IV into three more subfractions. Immunodiffusion tests with rabbit anti-total extract serum revealed three precipitation lines corresponding to fraction I and II and none with III or IV.

INTRODUCTION

Vaccination against schistosomoses with homogenates has started some 50 years ago. As early as 1930, OSAWA [10] challenged dogs which were previously given intravenous injection either of cercariae or of adult **S. japonicum** worm and recovered fewer worms than in control dogs. The symptomatology was also found to be milder in the vaccinated animals. Similar results were reported by KAWAMURA [4] while vaccination of Rhesus monkeys with preparations of adult **S. japonicum** worms was not successful [20], mice were found to be protected (20 to 50%) by a similar preparation of adult **S. japonicum** homogenate [5,17].

Though various types of extracts from **S. mansoni** adult worms have been further tested in order to induce protection and to elucidate its mechanisms of immunogenicity, attempt to protect rabbits has not been performed [1,8,16].

Reported here are results of preliminary experiments on rabbits immunization with a saline extract of adult **S. mansoni** worms, and partial physico-chemical caracterization of this extract.

MATERIAL AND METHODS

Preparation of the extract

Schistosoma mansoni (LE strain) were maintained at the laboratory in **Biomphalaria glabrata** and Swiss mice. Adult worms were obtained by perfusion of the liver and mesenteric vessels [10] with 0.15M NaCl solution buffe-

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red with sodium phosphate at pH 6.8 (PBS). The worms collected were rinsed briefly in two changes of PBS. Adult worm extract was obtained from 1.0g of fresh worms stored frozen in 10 ml of PBS for 10 days. The suspension was thawed and filtered with a steel net at room temperature. The filtrate was centrifuged at 10,000g for 60 min at 4°C and the supernatant was then kept frozen until use. This extract was referred to as SE. Other extracts were prepared by storing separately male and female worms initially for 30 min and then for 60 min in similar saline buffered solution.

Immunization of animals

Four rabbits were injected subcutaneously twice with 0.6 ml of SE (diluted with PBS to 1 mg protein/ml) emulsified in complete Freund's adjuvant over a 7 days period, followed by a boost of 1 mg of antigen injected intraperitoneally 21 days after. One control rabbit was injected with mouse serum in a similar scheme in order to check contamination by antigens of host origin. Another rabbit was used as an infection control. The rabbits were periodically bleeded for sera collection.

Double diffusion technique

The hyper-immune sera obtained, rabbit-anti-SE (RAS) and rabbit-anti-mouse-serum (RAMS), were tested in double immunodiffusion [11], using SE as antigen. Slides were covered with 2 ml of 1% indubiose in PBS, pH 6.8. Antigen and sera were allowed to diffuse for 24 hours. After diffusion the slides were washed, dried and stained with 0.25% Coomassie Brilliant Blue made up in a solution of 45 ml of metanol, 45 ml of water and 10 ml of glacial acetic acid, rinsed with the same solution and photographed.

Cytotoxic assay of immune sera

Schistosomula were prepared **in vitro** according to RAMALHO-PINTO et al. [13]. Aliquots of 10 ml of intact cercariae (1.500/ml) were cooled in an ice bath for 10 min, centrifuged at low speed for 1 min and the supernatant was decanted. The packed cercariae were suspended in 2 ml of Earle's saline solution containing 0.5% lactalbumin hydrolysate, 0.1% glucose, 200 units of penicillin and 100 mg of streptomycin (Elac) and agitated for 45 sec in a Vortex Jr. mixer. For removing the cercariae tails, 2 ml suspension was transferred to a conical centrifuge tube containing 10 ml of Elac. After 10 min, the tail-rich supernatant was decanted and the sedimented bodies ressuspended in 10 ml of the solution. This procedure was repeated twice.

The resulting bodies, containing less than 5% of tails, were suspended in Elac and incubated under continuous shaking at 37°C for 90 — 120 min, by which the larvae became water-sensitive and were considered to be 2 hr-schistosomula [14]. Cytotoxic assay was carried out according to TAVARES [19]. The 2 hr schistosomula (50/ml) were incubated at room temperature under sterile conditions with 0.1 ml of heat inactivated (30 min, 56.°C) rabbit serum obtained from the four immunized rabbits and the control. After 30 min, larvae were washed three times with Elac, suspended in 0.5 ml of Elac plus 50% rabbit serum and 0.5 ml of either fresh or heat-inactivated guinea pig serum and incubated over-night at 37°C in an humid atmosphere of 5% CO_2. To determine the number of dead or damage schistosomula, aliquots of 0.05 ml — 0.1 ml were counted in a stereoscopic microscope. The results were expressed as percentage of dead or damaged schistosomula per total schistosomula counted [19].

Challenge infection

After 3 to 4 months of the immunization schedule, the 5 rabbits were infected with 700 cercariae percutaneous in the abdominal region [3]. Sixty days later the rabbits were sacrificed and checked by perfusion of the mesenteric and intrahepatic veins for the presence of adult worms [12].

Physico-chemical characterization of SE

The analysis of SE was carried out by column chromatography, spectrophotometry, molecular filtration and protein and sugar dosage.

Column chromatography

SE was fractionated on Sephadex G-100 and G-200 columns (0.9 x 56 cm) as specified. After

equilibration with PBS pH 6.8 0.15 M the samples were eluted with the same buffer. The volume of the extract used in loading the column never exceeded 2% of its total volume (Vt).

Spectrophotometry

SE and the fractions obtained by gel filtration were analysed in the ultraviolet region of the spectrum prior to and after deproteinization obtained by mixing equal volume of SE and TCA (to a final concentration of 0.5M), heating at 89°C for 20 min and centrifuging at 5.000 rpm [9].

Protein content

For protein determinations the LOWRY et al. method [6] and a modification of the biuret method of ELLMAN [2] at 300 nm, were used.

Total contents of sugar

The method of MESSINEO & D'AMRICO [7] at 395 nm was employed to determine the total amounts of aldohexoses and aldopentoses.

RESULTS

Immunization of animals

As can be seen in Table I, the four rabbits immunized with SE presented a significant decrease in the number of **S. mansoni** worms recovered by perfusion 60 days after the challenge infection. In fact, in two immunized rabbits no worms could be found, and in the other two, 3 and 15 worms, respectively, were recovered by the perfusion. The control rabbit presented 65 **S. mansoni** adult worms. The percentages of protection (number of worms found in the immunized rabbit x 100/number in the control) varied from 77 to 100.

TABLE I

S. mansoni worms recovered in rabbits immunized with SE and infected with 700 cercariae

Rabbit	Number of **S. mansoni**	% of protection
1	0	100.0
2	0	100.0
3	3	93.4
4	15	77 0
Control	65	0.0

The RAS sera presented 91% to 100% cytotoxic activity, while the RAMS sera showed no cytotoxic activity in various tests. The cytotoxicity against schistosomula **in vitro** presented by the RAS sera was first detected after 10 days from completion of the immunization scheme and remained at the same high level until the animals were sacrified.

Physico-chemical and immunological characterization of SE

SE presented 0.507 mg/ml of protein, 0.371 mg/ml of carbohydrates and also, nucleotides or nucleic acid material and/or by products of it (Fig. 1). Two or three bands were found in Ouchterlony immunodiffusion test, according to the different batches of SE employed (Fig. 2). The elution profile of SE after fractionation in Sephadex G-100 column can be seen in Fig. 3. Four peaks were observed. The first peak (tube 6) came out with the void volume (Vo). The material eluted in tubes 6, 9,12 and 19 corresponds to FI, FII, FIII and FIV, respectively.

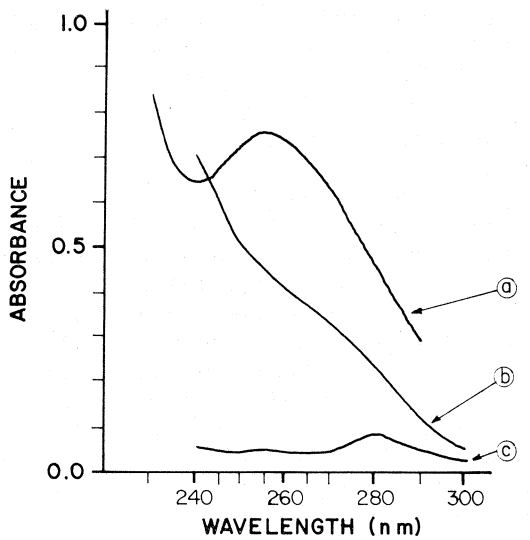

Fig. 1 — The ultraviolet absorption spectra of: SE after deproteinization with TCA at 80°C (a); FIV (b) and FI (c) of SE chromatogram in Sephadex G100

The last three fractions were retained by the column. All of them in similar experiments gave the same average partition coefficient (Kav). As an example, the Kav of FII was in all experiments very close to 0.45.

In Fig. 1 the spectra of FI (Fig. 1c) and FIV (Fig. 1b) are shown. The comparison of

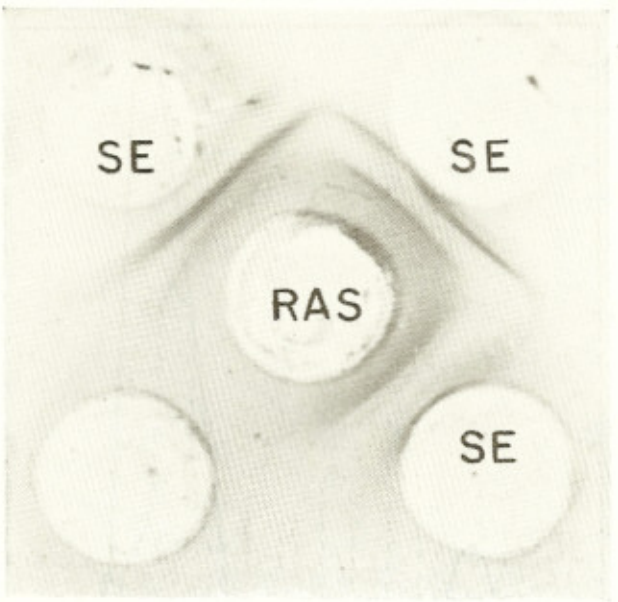

Fig. 2 — An Ouchterlony immunodiffusion plate: RAS — anti SE rabbit serum; SE — different batches of saline extracts.

(a) and (c) in Fig. 1 seems to indicate that all nucleic acid material is contained in FIV.

All fractions contained proteic material. The protein concentration was: FI — 3.5 μg/ml; FII — 4.2 μg/ml; FIII — 1.8 μg/ml and FIV — 3.0 μg/ml.

Carbohydrate analysis indicate that sugars are contained in FI (1.2 μg/ml) FIII (38.5 μg/ml) and FIV (36.6 μg/ml), but are not present in FII.

In Sephadex G-200 column the extract was resolved in additional peaks (Fig. 4). Immunological studies and absence of sugars indicate that the material eluted in tube 14 (Fig. 4) corresponds to FII eluted in tube 9 of Sephadex G-200 column (Fig. 3). Thus, it appears clear that FI in G-200 was resolved in two subfractions by absorbance at 274 nm in sugar analysis. Protein analysis resolved it in three subfractions.

The content of each tube was concentrated approximately fifteen times, after which FI and FII presented precipitation reaction with RAS, while FIII and FIV did not react at all (Fig. 5).

DISCUSSION

The first experiment with **S. mansoni** antigen prepared from mouse adult worms, were used for immunizing mice that showed a statistically significant degree of protection after the challenge infection (WATTS [12]). She used a saline extract of adult worms which had been frozen and dried, after 4 washing procedures. RITCHIE et al. [15] were unable to vaccinate mice using homogenates of cercariae, adult worms and eggs, separately or associated. In rats, protection was observed after consecutive daily injections of saline soluble antigens extracted from lyophilized **S. mansoni**, but control animals that received bovine serum albumin became equally protected [17]. Vaccination of Rhesus monkeys with homogenates of **S. mansoni** cercariae, adult worms or eggs was unsuccessful [18]. More recently, MURREL et al. [8] studied in mice and guinea-pigs different antigens, such as whole worm homogenates, freeze-thawed extract of adult worms, adult worm membrane antigens extracted with hypertonic 3M KCl, and also cercarial exoantigen or cercariae attennuated by ultraviolet irradiation. A variety of adjuvants was also employed, including Freund's complete and incomplete adjuvants, alumen, **Bordetella pertussis** and BCG. Although the authors reported that most animals produced moderate to high levels of cytotoxic antibodies, they could not establish a firm correlation with resistance to infection. Antigens solubilized by 3M KCl and the cercariae exoantigen gave a dis-

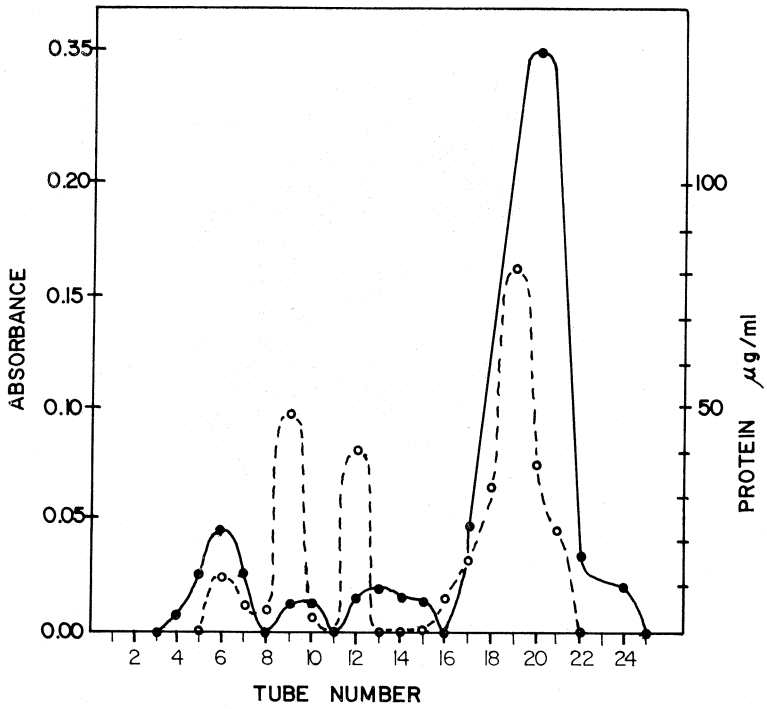

Fig. 3 — Elution profile of SE in Sephadex G100 column (0.9 x 56 cm). Each tube contains 1.6 ml
●-● absorbance at 274 nm
o-o protein content/ml by biuret test

creet degree of protection in one experiment out of three and two, respectively [8].

Based on these and other so far reported data, CLEGG & SMITH [1] in a review about prospects for the development of dead vaccines against helminth claimed "that very few of the attempts to vaccinate experiment animals with homogenates or extracts of larval or adult **S. mansoni** or **S. japonicum** have given more than marginal protection against challenge".

Our here presented results, although preliminary since based on experiments with small number of animals, evidence interesting findings. A high degree of protection was obtained in rabbits using an adult worm **S. mansoni** saline extract (SE). In fact, 2 animals became fully protected and 2 others presented 77.0% and 93.4% of reduction in the number of worms after challenge infection, as compared with the control animal. Furthermore, high cytotoxic activity of hyperimmune rabbit anti-SE serum (91% to 100%) was observed.

SE was obtained by simple keeping **S. mansoni** adult worms in storage fluid frozen during a few days. In other reported experiments, the storage fluid that contains proteins, carbohydrates, nucleotides, nucleic acid material or by-products of it, apparently could have been discarded by the several washings, as referred e.g. by WATTS [22] or MURREL et al. [8] in their antigen preparations. It is interesting to remark that the incubations of worms in saline for only 30 min was sufficient to release antigens, as was demonstrated by immunoprecipitation reaction using RAS (Fig. 6A).

SE contains two and sometimes three components which elicit and immunological response. The results with gel chromatography in column show that SE is heterogenous. The elution through Sephadex G-100 columns resolved SE in four major areas with significant absorbance at 274 nm. Proteins are present in these four areas, carbohydrates in all but fraction II, and nucleic acid bases were found only in fraction IV. Elution through Sephadex G-200 columns (Fig. 4) resolved other peaks in the corresponding absorbing areas of fraction I and IV.

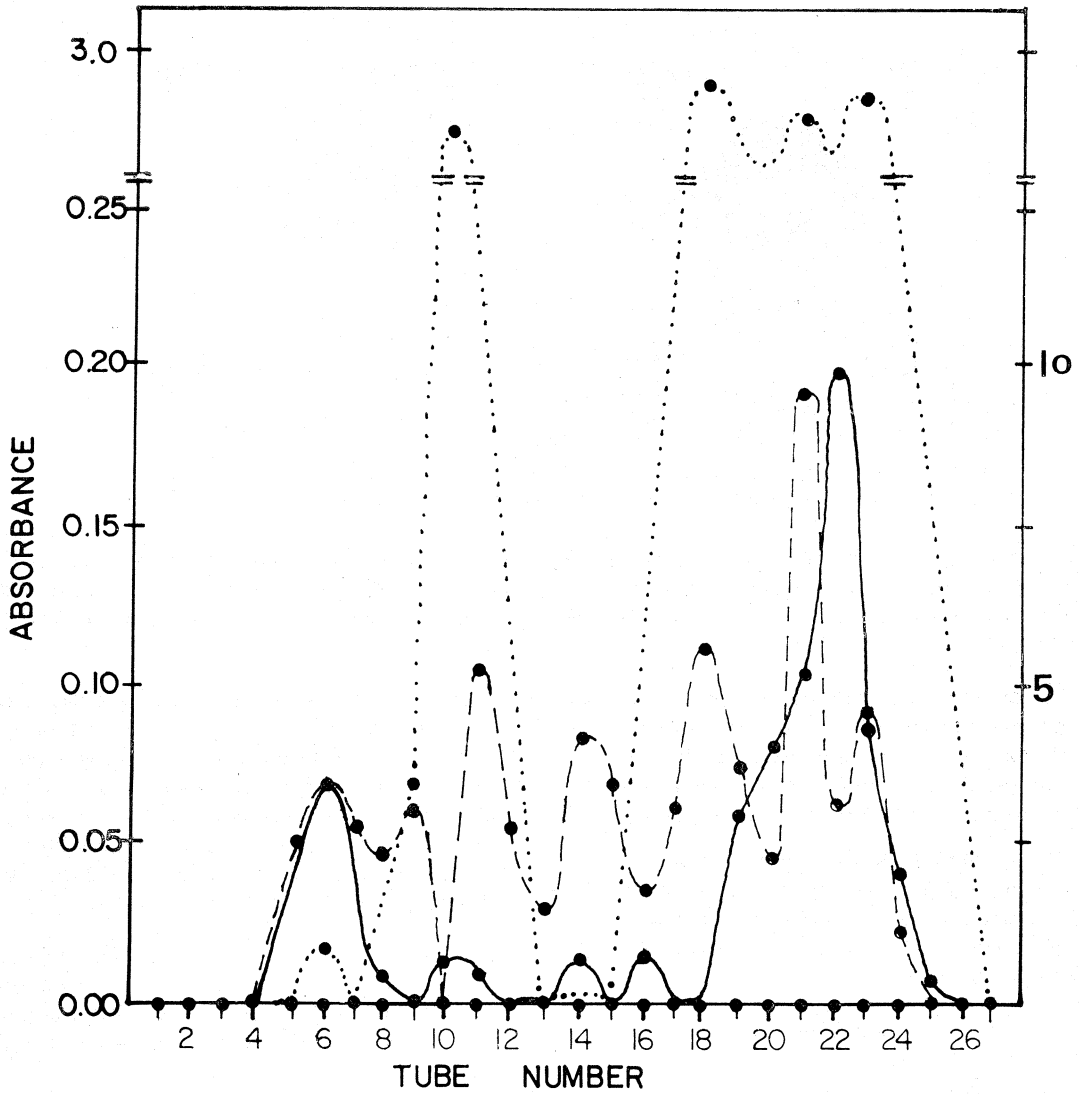

Fig. 4 — Elution profile of SE in Sephadex G200 column (0.9 x 56 cm). The volume of each tube is 1.6 ml
———— absorbance at 274 nm;
- - - - - - - - - - protein content/ml by biuret test;
..... sugars content/ml

Fraction I is eluted by both Sephadex columns with the void volume indicating a very high molecular weight. The other fractions are retained in the bed of Sephadex G-100 and must be of small molecular weight.

In immunodiffusion tests only fractions I and II gave strong precipitations lines with the rabbit anti-sera (Fig. 5). Two bands with these fractions were also observed using human sera from schistosomotic patients as antisera, in IEOP (Fig. 6 B). A lot of factors must be explored before final conclusion can be made about the value of SE as a vaccine for schistosomiasis mansoni. We to not know if the protection observed is due to the antigen alone, to the adjuvant or to the host peculiarities, or to the association of all these factors. Rabbit is considered as mediocre host for **S. mansoni** infection [21] based on the relatively low number of worms found in these animals at different periods after exposure, and also on the low number of mature **S. mansoni** eggs found in the faeces and intestinal walls. WARREN & PETERS [21] demonstrated that 4 weeks after the

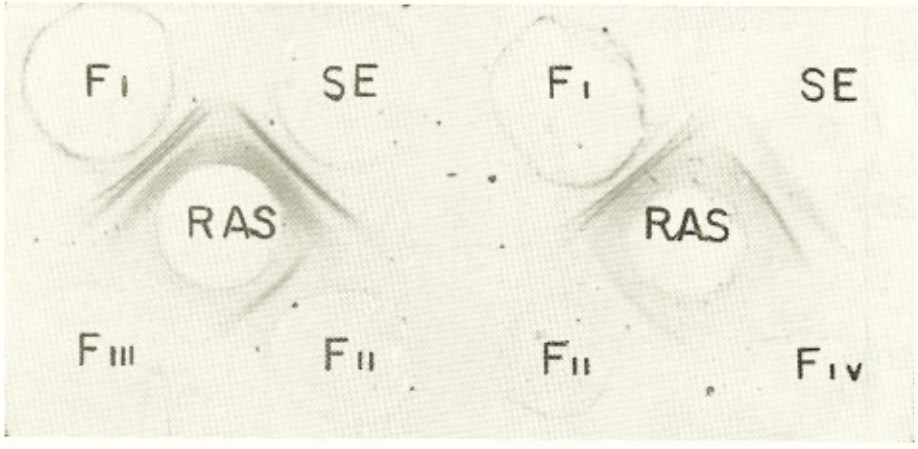

Fig. 5 — Immunodiffusion plate: RAS: rabbit anti-SE serum; FI,FII,FIII,FIV; SE components eluted in tubes 6,9,12 and 20, respectively from Sephadex G100 column.

maturation of the worms, in relation to the number of cercariae that penetrated, was 35.6% in rabbit, but after 8 and 16 weeks of infection, 15.6% and 6.4%, respectively.

Is SE only diminishing the period of "self cure" that one will expect to occur in these animals? On the other hand, what is the best animal model for immunization studies as compared to human: mice, rat, monkey or rabbit?

These questions, are now in study in our laboratory encouraged by the good results so far obtained with the saline **S. mansoni** adult worm extract that protected rabbits from the challenge infection.

RESUMO

Estudos preliminares com um extrato salino de Schistosoma mansoni **que induz proteção em coelhos**

Coelhos imunizados com extrato total de vermes adultos de **S. mansoni** apresentaram resistência total ou parcial quando infectados pelo **S. mansoni.** De fato, nos animais imunizados as porcentagens de redução do número de vermes em relação ao controle, foram de 100% em dois animais e nos outros dois de 77,0% e 93,4%.

Este extrato de vermes adultos obtidos por incubação em solução salina tamponada (pH 6,8), continha proteínas, carbohidratos e ácidos nucleícos e/ou derivados destes. Por cromatografia em coluna (Sephadex G-100) foram isoladas 4 frações. Utilizando-se a coluna de Sephadex G-200 a fração I mostrou ter 2 ou 3 subfrações e a fração IV, 3 subfrações.

Os testes de imunodifusão utilizando-se soro de coelho imunizado com este extrato total revelou 3 linhas de precipitação correspondentes as frações I e II e nenhuma com as frações III e IV.

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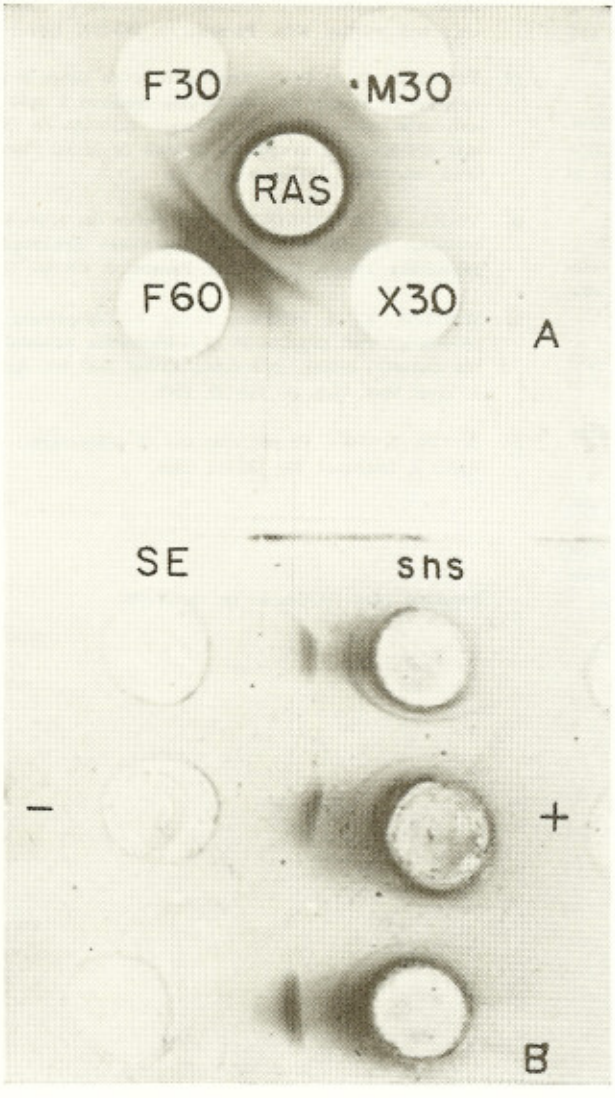

Fig. 6 — A) Immunodiffusion plate: RAS — rabbit anti-SE serum; M30 — extract from adult male schistosomes with 30 min. of incubation in saline solution; F30 and F60 — extracts from adult female schistosomes with 30 or 60 min. of incubation in saline solution; X30 — extract from a mixture of female and male schistosomes with 30 min. of incubation in saline solution. B) Immunoelectroosmophoresis (IEOP) plate: shs — schistosomotic human serum.

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