Rev. Inst. Med. trop. São Paulo
19(3):182-190, *maio-junho*, 1977

UDC 593.161.13

TRYPANOSOMA CRUZI SOLUBLE ANTIGEN. ROLE IN IMMUNOFLUORESCENCE, COMPLEMENT FIXATION AND INHIBITION OF HEMAGGLUTINATION TESTS

Maria Carolina Soares GUIMARÃES (1), Maria Tereza RIBEIRO (2) and Mario E. CAMARGO (1)

SUMMARY

Live **Trypanosoma cruzi** (Y strain) epimastigotes were extracted with phosphate buffered saline for 24 and 48 hours. Supernatant was positive for protein and sugar content. Immunodiffusion tests against rabbit anti-**T.cruzi** serum showed 2 lines which stained for protein and 2 lines which stained for glycoprotein. Analytical polyacrylamide gel electrophoresis showed 7 discs with Coomassie Brilliant Blue and 7 discs with Schiff's reagent and no staining for lipoproteins and mucopolisaccharides. The antigen was able to inhibit immunofluorescence and hemagglutination tests and to form complement fixing complexes with human and rabbit sera. Immunofluorescence tests, using the extracted parasites as antigen, showed greater loss ofmembrane staining after 48 hours extraction, when compared with non-extracted flagellates. After extraction, most parasites although alive displayed morphological changes.

INTRODUCTION

Glycoproteins are found as normal constituents of the cell membrane and have been shown to be present in exoantigens of **T. cruzi** [9] bearing complement fixation activity. PARDOE et al. [11] were able to extract from **Leishmania enrietti** a glycoprotein able to form complement fixing antigen-antibody complexes and inhibit hemagglutination tests.

In the present paper we have studied a glycoprotein complex obtained by saline extraction of **T. cruzi** epimastigotes capable of inhibiting immunofluorescence and hemagglutination tests performed with human sera and forming complement fixing immune complexes with both human and hyperimmune sera.

MATERIAL AND METHODS

Trypanosoma cruzi (Y strain) [12] epimastigotes grown in LIT medium [8] at 28.°C for 7 days were used throughout the study.

Saline extracted antigen (PBS-Ag)

Epimastigotes (1.14 x 10^9 cells/g of wet weight) were removed from culture medium and washed 3 times in PBS (phosphate buffered saline, 0.01 M, pH 7.2) spun down at 1,500 x g for 10 minutes at 4°C and the sediment parasites were extracted with PBS (1 g of wet sediment in 100 ml of PBS) at 4°C for 24 hours or 48 hours. Suspension was spun down at 39.000 x g/ 10 minutes/4°C. Supernatant was concentrated in a PM10 membrane filter (Amicon Corp.) to a final volume of about 2 ml. Protein content was assayed by the lowry method [10], human globulin taken as reference and sugar content by the anthrone method [17], glucose taken as reference.

SDS-extracted antigen

One hundred mg of lyophilized epimastigotes were submitted to treatment with an absolute alcohol-ether mixture and suspended in 4.0 ml of PBS, pH 8.0 at varying con-

(1) Professor Assistente, Instituto de Medicina Tropical de São Paulo, São Paulo, Brasil
(2) Técnica do Laboratório de Imunologia do Instituto de Medicina Tropical de São Paulo

centrations of SDS (sodium dodecyl sulfate) according to ZANETTA & GOMBOS[20]. The suspension was incubated at 28°C for 5 hours, dialyzed in several changes of PBS for 24 hours and concentrated to a final volume 2 ml. Protein was assayed by the Lowry method, and sugar by anthrone.

Immunofluorescence tests (IF)

Indirect immunofluorescence tests were performed according to CAMARGO[3]. Two kinds of antigens were used fixed to microscope slides: a) epimastigotes after PBS extraction (designated EA-IF) and b) non extracted epimastigotes grown for 7 days in LIT medium. In all tests and anti-human gammaglobulin conjugate (F/P = 8) was used.

Blocking of immunofluorescence tests with PBS-Ag

A human serum used in the laboratory as a positive standard for American Trypanosomiasis immunofluorescence tests (titer 1/320) was diluted 1/10, 1/50 and 1/100, and each dilution absorbed with a v/v amount of undiluted PBS-Ag overnight, at 4°C and used for immunofluorescence tests with both EA-IF and non-extracted flagellates.

Blocking of immunofluorescence tests with SDS-extracted antigen

The same human standard serum at the same dilutions as above was used. Equal volumes of diluted serum and either 4%, 2%, 1%, 0.5% or 0.1% dilutions of SDS-extracted antigen were mixed and left to stand at 4°C overnight. Absorbed serum was used for immunofluorescence tests with both EA-IF and non-extracted flagellates.

Immunodiffusion

Tests were done in 0.9% agar in 0.15M saline. Peripheral wells contained either PBS-Ag or SDS-extracted antigen and the center well contained a rabbit anti-**Trypanosoma cruzi** (Y strain) serum. Precipitation proceeded for 24/48 hours in a moist chamber at room temperature. Plates were washed in several changes of PBS for 24 hours. After drying, gels were stained for protein with Light Green[16] and for glycoprotein with p-phenylhydrazine HCl[16].

Inhibition of hemagglutination

Serum from an American Trypanosomiasis patient with a titer of 1/160 in "Chagas-HA test"[5] was diluted two-fold (1/10 on) in a plastic V well plate in 25 μl volumes. To each well, one drop of PBS-Ag was added, gently mixed and left overnight at 4°C. Controls and blanks were run in each experiment. One drop of formalin treated Chagas-sensitized human red blood cells was then added to each well. Readings were done after 60 minutes of incubation in a moist chamber at room temperature.

Complement fixation tests

Performed in plastic U-shaped well plates with one drop volumes (25 μl) of serum, complement and antigen diluted in triethanolamine buffer. Dubling dilutions of rabbit anti-**T. cruzi** serum (starting dilution 1/10) 4 CH50, and doubling dilutions (starting dilution 1:2) of PBS-Ag were used. Plates were incubated for 18 hours at 4°C and 30 minutes at 37°C. One drop of hemolysin-sensitized sheep red blood cells was added to each well. Plates were incubated for 60 minutes at 37°C with occasional agitation, re-incubated for 2 hours at 4°C and read immediately afterwards. Standards for positive and negative sera, complement and anticomplementary activity of 1:10 dilutions of sera were included. Hemolysis of 50% or less was taken as a positive result.

Analytical polyacrylamide gel electrophoresis (PAGE)

PBS-Ag was submitted to electrophoresis in the presence of sodium dodecyl sulfate according to WEBER & OSBORN[18]. Quadruplicate samples were removed from electrophoresis chamber when Bromophenol Blue had reached the bottom of the tube. Individuals samples were stained for protein with Coomassie Brilliant Blue[6], for glycoprotein with Schiff reagent[19], for lipoproteins with Sudan Black B[13], and for acid mucopolysaccharides with Toluidine Blue O[14].

Electron microscopy

The parasites were concentrated by low speed centrifugation and fixed in 2% glutaraldehyde buffered in 0.2 M phosphate, pH 7.2 for 30 minutes. After several changes in the same buffer plus 4% sucrose, the specimen was post-fixed in 1% Os O_4 for one hour, dehydrated and embedded in Araldite. The sections were stained with lead citrate and uranyl acetate and examined in a Zeiss EM 9A electron-microscope.

RESULTS

PBS-Ag

Extracts had an average concentration of 277 μg/ml of sugar and 153 μg/ml of protein after 24 hours extraction and 273 μg/ml and 310 μg/ml after 48 hours extraction. At the end of this extraction period roughly 70% of trypanosomes were still alive.

SDS-extracted Ag

At 4% concentration the SDS-extract had 8.7 mg/ml of sugar and 14.1 mg/ml of protein. Yields in glycoprotein and protein decreased with lesser concentrations of SDS.

Immunofluorescence tests

EA-IF displayed morphological changes on immunofluorescence tests when compared to tests performed with non-extracted flagellates, parasites being narrower or wider than non-extracted ones. For the same serum dilution, EA-IF showed loss of membrane staining as compared to the bright membrane staining of non-extracted flagellates (Figs. 1, 2 and 3) and staining intensity decreased with extraction time, changes being more noteworthy with parasites extracted for 48 hours (Fig. 3). This loss was accompained by a fall in standard serum titer from 1/320 to 1/80 for 24 hours extracted flagellates and to 1/20 for 48 hours-extracted flagellates. Immunofluorescence results (expressed as percent inhibition) of tests performed with standard serum absorbed with PBS-Ag and SDS-extracted antigen, with EA-IF and non-extracted flagellates fixed to microscope slides are shown in Fig. 8.

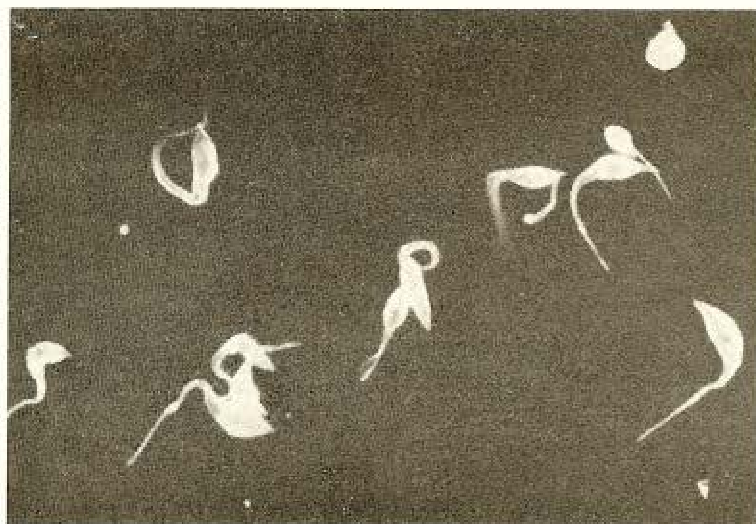

Fig. 1 — Epimastigotes showing characteristic membrane staining (400 X).

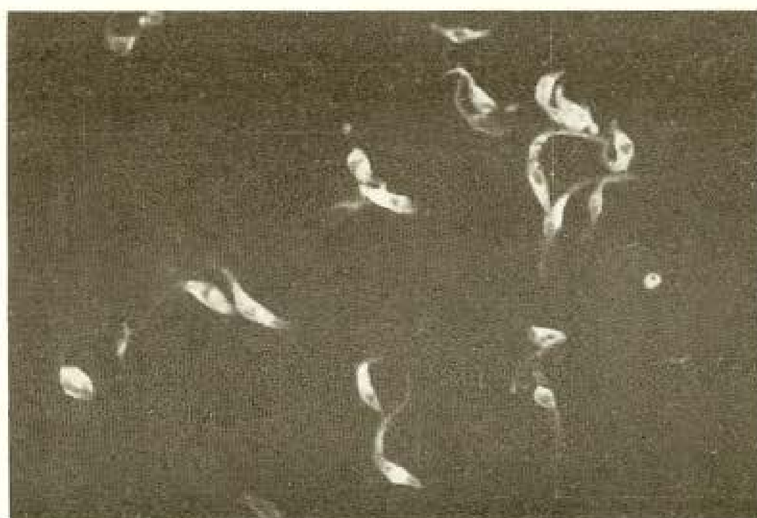

Fig. 2 — IF staining with 24-hours extracted epimastigotes. Serum at the same dilution in a Fig. 1. Intracytoplasm staining, posterior end evagination and loss of membrane staining are seen (400 X).

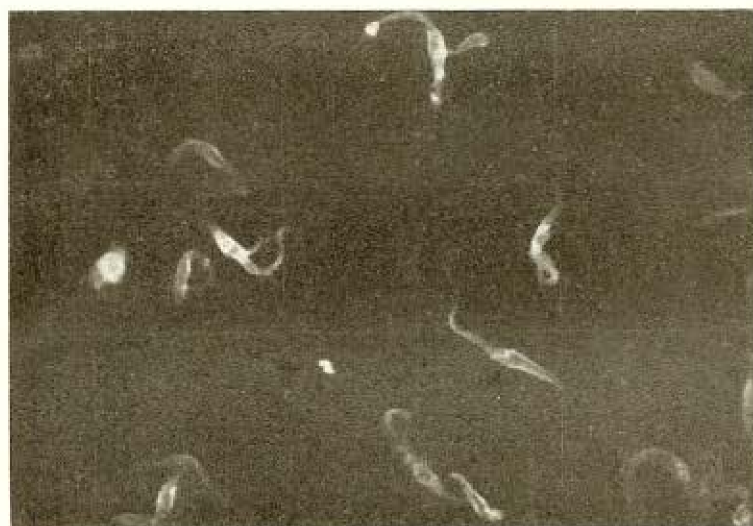

Fig. 3 — IF staining with 48 hours-extracted epimastigotes. Serum at the same dilution as in Figs. 1 and 2 (400 X).

Immunodiffusion

PBS-Ag displayed 2 precipitin lines against rabbit anti-**T. cruzi** (Y strain) serum with protein stain and 2 lines with glycoprotein stain (Figs. 4 and 5). One of the protein-stained lines showed non-identity with SDS-Ag.

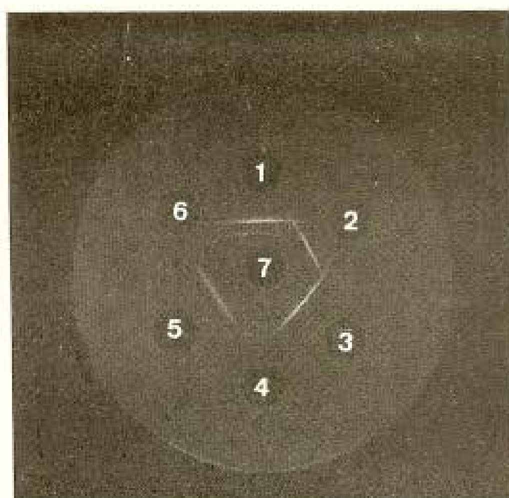

Fig. 4 — ID of PBS-Ag, protein stain: well 1, 3 and 5 — PBS-Ag extract 1; well 4 and 6 — PBS-Ag extract 5; well 2 — 4% SDS — antigen; well 7 — rabbit anti-**T. cruzi** serum.

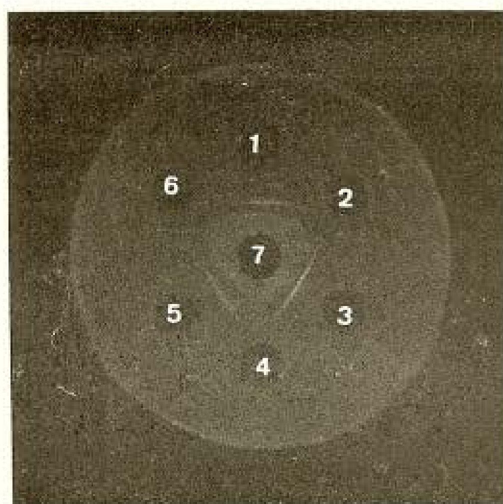

Fig. 5 — ID of PBS-Ag, glycoprotein stain; well 1, 3 and 5 — PBS-Ag extract 1; well 4 and 6 — PBS-Ag extract 5; well 2 — 4% SDS — antigen; well 7 — rabbit anti-**T. cruzi** serum.

Inhibition of hemagglutination

PBS-Ag was able to inhibit hemagglutination tests. The titer from a positive-standard serum fell from 1/160 to 1/10 titer which is regarded as non-specific according to the technique used. Sensitization of erythrocytes for HA tests with the PBS-Ag was not successful.

Complement fixation

PBS-Ag gave maximal complement fixa-
tion to the dilution of 1/256 against a rabb
anti-**T. cruzi** serum and of 1/2 against a hu-
man positive serum.

PAGE

PBS-Ag did not stain for acid mucopol
saccharide and lipoproteins.

Coomassie Brilliant Blue showed 7 dis
of increasing electrophoretic mobility. Stai
ing for glycoprotein coincided with prote
staining (Fig. 6).

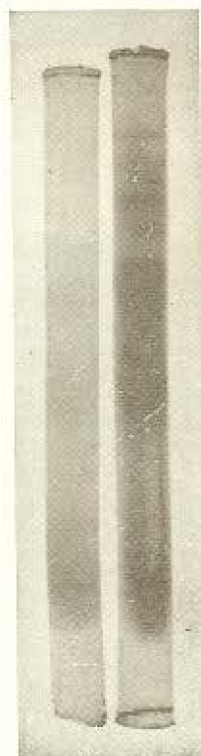

Fig. 6 — Polyacrylamide gel electrophoresis of PBS-A
Anode at the botton. Protein staining at right a
glycoprotein staining at left.

Electron microscopy

Flagellates showed an empty evaginati
at the posterior end, poorly preserved cyt
plasm, overall lack of organelle definition a
increased number of vacuoles (Fig. 7).

Fig. 7 — **Trypanosoma cruzi** showing evagination (E) at the posterior end. Cytoplasm is filled with vacuoles (V). Cytostome (C) and flagellum (F) are also shown. (45,000 X).

DISCUSSION

Removal by PBS from **T. cruzi** membrane of a mixture of proteins endowed with antigenic activity resulted in evident degenerative changes in the parasites, as described above. The "neck-like" evagination seen in extracted parasites in electron microscopy (Fig. 7) as well as in IF tests (Figs. 2 and 3) is thought to be due to a weakness in the membrane caused by the extraction procedure in a region where the number of microtubules is low since, according to BRACK[2] in order to allow for the tappered shape of the posterior end, the number of microtubules at that region decreases to 3 or 4. These changes, nevertheless, were compatible with life since after 48 hours extraction 70% of trypanosomes were still alive and could be cultured in LIT medium for at least 24 hours in the presence of 50,000 units of penicillin per milliliter of culture medium. The substances removed from the membrane were proteins, partly constituted by glycoproteins. Removal of the latter from the membrane did not increase proportionally with extraction time as total protein did and this discrepancy could be due to leakage of cytoplasmic protein(s) through membrane "holes" made by PBS extraction.

The antigen(s) removed by PBS seem to be the one(s) responsible for immunofluorescence tests since a human standard serum with a previous titer of 1/320 when titrated with 24 and 48 hours-extracted flagellates showed a reduction in titer of 75% and 93.5% respectively (Fig. 8). also, at a given dilution, intensity of staining was much lower for 48 hours-extracted flagellates than for 24 hours-extracted ones which on its turn was also lower than staining intensity displayed by non-extracted parasites.

SDS-extracted antigen and PBS-Ag share some but not all antigenic determifluorescence, since lesser concentration of nants, as shown by inhibition of immuno-SDS did not inhibit immunofluorescence to the extent PBS-Ag did. This point is reinforced by the finding that although all SDS-extracts had a higher protein and sugar content than PBS-Ag, when EA-IF and non-

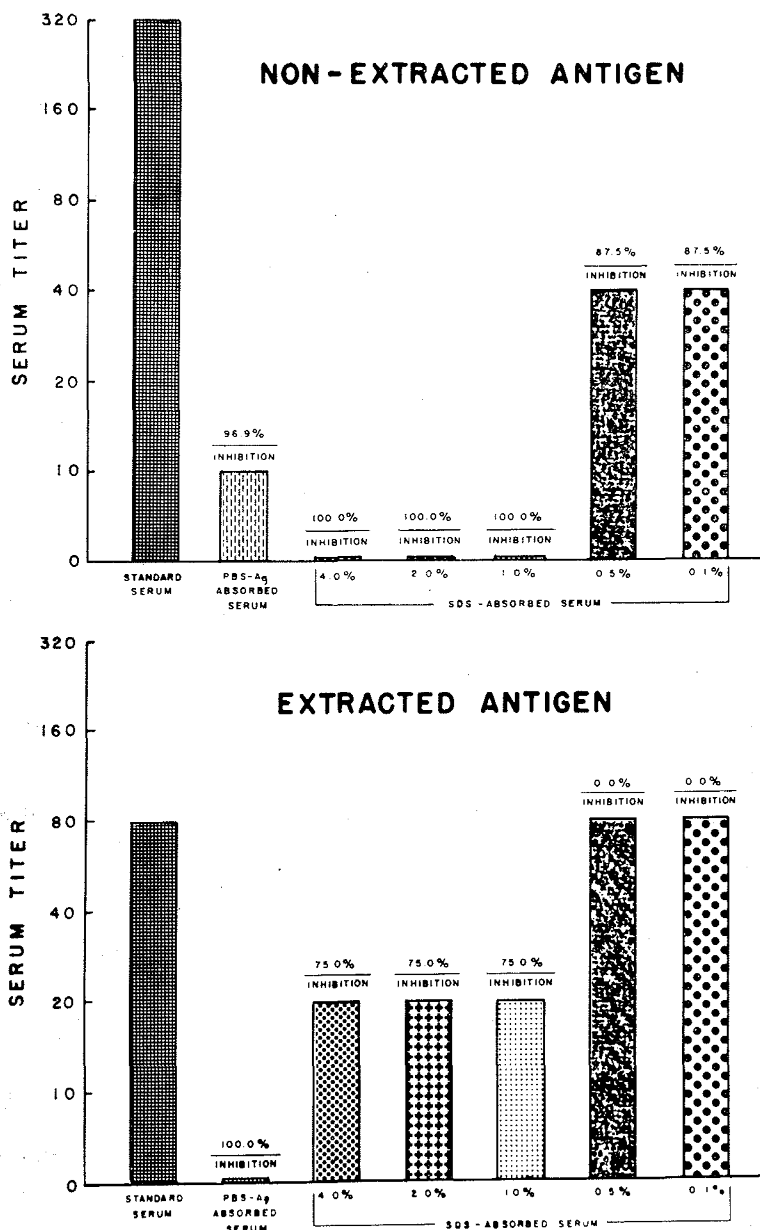

Fig. 8 — Comparison of IF-titer, expressed as percent of fluorescent inhibition, with human serum absorbed with PBS-Ag, and SDS-antigen. Fixed to the slides were non-extracted and extracted flagellates (EA-IF).

extracted flagellates results were compared, PBS-Ag inhibited immunofluorescence 96.9% and 100% respectively whereas SDS antigens inhibited immunofluorescence tests to a lesser extent or none at all (Fig. 8).

The antigen(s) extracted from the membrane was (were) able to form complement fixing Ag-Ab complexes with human and hyperimmune sera but unable to sensitize HRBC for hemagglutination tests although able to

inhibit hemagglutination. This is an interesting finding since according to previous paper separation of hemagglutination and complement fixation activities of **T. cruzi** extracts had been achieved only through chromatographical procedures [1,4,9].

RESUMO

Antígeno solúvel de Trypanosoma cruzi. **Seu papel nas reações de imunofluorescência, fixação do complemento e inibição de hemaglutinação**

Epimastigotas vivos de **Trypanosoma cruzi** (cepa Y) foram extraídos com salina tamponada por fosfatos, por 24 e 48 horas. No sobrenadante após extração, houve reação positiva para as proteínas e açúcares. Reação de imunodifusão usando-se soro de coelho anti-**Trypanosoma cruzi** mostrou duas linhas com coloração positiva para proteínas e duas com coloração para glicoproteínas. Eletroforese analítica em gel de poliacrilamida mostrou sete discos com Coomassie Brilliant Blue coincidentes com a coloração para glicoproteínas. O antígeno se mostrou capaz de inibir reações de imunofluorescência e hemaglutinação e de formar imunecomplexos fixadores de complemento com soros humanos e de coelho. Os testes de imunofluorescência usando-se os flagelados extraídos como antígeno, mostraram maior decréscimo de título quando comparados com antígenos feitos com parasitas não extraídos. Após, extração, a maioria dos parasitas embora vivos mostravam alterações morfológicas.

ACKNOWLEDGEMENT

The Authors which to thank Dr. Regina V. Milder for the electron microscopy data. The presente work was in part supported by grant SIP/08-059 from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

REFERENCES

1. BERGENDI, L.; KNIERIM, F. & APT, W. — **Trypanosoma cruzi**: immunological properties of a soluble extract of culture forms. **Exp. Parasitol. 28**: 258-262, 1969.

2. BRACK, C. — Elektronenmikroskopische Untersuchungen zum Lebenszyklus von **Trypanosoma cruzi. Acta Trop. 25**: 289-356, 1968.

3. CAMARGO, M. E. — Fluorescent antibody test for the serodiagnosis of American trypanosomiasis. Technical modification employing preserved culture forms of **Trypanosoma cruzi** in a slide test. **Rev. Inst. Med. trop. São Paulo 8**: 227-234, 1966.

4. CAMARGO, M. E.; GUIMARÃES, M. C. S.; PERES, B. A. & HOSHINO-SHIMIZU, S. — Antigenic fractionation and the development of new antigens. In: International Symposium on «New Approaches in American Trypanosomiasis Research, Belo Horizonte. **Ann. Amer. Tryp. Research 318**: 227-231, 1975.

5. CAMARGO, M. E.; HOSHINO, S. & SIQUEIRA, G. R. V. — Hemagglutination with preserved sensitized cells, a practical test for routine serologic diagnosis of American Trypanosomiasis. **Rev. Inst. Med. trop. São Paulo 15**: 81-85, 1973.

6. CROSS, G. A. M. — Identification, purification and properties of clone-specific glycoprotein antigens constituing the surface coat of **Trypanosoma brucei. Parasitology 71**: 393-417, 1975.

7. DAVIS, B. J. — Disc electrophoresis. II — Method and application to human serum proteins. **Ann. N.Y. Acad. Sci. 121**: 404-427, 1964.

8. FERNANDES, J. F. & CASTELLANI, O. — Growth characteristics and chemical composition of **Trypanosoma cruzi. Exp. Parasitol. 18**: 195-202, 1966.

9. GONZALEZ-CAPPA, S. M. & KAGAN, I. G. — Antigenicity of fractions of a somatic antigen of **Trypanosoma cruzi. J. Parasitol. 59**: 1080-1084, 1973.

10. LOWRY, O. H.; ROSENBROUGH, N. J.; FARR, A. L. & RANDALL, R. J. — Protein measurement with the folin phenol reagent. **J. Biol. Chem. 193**: 265-275, 1951.

11. PARDOE, G. I.; JAQUET, H.; HAHN, R. & WOJNAROWSKI, W. — The immunochemistry of surface antigen of **Leishmania enrietti. Behring Institute Mitteilungen 58**: 30-39, 1975.

12. PEREIRA DA SILVA, L. H. & NUSSENZWEIG, V. — Sobre uma cepa de **Trypanosoma cruzi** altamente virulenta para o camundongo branco. **Folia Clin. Biol. 20**: 191-208, 1953.

13. PRAT, J. P.; LAMY, J. N. & WEILL, J. D. — Coloration des lipoprotéines aprés électrophorèse en gel de polyacrylamide. **Bull. Soc. Chim. Biol.** (Paris) **51**: 1367, 1969.

14. RENNERT, O. M. — Disk electrophoresis of acid mucopolysaccharides. **Nature** (London) **213**: 1133, 1967.

15. TARRANT, C. J.; FIFE Jr., E. H. & ANDERSON, R. I. — Serological characteristics and general chemical nature of the **in vitro** of **T. cruzi. J. Parasitol. 51**: 277-285, 1965.

GUIMARÃES, M. C. S.; RIBEIRO, M. T. & CAMARGO, M. E. — **Trypanosoma cruzi** soluble antigen. Role in immunofluorescence, complement fixation and inhibition of hemagglutination tests. **Rev. Inst. Med. trop. São Paulo 19**:182-190, 1977.

16. URIEL, J. — Color reactions for the identification of antigen-antibody precipitates in gels. In: Williams, C. A. & Chase, M. W. (ed.) Vol. III. **Methods in Immunology and Immunochemistry.** New York, Academic Press, 1971, p. 294.

17. VILLELA, G. G.; BACILA, M. & TASTALDI, H. — **Técnicas e Experimentos de Bioquímica.** Rio de Janeiro, Guanabara-Koogan, 1973, p. 137.

18. WEBER, K. & OSBORN, M. — The reliability of molecular weight determinations by dodecyl-sulfate-polyacrylamide gel electrophoresis. **J. Biol. Chem. 244:** 4406-4412, 1969.

19. ZACHARIUS, R. M.; ZELL, T. E.; MORRISON, J. H. & WOODCOCK, J. S. — Glycoprotein staining following electrophoresis on acrylamide gels. **Analyt. Biochem. 30:** 148-152, 1969.

20. ZANETTA, J. P. & GOMBOS, G. — Affinity chromatography on Con A-Sepharose of synaptic vesicle membrane glycoproteins. **FEBS Letters 47:** 276-278, 1974.

Recebido para publicação em 15/12/1976.