Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. 83(4): 513-517, out./dez. 1988

ANALYSIS OF *TOXOPLASMA GONDII* PROTEINS AFTER TRITON X-114 SOLUBILIZATION AND HYDROPHOBIC CHROMATOGRAPHY

SALVATORE GIOVANNI DE SIMONE*/**, HELENA C. B. GUEDES* & IZIDRO BENDET*

* Instituto Oswaldo Cruz, Departamento de Imunologia, Caixa Postal 926, 20001 Rio de Janeiro, RJ, Brasil
** Departamento de Biologia Celular e Molecular, Universidade Federal Fluminense, Niterói, RJ, Brasil

The distribution of the surface proteins of Toxoplasma gondii *radiodinated were studied using the phase separation technique and ability of binding in the phenyl-Sepharose column. Eight polypeptides with Mr 22 to 180 distributed exclusively in the detergent rich-phase, while six polypeptides with mol. wt. 15,000 to 76,000 distributed exclusively in the detergent poor-phase. Two polypeptides with 15,000 and 70,000 distributed in both phase. All the polypeptides present in the detergent rich-phase binding in the phenyl-Sepharose column, and can be isolated in two peak according with their relative hydrophobicities.*

Two polypeptides hydrophobic with Mr 60 and 66 recognized by human serum were isolated by the association of the two technique. Our result showed that the surface proteins of T. gondii *present different degrees of hydrophobicity and that the use of hydrophobic interaction chromatography after Triton X-114 extraction may be an important isolation method of membrane proteins.*

Key words: *Toxoplasma gondii* – Triton X-114 – hydrophobicities – membrane proteins – hydrophobic chromatography

Toxoplasma gondii is a coccidian protozoan which represents one of the major etiological agent of protozoan infections. Current biochemical research on this parasitic disease is concerned with isolation of antigens involved in the immune response of the host, and the search for specific antigens for diagnostic purpose. Extraction of *T. gondii* radiodinated proteins with detergent, such as deoxycolate, SDS or Triton X-100 yelded antigen preparations which were used for immunoprecipitation studies (Handman et al., 1980; Johnson, 1985) or protein profiles (Johson et al., 1981, 1983, Kasper et al., 1983). These detergents permit the solubilization of most of the membrane proteins, however, do not discriminate between proteins which strongly interact with the membrane lipid bilayer. Previous studies have shown that tachyzoites of *T. gondii* are surrounded by a typical plasma membrane and that below it there is a complex formed by two opposed unit membranes (Cintra & De Souza, 1985). Besides this, the presence of surface carbohydrates have not been shown (Handman et al., 1980; Hoshino-Shinizu et al., 1980; Cintra et al., 1986). These observations are of interest since these parasites seem to represent an exception to general idea that all eucaryotic cells have glycoconjugates associated to the plasma membrane. We used two different hydrophobic isolation techniques in order to learn more about the plasma membrane proteins of tachyzoite.

MATERIAL AND METHODS

Parasite – The virulent RH strain of *T. gondii* was grown in the peritoneal cavities of mice and the tachyzoites were purified from peritoneal exudates cells using an polycarbonate membrane (Nuclepore Corporation Plasanton California, USA) as described by Dahl & Johson (1983).

Radiodination procedures – Tachyzoite surface proteins were radiolabelled in accordance with Markwell & Fox (1978) by incubation 1-2 x 10^8 washed viable parasites in cell culture medium (RPMI medium 1640, Grand Island Biological Co., NY, USA) with 500 μCi of iodine ($Na^{125}I$, Amersham, UK) and 125 μg of glycoluril (Iodo-gen, Pierce Chemical Co., USA) for 10 min at 4 ^{o}C.

Supported by the National Council for Scientific and Tecnological Development, Brasil – CNPq.

Receveid May 23, 1988.
Accepted September 26, 1988.

Detergent solubilization and phase separation – The iodinated cells were solubilized in 1% Triton X-114 (Sigma Chemical Company, St. Louis, USA) precondensed twice as described by Bordier (1981) in Tris-saline (TS) buffer (10 mM Tris, 150 mM NaCl pH 7.4) containing 1 mM PMSF, 1 mM EDTA and 2 mM iodoacetamide for 30-40 min at 0-4 °C after washing (four times 1000 g, 5 min). Supernatants, were collected and subjected to phase separation according to Bordier (1981). Amphiphilic and hydrophilic proteins were obtained in the detergent rich-phase (DRP) and detergent poor-phase (DPP) respectively as described earlier (Bordier, 1981).

Phenyl-Sepharose chromatography – As the high ionic strength lower the cloud point of Triton X-114, the samples DRP and DPP were previousely mixed with the same volume of Triton X-100 (10%) to prevent the condensation of micelles (Bordier, 1981). Thereafter, the aqueous and detergent phases of radiodinated tachyzoites were adjusted to 0.05% of detergent by dilution in TS buffer containing 0.8 M ammonium sulfate (TSA). The samples were applied to a column (0.9 x 22 cm) of phenyl-Sepharose CL-4B (Pharmacia Fine Chemical, Uppsala, Sveden) previousely equilibrated in TSA. The columns were washed successivelly with 40 ml TSA and with 40 ml TS buffer. The adsorbed proteins were eluted with a linear gradient of 0.1% to 2% Triton X-100 (60 ml each) in TS buffer. Radioactivity in each fraction was measured using a Beckman γ-counter. Triton X-100 concentration in each fraction was estimated by absorbance using E = 2.32 (Robison & Tanford, 1975).

SDS-gel electrophoresis and autoradiography – SDS-PAGE was performed as described by Laemmli (1970) and after silver staining (Niellsen & Brown, 1984), the gels were autoradiographed using XAR-5X-ray film and Kodak-X-O-Matic regular intensifying screens. Molecular weights were estimated by comparasion with gels loaded with proteins of known molecular weight (Sigma Chemical Co., USA, MW-SDS-70 and 200 kits).

Immunoprecipitation – Immunoprecipitation of the amphiphilic proteins was accomplished by overnight incubation with 50 μl of neat human convalescent serum, (indirect immunofluorescence 1:4056) followed by a 30 min incubation at room temperature with 70 μl of a 10% suspension of *Straphylococcus aureus*. The immunocomplex-coated *Staphylococci* were washed twice with 10 mM Tris-HCl buffer, pH 8.6, containing 0,1% SDS (w/v) and 0,3 M NaCl. The precipitates were resuspended in SDS-PAGE sample buffer (Laemmli, 1970) centrifuged at 10,000 g for 30 min and the supernatants subjected to SDS-PAGE.

RESULTS

Phase-separation – Proteins that have a hydrophobic domain have the property of partitioning into the detergent rich phase of Triton X-114 solution above the cloud point of the detergent (Bordier, 1981). When the proteins of *T. gondii* present into the DRP and DPP were subjected to electrophoresis (Fig. 1), stricking difference in the distribution of proteins in the two phase were obtained. Three intensively radiolabelled bands with mol. wt 22,000, 30,000, 40,000 and four minor intensive bands with mol. wt 50,000, 60,000,

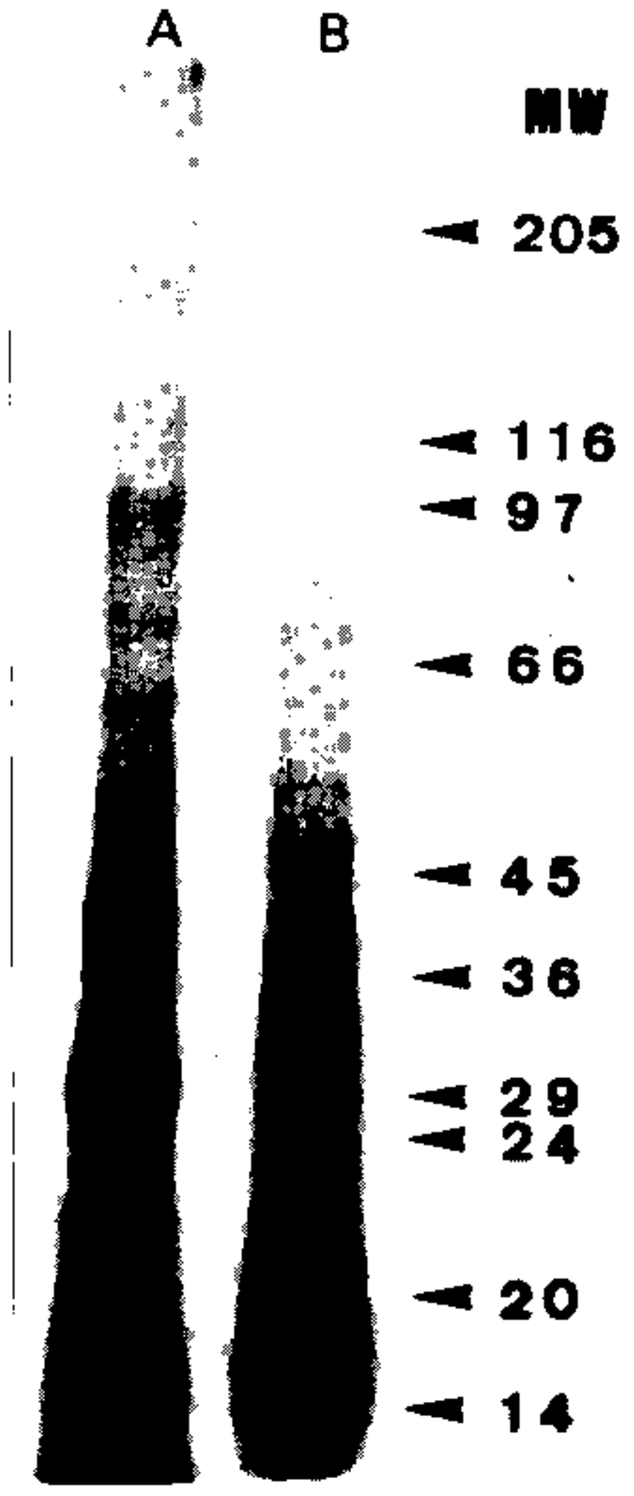

Fig. 1: phase separation autoradiographs of SDS-PAGE gel electrophoresis analysis of surface radiodinated tachyzoite. A, proteins present in the hydrophobic phase; B, proteins present in hydrophilic phase. The number represent stained markers.

97,000 and 180,000 were seen exclusively in the DRP (Fig. 1a). Contrasting, six other minor bands with mol. wt 21,000, 23,000, 32,000, 38,000, 46,000 and 76,000 were seen exclusively in DPP (Fig. 1b). There was an overlap of proteins of Mr 15 and Mr 70 in both phase.

Hydrophobic interaction chromatography (HIC) and immunoprecipitation studies – To confirm the presence of hydrophobic domain in the proteins and to evaluate their relative hydrophobicities, aqueous and detergent phases from Triton X-114 solubilized extracts of labelled tachyzoites of *T. gondii* were each analysed separately on the phenyl-Sepharose column.

Approximately 98% of the counts from the DPP were recovered in the unbound fraction and in the washing volume (Fig. 2B). In contrast, the most radioactive counts in the detergent phase were bound to the column and subsequentely eluted in two peaks (Fig. 2A). The peak II eluting at 0,35% Triton X-100 and the peak III at 0,93%. The counts not bounded probably represent iodine not bound to proteins as no count were obtained with TCA precipitation.

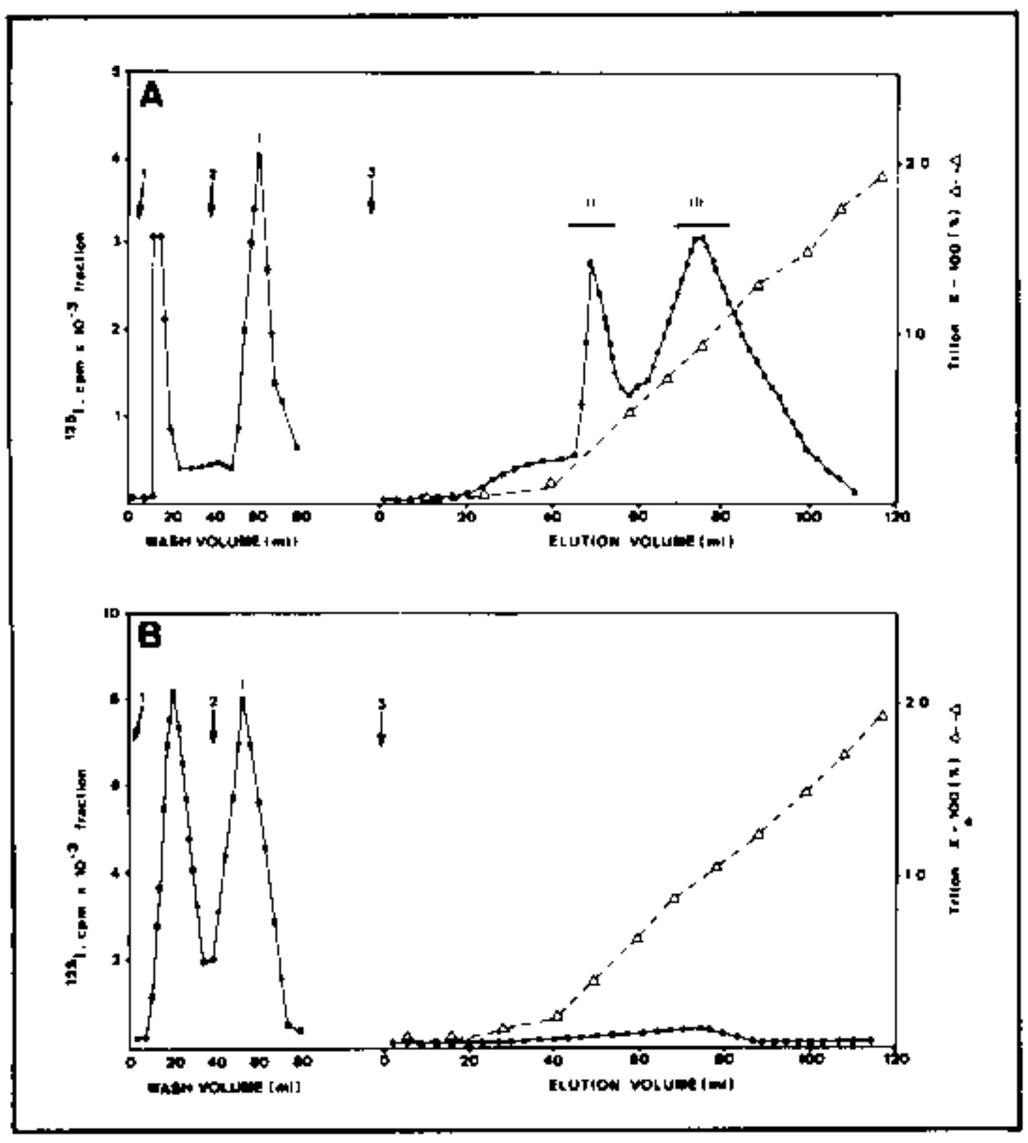

Fig. 2: chromatography of a DRP and DPP of ^{125}I-surface labelled tachyzoite proteins pn phenyl-Sepharose. The columns (0,9 x 22 cm) were washed with 40ml TS + 0.8M ammonium sulfate (1), then with Trissaline buffer (2). Bound proteins were eluted (Δ – – – Δ) with a 0.1 to 2% linear gradient of Triton X-100 (60ml each). Fractions were counted (• – • – • –) and Triton concentration was assessed by measuring absorbance at 275nm. (A) DRP and (B) DPP.

To identify each of the surface-peptide peak, fractions under the number bars (Fig. 2) were pooled, exaustively dialysed against water and concentrated by pervaporation and subjected to immunoprecipitation with a human convalescent serum. The autoradiography of the gels showed that the peak I present minor surface cell proteins with mol. 60,000 and 66,000 (Fig. 3, lane a). The peak II containing principally proteins with m.w. 15, 25 and 40,000 (Fig. 3, lane b) and the peak III, the major surface cell proteins with m.w. 30,000 (Fig. 3, lane c). In addition, the peptides with m.w. 60,000 and 66,000 isolated in the peak I can be also detected by silver coloration (data not shown).

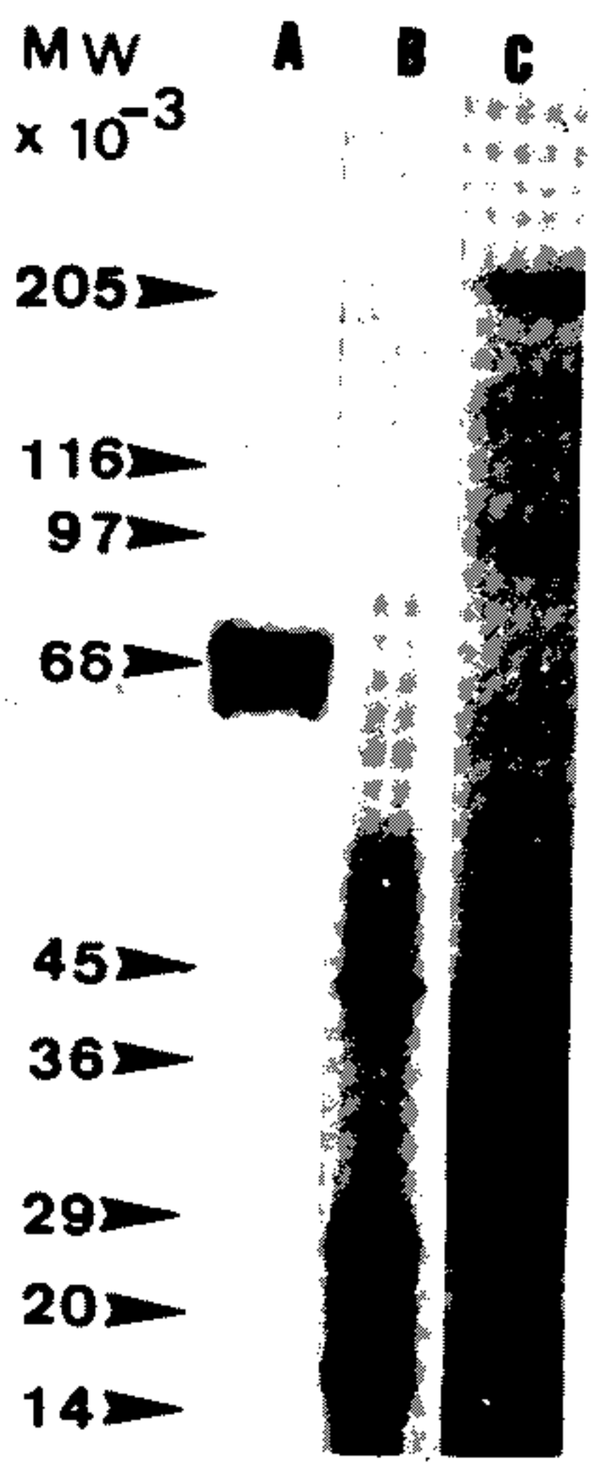

Fig. 3: autoradiography of proteins from DRP eluted from phenyl-Sepharose column and immunoprecipitated with toxoplasmosis patient serum. A, peak I (wash volume); B, peak II and C, peak III.

DISCUSSION

Although surface proteins can readly be identified by radiodination, their purification generally poses problems that do not have to be faced when dealing with cytoplasmic proteins. Proteins at the cell surface are often anchored to or penetrate through membranes. These

integral membrane proteins have extensive hydrophobic regions, making them insoluble in water and thus difficult to purify and characterize. As the phase separation technique offer a simple step for isolation of the more hydrophobic proteins from the less hydrophobic ones (Bordier, 1981) we used this technique to learn more about the proteins in tachyzoite of *T. gondii.* Proteins with Mr 22, 30, 40, 50, 60, 97, 180 KDa remained in the DRP whereas the 21, 23, 32, 38, 46, 76 KDa proteins were essencially recovered in the DPP. This results showed that only a selected group of toxoplasma proteins partition with the detergent.

It is interesting to point out in the most of parasites analysed up to now proteins have been detected mainly in the DRP (Etges et al., 1985; Bordier et al., 1986). In the *Orithidia species* (Giovanni De Simone et al., 1987) or in the case of *Plasmodium gallinaceum* (Kumar, 1985) only one or two different polypeptides were seen in the detergent poor phase. In agreement with the phase separation data the HIC also demonstrated that all proteins present in the DRP are highly hydrophobic, while that present in the DPP are essencially hydrophilic or less hydrophobic. The HIC may be used as an alternative technique to concentrate and isolate hydrophobic membrane proteins from the hydrophilic ones.

It is also interesting to point one that the association of phase-separation technique with the HIC permit to isolate two minor hydrophobic polypeptides present in the cell membrane tachyzoite which are detected by convalescent serum. The phase separation also resolt two polypeptides with 30 and 32 KDa, one present in the DRP and other in the DPP showing that are distint molecular species. The p30 phase-separated in the DRP appear to be the major surface polypeptide isolated using monoclonal antibodies (Kasper et al., 1983). The presence of hydrophobic region in this polypeptide has been suggested by charge shift electrophoresis (Kasper et al., 1983). We confirmated using the phase-separation technique and the HIC that this protein presents extensive hydrophobic region. It is released from the matrix column using only high detergent concentrations (Fig. 2) suggesting strongly that is an integral membrane protein. However, the same is not the case to the proteins 60-66,000 eluted from the column with the decreasing ionic strengh. Wheter the high hydrophobicity of the major amphiphilic molecule is dependent of the extensive presence in their chain of region rich in hydrophobic residues or due to an anchor lipidic as described in numerous membrane proteins of procaryotic and eucaryotic cells (Schlesinger, 1981; Schmidt, 1983; Cross, 1987; Selfton & Buss, 1987) remains to be determined. On the other hand we have showed that the employment of HIC after Triton X-114 extraction may constitute a step of amphiphilic membrane proteins fractionation.

Finally, it is important to emphasize that although the toxoplasma present a different organization of plasmatic membrane, the technique applied in this study are of value to isolate an specific number of membrane proteins important immunologicaly, as well as to study their relation between structure and function.

RESUMO

Análise de proteínas do *Toxoplasma gondii* após solubilização com Triton X-114 e cromatografia hidrofóbica – A distribuição das proteínas superficiais de *Toxoplasma gondii* radiodinadas, foram estudadas usando a técnica de separação de fases e a capacidade de ligação em coluna de fenil-Sepharose. Oito polipeptídeos com peso molecular entre 22 e 180.000 distribuíram-se exclusivamente na fase rica em detergente enquanto seis polipeptídeos com PM entre 15.00 e 76.000 distribuíram-se exclusivamente na fase pobre em detergente. Dois polipeptídeos com 15.00 e 70.000 distribuem-se em ambas as fases. Todos os polipeptídeos presentes na fase rica em detergente foram retidos por coluna de fenil-Sepharose e isolados em dois picos de acordo com sua hidrofobicidade relativa.

Dois polipeptídeos hidrofóbicos com PM 60.000 e 66.000 reconhecidos por soro humano, foram isolados pela associação das duas técnicas. Os nossos resultados mostram que as proteínas de superfície do *T. gondii* possuem diferentes graus de hidrofobicidade e que o emprego da cromatografia de interação hidrofóbica após extração com Triton X-114, pode ser um importante método de isolamento de proteínas de membrana.

Palavras chave: *Toxoplasma gondii* – Triton X-114 – hidrofobicidade – proteínas de membrana – cromatografia hidrofóbica

REFERENCES

BORDIER, C., 1981. Phase separation of integral membranes proteins in Triton X-114 solution. *J. biol. Chem., 256:* 1604-1607.

BORDIER, C.; ETGES, R. J.; WARD, J.; TURNER, M. J. & CARDOSO DE ALMEIDA, M. L., 1986. Leishmania and Trypanosoma surface glycoproteins have a common glycophospholipid membrane anchor. *Proc. natn. Acad. Sci. USA, 83:* 5988-5991.

CINTRA, W. M. & DE SOUZA, W., 1985. Distribution of intramembranous particles and filipin-sterol complexes in the cell membrane of *Toxoplasma gondii. Eur. J. Cell. Biol., 37:* 63-69.

CINTRA, W. M.; SILVA-FILHO, F. C. & DE SOUZA, W., 1986. The surface charge of *Toxoplasma gondii;* a cytochemical and electrophoresis study. *J. Submicros. Cytol., 18:* 773-781.

CROSS, G. A. M., 1987. Eukaryotic protein modification and membrane attachment via phosphatidylinositol. *Cell, 48:* 179-181.

DAHL, R. J. & JOHNSON, A. M., 1983. Purification of *Toxoplasma gondii* from host cell. *J. Clin. Pathol., 36:* 602-604.

ETGES, R. J.; BOUVIER, J.; HOFFMAN, R. & BORDIER, C., 1985. Evidence that the major surface proteins of three *Leishmania* species are structuraly related. *Molec. Biochem. Parasitol., 14:* 141-147.

GIOVANNI DE SIMONE, S.; SOARES, M. J.; DE SOUZA, W. & GOLDENBERG, S., 1987. Identification and partial characterization of plasma membrane polypeptides of *Crithidia guilhermei, Crithidia deanei* and *Crithidia oncopelti. Comp. Biochem. Physiol., 88B:* 1091-1096.

HANDMAN, E.; GODING, J. W. & REMINGTON, J. S., 1980. Detection and characterizati on of membrane antigens of *Toxoplasma gondii. J. Immunol., 124:* 2578-2583.

HOSHINO-SHINIZU, S.; MINEO, J. R., & CAMARGO, M. E., 1980. Lectin used in the purification process of *Toxoplasma gondii* tachyzoites. *J. Parasitol., 66:* 989-991.

JOHNSON, A. M., 1985. The antigenic structure of *Toxoplasma gondii.* A review. *Pathology, 17:* 9-19.

JOHNSON, A. M.; McDONALD, P. J. & NEOH, S. H., 1981. Molecular weight analysis of the major polypeptides and glycopeptides of *Toxoplasma gondii. Biochem. Biophys. Res. Commun., 100:* 934-943.

JOHNSON, A. M.; McDONALD, P. J. & HEOHN, S. H., 1983. Molecular weight analysis of soluble antigens from *Toxoplasma gondii. J. Parasitol., 69:* 459-464.

KASPER, L. H.; CRABB, J. H & PFERFFERKORN, E. R., 1983. Purification of a major membrane protein of *Toxoplasma gondii* by immunoabsorption with a monoclonal antibody. *J. Immunol., 130:* 2407-2412.

KUMAR, N., 1985. Phase separation in Triton X-114 of antigens of transmission blocking immunity in *Plasmodium gallinaceum. Molec. Biochem. Parasitol., 17:* 343-358.

LAEMMLI, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227:* 680-685.

MARKWEKL, M. A. K. & FOX, C. F., 1978. Surface-specific iodination of membrane proteins of viruses and eucaryotic cells using 1,3,4,4-tetrachloro-3, 6-diphenyl-glycoluril. *Biochemistry, 17:* 4807-4817.

NIELLSEN, B. L. & BROWN, L. R., 1984. The basis for colored silver-protein complex formation in stained polyacrylamide gel. *Analyt. Biochem., 141:* 311-315.

ROBINSON, N. C. & TANFORD, C., 1975. The binding of deoxycholate, Triton X-100, sodium dodecyl sulfate, and phosphatidylcholine vesicles to cytochrome b5. *Biochemistry, 14:* 369-378.

SCHLESINGER, M. J., 1981. Proteolipids. *Ann. Rev. Biochem., 50:* 193-206.

SCHMIDT, M. F. G., 1983. Fatty acid binding*A new kind of protranslation modification of membrane proteins. *Curr. Trop. Microbiol. Immunol., 102:* 101-129.

SELFTON, B. M. & BUSS, J. E., 1987. The covalent modification of eucaryotic proteins with lipid. *J. Cell. Biol., 104:* 1449-1453.