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I N D I C E

8. Guerra, G. and Cañedo, L. "Proteins of Cysticercus cellulosae" en Van den Bossche, H. (Ed.) "Biochemistry of parasites and Host-parasite relationships". Elsevier/ North Holland, Biomedical Press 1976, p. 109-115.

9. Flisser, A., Plancarte, A., Espinoza, B., Woodhouse, E., Rivera L., Trueba, J., Yakoleff Greenhouse, V., Sierra, A., Laclette, J.P., Guerra, G., Cañedo, L. and Larralde, C. "The immune status of patients with cysticercosis". Afr. J. Clin. Exp. Immunol. 3:183-190, 1982.

10. Guerra, G., Flisser, A., Cañedo, L., and Laclette, J.P. "Biochemical and immunological characterization of antigen purified from cysticerci of Taenia solium" en Flisser, A., Willms, K., Laclette, J.P., Larralde, C., Ridaura, C., and Beltran, E. (Eds.) "Cysticercosis present state of Knowledge and Perspectives". Academic Press 1982, p. 437.

11. Laclette, J.P., Guerra, G., Cañedo, L. "On the mechanism of action of Mebendazole". IV International Congress of Parasitology. Section D, p. 12. Warsawa, Poland. Agosto, 1978.

12. Laclette, J.P., Merchant, M.T., Willms, K. and Cañedo, L. "Paracrystalline bundles of large tubules, induced in vitro by Mebendazole in Cysticercus cellulosae". Parasitology 83: 513-518 (1981).

INTRODUCCION

A principios de 1972 se iniciaron los trabajos tendientes a definir la metodología y plantear los problemas más importantes a resolver en la cisticercosis cerebral. Los trabajos que constituyen esta tesis, forman parte de esas investigaciones y exploran las siguientes áreas:

En la primera etapa (artículos 1 y 2) se definen las condiciones óptimas para mantener *in vitro* metacéstodos de *T. solium* viables en condiciones estables por períodos prolongados, controlando voluntariamente su evaginación y transformación a las primeras etapas de la forma adulta. Colateralmente de estos estudios, se dedujo un modelo para explicar el mecanismo de fijación de la larva al intestino.

El siguiente trabajo (artículo 3) tuvo como objetivo, hacer el estudio morfológico completo de la pared de la vesícula del metacéstodo de *T. solium*, ya que sólo existían descripciones parciales de esta estructura y este conocimiento era necesario para poder hacer experimentos fisiológicos o bioquímicos racionales.

Del trabajo anterior se dedujo que el *C. cellulosae* no tiene sistema digestivo o respiratorio, y sólo cuenta con un sistema excretor primitivo, por lo tanto, su relación con el medio ambiente que le rodea tiene que realizarse en su mayor parte a

través de la pared de la larva.

Para explorar esta idea, se hicieron experimentos con el fin de conocer si la pared del cisticerco de T. solium actúa como una barrera fisiológica en el huésped bajo condiciones normales de infección. La evidencia a favor de esta hipótesis se muestra en el cuarto artículo.

La siguiente fase estuvo orientada a definir a través de un estudio histoquímico y de análisis orgánico sistemático los cambios que ocurren en las diferentes etapas de calcificación del cisticerco de T. solium en el cerebro humano. (Artículo 5). El trabajo anterior, fue continuado por un estudio estructural y fisicoquímico con el fin de definir el papel del huésped y del parásito en el proceso de calcificación. Los resultados se muestran en el sexto artículo.

La evidencia estructural y fisicoquímica acerca del papel que juegan las fibras extracelulares de la pared del metacéstodo de T. solium en la formación del tejido calcificado, hizo interesante el intentar su aislamiento con el fin de conocer la clase de proteína de que se trataba. Para ello, se llevó a cabo un análisis preliminar de las proteínas del Cysticercus cellulosae generando dos resultados relevantes: El primero fue la respuesta directa a esta pregunta, lográndose la purificación parcial de una proteína parecida a colágena, que fue posteriormente caracterizada como tal en nuestro laboratorio (Torre-Blanco, A. y Toledo, I. "The isolation, purification and characterization

of the collagen of Cysticercus cellulosae". J. Biol. Chem. 256, 5926, 1981). El segundo resultado consistió en la obtención de una fracción proteíca soluble en ácido, que presenta una imagen globular al microscopio electrónico y que en presencia de ATP polimeriza formando una red. Estos resultados se muestran en el séptimo artículo. Esta fracción proteíca soluble en ácido, resultó relevante al asociarla con los trabajos realizados independientemente por C. Larralde y colaboradores orientados a caracterizar la respuesta inmune en los enfermos de cisticercosis. En estos trabajos, se muestra que al realizar una electroforesis a pH.8.6 del suero de pacientes cisticercosos contra un estracto crudo de cisticercos, se detectan 8 bandas de precipitación, siendo la banda isoeléctrica (Antígeno B), la más frecuentemente reconocida (Flisser,A., Woodhouse, E. and Larralde, C. "Human cysticercosis: antigens, antibodies and non-responders". Clin. Exp. Immunol. 39:27, 1980).

Este hallazgo fue repetido posteriormente en pacientes con cisticercosis cerebral y se reporta en el octavo artículo. La asociación entre la fracción soluble en ácido descrita en el artículo 7 y el antígeno B caracterizado por Larralde y colaboradores, ocurrió al identificar una banda de precipitación correspondiente al antígeno B en una electroforesis de la fracción soluble en ácido paracialmente purificada, contra el suero de pacientes cisticercosos. Estos resultados hicieron de la fracción soluble en ácido, un candidato adecuado para ser uti-

lizado en pruebas de diagnóstico serológico o bien como agente inmunizante. La caracterización bioquímica e inmunológica del antígeno B se reporta en el artículo noveno.

La reciente aparición de una nueva droga antihelmíntica el Mebendazol (metil-5-benzoil benzimidazol-2-carbamato) permitió estudiar su interacción con tubulina. Nota 10 (posteriormente publicado en: Laclette, J.P., Guerra, G. y Zetina, C. "Inhibition of tubulin polymerization by Mebendazole". Bioch. Bioph. Acta 92:417, 1980), usando esta droga, paralelamente se estudió su efecto en cisticercos de T. solium mantenidos en medio de cultivo. Los cambios observados en la larva se reportan en el artículo 11.

La investigación sobre cisticercosis se encuentra actualmente en expansión. (Flisser, A., Willms, K., Laclette, J.P., Larralde, C., Ridaura, C. y Beltrán, E. (Eds.) "Cysticercosis present state of Knowledge and Perspective", Academic Press 1982). Estos resultados representan un estímulo para continuar el estudio de este problema con el fin de poder comprender y controlar esta enfermedad.

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EVAGINATION OF THE METACESTODE OF *TAENIA SOLIUM*

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I. INTRODUCTION

The transformation of the cyclophyllidean metacestode to the adult form occurs after the cyst has been ingested by the definitive host. During the transit through the stomach and the initial part of the small intestine, the larva evaginates from the surrounding bladder, permitting the scolex to attach to the intes-

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tinal wall of the host. Once fixed, the parasite grows and matures until the egg-producing phase of the adult is reached.

Because the study of the evagination of metacestodes in the gut of the host presents obvious difficulties, a very common practice has been to simulate *in vivo* conditions *in vitro*. By this means, several factors such as proteolytic enzymes, bile salts, osmotic pressure, pH, and temperature have been determined to be essential for the evagination of some hymenolepidid [1–4] and taeniid [4–7] metacestodes. The particular requirements of each parasite could be a factor in determining host specificity [8].

In the case of the metacestode of *Taenia solium*, Rothman reported that pepsin and bile salts, but not trypsin, are required for evagination [5]. In contrast, Melgar found that a pancreatic extract was very effective in promoting the evagination of these larvae, whereas gastric juice was not [9]. The best results were obtained when the cysts were incubated in a mixture of pancreatic extract and bile. In view of the apparent disagreement between these data, we decided to re-examine the requirements for the *in vitro* evagination of these metacestodes.

In this paper we report the "spontaneous" evagination of cysticerci of *T. solium* when maintained for long periods of time (> 24 h) in standard culture medium. The promotion of evagination over a much shorter period of time (< 20 min) by human bile and trypsin, but not by artificial gastric juice, is also described. The evagination process and the movements of the excysted scolex were studied by light microscopy, and scanning electron micrographs of selected stages were prepared. From these data, we propose a hypothetical model for the attachment of the evaginated cysticerci to the intestinal wall of the definitive host.

II. MATERIALS AND METHODS

Materials. Infested pigs were obtained immediately after slaughter from abattoirs in Mexico City. Cysticerci were dissected from the skeletal muscle and only those with fluid-filled intact bladder walls were selected. Sample cysticerci from each pig were squashed between two microscope slides in order to confirm the presence of an armed rostellum using light microscopy; in no case were unarmed cysts found in these animals.

Human bile was obtained from patients at the Gastroenterology Department of the Hospital General, Centro Médico Nacional, I.M.S.S. Minimal essential medium (MEM) and fetal calf serum were purchased from Difco. Tyrode's buffer trypsin (twice crystallized), and soybean trypsin inhibitor (type 1-S) were obtained from Sigma. All other reagents were of analytical grade.

In vitro *evagination and maintenance of the larvae.* After having been dissected, the cysticerci were maintained for 3 h in culture flasks containing MEM medium, pH 7.4, supplemented (per ml) with fetal calf serum (20 mg), glucos

(4 mg), penicillin G (50 units), streptomycin sulfate (50 μg), and gentamycin (30 μg). This mixture is referred to as the culture medium. The larvae were maintained at 37°C, under an atmosphere of 95 % air–5 % CO_2 at 100 % humidity. Groups of 25 larvae were formed. In several groups, cuts were made in the bladder walls at a point opposite the invaginated scolex in order to ensure the access of macromolecules to the interior. All larvae were washed twice in Tyrode's buffer, pH 7.3. Duplicate groups were incubated separately for 20 min at 37°C in flasks containing 50 ml of Tyrode's buffer alone or with one of the following: 25 % (v/v) complete human bile, or 0.1 % (w/v) trypsin, or 0.1 % (w/v) heat-inactivated trypsin, or 0.1 % (w/v) trypsin plus 0.5 % (w/v) soybean trypsin inhibitor. Larvae were also incubated in artificial gastric juice prepared according to Silverman [10]. All flasks were agitated on a shaker at 125 rpm. After incubation, the percent of evaginated cysticerci in each group was calculated. Nonevaginated cysticerci from each group of incised larvae were washed separately in Tyrode's buffer and reincubated for 20 min at 37°C in the same buffer plus 0.1 % (w/v) trypsin in order to assay the ability of the larvae to evaginate.

"Spontaneous" evagination in the absence of human bile or enzymatic agents was studied in groups of cysticerci maintained over prolonged periods of time (> 24 h) in culture medium under the same conditions as described above.

All evaginated cysticerci were transferred to and maintained in culture medium in which the fetal calf serum had been increased to 100 mg/ml. The culture medium was replaced every 24 h. The evagination process, as well as the behavior of the excysted larvae, was studied using light microscopy.

Scanning electron microscopy. Specimens representative of the different stages of evagination and post-evagination were processed for scanning electron microscopy following the methods described by Echlin [11]. The larvae were fixed in Karnovsky's fixative [12] at 4°C for 2 h, postfixed with 2.5 % (v/v) osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, at 4°C for 1 h, and serially dehydrated with acetone. The samples were dried in a model CPA II critical point dryer (Technics Inc.). Gold-coated samples were examined with a JSM-35 JEOL scanning electron microscope.

III. RESULTS

A. In Vitro *Evagination of the Cysticerci*

The results of the study on the requirements for the evagination of the cysts are shown in Table I. In those with an intact bladder wall, complete human bile induced the evagination of 82 % of the larvae. In contrast, purified bile salts of cholic, deoxycholic, and taurocholic acid were ineffective when assayed separately (data not shown). Similarly, neither trypsin nor artificial gastric juice in-

TABLE I. In Vitro *Evagination of* Taenia solium *Cysticerci with Intact or Incised Bladder Walls*[a]

Treatment	*Intact* (%)	*Incised* (%)	*Reincubation*[b] (%)
Buffer	0	0	100
Human bile	82	56	98
Artificial gastric juice	0	0	0
Trypsin	0	100	
Trypsin + Inhibitor		2	100
Heat-denatured trypsin		0	100

[a] *Groups of 25 cysticerci were incubated in duplicate for 20 min at 37 °C.*

[b] *Cysticerci with an incised bladder wall that did not evaginate during the first incubation were washed and reincubated in the presence of trypsin.*

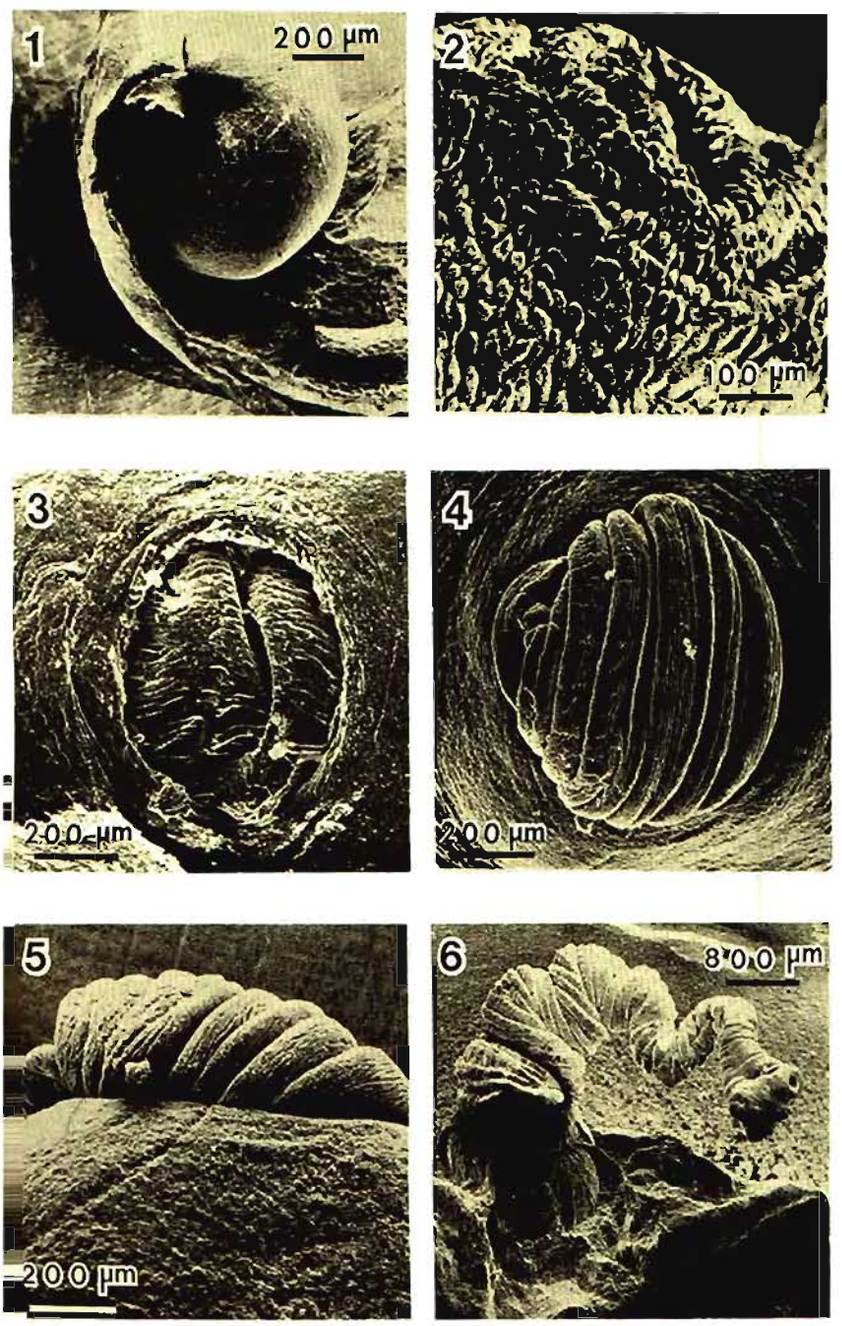
duced the evagination of intact larvae. However, when the cysticerci had an incised bladder wall, trypsin was 100% effective in promoting evagination, whereas the efficiency of complete human bile dropped to 56%. The effect of trypsin was abolished either by the use of a specific inhibitor or by heat denaturation. Artificial gastric juice had no effect on the incised cysticerci. All nonevaginated larvae with an incised bladder wall, except those treated with artificial gastric juice, were able to evaginate during reincubation in the presence of trypsin. The induced evaginated cysticerci were transferred to culture medium where they remained viable for at least 2 months.

The evagination of 10–20% of the cysticerci maintained in culture medium occurred "spontaneously" after 24–96 h of incubation. The "spontaneously" evaginated cysts remained viable – as judged by the motility of the scolex – for at least 3 weeks, and during this time their general morphology was observed to be normal.

B. Description of the Evagination Process

No differences in the sequence of evagination between "spontaneous" and induced evaginated cysticerci were observed. Consequently, the following description represents the general features of both processes.

The cysticercus inside the bladder (Fig. 1) began its evagination with the opening of the spiral canal whose entrance is located on the outer surface of the bladder (Figs. 2 and 3). Evagination took place through an orderly and gradual unwinding of the invaginated structures, commencing with the surface nearest the external opening and followed sequentially by the transversally wrinkled spiral canal, the immature neck or stalk region, and the scolex (Figs. 4–6). The process was accompanied by very active movements on the part of the larva.



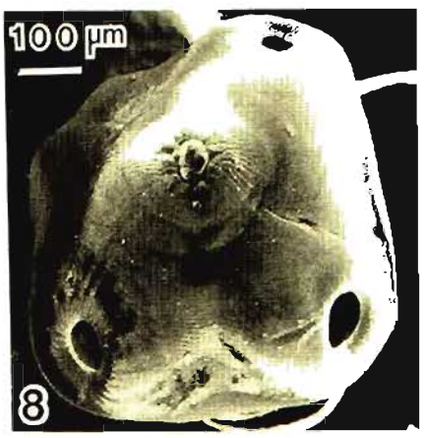
GS. 1–6. Scanning electron micrographs of different stages of the in vitro *evagination of ? metacestode of* Taenia solium. *1. Inside view of the invaginated cysticercus within the ıdder. 2. Outside view of the opening of the spiral canal located on the external surface the bladder wall. 3 and 4. Frontal views of the progressive evagination of the spiral canal. Lateral view of the progressive evagination of the spiral canal. 6. Lateral view of a fully ıginated cysticercus.*

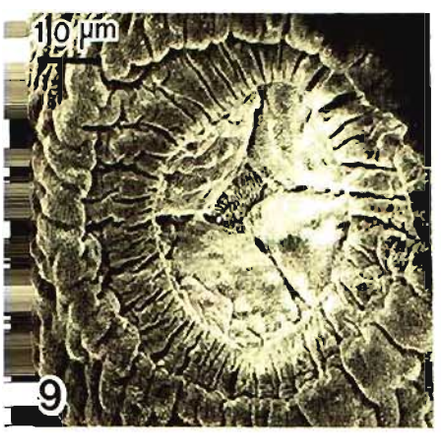
C. Movements of the Evaginated Cysticerci

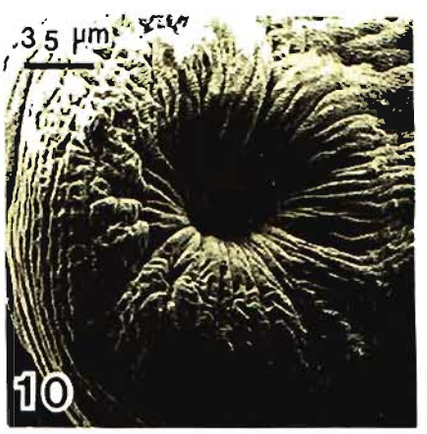
Several differences were observed between the "spontaneous" and induced evaginated cysticerci. Little or no growth occurred in the former, whereas the latter tripled their size during the first 2 weeks of culture. The induced evaginated cysts were very motile, while the motility of the "spontaneously" evaginated metacestodes diminished after 3–4 days. However, during the initial days of culture there were no important differences in the activity displayed by both groups.

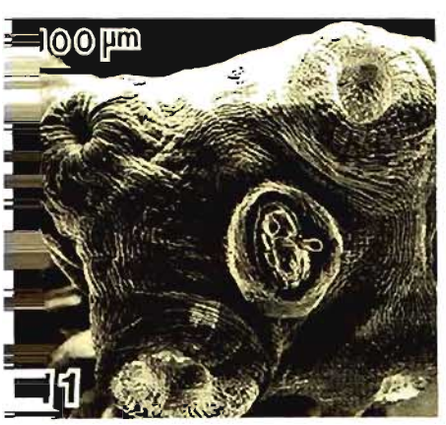
The evaginated cysticerci presented a series of concurrent movements that are described separately according to each structural component involved. 1) The behavior of the evaginated spiral canal beneath the stalk region was characterized by very active lateral and circular movements, as well as by a telescopelike retraction and protrusion. The latter permitted the cysticerci to reinvaginate the region of the spiral canal closest to the bladder wall (Fig. 7). 2) The stalk also presented lateral and circular movements, providing additional motility to the scolex. The movements of the stalk were independent of those of the spiral canal. 3) The scolex showed a striking, repetitive pattern of activity that we have used to support the model of attachment presented in Section IV. Following complete evagination, the scolex displayed no rostellar hooks (Fig. 8) and the movements of the unarmed scolex were restricted to the four suckers. The early movements of the suckers were contractile, much like those of sphincter muscles. Initially, the "mouths" of the suckers were in an open position (Fig. 9). As the sphincterlike contractions controlling the diameter of the sucker mouth increased (Fig. 10), other types of movements were initiated. These movements probably carried out by another set or sets of muscles posterior to those surrounding the sucker mouth, consisted of rhythmic and independent contractions which had the effect of extending each sucker away from the center of the scolex (Fig. 11). They appeared to be related to the initial protrusion of the apical organ of the rostellum, from which the hooks later emerged (Figs. 12 and 13).

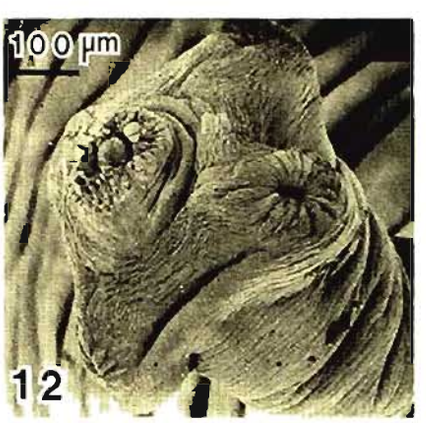
The mechanism by which the hooks began to come forth from the rostellum could not be clearly determined; however, it appeared that the muscular activity in the vicinity of the rostellum and the mechanical pressure exerted by the independent contractions of the four suckers were the main forces. Figure 14 shows that the rostellar hooks had to pierce a superficial enveloping tissue before becoming exposed for the first time. Figure 15 demonstrates that this tissue has microvilli, characteristic of the tegumentary surfaces of the cyst. The hooks became progressively exposed, assuming a position perpendicular to the long axis of the worm (Figs. 13, 16, and 17). The scolex maintained the appearance shown in Figure 17 for only 1–2 seconds, followed by the retraction of the hooks so that only their tips were visible. The telescopelike movements of the spiral canal also were made at this moment, allowing the cysticercus to assume the shape shown in Figure 18, characterized by an almost completely invaginated spiral canal and a scolex with retracted but visible rostellar hooks. The ei











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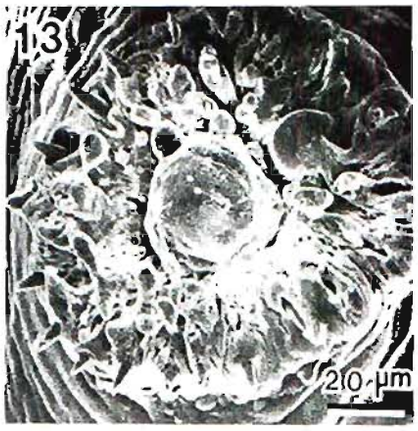
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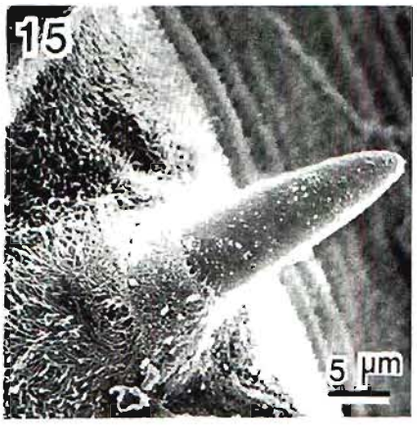
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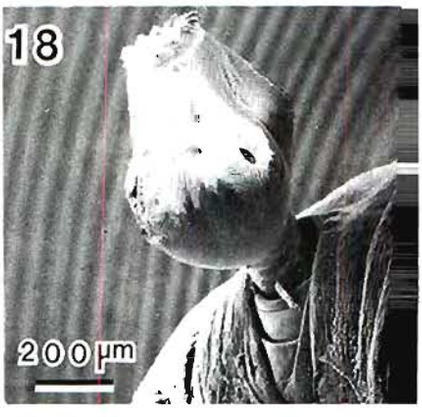
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tire pattern of movements from the outward extension of the spiral canal to the retraction of the rostellar hooks and the reinvagination of the spiral canal was repeated many times during the weeks in culture prior to the death of the larvae.

IV. DISCUSSION

Two sets of cysticerci of *T. solium* were used in studying the conditions conducive to *in vitro* evagination. The first group comprised intact cysticerci, while the second was made up of larvae with incised bladder walls which assured contact between macromolecules in the culture medium and the invaginated scolex. In our experience, both human bile and trypsin were fairly good inducers of evagination. The former was most effective on cysts with an intact bladder wall, while the latter did not affect these cysticerci. However, trypsin was 100 % effective on the incised cysts. These data indicate that the site where trypsin exerts its proteolytic action must be located within the bladder. As the trypsin-induced evaginated cysts remained viable for at least 8 weeks in the culture medium, the use of this enzyme appears to be a gentle and reliable method for obtaining evaginated cysticerci and could possibly be employed in studies on the *in vitro* development of the adult form. Artificial gastric juice was ineffective in both intact and incised larvae.

The sequence of events that we observed during evagination of *T. solium* metacestodes is in agreement with the description made by Šlais [13]. However, we also found that this process is at least partially reversible.

Once excysted, the immature worm has a limited amount of time to become attached to the intestinal wall of the host. As there is both a continual flow and movement of the contents of the stomach and small intestine, this attachment is likely to be critical for the permanent establishment of the larvae within the host. The specific pattern of movements manifested by the evaginated cysticerci appears to be directed to this goal.

FIGS. 7–18. Scanning electron micrographs of representative positions assumed by actively moving evaginated cysticerci. 7. Lateral view of an evaginated cysticercus with a partially invaginated spiral canal resulting from an infolding telescopelike movement. 8. Frontal view of the scolex in a recently evaginated cyst. The four suckers are relaxed and the apical organ has not begun to protrude. 9 and 10. Frontal views of open and contracted suckers. 11. Independent contractions of the suckers. The contracted sucker in the upper left of the figure is oriented away from the center of the scolex. The apical organ is beginning to emerge. 12. Semifrontal view of a scolex with four contracted suckers. The apical organ is emerged and the rostellum protrudes upwards. 13. Frontal view of the same cysticercus as in Figure 12 at a higher magnification, showing the rostellar hooks as they first appear. 14. Detailed view of the emerging rostellar hooks. The upper hook has already pierced the enveloping tissue, while the lower hook has not. 15. Microvilli of the pierced enveloping membrane at the base of a rostellar hook. 16. A later stage as the hooks are becoming exposed. 17. Lateral view of a scolex with fully exposed rostellar hooks. Note the contracture of the rostellum and four suckers. 18. Lateral view of a scolex after retraction of the rostellar hooks. The spiral canal is almost totally reinvaginated.

Although the attachment of the evaginated larvae seems to be a crucial event in the life cycle of *T. solium*, a detailed description of this process is not presently available, to our knowledge. Thus, based on the observations described above, we wish to advance a hypothetical model concerning the activity of the evaginated cysts that enables them to become attached to the interior surface of the intestine of the definitive host.

A schematic representation of this model is depicted in Figure 19. The lateral, circular, and telescopelike movements of the evaginated larva seem to be oriented to making contact with the host. Thus, when the scolex is close to the intestinal epithelium, the projected suckers may attach themselves to the surface (Fig. 19A). If the attachment is broken, this process can be repeated several times until all four suckers are firmly adhered (Fig. 19B). It is possible that the suckers are attached strongly enough to allow the penetration of the rostellar hooks by means of a musculature centrally located beneath the rostellum. The

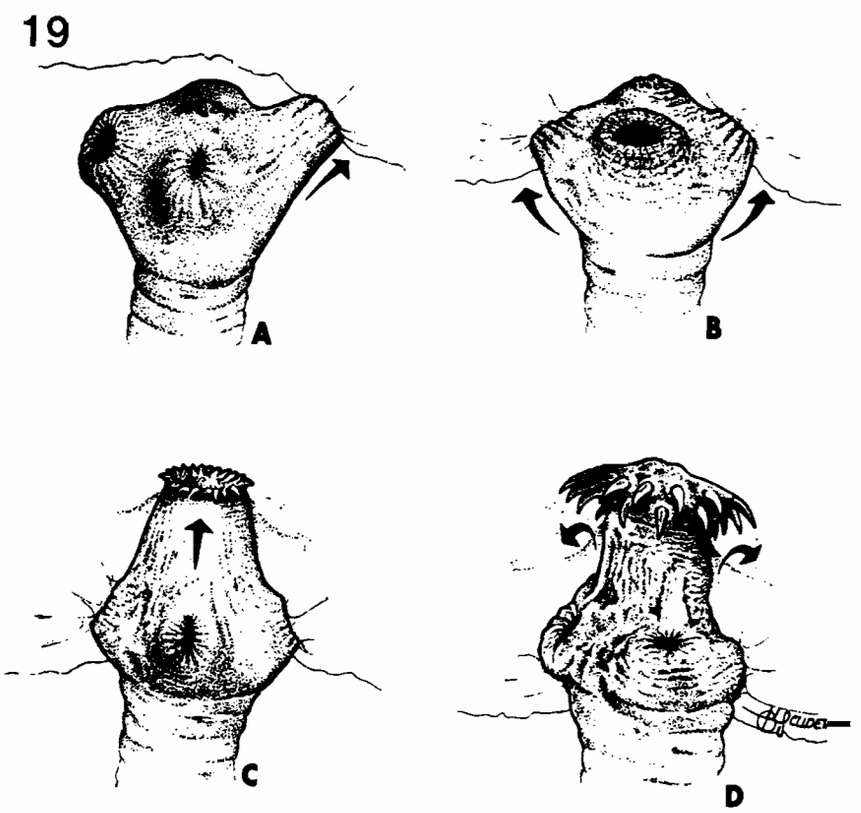

FIG. 19. Schematic representation of a proposed model for the attachment of the metacestode of T. solium *to the host intestinal wall. See Section IV (Discussion) for a detailed explanation.*

pressure exerted by the contractions of this musculature could enable the hooks to pierce the intestinal wall (Fig. 19C) and to rotate 90° with respect to the long axis of the worm, until the tips of the hooks are directed toward the surface of the epithelium, thereby anchoring the scolex (Fig. 19D). This event would fix the larva in a permanent and stable position in an environment favorable for its development into a mature worm.

It should be pointed out that this model is hypothetical due to the difficulty in obtaining direct evidence. However, our proposal, although based on different observations, is in agreement with a suggestion advanced by Featherston in regard to the attachment of *T. hydatigena* [14].

REFERENCES

1. Goodchild, C.G. and Davis, B.O., Jr. *Hymenolepis microstoma* cysticercoid activation and excystation *in vitro* (Cestoda). *J. Parasitol.* 58: 735, 1972.
2. Lippens-Mertens, F. and De Rycke, P.H. Excystation of *Hymenolepis microstoma*. II. Relative influences of proteolytic enzymes and bile salts. *Z. Parasitenkd.* 42: 61, 1973.
3. Sinha, D.P. *In vitro* excystation of cysticercoids of *Hymenolepis nana*. *Ann. Zool.* 9: 41, 1973.
4. Voge, M. Cestoda. *In* "Methods of Cultivating Parasites *In Vitro*" (A.E.R. Taylor and J.R. Baker, eds.), pp. 193–225. Academic Press, London, 1978.
5. Rothman, A.H. Studies on the excystment of tapeworms. *Exp. Parasitol.* 8: 336, 1959.
6. De Rycke, P.H. and Van Grembergen, G. Etude sur l'evagination de scolex d'*Echinococcus granulosus*. *Z. Parasitenkd.* 25: 518, 1965.
   De Rycke, P.H. and Van Grembergen, G. Study on the evagination of *Cysticercus pisiformis*. *Z. Parasitenkd.* 27: 341, 1966.
7. Smyth, J.D. and Haslewood, G.A. The biochemistry of bile as a factor in determining host specificity in intestinal parasites, with particular reference to *Echinococcus granulosus*. *Ann. N.Y. Acad. Sci.* 113: 234, 1963.
   Melgar, R.L. Estudio de varios sustratos para evaluar la evaginación de *Cysticercus cellulosae*. Thesis, Facultad de Medicina Veterinaria y Zootecnia, Universidad de San Carlos de Guatemala, 1964.
   Silverman, P.H. Studies on the biology of some tapeworms of the genus *Taenia*. I. Factors affecting hatching and activation of taeniid ova, and some criteria of their viability. *Ann. Trop. Med. Parasitol.* 48: 207, 1954.
   Echlin, P. The application of scanning electron microscopy to biological research. *In* "New Developments in Electron Microscopy" (H.E. Huxley and A. Klug, eds.), pp. 135–172. The Royal Society, London, 1971.
   Karnovsky, M.J. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy (Abst.). *J. Cell Biol.* 27: 137a, 1965.
   Šlais, J. "The Morphology and Pathogenicity of the Bladder Worms *Cysticercus cellulosae* and *Cysticercus bovis*," 144 pp. Dr. V. Jung N.V., The Hague – Academia, Prague, 1970.
   Featherston, D.W. *Taenia hydatigena* – V. Surface structure of the adult worm and evaginated scolex. *Int. J. Parasitol.* 5: 615, 1975.

IN VITRO STUDY OF THE EARLY TRANSITION OF *TAENIA SOLIUM* FROM METACESTODE TO ADULT

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I. INTRODUCTION

The *in vitro* development of taeniid metacestodes from oncospheres has been described for *Echinococcus granulosus, Taenia hydatigena, T. ovis, T. pisiformis, T. serialis* [1], *T. taeniaeformis* and *T. saginata* [2]. However, the culture of *T. solium* larvae has not been achieved, possibly due to the difficulty of obtaining an adequate supply of oncospheres. Moreover, to our knowledge, no attempt has been made to develop an "*in vitro*" culture of adult taeniae from the metacestodes. In order to study the development of the life cycle *in vitro*, we undertook the culture of *T. solium* from the metacestode -- a procedure that has been partially developed for *T. crassiceps* [3], *T. pisiformis* [4], and *E. granulosus* [5]. The preliminary results are presented here.

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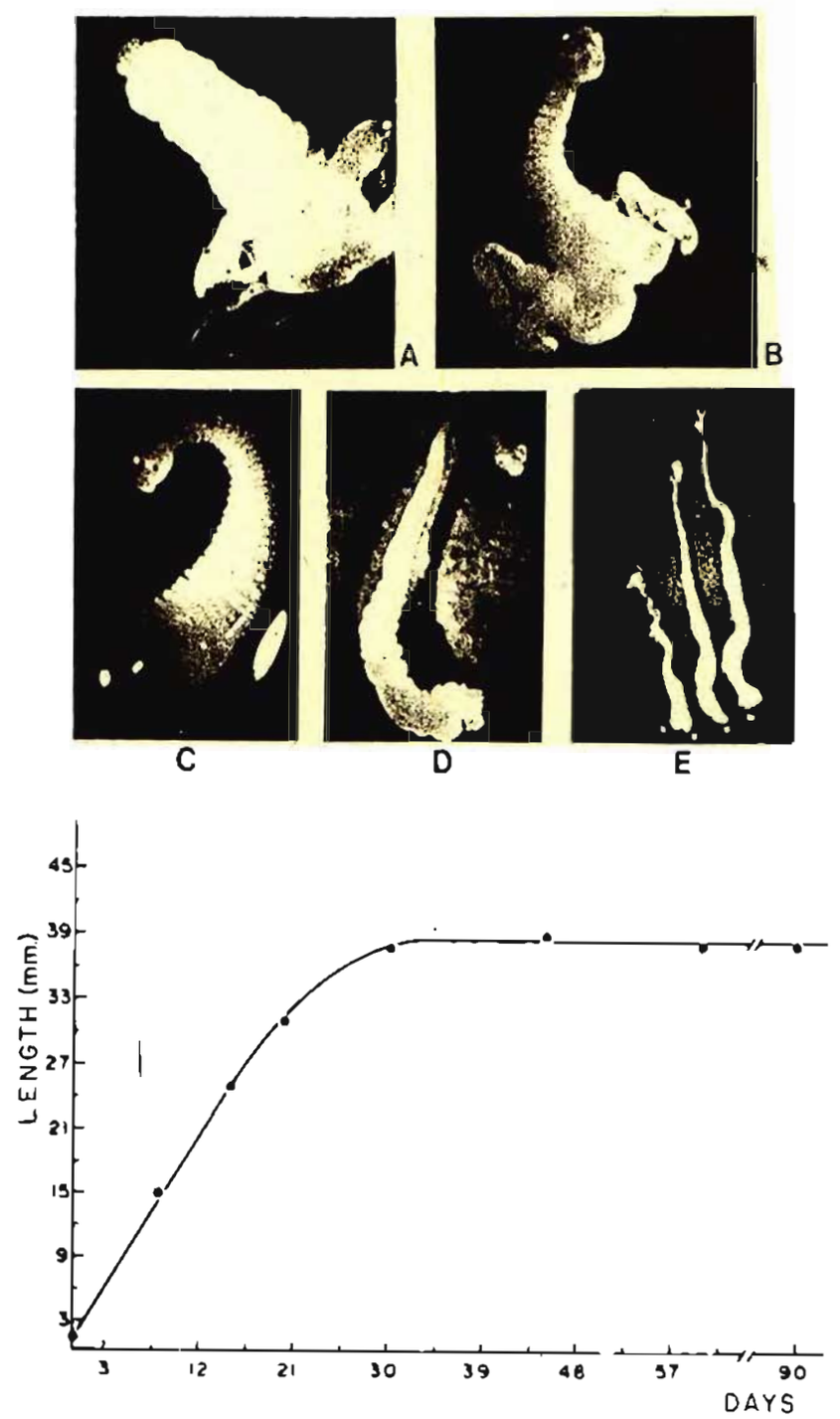

Figure 1

II. MATERIAL AND METHODS

Cysticerci were dissected from the skeletal muscles of infested pigs immediately after death in slaughterhouses in Mexico City. Only those larvae with an intact bladder wall filled with fluid were selected. Cysts from each animal were studied at random using the light microscope for the presence of a rostellum; in no case were unarmed cysts observed. The cysts were incubated in Erlenmeyer flasks for 3 h at 37 °C in an atmosphere of 95 % air–5 % CO_2 and 100 % humidity. The flasks contained minimal essential medium (MEM; Gibco), pH 7.4, supplemented (per ml) with glucose (4 mg), penicillin G (50 units), streptomycin sulfate (50 μg), and gentamycin (30 μg). Following incubation the bladder walls were eliminated and the wall-less larvae were incubated in the above culture medium supplemented with 0.1 % trypsin (2X crystallized; Sigma) in order to promote their evagination [see chapter by L. Cañedo *et al.* in this volume]. The medium was then decanted and the evaginated larvae were washed four times with culture medium before transfer to flasks containing culture medium supplemented with 10 % fetal calf serum. The larvae were grown in the incubator under the same conditions as described above.

III. RESULTS AND DISCUSSION

The growth of the evaginated larvae is summarized in Figure 1. Even if the larvae survived for longer than one month, at which time they had attained an average length of 39 mm, no further growth was observed. Several substrates were tested for their ability to stimulate the attachment of the evaginated larvae and thus promote further growth. These included coagulated human serum, collagen gel (Calbiochem), a tissue culture of muscle cells [6], fibroblasts [7], a fragment of human intestine obtained during autopsy immediately after death and maintained *in vitro*, and a section of dialysis tubing. Additionally, one-week-old evaginated larvae were implanted in the lumen of the small intestine of newborn dogs and a spider monkey. None of these experiments resulted in the continued growth of *T. solium* to the adult stage.

It is important to continue the efforts to cultivate *T. solium* under different conditions and in different hosts, including immunodepressed animals, in order

FIG. 1. Growth of evaginated larvae of Taenia solium *at varying times during* in vitro *culture: after 20 min of culture, 1.5 mm length* (A)*; 24 h, 2–3 mm* (B)*; 3 days, 6.0 mm* (C)*; 12 days, 20 mm* (D)*; 15 days (25 mm), 20 days (32 mm), and 30 days (39 mm)* (E, *from left to right*)*. Bottom graph shows the growth curve of the evaginated larvae of* T. solium *cultured in Erlenmeyer flasks containing culture medium supplemented with 10 % fetal calf serum at 37 °C in an atmosphere of 95 % air–5 % CO_2 and 100 % humidity.*

to define what role the attachment to the intestinal wall has in promoting further growth to the adult stage, as well as to determine the metabolic requirements of this developmental stage. This work represents an initial attempt in this direction.

REFERENCES

1. Heath, D.D. and Smyth, J.D. *In vitro* cultivation of *Echinococcus granulosus, Taenia hydatigena, T. ovis, T. pisiformis* and *T. serialis* from oncosphere to cystic larva. *Parasitology* 61: 329, 1970.
2. Heath, D.D. and Elsdon-Dew, R. The *in vitro* culture of *Taenia saginata* and *Taenia taeniaeformis* larvae from the oncosphere, with observations on the role of serum for *in vitro* culture of larval cestodes. *Int. J. Parasitol.* 2: 119, 1972.
3. Esch, G.W. and Smyth, J.D. Studies on the *in vitro* culture of *Taenia crassiceps. Int. J. Parasitol.* 6: 143, 1976.
4. Heath, D.D. Resistance to *Taenia pisiformis* larvae in rabbits: immunization against infection using non-living antigens from *in vitro* culture. *Int. J. Parasitol.* 6: 19, 1976.
5. Smyth, J.D., Miller, H.J. and Howkins, A.B. Further analysis of the factors controlling strobilization, differentiation, and maturation of *Echinococcus granulosus in vitro. Exp. Parasitol.* 21: 31, 1967.
6. Ramírez, O. and Alemán, V. Efecto de los factores en los medios condicionados sobre proliferación de músculo *in vitro. Ciencia (Mexico)* 28: 67, 1973.
7. Burridge, K. A comparison of fibroblast and smooth muscle myosins. *F.E.B.S. Lett.* 45: 14, 1974.

ULTRASTRUCTURE OF THE BLADDER WALL OF THE METACESTODE OF *TAENIA SOLIUM*

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I. INTRODUCTION

Partial descriptions of the bladder wall of the metacestode of *Taenia solium* based on light and electron microscopy have been reported [1–5]. Some constituents of this structure have been inferred from studies of other metacestodes on the basis of the similar cytological organization shared by all members of this group [6; see chapter by R.D. Lumsden *et al.* in this volume]. Due to the fact that a complete morphological study of the bladder wall of *T. solium* larvae is not available at the present time, we give a general description of this structure based on electron microscopic studies.

II. MATERIAL AND METHODS

Cysticerci of *T. solium* were obtained from the muscles of parasitized pigs immediately after death. For transmission electron microscopy studies, an incision was made in the bladder wall of the whole larva and the cyst was fixed for 1 h in 2.5 % glutaraldehyde in 0.1 M veronal buffer, pH 7.4. The tissue was postfixed for 1 h with 1 % osmium tetroxide in 0.1 M veronal buffer, pH 7.4, dehydrated, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate [7, 8]. In studying the glycocalyx the procedure was the same except that the tissue fragments were postfixed for 2 h in 1 % osmium tetroxide plus 0.05 % ruthenium red. These sections were examined unstained or lightly contrasted with lead citrate. The sections were observed with a Hitachi HU 11A or a JEOL 100-B electron microscope. All representative areas were photographed on Kodak lantern slides or Kodak thick-base film.

For scanning electron microscopy studies, the larvae were processed following the methods described by Echlin [9]. A JEOL J5M35 scanning electron microscope was used to examine the samples. Representative images were recorded on Polaroid film.

The extracellular fibers were purified following the procedure outlined by Guerra *et al.* [10].

III. RESULTS

The thickness of the bladder wall varied between 70–100 μm. The exterior surface was covered with microvilli (Figs. 1 and 2) and the inner surface was irregular and made up of cellular and extracellular elements (Fig. 3). Six different cellular systems were identified within the wall: the tegument associated with tegumentary cytons [see chapter by R.D. Lumsden *et al.* in this volume]; storage cells [11]; a smooth muscle system represented by the myocytons [12]; fiber-

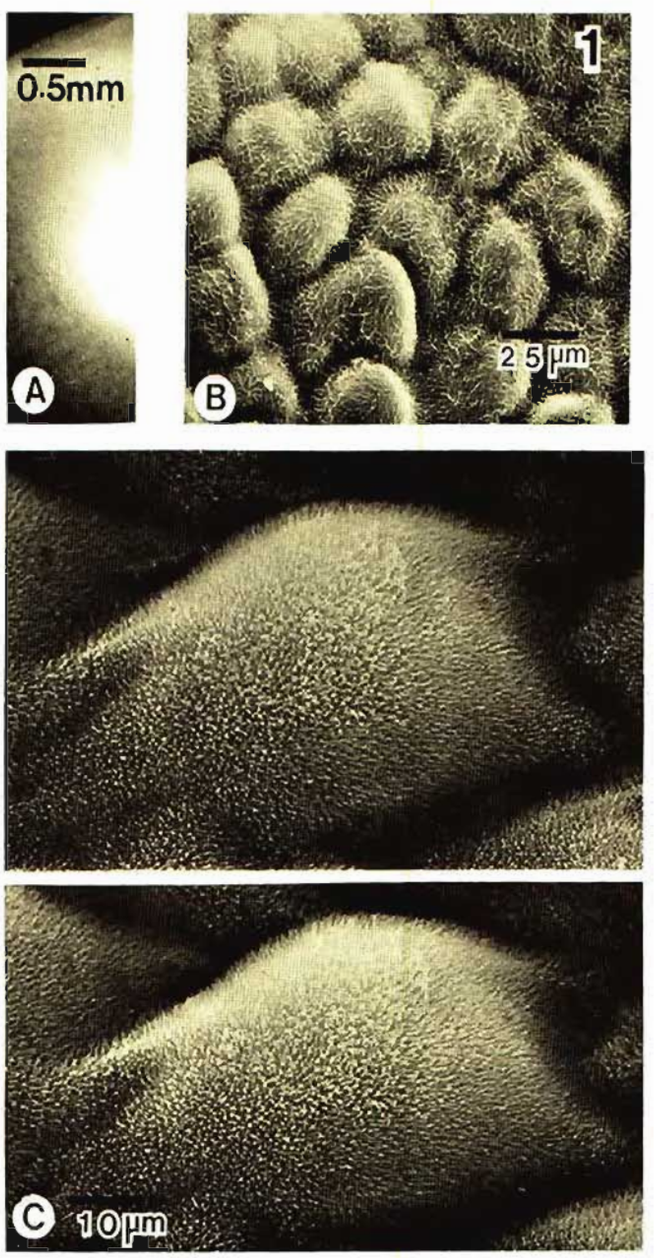
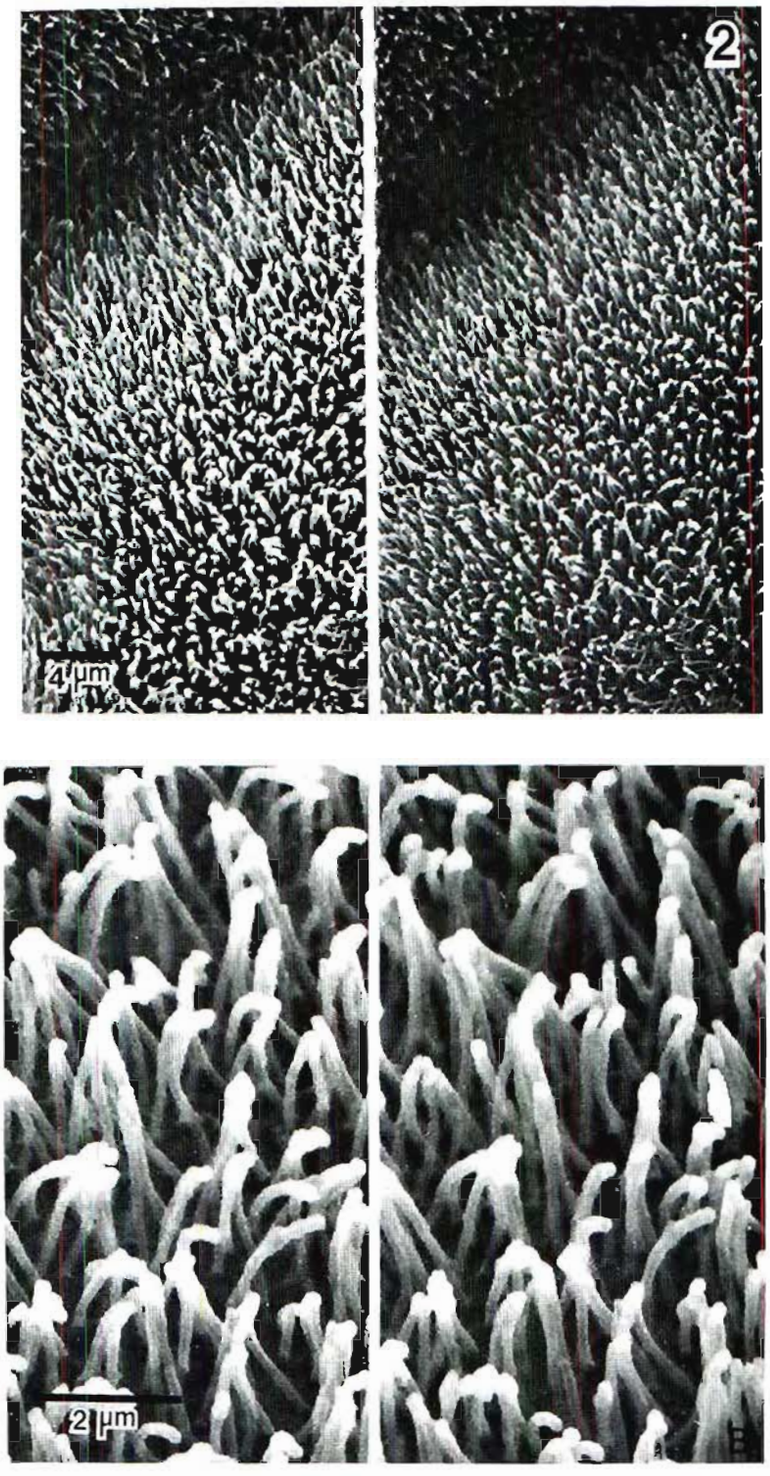


FIG. 1. Light (A) *and scanning electron* (B) *micrographs of the surface of the bladder wall of a* Taenia solium *metacestode.* C. *Stereoscopic view of a bulblike projection on the surface of the bladder wall.*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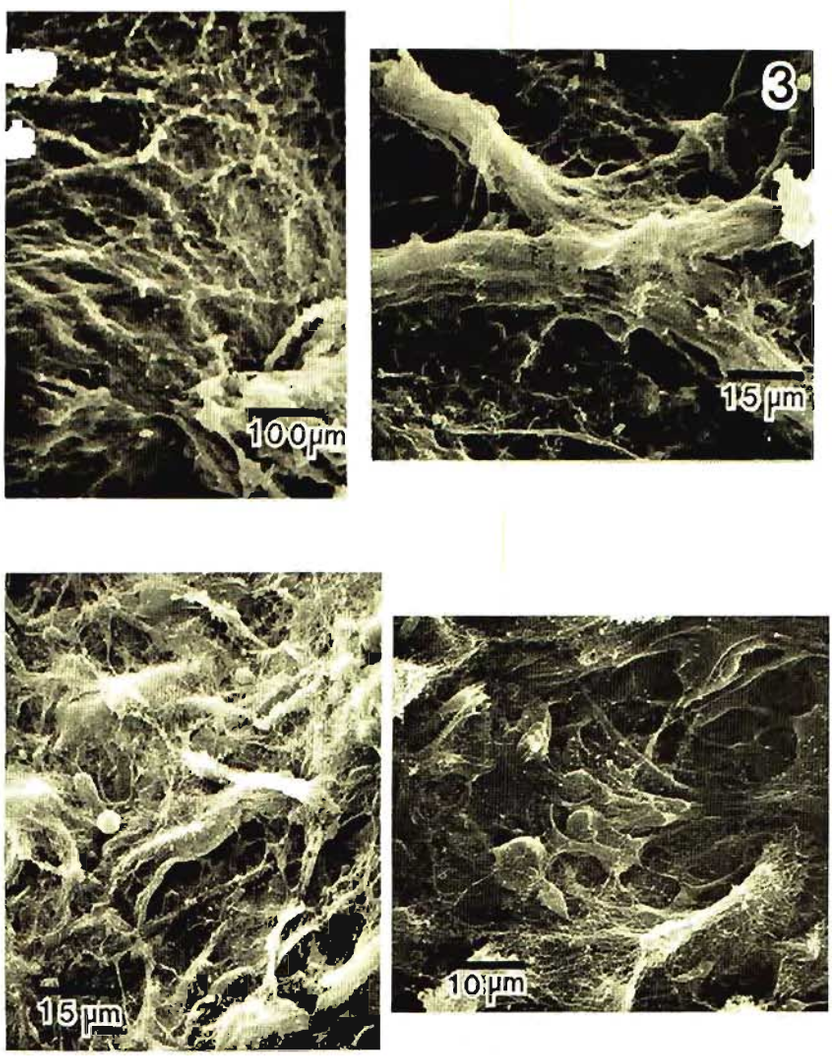


FIG. 3. Scanning electron micrographs of several regions on the inner surface of the bladder wall. The elongated and spherical structures presumably correspond to excretory ducts and cell bodies, respectively.

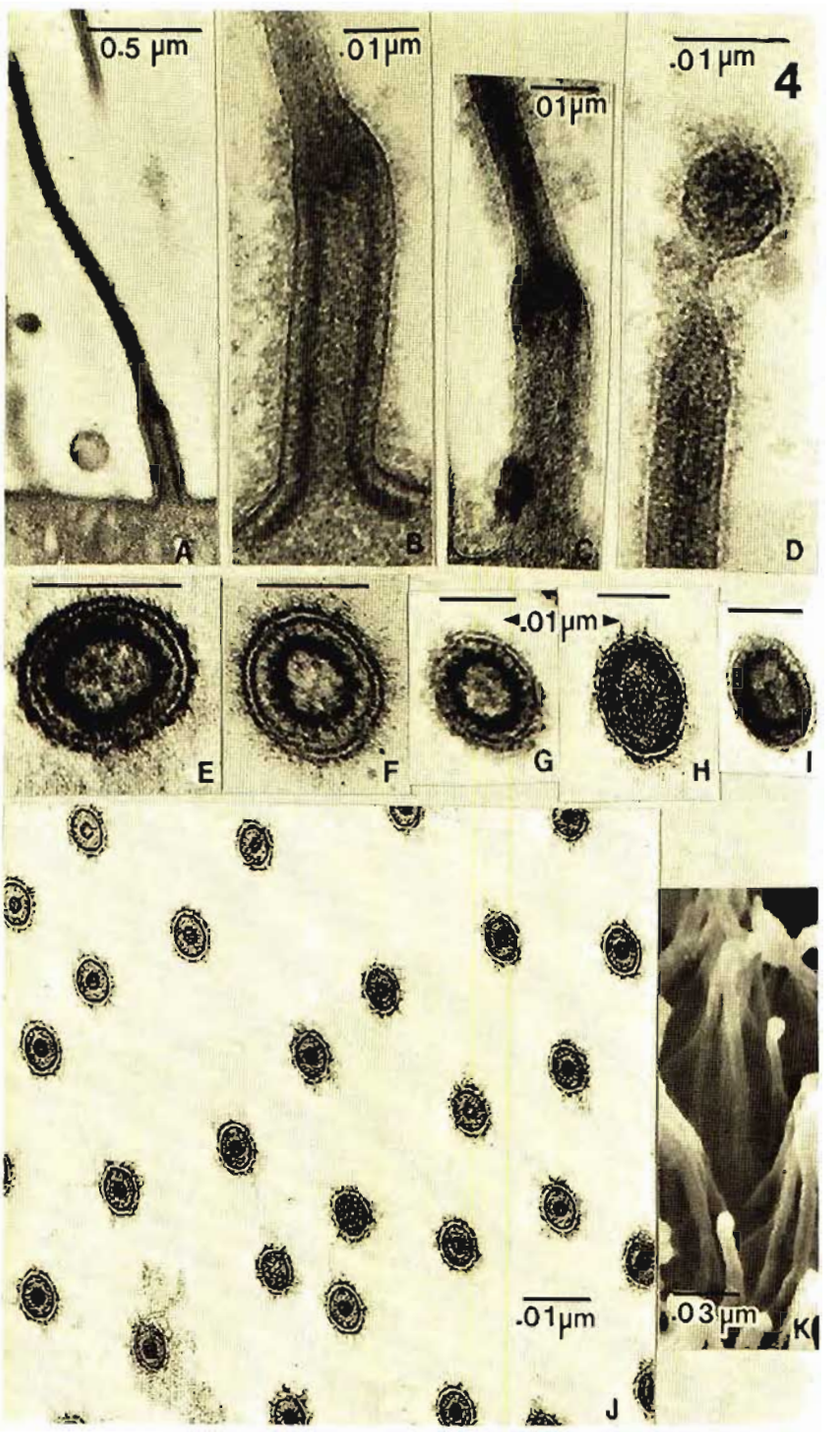
FIG. 2. Stereo images at two different magnifications of the microvilli on the outer surface of the bladder wall of a T. solium *larva.*

producing cells [13]; an excretory system formed by flame and duct cells [3, 13; see chapter by L. Cárdenas-Ramírez *et al.* in this volume]; and a nervous system identified by nerve axons [see chapter by L. Cárdenas-Ramírez *et al.* in this volume]. The only extracellular elements found were collagen fibers [see chapter by A. Torre-Blanco in this volume].

The external surface of the bladder wall had a bulblike appearance (Fig. 1). These swellings were presumably formed by the contraction of a circular musculature located at the base of each bulblike projection. Using light microscopy and polarized light it was possible to observe that the muscles were in constant motion, making these structures more or less prominent (Fig. 1). The tegumentary surface was covered with microvilli (Figs. 1 and 2), thin cylindrical structures which projected vertically about 2.0 μm and were separated from each other at the base by a distance of 0.5 μm (Figs. 4A, 5C,E and 6). The microvilli had two distinctive parts: a wide base, 0.1 μm in diameter and 0.5 μm in height, and an apical region which had an external diameter of 0.7 μm and a length of 1.5 μm (Fig. 4). The apical section terminated in a papillaelike spherical structure, 0.7 μm in diameter (Fig. 4D). The microvilli were limited by a plasma membrane, 0.01 μm thick. Inside the basal portion of this structure was a cylindrical core of longitudinally arranged microfilaments, 0.5 μm in diameter [see chapter by R.D. Lumsden *et al.* in this volume] (Fig. 4A,B,E,F,G). At the base of the microvilli, the microfilaments continued under the plasma membrane of the tegument (Fig. 4A,B). In the distal portion of the basal shaft, the core of the microfilaments ended in an ellipsoidal structure called the base plate by Lumsden *et al.* [see chapter by R.D. Lumsden *et al.* in this volume] (Fig. 4A,B, C,H,I). From the base plate the microfilaments continued along the center of the apical portion of the microvilli (Fig. 4C,D,J).

The external surface of the plasma membrane was covered with a loose fuzzy layer of glycocalyx which interconnected the microvilli by means of long thin filaments [14] (Fig. 5A,B). These structures were enhanced by the addition of 0.05 % ruthenium red to the osmium tetroxide postfixative solution (Fig. 5C, D). Using this procedure an electron-dense layer ranging from 10–15 nm in

FIG. 4. A. *Longitudinal section of a microvillus showing the internal core of the microfilaments and the base plate.* B. *Longitudinal magnified view of the basal shaft of a microvillus showing the extension of the microfilament core from beneath the tegumental membrane and its termination at the base plate. The glycocalyx appears as a fuzzy layer on the surface of the plasma membrane.* C. *Longitudinal section of the basal shaft showing a different view of the base plate.* D. *An enlargement of the apical portion of a microvillus demonstrating the papillaelike ending, the core of microfilaments, and the glycocalyx coat.* E, F, G. *Transverse sections taken at different locations along the length of the basal shaft of a microvillus showing the organization of the microfilament core.* H, I. *Sections of the base plate.* J. *Transverse section of a group of microvilli demonstrating the internal organization of the apical portion.* K. *Scanning electron micrograph of a group of microvilli. The different parts of a microvillus demonstrated by this technique correspond to those found in the sectioned material.*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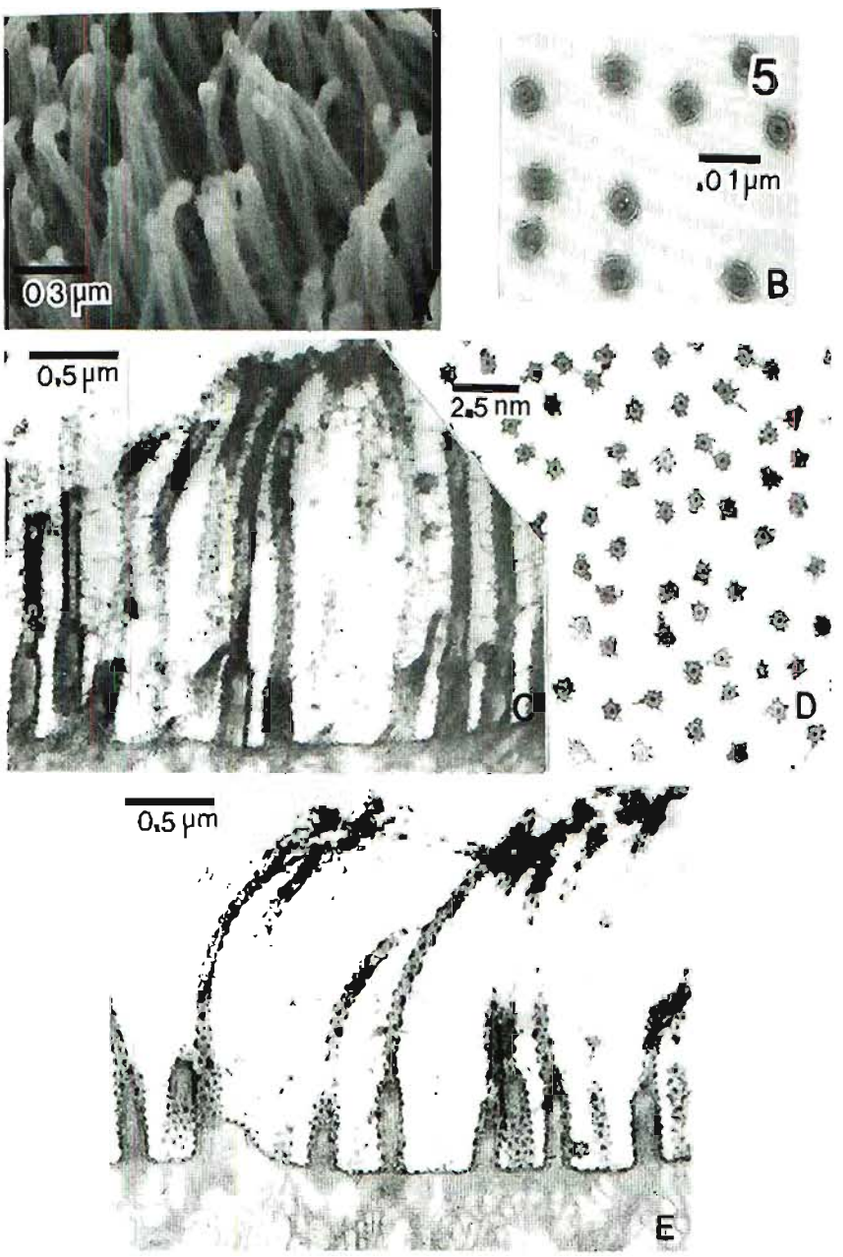


FIG. 5. A. *Scanning electron micrograph of the surface of the bladder wall showing long, thin filaments of the glycocalyx interconnecting the microvilli.* B. *Section of the apical portion of a group of microvilli demonstrating the long, thin filaments and the glycocalyx coat.* C, D. *Same structures as in* B *enhanced by the addition of 0.05 % ruthenium red to the osmium tetroxide postfixative solution showing hemispherical electron-dense structures on the surfaces of the microvilli.* E. *Unstained section of the external portion of the tegument demonstrating the helical patterns adopted by the glycocalyx on the surface of the microvilli, and their distribution on the surface of the plasma membrane. The same enhancement procedure as in* C *and* D *was utilized.*

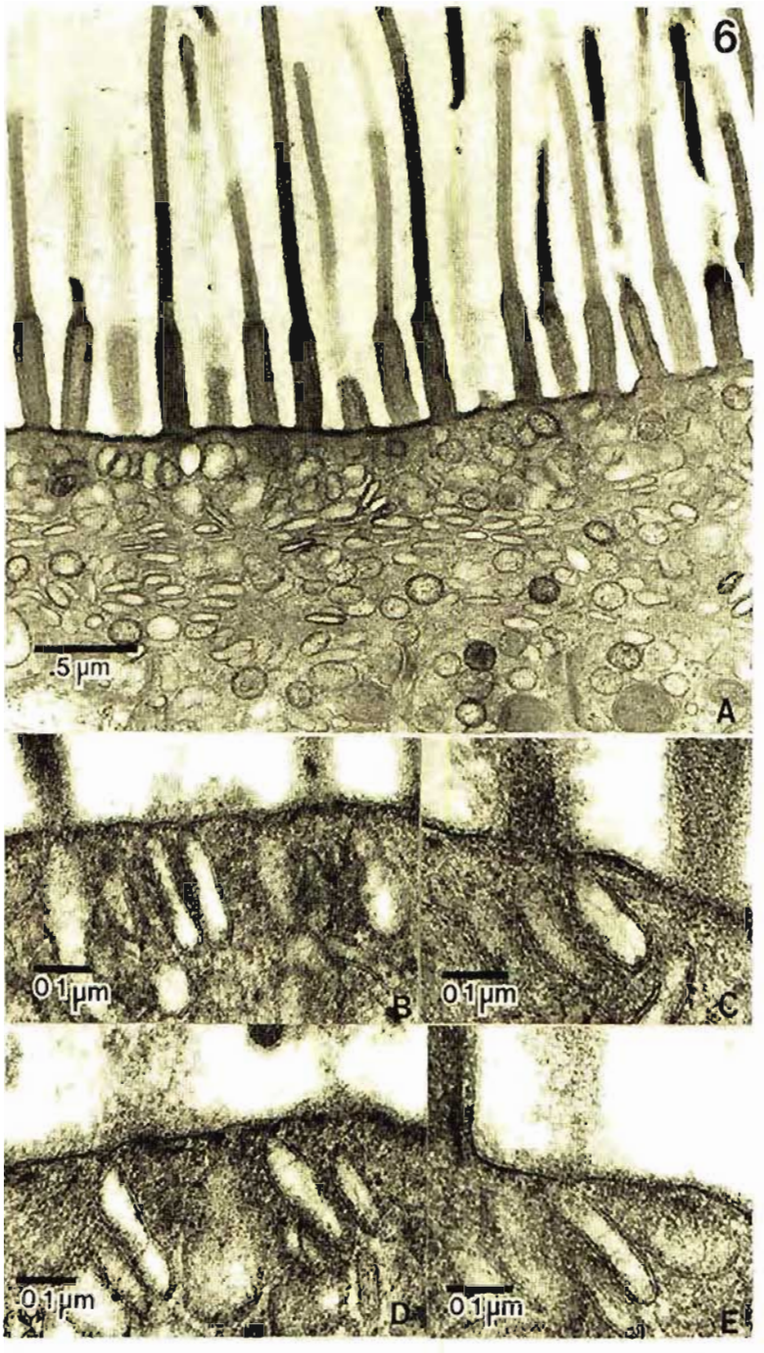


FIG. 6. A. *Section of the tegument showing the distribution and morphology of the vesicles and mitochondria.* B, C, D, E. *Vesicles can be seen to be open at the surface, apparently emptying their contents into the exterior.*

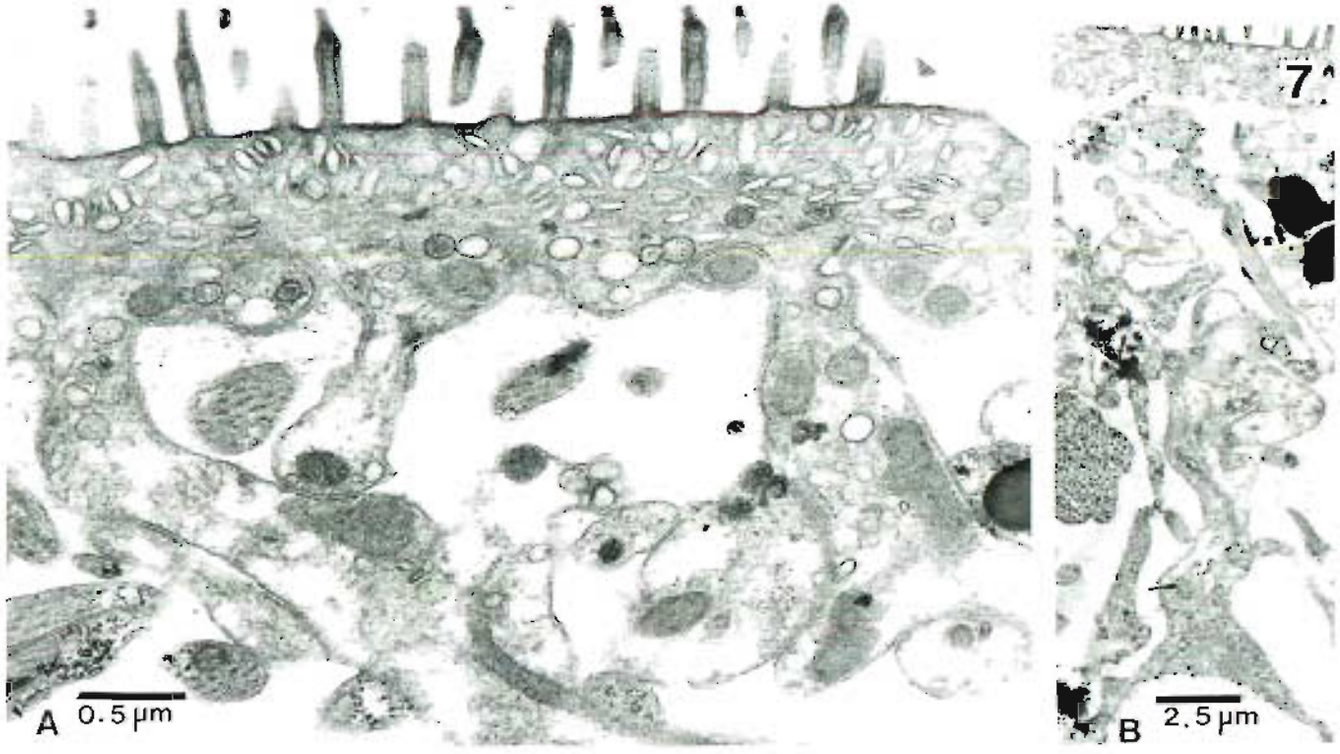


FIG. 7. A. *Section of the external portion of the bladder wall. The basal membrane under the tegument is interrupted by infoldings of cytoplasmic processes that join the tegument with deeper nucleated cell bodies.* B. *A structure which appears to be an intermuscial cytoplasmic process of a tegumental cell is issuing from a collector duct, oriented toward the external band of the tegument.*

thickness was observed on the surface of the plasma membrane with round deposits, 0.02 μm in diameter, scattered in some areas. In addition to the long, thin filaments connecting the microvilli, an arrangement of electron-dense hemispherical elements, 0.03 μm in diameter, with a helical distribution pattern were observed on the surface of the microvilli (Fig. 5C,D,E).

Under the external plasma membrane, the tegument was filled with ellipsoidal vesicles of different sizes, whose diameters ranged from 0.05 x 0.08 μm to 2.6 x 3.0 μm. Some of them were more electron-dense than others. Occasionally, the membranes of the vesicles merged with the plasma membrane and opened onto the external surface, appearing to empty their contents into the exterior (Fig. 6). The inner limit of the tegument was a continuous protoplasmic band, 1.0 μm wide, supported by a basal membrane of connective tissue (Fig. 7). This membrane was interrupted by infoldings of the syncytium, forming cytoplasmic processes that connected the tegument to the nucleated cell bodies located 15–20 μm below the surface. These processes were normally filled with vesicles, mitochondria, and microtubules (Fig. 7).

Before making a description of the different types of cells identified in the bladder wall, the characteristics shared by all of them in the perinuclear region can be summarized as follows. The smallest diameter of sections that included the nucleus and nucleolus, which was the most constant measurement for any section of the nucleated cell bodies, averaged between 3.2–5.0 μm. The big nucleus, 2–3 μm in diameter, contained a prominent nucleolus, 0.5–0.7 μm in diameter, dense granules of chromatin near the nuclear membrane, and a granular nucleoplasm. The cellular organelles and inclusions common to all the cells observed were free ribosomes, a Golgi apparatus, mitochondria, microtubules, and small vesicles. The rest of the cytoplasm showed specific features that characterized each cell.

The perinuclear cytoplasm of the tegumentary cytons had a very similar appearance to the tegument, containing a great number of vesicles, a well-developed Golgi apparatus, microtubules, ribosomes, and mitochondria [13] (Fig. 8B). The nucleated cell bodies of the storage cells [11] were located near to those of the tegumentary cytons. The rest of the cytoplasm of the storage cells, which was distributed throughout the bladder wall, contained large quantities of α-glycogen and lipid droplets. The cytoplasmic membranes of the storage cells were in contact with the cytoplasmic membranes of all the other cellular types that were identified (Fig. 8A).

The excretory system was characterized by two types of cells: flame cells and duct cells [13; see chapter by L. Cárdenas-Ramírez *et al.* in this volume]. The former were distinguished by the presence of a variable number (60–130) of hexagonally arranged cilia with a typical 9 + 2 arrangement of microtubules (Fig. 9). The cilia extended for 6.5 μm inside the collector duct which was 1.0 μm in diameter (Fig. 9). This duct appeared to be formed by the interdigital projections of the cytoplasm of the flame and duct cells (Fig. 9). Further from

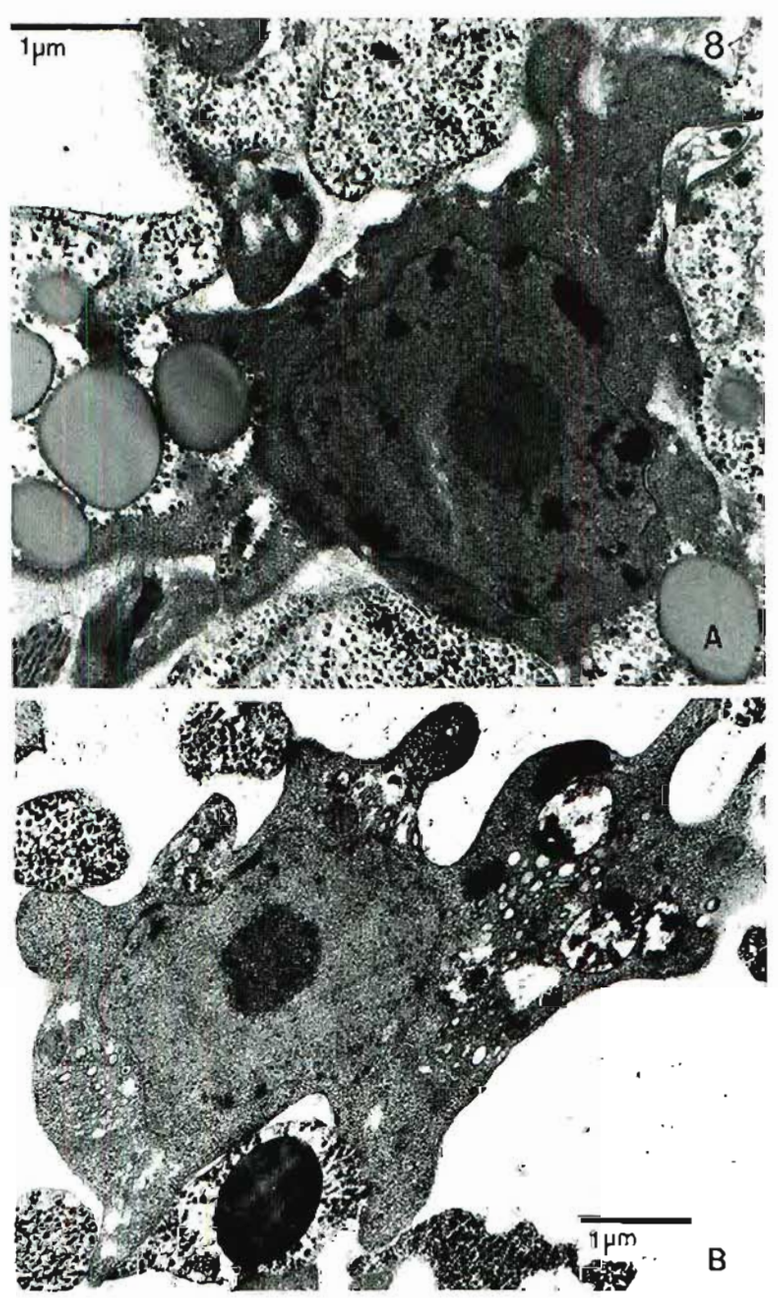


FIG. 8. Sections showing closely juxtaposed nucleated cell bodies in the bladder wall. A. *A storage cell, α-glycogen, and lipid droplets are surrounded by ribosome-rich perinuclear cytoplasm.* B. *A tegumental cell characterized by vesicles, mitochondria, and ribosomes.*

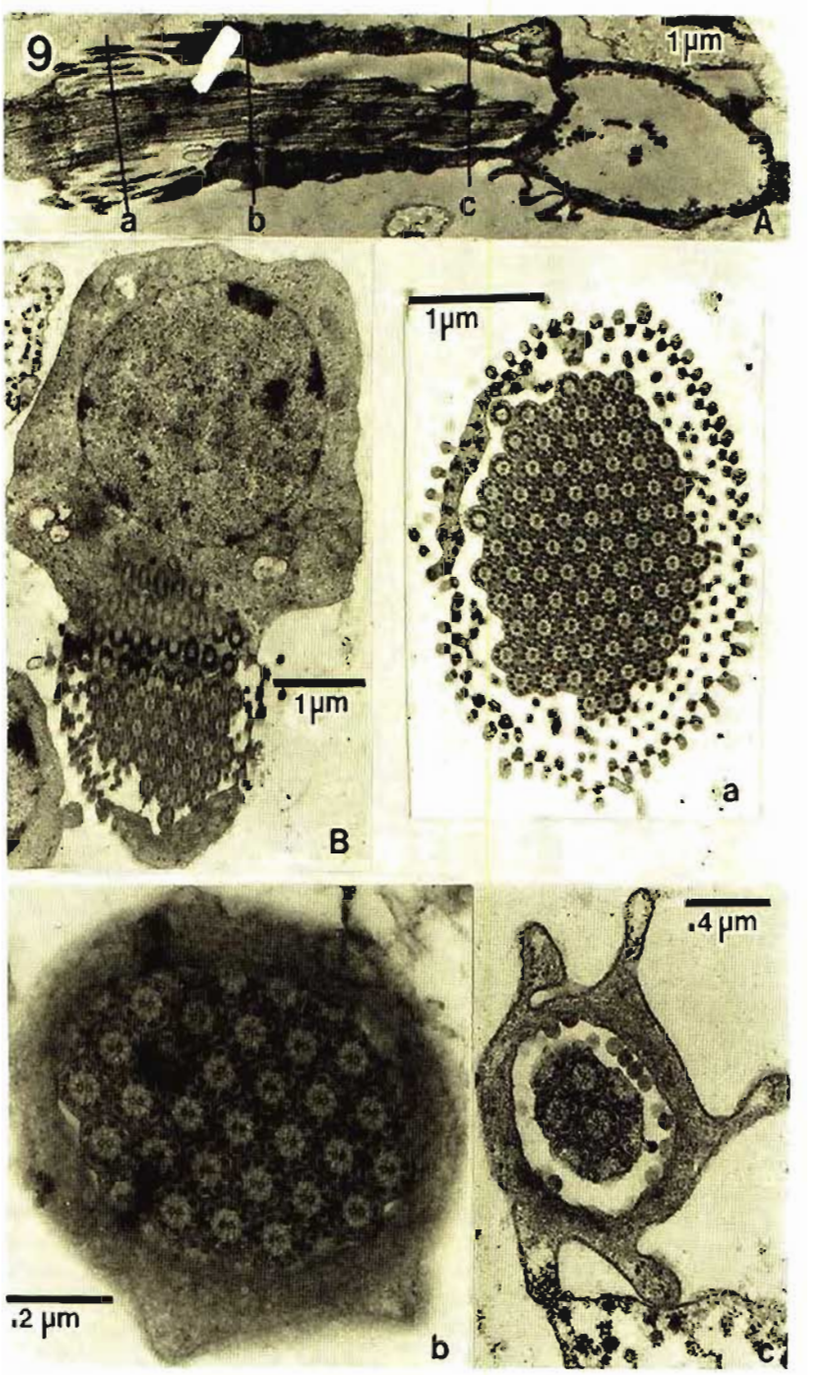


FIG. 9. A. *Micrograph of the cilia of a flame cell located inside a collector duct that has been sectioned at three different locations: the interdigital region between the flame and duct cells* (a); *in a region of the collector duct filled with cilia* (b); *and in an area of the collector duct showing the tip of the cilia* (c). B. *Nucleated cell body of a flame cell.*

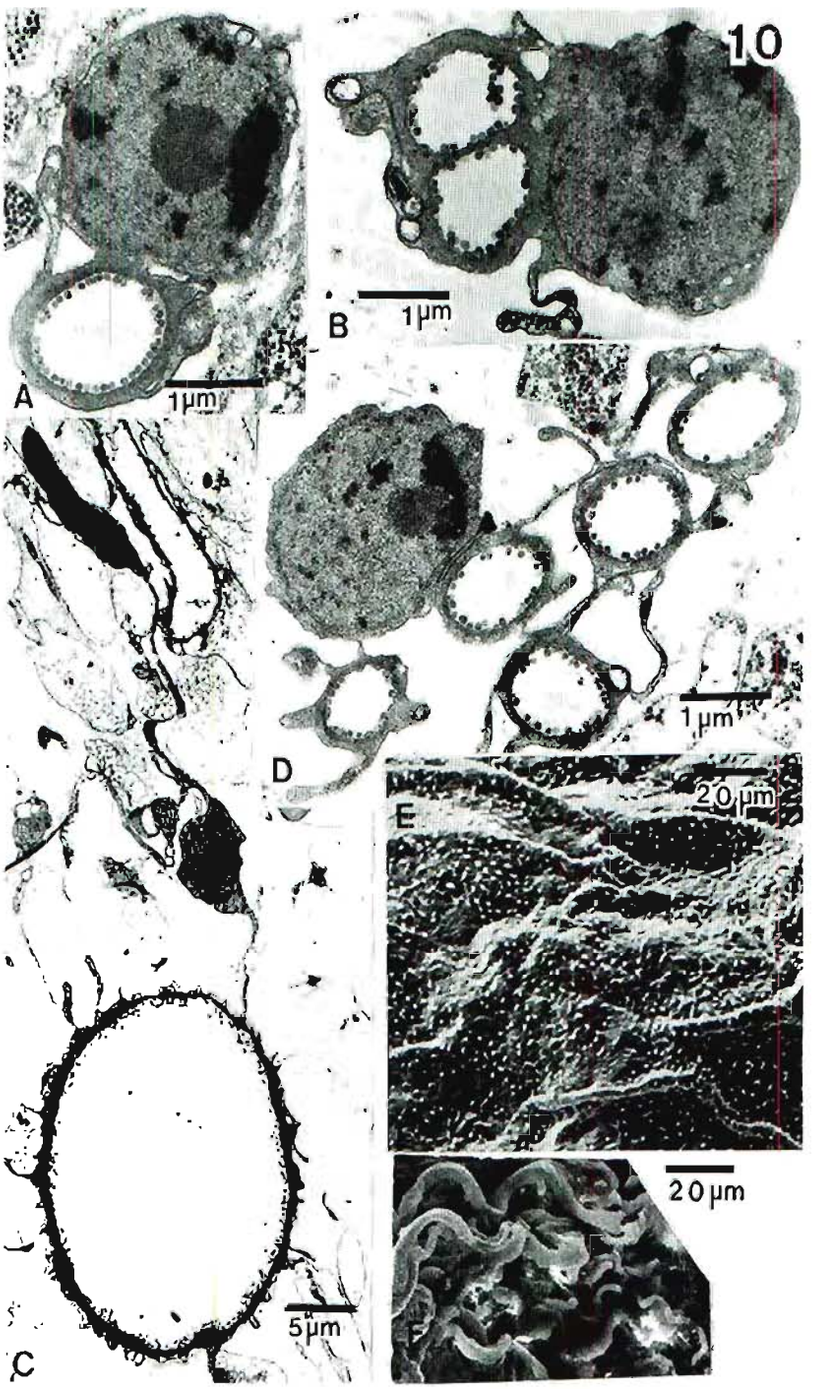


FIG. 10. A, B, C, D. *Sections of the nucleated cell bodies of duct cells. Two nucleated cell bodies can be seen to be joined together and also to be connected to two different collector ducts through cytoplasmic bridges, forming a syncytium* (C). *Profiles of collector ducts are shown at both the external* (E) *and internal* (F) *surfaces of the bladder wall.*

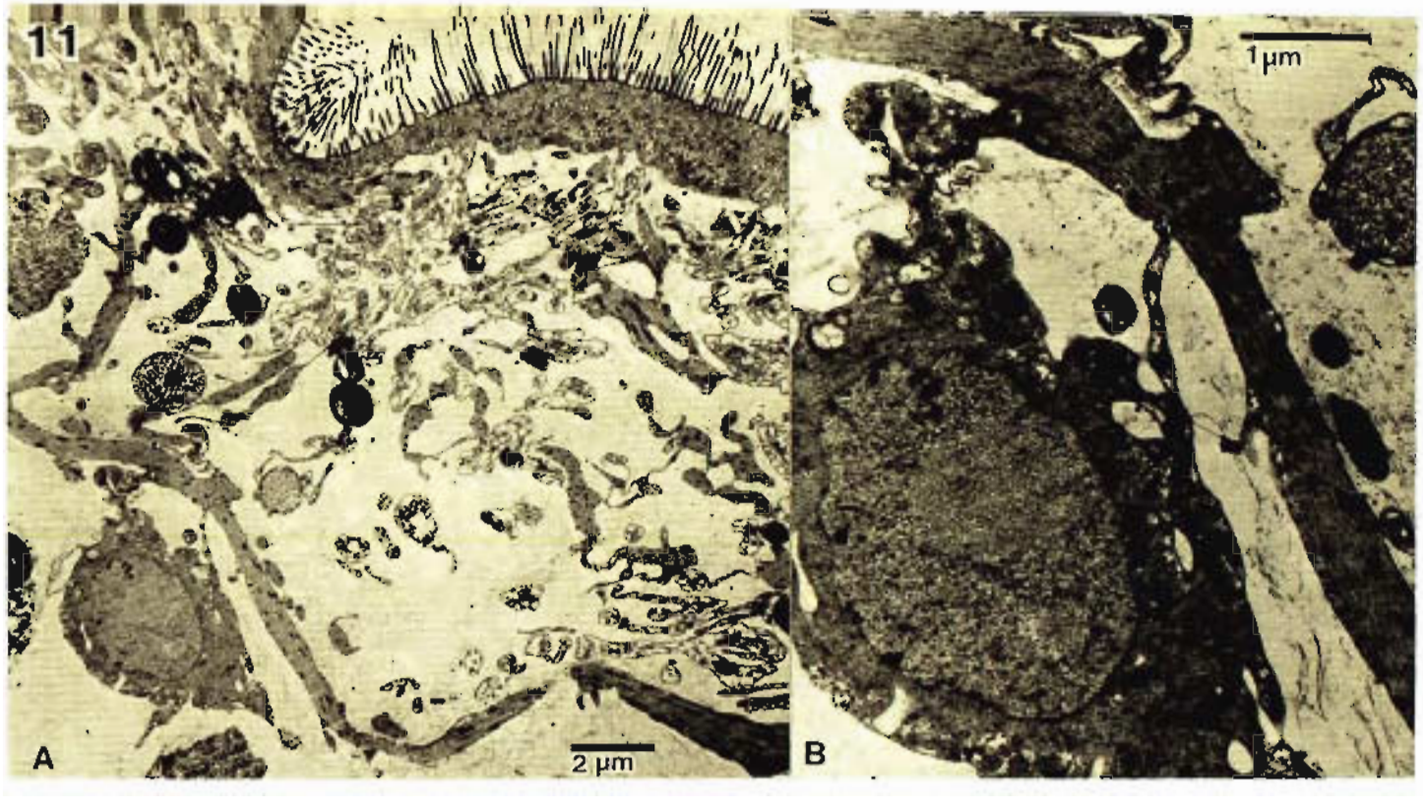


FIG. 11. A. *A nucleated cell body of a myocyton can be seen to be joined through cytoplasmic bridges to a bundle of myofibrils.*
B. *Enlargement of* A.

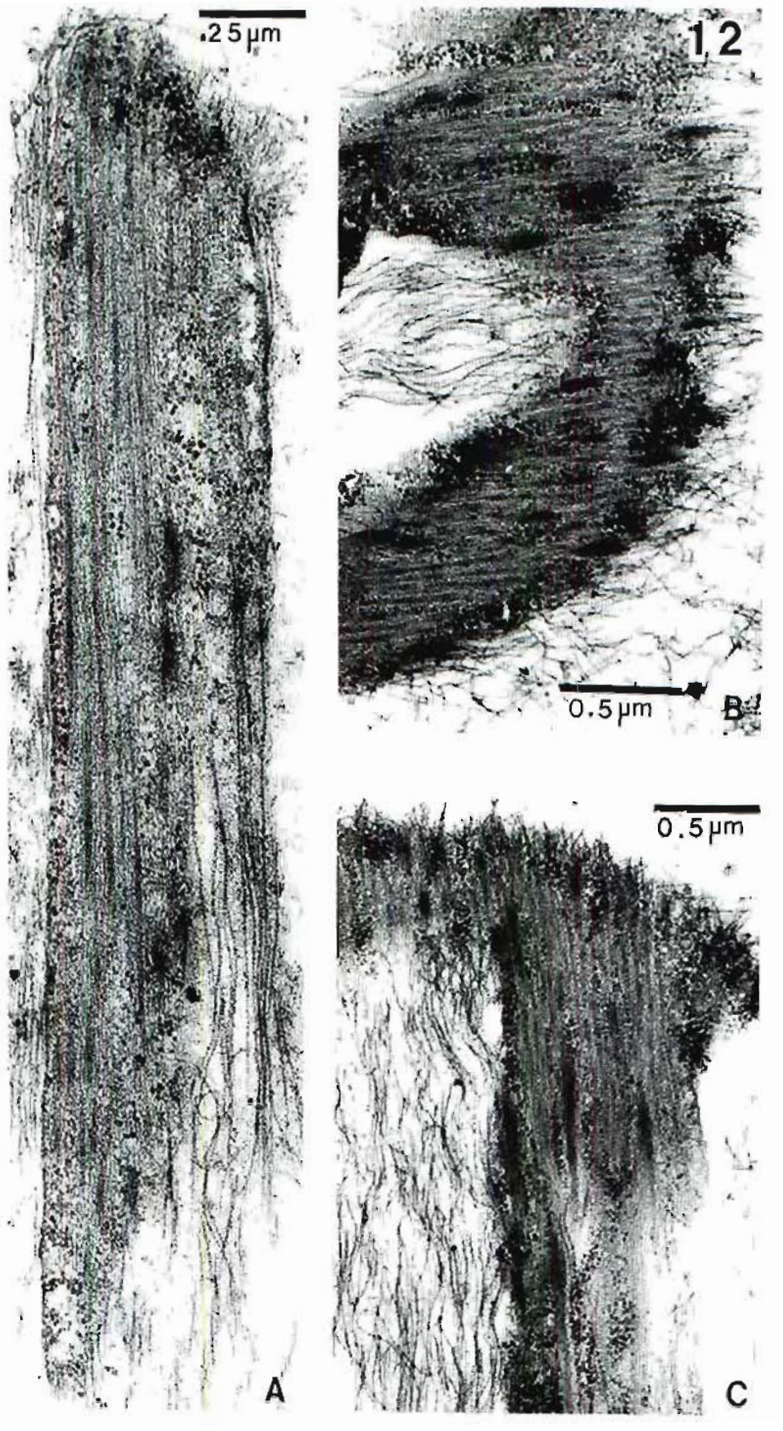


FIG. 12. The myofibrils in the bladder walls of T. solium *larvae are closely associated with extracellular fibers.*

the flame cell, the collector ducts appeared to be formed by a syncytial arrangement of the duct cells. Their lumen increased as they left the flame cell, reaching a diameter of up to 15 μm (Fig. 10), giving origin to prominent tubular structures on both surfaces of the bladder wall (Fig. 10E,F).

The nucleated cell bodies of the myocytons were connected to the myofibrils by cytoplasmic bridges, giving origin to a syncytium (Fig. 11B). The muscle cells found in the larvae of *T. solium* had a morphology which has been described for this genera [11], and their distribution in the bladder wall corresponded to that described by Voge [3]. Bundles of muscle fibers ran between the basal membrane of the tegument and the nucleated cell bodies of the tegumentary cytons and storage cells (Fig. 11A). Bands of myofibrils that were perpendicularly oriented with respect to the surface were occasionally seen.

The layers of muscle fibers were very closely associated with the collagen fibers (Fig. 12). The connective tissue surrounding the muscle presumably acted as a point of attachment for particular groups of myofibrils. The extracellular fibers have been characterized as a special kind of collagen fiber [see chapter by A. Torre-Blanco in this volume] and have been partially purified [10] (Fig. 13C).

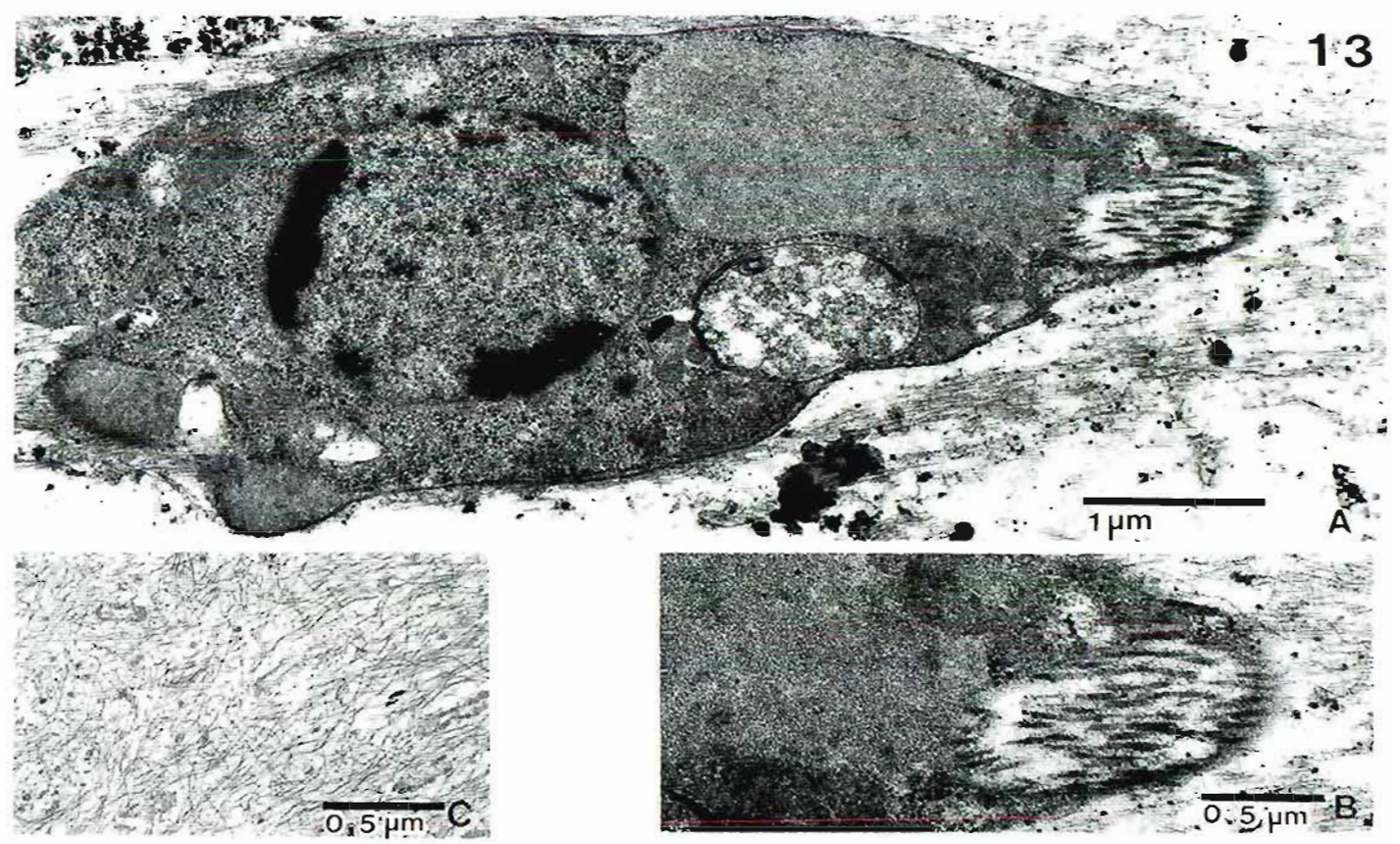
The term fibroblast has been used here to identify structures that have nucleated cell bodies containing a moderate amount of perinuclear cytoplasm and vacuoles filled with fine granular material (Fig. 13). The vacuoles displayed cigar-like structures (250 Å wide), some of which were located at the level of the cytoplasmic membrane. Occasionally, extracellular fibers were associated with these structures (Fig. 13A,B).

Sensory neural elements associated with the body surface of metacestodes [see chapter by L. Cárdenas-Ramírez *et al.* in this volume] were not observed in the hundreds of sections examined for this purpose; only parts of nerve axons were occasionally seen (data not shown).

IV. DISCUSSION

The bladder wall of a *T. solium* cysticercus is a complex organ designed to protect and maintain the invaginated larva. This structure directly interacts with the host, and most of the functions of the larva that are related to the environment such as alimentation, waste elimination, and defense against the immunological apparatus of the host [see chapter by R.D. Lumsden *et al.* in this volume] presumably are performed through this organ.

The surface of the bladder wall of an average cysticercus (1.4 x 0.7 cm) has an approximate area of 2.49 cm^2. This surface is increased about 136 times by the microvilli. The external cytoplasmic membrane of the bladder wall is protected by a layer of glycocalyx, possibly secreted by the vesicles located in the tegument. Although some aspects of this phenomena are still unclear, the secretory role of the vesicles in *T. solium* metacestodes is supported by the following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observations: 1) mebendazole (methyl 5-benzoyl-2-benzimidazolecarbamate), a synthetic drug that interferes with the microtubular function of the parasite, causes vesicles to accumulate in the nucleated cell bodies of the tegumentary cytons [15]; 2) some vesicles merge with the protoplasmic membrane and open onto the external surface, empyting their contents into the exterior (Fig. 6); and 3) live cysts incubated for several days in the presence of different markers including cobaltous chloride, ruthenium red, cationized ferritin, methylene blue, and carbon particles show an accumulation of these materials only at the surface of the glycocalyx, but no traces are found inside the vesicles even if they are opened onto the surface [unpublished observations]. The functions that the secreted material might have in the immunological response are still not defined.

The glycogen and lipids contained in the storage cells of *T. solium* larvae are derived from the environment. The regulatory mechanisms by which the metacestode metabolizes these substrates are yet to be studied in detail.

The specific mechanism by which collagen fibers are produced is suggested by the structural evidence presented in Figure 13A,B. Whether the fibroblasts constitute an independent line of cells or represent only specialized regions of the muscle or duct cells is not clear at the present time.

The elimination of waste by the larva appears to be carried out by means of a primitive excretory system. The incorporation of markers inside the bladder wall or cyst fluid using microneedles and a micromanipulator, followed by the study of their elimination through the tubular system, could help to elucidate its function.

FIG. 13. A. *Section of the nucleated cell body of a fibroblast.* B. *Detail of* A. *The cigar-like structures appear to be formed in the vacuole close to the fine granular material. Extracellular fibers are associated with them at the level of the cytoplasmic membrane.* C. *Partially purified extracellular fibers.*

REFERENCES

1. Šlais, J. "The Morphology and Pathogenicity of the Bladder Worms *Cysticercus cellulosae* and *Cysticercus bovis*," 144 pp. Dr. V. Jung N.V., The Hague – Academia, Prague, 1970.
2. Šlais, J. Functional morphology of cestode larvae. *Adv. Parasitol.* 11: 395, 1973.
3. Voge, M. Observations on the structure of cysticerci of *Taenia solium* and *Taenia saginata* (Cestoda: Taeniidae). *J. Parasitol.* 49: 85, 1963.
4. Tay, J. Estudio del *Cysticercus cellulosae* al microscopio electrónico. I. Membrana cuello y escólex. *Rev. Lat. Am. Microbiol.* 14: 107, 1972.
5. González Angulo, A., Hernández Jáuregui, P., Rabiela, M.T. and Ruíz de Chávez, I. Comparative scanning electron microscopy of the outer wall of the bladder of *C. cellulosae* and *C. racemosus. Proceedings. Electron Microscopy Society of America 34th Annual Meeting*: 210, 1976.
6. Lee, D.L. The structure and composition of the helminth cuticle. *Adv. Parasitol.* 4: 187, 1966.
7. Reynolds, E.S. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17: 208, 1963.
8. Cañedo, L. and González del Pliego, M. Estudios preliminares de la estructura del *C. racemosus. Archivos Mexicanos de Anatomía* 15: 59, 1978.
9. Echlin, P. The application of scanning electron microscopy to biological research. *In* "New Developments in Electron Microscopy" (H.E. Huxley and A. Klug, eds.), pp. 135–172. The Royal Society, London, 1971.
10. Guerra, G., Cañedo, L. and Rojkind, M. Proteins of *Cysticercus cellulosae. In* "Biochemistry of Parasites and Host-Parasite Relationships" (H. Van den Bossche, ed.), pp. 109–115. North-Holland, Amsterdam, 1976.
11. Lumsden, R.D. Macromolecular structure of glycogen in some cyclophyllidean and trypanorynch cestodes. *J. Parasitol.* 51: 501, 1965.
12. Lumsden, R.D. and Byram, J., III. The ultrastructure of cestode muscle. *J. Parasitol.* 53: 326, 1967.
13. Ramírez, E., Merchant, M.T. and Cañedo, L. Fine structure of the bladder wall of *Cysticercus cellulosae. Third International Symposium of Parasitology* 1: 405, 1974.
14. Sosa, A., Girón, H., Alva, S. and Calzada, L. Presence and nature of a glycocalyx-like coat on the external vesicular membrane of *Cysticercus cellulosae. Life Sci.* 21: 1021, 1977.
15. Laclette, J.P., Guerra, G. and Zetina, C. Inhibition of tubulin polymerization by mebendazole. *Biochem. Biophys. Res. Commun.* 92: 417, 1980.

BLADDER WALL OF *Cysticercus cellulosae* AS A PHYSIOLOGICAL BARRIER

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I. INTRODUCTION

Taenia solium metacestode does not have a digestive or a respiratory system and posseses only a very primitive excretory apparatus (1); therefore, its relationship with the environment has to be done mostly through the bladder wall. Very little is known about the physiology of this parasite (2), preliminary experiments done with complete viable larvae obtained from infected pigs by Puyou, Grau and Cañedo (unpublished), showed active intake of calcium and low oxigen consumption. The purpose of this preliminary report is to provide evidence that supports this hypothesis suggesting that the bladder wall acts as a physiological barrier.

II. MATERIALS AND METHODS

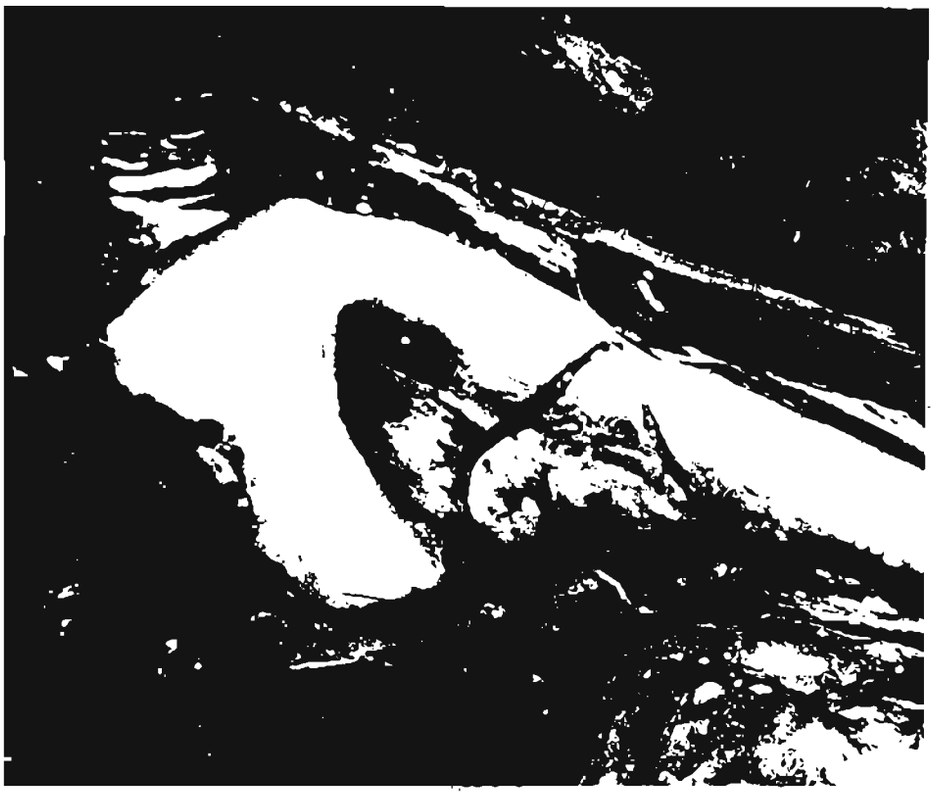
a) Biological material:

Landrace infected pigs growing in farms, were adapted to the laboratory environment. Physical examination, record of temperature, heart and respiratory frequency, analysis of blood, urine and feces, were performed as frequently as necessary until stable normal conditions were achieved. When necessary, treatment to other infections and non-cestodes parasitic diseases were applied. Commercial "Purina" food was administered *ad libitum* and four hours prior surgery, 400 gr. of butter were fed to each pig to allow proper identification of the thoracic duct. Intravenous administration of Ketalar (2-(o-chlorophenyl)-2(Methylamina) cyclohexanone hydrochoride) (2 mg/Kg) was used as an anesthetic.

The thoracic duct was clearly exposed close to the aorthic hiatus (Fig. 1). In order to obtain the lymph sample, a canule was inserted with a single shot without damaging the rest of the thoracic duct in order to avoid contamination with other fluids. The lymph was placed in a tube with heparin, centrifuged to separate the cellular components and the super-

Fig. 1

Thoracic duct exposed close to the aorthic hiatus (3X).



natant quantified before it was lyophilized. The blood sample was obtained from dissected peripheral veins and divided into two portions. The first sample was placed in a tube containing heparin as anticoagulant, centrifuged to separate the cell package and the remaining supernatant was quantified lyophilized in a dry clean, wighted flask, and marked as plasma. The second sample was placed in a tube without anticoagulant, after a contracted clot was observed the tube was centrifuged and the supernatant measured and lyophilized in a similar way as the previous sample and marked as serum.

The cyst fluid was obtained by piercing the bladder wall of a decapsulated cyst with a hypodermic needle. The cyst fluid obtained was placed in a clean tube and passed through a 0.45μ millipore filter to separate cellular debris, the filtered liquid was quantified and lyophilized before analysis.

Once the death of the animal occurred, the remaining infected muscle was divided in three portions. One was kept at room temperature for six hours; the second was placed 24 hours at 4 C, and the third sample frozen at -4 C during 12 hours. From these samples, decapsulated cysts were obtained, the fluid extracted and stored prior to analysis in the same manner as discussed previously.

A Cysticercus racemosus located in the subarachnoid space was obtained from a patient from the Instituto Nacional de Neurologia through the courtesy of Dr. Gómez Llata. The cerebrospinal fluid from this patient was obtained at the beginning of surgery before it was contaminated with blood, the material was centrifuged to eliminate the cellular components and the supernatant measured and lyophilized. The cyst fluid was obtained by piercing the bladder wall with a hypodermical needle while the parasite still was in the brain of the patient and placed in a clean flask. The fluid was passed through a 0.45μ millipore

filter to separate cellular debris, measured, placed in a dry clean weighted flask and then lyophilized.

b) Chemical analysis:

The protein concentration was determined by the Lowry procedure modified by Hartree (3). Carbohydrates were identified by the anthrone method of Morris (4), by the glucose oxidase procedure (5), by thin layer chromatography (6), and by gas chromatography (7). The total lipids were isolated and quantified according to Folch (8), lecithin by thin layer chromatography and phosphate quantification (9,10,11). Triglicerides values were obtained following the procedure of Van Handel (12), cholesterol after Zak (13), DNA according to Dische (14), and RNA after Mejbaum (15). Element determination was carried out by atomic absorption in a Perkin Elmer spectrophotometer Mod. 303 and a Astrup micro equipment was used for Ph determination.

III. RESULTS

The data are summarized in the following tables.

well at type III collagen; he suggested that antigen B may interfere with this enzyme and alter the deposition of connective tissue, thus favoring the parasite. Williams suggested that binding studies of antigen B to collagen be carried out with a nonfluoresceinated sample, and Mitchell proposed that the host-parasite interface be analyzed to see whether it is saturated with antigen B released by the parasite.

Cañedo presented some experiments done with F. García-González, M. Romero, and P. Díaz§ on the chemical composition of the vesicular fluid of four cysticerci compared with the serum, plasma, and lymph of the same pig. Carbohydrates, lecithin, cholesterol, sodium, and chloride had similar values in these fluids; proteins, DNA, and RNA were lower in the cyst fluid; and lipids, potassium, calcium, and magnesium tended to be higher (Table I). Also, the cerebrospinal fluid of a patient suffering from infection with a racemose cysticercus was compared with the vesicular fluid from the same metacestode (Table II). The authors suggested that these results indicate that the bladder wall of cysticerci functions as a physiological barrier for small and large molecules.

With respect to the use of cell-free systems for the identification of antigens of the cysticerci of *T. solium*, Díaz de León showed that by using antipig IgG, a molecule coded by the parasite's RNA is precipitated. Heath, Mitchell, and Williams raised the question of whether the rabbit antisera used in these experiments did not contain antiparasite antibodies that could cross react with the parasite. As an example, Williams mentioned that a goat antirat IgG purchased from Miles reacted with several *Taenia* species and with *Moniezia*, suggesting that the goat had a natural infection with the latter parasite. The porcine IgG used for immunization could have contained immune complexes so that the rabbit produced antipig IgG as well as antiparasite antibodies. Willms agreed that commercially obtained antisera should be carefully screened for cross-reacting antibodies; however, normal rabbit serum used in her laboratory did not precipitate pig IgG, cysticercus antigen, or the *in vitro* translated proteins. The pig serum used for obtaining the IgG fraction as well as the sera from rabbits immunized with this fraction also did not precipitate cysticercus antigens.

During the presentation of his paper, Heath explained in detail the methods he used for obtaining taeniid eggs from feces. In response to Rickard who questioned whether *T. solium* proglottids liberate eggs into feces, Soulsby said that this species of *Taenia* has an intraproglottideal muscle which keeps the segment closed when the proglottid is separated. Pawlowski commented that the eggs probably are released because patients with taeniasis can be detected by copro-

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TABLE I. Average Chemical Composition of Serum, Plasma, and Thoracic Lymph from Four Cysticercotic Pigs and of the Cyst Fluid from Taenia solium *Larvae Present in the Muscles of these Animals*[a]

Component[b]	*Serum*	*Plasma*	*Lymph*	*Cyst Fluid* in vivo	*Cyst Fluid from Infested Muscle Stored at*[c]*:*		
					1	*2*	*3*
Proteins	*6.6*	*6.6*	*5.7*	*1.2*	*1.302*	*1.465*	*1.435*
Carbohydrates	*112.5*	*132.5*	*100.0*	*95.0*	*93.8*	*120.0*	*100.0*
Lipids	*330*	*380*	*1505*	*700*	*500*	*470*	*NM*[d]
Lecithin	*32.2*	*31.8*	*35.0*	*29.4*	*NM*	*27.3*	*NM*
Cholesterol	*136*	*135*	*110*	*108*	*109*	*115*	*107*
DNA	*53.5*	*50.6*	*65.0*	*26.5*	*27.2*	*42.5*	*26.9*
RNA	*93.0*	*90.5*	*91.5*	*54.0*	*57.3*	*67.0*	*59.5*
Na^+	*411.0*	*404.6*	*368.2*	*450.0*	*481.2*	*438.0*	*350.0*
K^+	*41.5*	*37.0*	*28.0*	*56.0*	*50.3*	*80.1*	*134.0*
Ca^{++}	*12.1*	*9.4*	*9.5*	*15.6*	*30.5*	*32.0*	*23.0*
Mg^{++}	*1.9*	*2.0*	*2.0*	*4.1*	*6.4*	*6.9*	*7.5*
Cl^-	*257.8*	*260.2*	*249.7*	*280.0*	*375.6*	*309.3*	*273.0*
pH	*7.4*	*7.4*	*7.5*	*7.7*	*7.0*	*7.6*	*7.6*

[a] *Work carried out by Francisco García-González (Universidad Autónoma de Zacatecas, Zacatecas, Zacatecas, Mexico), Margarita Romero, Pedro Díaz and Luis Cañedo (Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México, Cuautitlán, Mexico, Mexico).*

[b] *Proteins in the serum, plasma, lymph and cyst fluid* in vivo *are expressed as gram percent of dry weight, while proteins in the cysts stored under various conditions are expressed as mg/ml; all other components are expressed as milligram percent of dry weight.*

[c] *The different conditions of storage of the muscles from which the cysts were taken were: 1, 6 h at room temperature; 2, 24 h at 4 °C; 3, 12 h at −4 °C.*

[d] *NM, not measured.*

TABLE II. Chemical Composition of the Vesicular Fluid from a Racemose Form of Taenia solium *Metacestode Found in Human Brain and of Cerebrospinal Fluid (CSF) from the Same Patient*[a]

Component[b]	*Cyst Fluid*	*CSF*
Proteins	*1.27*	*1.34*
Total lipids	*7*	*3*
Cholesterol	*0.36*	*0.23*
Carbohydrates	*8.2*	*11.9*

[a] *See footnote in Figure 1 for the names and affiliations of the authors of this table.*

[b] *Proteins are expressed as mg/ml; all other components are expressed as milligram percent of dry weight.*

IV. DISCUSSION

It is well known that the non-cellular portion of the blood or plasma is part of the extra-cellular fluid and communicates continually with the interstitial fluid through pores in the capillaries. The chemical components of the plasma has been reported as almost identical to the interstitial fluid found between tissue cells except in its protein content. Plasma contains about 7%, whereas interstitial fluid contains an average of 2% (16). This difference is explained by the colloid osmotic pressure exerted by the plasma proteins minimizing its leakage through the capillary pores into the interstitial spaces.

Lymph, on the other hand is the interstitial fluid that flows into the lymphatics and has almost identical composition as the interstitial fluid in that part of the body from which the lymph flows. The thoracic lymph is a mixture from all lymph areas of the body and, therefore, represents an average of its composition. Finally, serum is the blood fluid that remains after clot contraction and differs from plasma in the sense that all of its fibrinogen and most of the other clotting factors have been removed. Therefore, it is valid to compare the chemical composition of plasma with that of the interstitial fluid keeping the serum and lymph values as control.

From this premise, we observed differences in the chemical composition of the cyst fluid and the interstitial fluid of the pig muscle with which it is in contact. Protein concentration is similar to the theoretical value of the interstitial fluid. DNA and RNA are only half of the concentration inside the cyst than the values obtained for plasma and lymph. Lipids values are higher inside the cyst when compared to the plasma values. (The lipid values of the lymph are artificially increased due to the butter administered prior to surgery). Ions and Ph also showed higher values inside the bladder than those of the plasma.

When the bladder wall is damaged by any of the procedures described,

the chemical composition of the cyst fluid differs from its *in vivo* values, suggesting that the integrity of the bladder wall under normal physiological conditions is necessary to keep the values of the cyst fluid constant. It is interesting to mention that the ions that increases when the bladder wall is damaged correspond to those that participate in the calcification process. Some differences although minimal, are also shown in the preliminary data of the chemical analysis of the bladder fluid of *Cysticercus racemosus* and the cerebro spinal fluid with which it is in contact.

Although the previous preliminary data are non conclusive, they agree with the hypothesis that the bladder wall of *Cysticercus cellulosae* acts as a physiological barrier to the surrounding media under normal physiological conditions.

REFERENCES

1. Ramírez-Bon, E., Merchant, M.T., González del Pliego, M. and Cañedo. "Ultrastructure of the bladder wall of the metacestode of Taenia solium" en Flisser, A., Willms, K., Laclette, J.P., Larralde, C., Ridaura, C., and Beltran E. (Eds.) en "Cysticercosis present state of Knowledge and Perspectives". Academic Press 1982 p. 363-373.

2. Romero, M. and Cañedo, L. "Some aspects of the chemical composition of the fluid of Cysticercus cellulosae". Proceedings of the Third International Congress of Parasitology. Munich 3: 1485-1486, 1974.

3. Hartree, F.E. "Determination of protein: A modification of the Lowry Method that gives a linear photometric response". Anal. Biochem. 48, 422-427, 1972.

4. Morris, D.F. "Quantitative determination of Carbohydrates with Dreywood's Anthrone Reagent". Science. 107, 254-255, 1948.

5. Saifer, A. "The photometric microdetermination of blood glucose with glucose oxidase". J. Lab. and Clin. Med. 51, 448-453, 1958.

6. Arreguin, B. and García, G.F. "Silica Gel chromatography of sugars". (Manuscript in preparation).

7. Sweeley, C.C., Bentley, R., Makita, M. and Wells, W.W. "Gas liquid chromatography of trimethylsilyl derivatives of sugars and related substances". J. Am. Chem. Soc. 85, 2497-2507, 1963.

8. Folch, J. "A single method for the isolation and purification of total lipids from animal tissues". J. Biol. Chem. 226, 497, 1957.

9. Gliter, C. Anal. Biochem. 50, 324, 1972.

10. Keenan, R.W. Anal. Biochem. 23, 555, 1968.

11. Bartlett, G.R. J. Biol. Chem. 234, 466, 1959.

12. Van Handel. Clinical Chemistry. 7, 249, 1961.

13. Zak, B. "Simple rapid microtechnique for serum total cholesterol Am. J. Clin. Pathol. 27, 583-588, 1957.

14. Dische, Z. Microchemie 8, 4, 1930.

15. Mejbaum, W.Z. Phisiol. Chem. 258, 117, 1959.

16. Guyton, A.C. Textbook of Medical Physiology. Saunders Company 1976, p. 224.

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HOST-PARASITE RELATIONSHIP OF CYSTICERCIASIS IN MAN

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INTRODUCTION

Knowledge of the host-parasite relationship is indispensable for comprehension of the mechanism of this illness and for rational employment of chemotherapy. Its systematic study includes: a) the infestation mechanism producing permanent presence of the pathogen in the host, b) the state of equilibrium between the host and the parasite, which does not produce clinical manifestations, c) the state of illness. The defense mechanisms of the host: local (physical, biochemical), and general (humoral, cellular), intervene in the establishment of this relation, as do the agression mechanisms of the parasite (invasivity, migration, localization).

In cerebral cysticerciasis, patients are frequently found with cysticerci in various states of degeneration, in which there is practically no inflammatory response, and clinical manifestations of the illness are minimal or inexistent. On the other hand, there are cases in which a small number of larvae, independently of their localization or viability, generate a grave inflammatory reaction with severe clinical manifestations. It is inferred from this finding that the parasite does not produce identical reactions in different hosts.

As a preliminary attempt to understand the host-parasite relationship, a description is made of the histopathological and chemical changes which occur in lesions produced by this disease, emphasizing the change observed due to the calcification process. The following analyses are presented: a) Cerebral cysticerciasis lesions in several localizations and different states of viability of the larvae, in patients selected at random. b) The most important organic components, in those cases where no structures recognizable under the electron microscope exist (calcified cysticerci). c) Some degenerative changes which occur in C. racemosus, a structure which has been postulated as a degenerated form of C. cellulosae.

MATERIALS AND METHODS:

Origin and Preparation of Biological Material: From 19 cases of autopsies, larvae with adjacent tissue of meningeal, parenchymatous and ventricular lesions were obtained and processed for histological analysis. Tissue was selectively

stained for collagen (Masson) amyloid (Congo red), mucopolysaccharides (Pas) and calcium (Von Kossa), a systematic study of changes in the structure of the cysticerci and adjacent tissue was performed. Some fragments were fixed at 4°C for 1 hr in a solution of 2.5% glutaraldehyde in 0.1M buffer pH 7.4. The tissue was fixed for 1 hr in 1% osmium tetraoxide in the same buffer, and included in epon. Sections were stained with uranyl acetate and lead citrate (1) and observed in a Jeol 100B electron microscope. All representative areas were photographed on Kodak thick-base film and compared. Larvae of C. racemosus were obtained during brain surgery on three infested patients. In these parasites, three zones with different consistencies and colors were distinguished macroscopically: a white opaque area similar to the membrane of viable C. cellulosae, and two areas with hardened tissue, one with a yellow and the other with a brown color. Small fragments were cut from each of the above areas and processed for electron microscopy examination as described.

Chemicals: Polydase E.C. No. 3.1.3.2 (ortophosphoric mono ester phosphohydrolase (Schwartz Bio Research). All other reagents were of analytical grade.

Methods of Extraction and Analysis: Extraction of lipids was performed using Folch's method (2); Metcalfe's technique was followed for methylation of the extracted lipids and standards (3). Cholesterol crystals were obtained from samples of calcified cysticerci with the aid of a stereomicroscope. 1 ml of Regisil-TMS was added to 10 mg crystals, the mixture incubated at 55°C for 2 1/2 hrs, and the precipitate was eliminated by centrifuging before analysis by gas chromatography. Carbohydrate extraction was performed precipitating the protein with trichloracetic acid at 15%, and incubating the supernatant with polydase at room temperature for 4 hrs; the enzyme was inactivated by heating, and the suspension clarified by centrifuging; the obtained supernatant was passed through ion exchange resins and the fractions were liophilized, preparations of silyl derivatives was performed by use of TRI-SIL. The following methods were applied for determination of the main components of the calcified cysticerci: determination of the protein concentration: Hartree (1), nitrogen content: Microkjeldahl, nucleic acids: Spirin (4), carbohydrates: Loewus (1), glucose oxidase, osazones, fine layer chromatography and gas chromatography; total lipid Folch (2), cholesterol Zak (5), and fine layer chromatography; ion determination (Ca^{+2}, Na^{+}, K^{+}, Mg^{+2}) was by atomic absorption (Perkin Elmer Mod. 303). For gaseous-phase chromatography, a Beckman GC-72 chromatograph with flame ionizatio detector was used.

RESULTS

Results of analysis of cysticerci lesions at various localizations are shown in Table 1.

TABLE 1

HISTOPATHOLOGICAL CHANGES IN LESIONS OF CEREBRAL CYSTICERCIASIS WITH DIFFERENT LOCALIZATIONS

L	Cysticerci								Fibrous Capsule				Adjacent Tissular Elements				
	1	2	3	4	5	6	7	8	4	5	6	7	GM	WM	LM	E	VA
V	+			3+	+	+	+		4+	+	+	4+	9	9	*	11	12
P	2+			3+	3+	3+	3+	3+	4+	3+	3+	4+	10	9	2	*	13-15
S	3+			3+	3+	3+	3+	3+	4+	3+	3+	4+	10-7	9	2	*	13-14-15
B	3+	-	-	3+	3+	3+	3+	3+	4+	3+	+	4+	9	*	2-7	*	

(L) Localization of the lesion: (V) Ventricular; (P) Parenchymal; (S) Subarachnoidal; (B) Basal.

Histopathological Changes: (1) Necrosis; (2) Fibrosis; (3) Presence of amyloid; (4) Hyalinization; (5) Cholesterol; (6) Mineralization; (7) Lymphocyte infiltration; (8) Granulomatous inflammation; (9) Gliosis; (10) Compression, with neuronal degeneration; (11) Ependymitis granularis; (12) Vascular proliferation; (13) Endarteritis; (14) Necrosis; (15) Thrombosis. (*) No alteration; (-) Absent; (+) Rare; (2+) Infrequent; (3+) Frequent; (4+) Very frequent.

Adjacent tissular elements: (GM) Gray matter; (WM) White matter; (LM) Leptomeninges; (E) Ependyma; (VA) Vascular alterations.

In cysticerci fixed to meningeal or cerebral tissue, necrosis, hyalinization and cholesterol crystals were frequently found. The degree of calcification of the cysticerci was determined in accordance with their calcium content (Von Kossa); fibroblasts and collagen were searched for by histological methods (Masson and birefringenece to polarized light) and by electron microscopy, no signs of fibrosis being found. In the lesions studied, the characteristic green polarization birefringence upon Congo red staining exhibited by the fibrillar amyloid proteins was not observed, nor was the presence of ribbon-like extracellular proteinaceous material 7 to 8 nm wide by 100 to 200 nm long detected by electron microscopy. With the exception of free ventricular cysticerci, in all other cases a fibrous limiting capsule formed by proliferating capillaries, fibroblasts, collagen fibers, lymphocytes and a few macrophages was found; capsules frequently showed local areas of hyalinization, calcification and cholesterol crystals. Ventricular cysticerci showed lymphocyte infiltration, in other localizations, granulomatous inflammation was found in the cyst, this process was minimal with viable larvae. Gliosis was found in nerve tissue; the gray matter also showed compression of tissular elements, with neuronal degeneration. In cases of ventricular cysticerciasis, ependymitis was observed, and in the other localizations, fibrosis of the adjacent leptomeninges. Vascular changes: endarteritis, thrombosis and necrosis of the middle layers

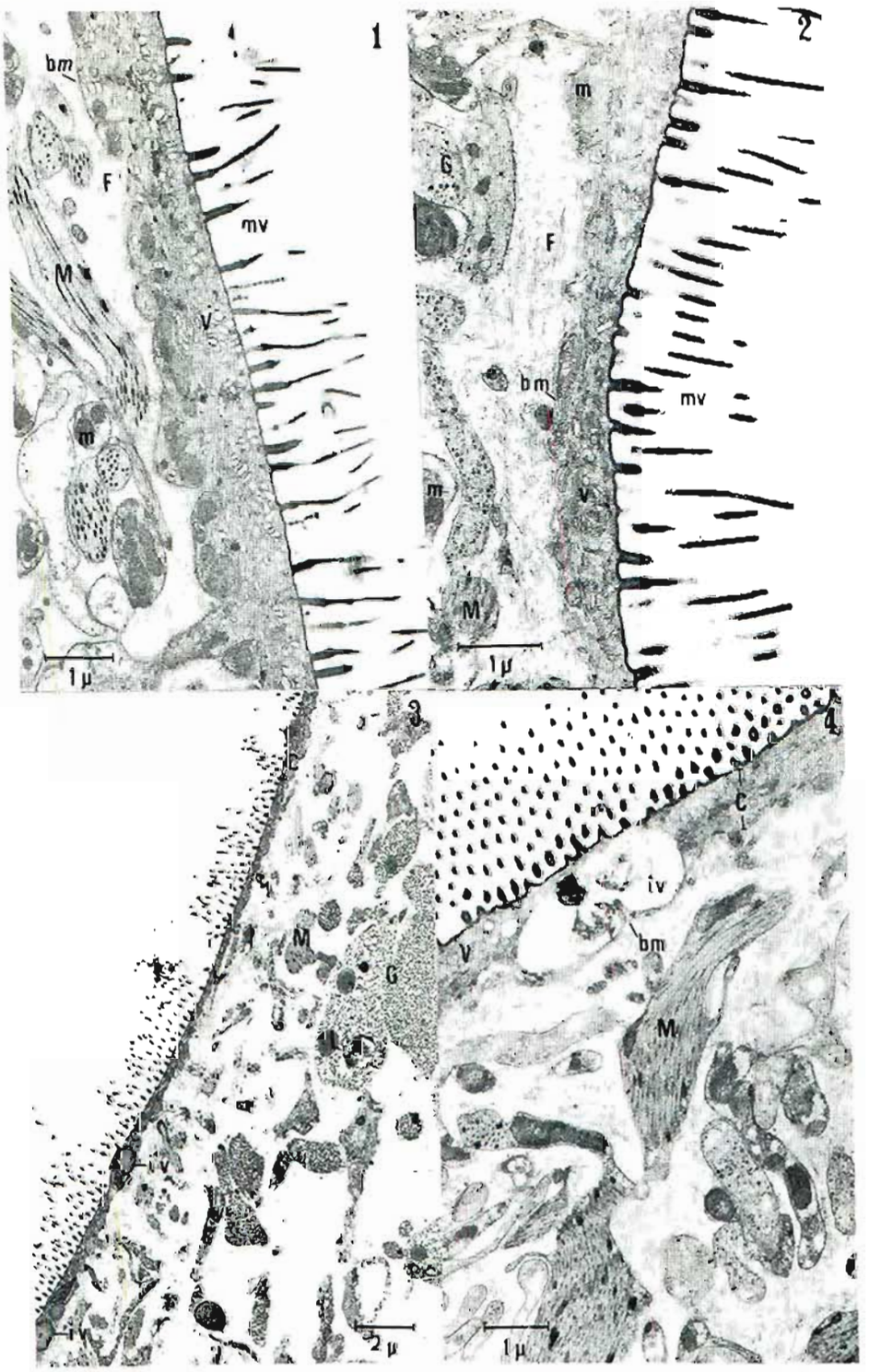

Plate 1: (1) Wall of C. cellulosae from pig muscle, external zone. Wall of C. racemosus from different areas. (2) Opaque white, (3) brown, (4) transparent

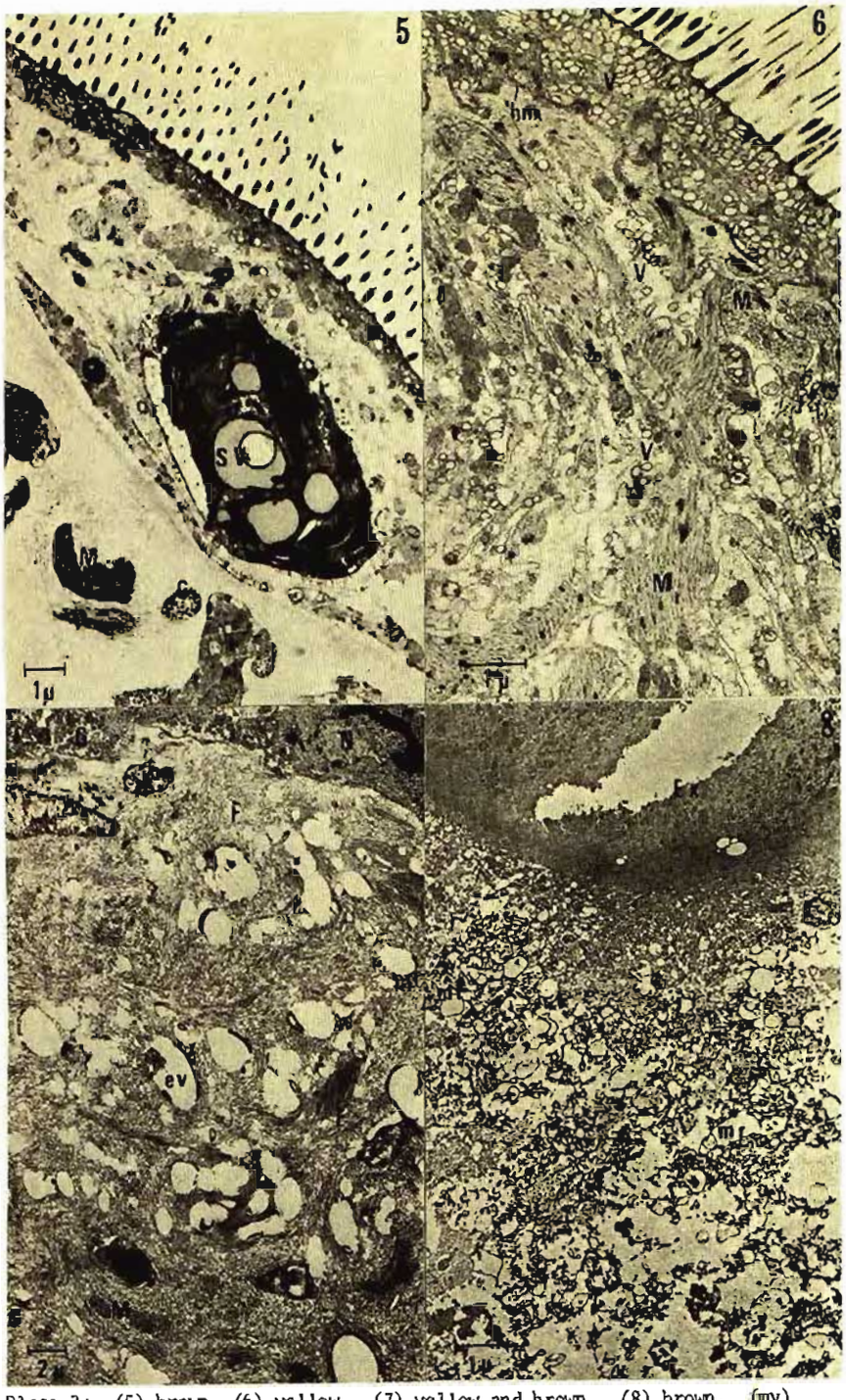

Plate 2: (5) brown, (6) yellow, (7) yellow and brown, (8) brown. (mv) microvilli, (V) vesicles, (bm) basal membrane, (M) muscle, (F) fibers, (m) mitocondria, (G) glycogen, (C) cuticle, (iv) intracuticular vacuole, (SV) subcuticular vacuole containing highly electrodense material, (L) lipids, (N) nucleous, (ev) empty vacuoles, (Ex) external part of the wall, (mr) membrane residue.

were observed in all cases, with the exception of ventricular localizations which showed only some vascular proliferation.

Protein is the most important organic component of calcified C. cellulosae, and constitutes between 20 and 30% of the total; this data agreed with the nitrogen levels found 3 - 5%. Cholesterol constitutes 2.6% of the total of the sample; the composition of fatty acids identified in the total lipids of calcified C. cellulosae were: caprylic, capric, lauric, myristic, pentadecylic, palmitic, palmitoleic, margaric, stearic, oleic and linoleic. Carbohydrate content constitutes 1.3% of the sample of calcified C. cellulosae; its concentration was determined by the following methods: antrone, glucose oxidase, preparation of osazones, fine-layer chromatography, and gas chromatography, glucose being identified as the sole carbohydrate. The remaining analyzed material was inorganic matter forming the structure of amorphous phosphate, hydroxyapatite, and whitlockite (6). Preliminar comparative analysis between the structure of C. cellulosae and the opaque white area of C. racemosus showed quantitative, but not qualitative differences between the two larvae Plate 1 (1), and (2). The other areas of C. racemosus showed changes of their structure when viewed through the electron microscope. One of said changes is the presence of vacuoles distributed over the entire wall, containing electrodense and/or optically homogeneous material, with an appearance similar to that of the vacuoles of lipids found in cells containing glycogen Plate 1 (3), (4) and Plate 2 (5). Signs of alterations of cell structure were also observed, such as an increase of the number of vesicles of the cuticle Plate 2 (6). In other areas, the destruction of tissular elements is more marked, being found only remnants of cellular structures, lipid vacuoles, fibers and empty vacuoles or just residual membranes Plate 2 (7) and (8).

DISCUSSION

The histopathological study showed that the type of tissue reaction in the lesions depends on the local characteristics of the elements adjacent to the lesion. The quantitative study of the evolution of the inflammatory and immunological processes requires an experimental model; the changes described are useful for establishment of the rational basis of such study.

A common phenomenon found in the lesions analyzed was the presence of areas with differing degrees of calcification; some of the experiments reported were oriented to define the changes which take place during this process. Calcification depends on two general processes: a) systemic conditions, including ions and other elements which are transported to the site where calcification occurs. b) The local mechanism, which includes the enzymatic and non-enzymatic factors participating in the chemical processes responsible for initiating and regulating the incorporation of calcium and phosphate in aggregates such as

amorphous calcium phosphate, hydroxyapatite and whitlockite. The change of free ions to crystalline structures may occur spontaneously at neutral pH from solutions containing high concentrations of the corresponding ions. From this mixture, amorphous calcium phosphates are precipitated. However, if supersaturation exists with respect to hydroxyapatite but the ionic activity required for spontaneous precipitation of amorphous phosphate is absent, ions are spontaneously converted to the complex hydroxyapatite crystal (7). Alternatively when solutions whose ionic concentration is under supersaturation are placed in contact with fibrillar proteins such as collagen or glycoprotein; the surface of the fibrils constitutes a matrix serving as a center for nucleation for hydroxyapatite crystals. C. cellulosae lesions in process of calcification shows fibers similar to those of the extracellular structure found in viable larvae; in addition, it was demonstrated the presence of amorphous calcium phosphate hydroxyapatite and whitlockite, which are intimately bound in a proteic matrix constituting the most important organic component; cholesterol crystals are trapped among these compounds (8). In inflammatory processes, including those evolving towards calcification, it is possible to identify, during cellular activity, macrophages, leucocytes and phagocytosis of tissular wastes, followed by necrosis and proliferation and invasion of the lesion by histiocytes and fibroblasts. These latter cells produce collagen fibers and mucopolysaccharides, which in some cases of ectopic calcification could serve as a matrix for ion deposits. In these cases studied, experiments oriented towards establishing the presence of cells and collagen of the host in the degenerated cyst, or the possibility that the cysticerci accumulates in its interior amyloid fibrils derived from the N-terminal variable segment of immunoglobulin light chains, were negative. Changes found in C. racemosus show conservation and increase of lipid vacuoles and extracellular fibers in very advanced states of degeneration. Vacuoles also appear containing electrodense and/or optically homogeneous material, similar to lipid vacuoles.

Considerations has recently been given to the possibility that those components found in lesser amounts of calcified tissue could play an important role in initiation of calcification; and among said components, lipids have attracted the greatest attention. The presence of said molecules in mineralized tissue has been amply proven (9), the union of lipids to the mineral ions of the apatite lattice by coordinate linkages, and to the organic matter, forming bonds with protein fibrils, has been postulated as the mechanism responsible for the union between the organic and inorganic phases of calcified tissue (9). The present data shows increased amounts of cholesterol and fatty acids in the site of calcification. Although the study of the complete composition of proteins and lipids of calcified C. cellulosae has not yet been finished, the foregoing data suggest the possibility that the protein and lipid components of cysticerci are

involved in the calcification process. With existing data, the presence of glucose in cysticerci may be associated with the glycogen found in the cysticerci; this polysaccharide is observed even in advanced stages of degeneration Plate 2 (7), but it is not known if it also proceeds from other molecules.

We thank Dr. Alfredo Feria for the valuable suggestions with the histophatological study and also to Dr. Armando Acosta for his help and suggestions with the gas-chromatography and atomic absorption analysis.

REFERENCES

1. Guerra, G. et al (1976) This symposium see references 1, 2, and 7.
2. Folch, J. et al (1957) A single method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497.
3. Metcalfe, L.D. and Schmitz, A.A. (1966) Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Anal. Chem. 38, 514-515.
4. Spirin, A.S. (1958) Spectrophotometric determination of total nucleic acid content. Biokhimiia 23, 617-622.
5. Zak, B. (1957) Simple rapid microtechnic for serum total Cholesterol Am. J. Clin. Pathol. 27, 583-588.
6. Garrido, F., Cañedo, L. Manuscript in preparation.
7. Howell, D.S. (1971) Current concepts of calcification. J. Bone and Joint Surg. 53-A, 250-258.
8. Grau, E. et al (1974) On the calcification of C. cellulosae. Third. Int. Symp. of Parasitology 1, 396.
9. Shapiro, I.M. (1973) The lipids of skeletal and dental tissues: Their role in mineralization. In Biological Mineralization Zipkin I (ed) John Wiley 117.

CALCIFICATION OF THE CYSTICERCI OF *TAENIA SOLIUM* IN THE HUMAN BRAIN

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I. INTRODUCTION

The transformation of soluble forms of calcium and phosphate into amorphous calcium phosphate and hydroxyapatite is known to take place in specialized vertebrate tissues parallel to systemic and local changes in physiological activity [1]. These amorphous and crystalline structures are embedded in an interstitial matrix made up of collagen, carbohydrate-containing macromolecules, and lipids [2]. While extracellular deposition of calcium salts normally takes place in bones and teeth, soft tissue calcification is the result of a pathological process [3, 4].

Although parasites usually remain uncalcified as long as they are alive, the calcification of several species in long-lasting infections has been reported [5]. One of the major unresolved questions about this process concerns the roles played by the host and the parasite in calcification. In this paper we present the results of a study on the changes that occur during the calcification of *Taenia*

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solium metacestodes in the human brain. The role of lipids and parasite extracellular fibers in the formation of the calcified tissue is discussed. Evidence is presented that indicates an apparent lack of participation of the calcareous corpuscles as nucleation centers. The results are compatible with the hypothesis that the process of calcification of *T. solium* larvae in the human brain is similar to that observed in other examples of ectopic calcification [3, 4].

II. MATERIAL AND METHODS

Biological material. Cysticerci in different stages of calcification were obtained from 19 infected autopsied human brains fixed in 10% formaldehyde. The larvae were classified according to their macroscopic appearance as viable, hyaline, or calcified. A cysticercus was identified as viable when no alterations of the normal structures, consisting of a bladder wall that enclosed a single invaginated scolex and a central cavity filled with fluid (Fig. 1 A), were found. The hyaline cysts were characterized by the replacement of the parasite structures by homogeneous, eosinophilic, acellular material (Fig. 1 B). In the calcified cysticerci, this homogeneous material was hardened in such a way that the parasite looked like an irregularly shaped stone (Fig. 1 C). The metacestodes found in

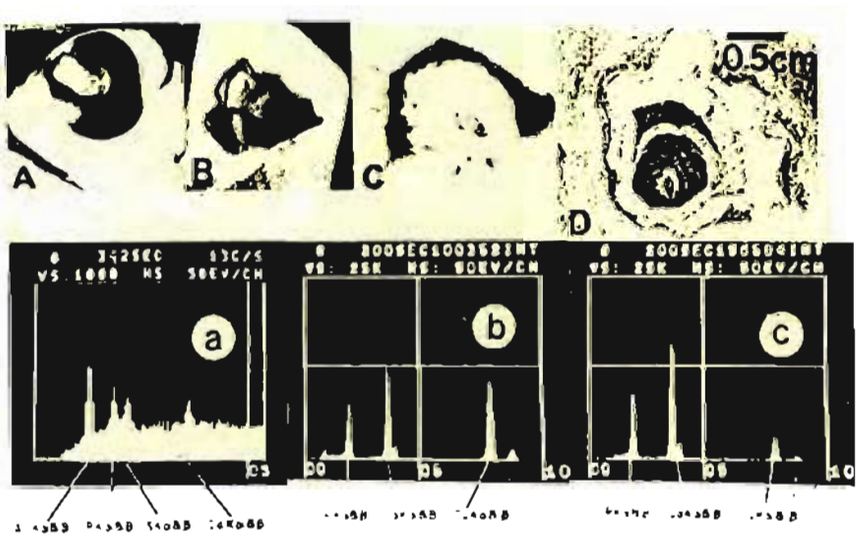


FIG. 1. Cysticerci of Taenia solium *(actual size) obtained from human brains post-mortem and fixed in formaldehyde in different stages of calcification: viable* (A), *hyaline* (B), *and calcified* (C). D. *Masson-stained section of a hyaline cysticercus showing the host-derived collagen capsule* [6]. a, b, c. *Elemental analysis of the larvae depicted in* A. B, *and* C, *respectively.*

brain tissue or the meninges (viable, hyaline, and calcified) were usually surrounded by a host-derived inflammatory capsule [6] (Fig. 1D).

Cysticerci were dissected from skeletal muscles of infected pigs immediately after slaughter. Only those with a fluid-filled intact bladder wall were selected. Calcareous corpuscles were extracted from viable *T. solium* metacestodes, and were washed in distilled water after isolation with ethylenediamine, following the method of Von Brand *et al.* [7]. The hyaline and calcified cysts were dissected, dried at room temperature, decapsulated, and subsequently separated under a stereoscopic microscope using electron microscopy tweezers into two components: a brown-white solid and transparent crystals. The solid was further separated into two homogeneous samples containing the brown and white components, which were lyophilized.

Light and electron microscopy. Paraffin-embedded tissue sections obtained from the three types of larvae were treated with Masson and von Kossa stains [8], and birefringence was studied under polarized light using a light microscope [9]. Samples were prepared for transmission electron microscopy according to the procedure outlined by Luft [10]. The white component was decalcified prior to sectioning. Electron micrographs were taken with a Hitachi HU-11A microscope on Kodak lantern slides. Samples were prepared for scanning electron microscopy following the methods described by Echlin [11]. Electron micrographs were taken with a Cambridge Stereoscan S4-10 microscope.

X-ray analysis. Elemental x-ray analysis of the brown and white components and of the transparent crystals was performed with a Cambridge Stereoscan S4-10 microscope equipped with a lanthanum hexaboride electron gun which emits more electrons than tungsten, giving good illumination with a small beam diameter. High resolution with a strong beam current is thus achieved, thereby improving the x-ray count rate. The x-ray analysis was performed with energy dispersive Kevex Ray 500 A and EDAX 707A x-ray spectrometers. Scanning electron microscope images were recorded on Polaroid film.

For the diffraction studies, x-rays corresponding to the nickel-filtered Kα line of copper were produced in a Coolidge tube operated at 30 kV and 20 mA of current supplied to the cathode. The specimens (reduced to powder and packed in quartz capillary tubes or on glass plates) were exposed to the x-rays in a Phillips diffractometer. A 114.6-mm diameter (Debye-Scherrer) Phillips camera was used to photograph the patterns which were measured according to the method of Pastrana and Cano [12]. The crystalline compounds were identified [13], and the interplanar distances were calculated and compared to those previously reported [13, 14]. The angles of completion of Bragg's Law were obtained directly from the diffractometer graphs or were calculated by vernier measurement of the base diameters of the diffraction cones recorded on Kodak x-ray film developed with Dektol.

Electron diffraction. The samples were placed on copper grids between two carbon films. Micrographs were taken with a Zeiss EM 9 A microscope operated at 60 kV. The diffraction patterns were recorded on Kodalith L.R. film (estar base), and were identified, indexed, and compared with those previously reported [13].

Atomic absorption. Samples for element determination were prepared by dissolving 100 mg of the lyophilized brown and white components in 1 ml of conc. HCl and adjusting the volume to 100 ml with deionized water. Lanthanum oxide was added to avoid interference with phosphorus. Atomic absorption was measured in a Perkin-Elmer model 303 spectrophotometer.

Gas chromatography. Transparent crystals were obtained from samples of calcified cysticerci with the aid of electron microscope tweezers using a stereomicroscope. One milliliter of Regisil-TMS (Pierce Chemical Company, Rockford, IL) was added to 10 mg of crystals, the mixture was incubated at 55 °C for 2.5 h, and the precipitate was eliminated by centrifugation. Gas chromatography analysis was performed using the technique described by Pierce [15].

Carbohydrate extraction of the brown and white components and the transparent crystals was carried out by precipitating the protein with 15 % trichloroacetic acid and incubating the supernatant with polydase (E.C. 3.1.3.2), orthophosphoric monoester phosphohydrolase (Schwartz BioResearch, Orangeburg, NY), at room temperature for 4 h. The enzyme was inactivated by heating, and the suspension was clarified by centrifugation. The supernatant was passed through ion exchange resins and the fractions were lyophilized. The carbohydrates present in the brown and white components were analyzed using gas chromatography following the method of Sweeley *et al.* [16].

Lipids were isolated from the brown and white components by the extraction method described by Folch [17]. The methylation of the extracted lipids and standards was carried out using the technique described by Metcalfe and Schmitz [18]. A Beckman GC-2 chromatograph equipped with a flame ionization detector was used for gas chromatography.

Nuclear magnetic resonance (NMR). Samples of the isolated transparent crystals were dissolved in chloroform and activated carbon was added. The mixture was centrifuged, and the supernatant was evaporated and resuspended in deuterated chloroform. The NMR spectrum was determined using a Varian HA-100 spectometer equipped with Hewlett-Packard 200 and 200 AD audio oscillators. Trimethylsilyl was used as an internal reference.

Infrared absorbance studies. Infrared studies were carried out on the brown and white components, as well as on the transparent crystals. The samples were dissolved in solid KBr, reduced to powder, and compressed at 490 kg/cm^2 until a pellet was formed. The absorbance infrared spectra from the KBr pellets were

recorded using a Perkin-Elmer 521 grating infrared spectrophotometer equipped with a double beam and attenuator.

Determination of the main organic components of the calcified cysts. Protein concentration was determined by the Lowry method as modified by Hartree [19]. Nitrogen content was quantified by the microkjeldahl procedure described by Miller and Houghton [20]. The nucleic acid content was measured spectrophotometrically using the method of Spirin [21], and the carbohydrates were identified and quantified by the following techniques: the anthrone method of Loewus [22]; the glucose oxidase procedure [23]; and gas-liquid chromatography [16]. Total lipids were quantified by gas chromatography [18]. Cholesterol was identified by the appearance of the crystals under light and scanning electron microscopes, thin-layer chromatography [24], x-ray diffraction studies according to Pastrana and Cano [12], gas chromatography [15], and nuclear magnetic resonance [25, 26]. Hydroxyproline content was determined using the colorimetric method of Woessner [27].

III. RESULTS

A. Inorganic Components of Hyaline and Calcified Cysts

In order to confirm whether the different larvae selected for this study on the basis of their macroscopic appearance represented progressive stages of a calcification process, an energy dispersive x-ray analysis of intact viable, hyaline, and calcified larvae was performed. A significant increase in the calcium and phosphorus content of the hyaline and calcified samples compared to the viable larvae was found (Fig. 1 a,b,c).

Analysis of the inorganic constituents of hyaline and calcified *T. solium* larvae showed that the brown component of the brown-white solid obtained from these calcified cysts (Fig. 2A) contained mainly amorphous calcium phosphate [28] (Fig. 2B, upper profile), with small quantities of crystalline material. The crystalline substance was concentrated in the white component of the brown-white solid [28] (Fig. 2B, lower profile) and contained only hydroxyapatite and whitlockite (Fig. 2C,D,E). A uniform distribution of calcium and phosphorus was found in the calcified larvae, but these minerals were less concentrated in the transparent crystals (Fig. 3A–D).

The calcareous corpuscles localized in the scolex (Fig. 4A,C) are naturally formed inorganic deposits found in cestode larvae, and in some species account for almost half of the total dry weight [7]. Calcium was the major mineral component of the calcareous corpuscles in *T. solium* larvae from human brain and pig muscle, and no phosphorus was detected (Fig. 4B,D). The crystalline structures observed in the isolated calcareous corpuscles corresponded only to calcium carbonates (Fig. 4E).

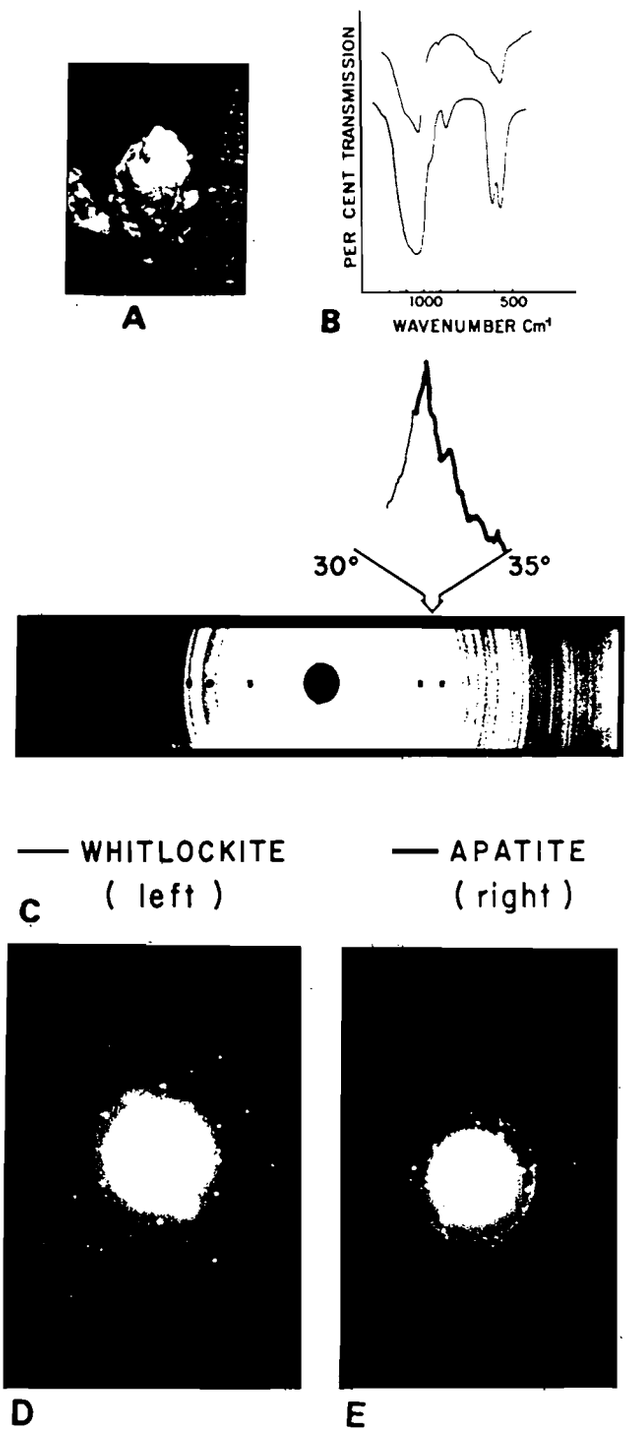

A
PER CENT TRANSMISSION
1000
500
WAVENUMBER Cm⁻¹
B
30°
35°
— WHITLOCKITE
(left)
— APATITE
(right)
C
D
E

Figure 2 A–E

B. Organic Components of Hyaline and Calcified Cysts

The analysis of the organic components of the hyaline and calcified larvae included a search for vertebrate fibroblasts and collagen by histological methods [8, 9] and by electron microscopy. However, there was no sign of viable vertebrate cells or collagen structures in the samples analyzed.

Electron microscopy of several hyaline and calcified cysts showed progressive degenerative changes with preservation of extracellular fibers whose morphology was very similar to those observed in viable larvae (data not shown). The degenerative changes increased as the cyst was transformed from a hyaline to a calcified form, and were very similar to those reported for the bladder wall of racemose *T. solium* metacestodes (*Cysticercus racemosus*), a form of cysticercus which has been postulated to be aberrant [29].

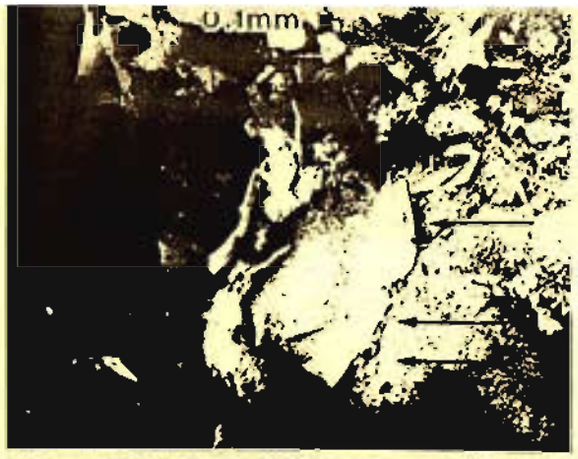
Hartree determination of decapsulated hyaline and calcified larvae showed that 30–40 % of the material was composed of protein, which correlated with the 3–5 % nitrogen content determined by the microkjeldahl method.

The extracellular fibers found in hyaline and partially calcified cysts have been partially purified from the bladder wall of *T. solium* cysticerci isolated from pig muscle [30]. Torre-Blanco has recently isolated, purified, and characterized these fibers as collagen [see chapter by A. Torre-Blanco in this volume]. In contrast to other typical collagens, this protein has an extremely low hydroxyproline content (5 μg/mg protein) – an amount similar to that found in our samples of calcified material (0.95–1.05 μg/mg protein), and roughly two orders of magnitude lower than is found in mammalian collagen (135 μg/mg protein).

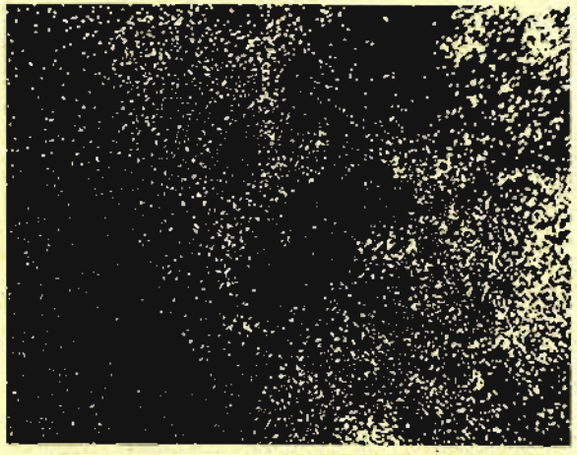
Infrared analysis of the brown and white components of calcified *T. solium* larvae revealed a pattern similar to that given by mammalian collagen, including the transformation of the amide bands in the presence of phosphates (Fig. 5). Although this method is not very specific for protein identification, at least it showed that a protein matrix was present in the calcified cysts.

Cholesterol constituted 2.6 % of the lyophilized brown and white components and was the only organic material found in the transparent crystals from the calcified material (Fig. 6). In addition, the brown and white components contained less than 1 % fatty acids (Fig. 7), and carbohydrates, represented solely by glucose, made up 1.3 % of these lyophilized samples (Fig. 8). Nucleic acids were below the limits of detection [29].

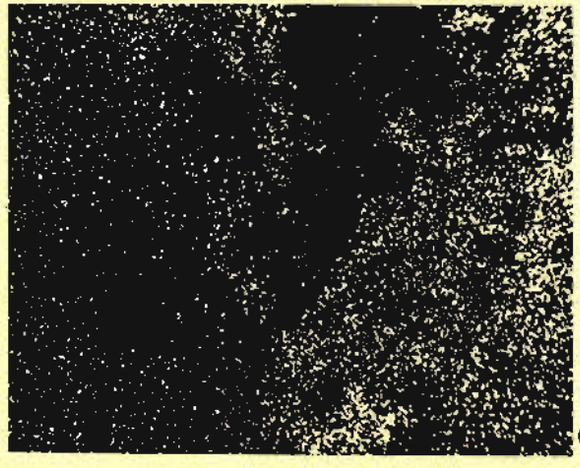
FIG. 2. Analysis of the inorganic constituents of calcified T. solium *larvae.* A. *Brown-white component obtained from dried calcified larvae (actual size).* B. *Bands observed in the 1200–400 cm^{-1} region of the infrared spectra. Upper profile: the material found in the brown component coincides with the pattern of amorphous calcium phosphate. This component also appears amorphous by x-ray and electron diffraction. Lower profile: material found in the white component is typical of crystalline apatitic calcium phosphate* [28]. C. *X-ray diffraction analysis of the crystalline structure found in the white component.* D, E. *Electron diffraction patterns of whitlockite and apatite, respectively.*



A



B



C

A–C

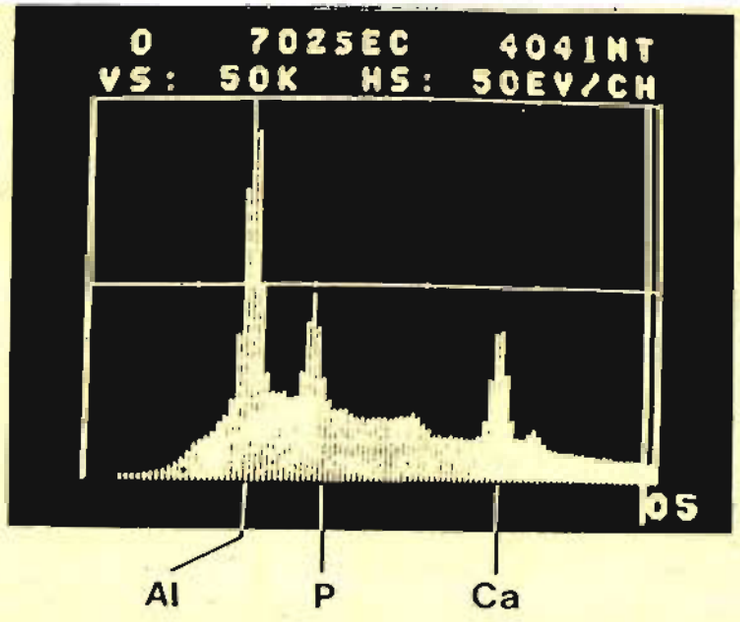


Figure 3 D

FIG. 3. A. *Transparent crystals (arrows) embedded in the brown-white component of a calcified* T. solium *larva.* B, C. *Scanning images of the material shown in* A *using the* Kα *emission lines of calcium and phosphorus, respectively. These elements are less concentrated in the transparent crystals.* D. *Elemental analysis of the material shown in* A.

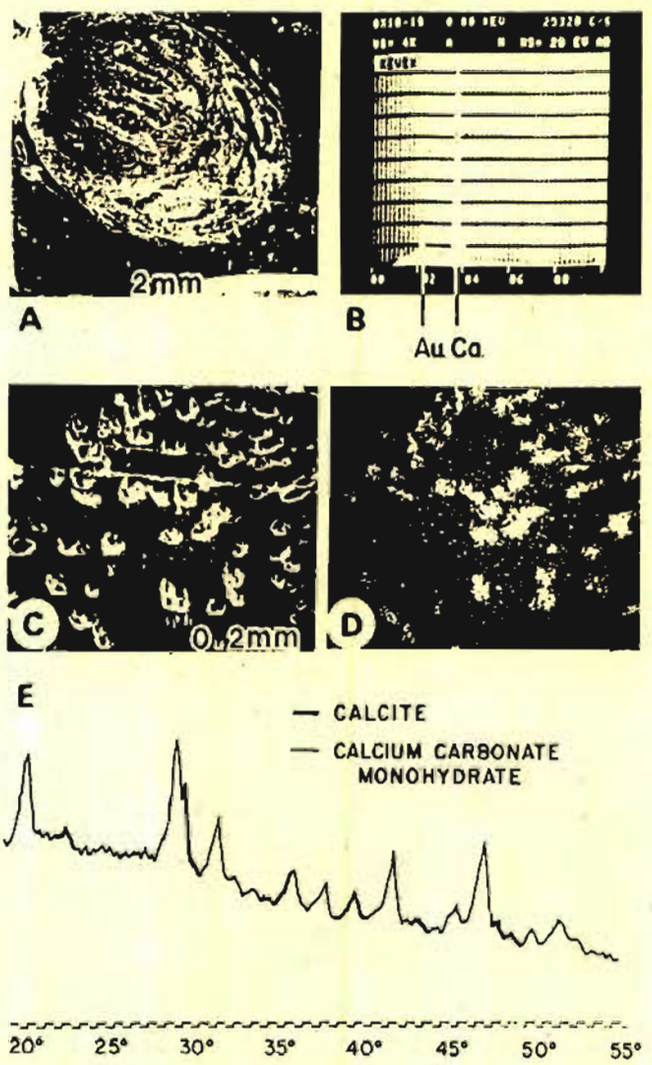


FIG. 4. A. *Section of a scolex of a* T. solium *larva that has been embedded in Epon.* B. *Energy dispersive x-ray analysis of the scolex shown in* A. C. *Higher magnification of the scolex shown in* A. D. *CaKα emission line scanning image of section shown in* C *demonstrating that the calcium is concentrated in the calcareous corpuscles.* E. *X-ray diffraction analysis of calcareous corpuscles isolated from* T. solium *metacestodes.*

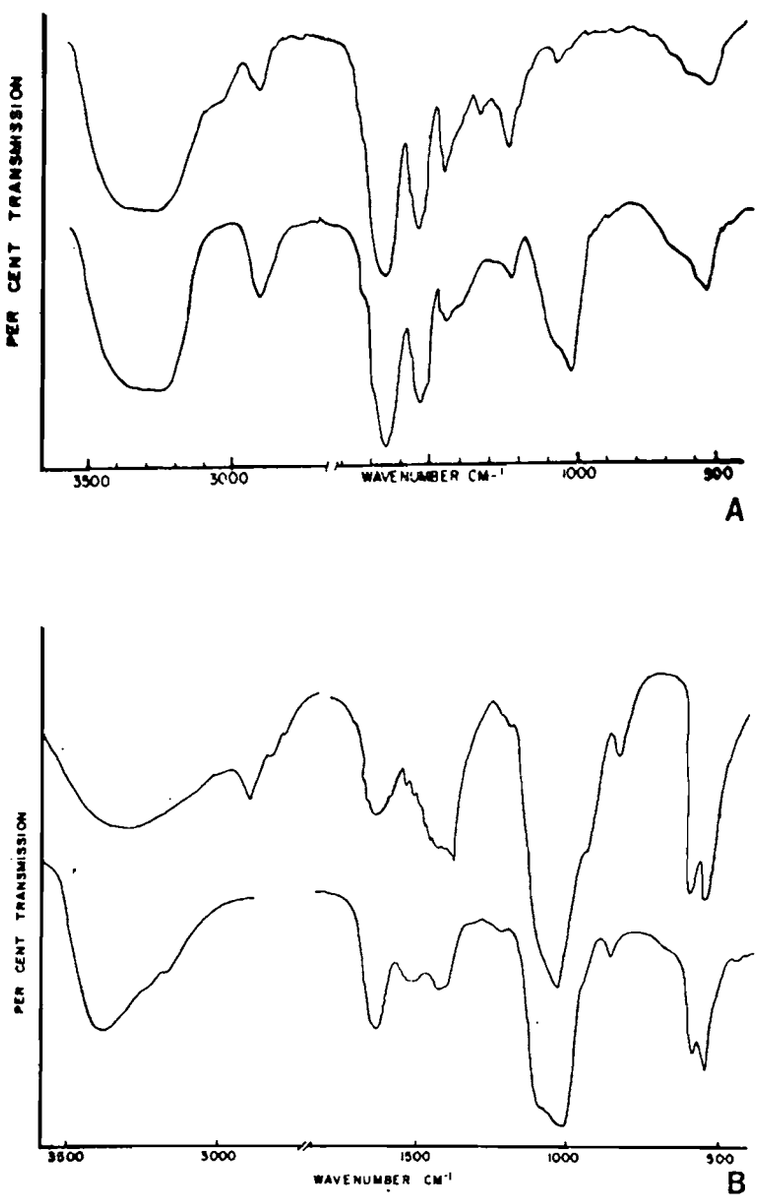


FIG. 5. Plots of percent transmittance vs. frequency showing the bands observed in the region between 3500–3000 cm^{-1} and 1800–500 cm^{-1} of the infrared spectrum. A. Infrared pattern of mammalian collagen (upper profile) and the brown component of calcified T. solium *metacestodes (lower profile). B. Transformation of amide bands in the presence of phosphates. Upper profile: white component of calcified* T. solium *larvae. Lower profile: mineralized mammalian collagen. The mineralized mammalian collagen profile is reproduced from [31], courtesy of Dr. J.D. Termine and John Wiley & Sons.*

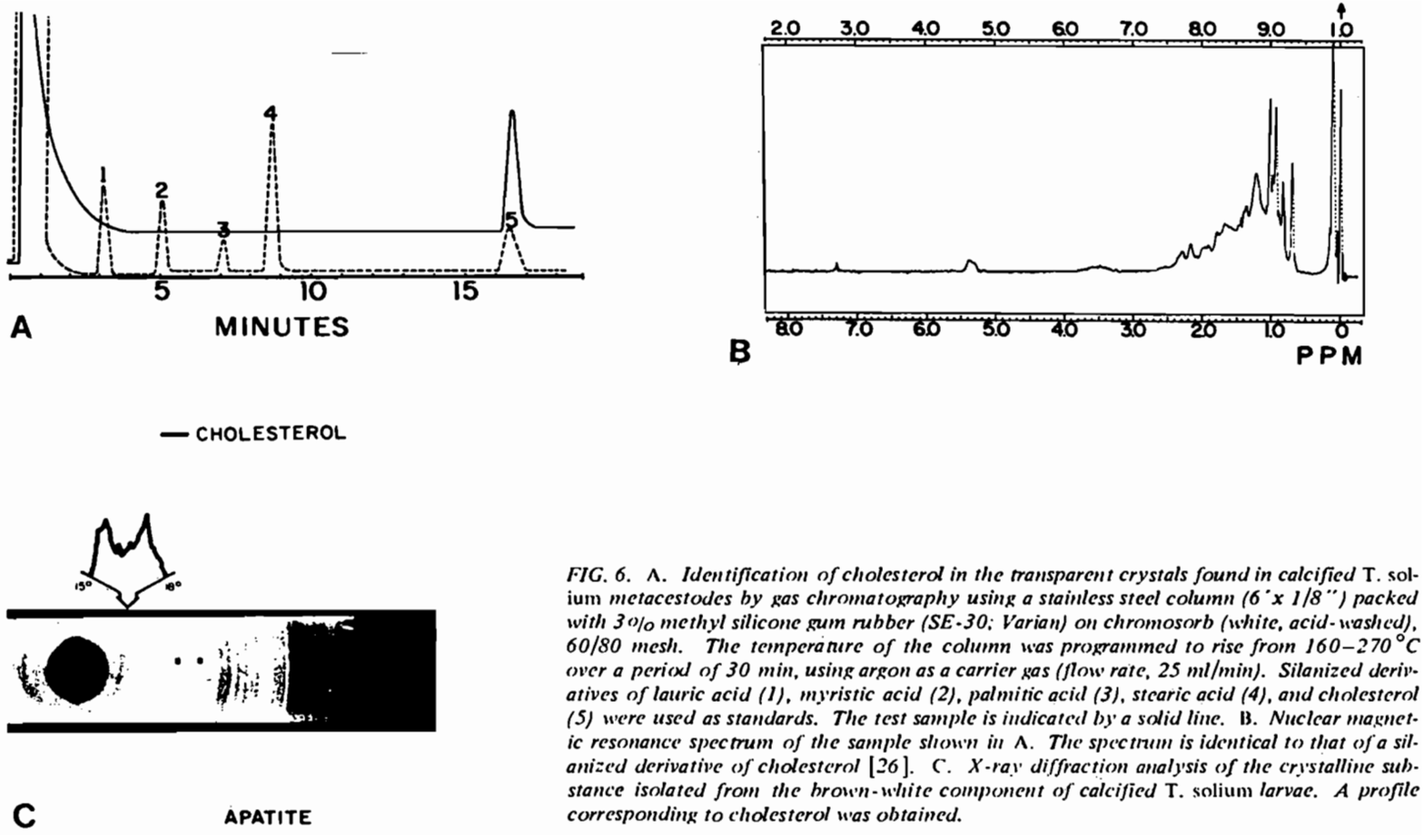


FIG. 6. A. *Identification of cholesterol in the transparent crystals found in calcified* T. solium *metacestodes by gas chromatography using a stainless steel column (6′ x 1/8″) packed with 3% methyl silicone gum rubber (SE-30; Varian) on chromosorb (white, acid-washed), 60/80 mesh. The temperature of the column was programmed to rise from 160–270 °C over a period of 30 min, using argon as a carrier gas (flow rate, 25 ml/min). Silanized derivatives of lauric acid (1), myristic acid (2), palmitic acid (3), stearic acid (4), and cholesterol (5) were used as standards. The test sample is indicated by a solid line.* B. *Nuclear magnetic resonance spectrum of the sample shown in* A. *The spectrum is identical to that of a silanized derivative of cholesterol* [26]. C. *X-ray diffraction analysis of the crystalline substance isolated from the brown-white component of calcified* T. solium *larvae. A profile corresponding to cholesterol was obtained.*

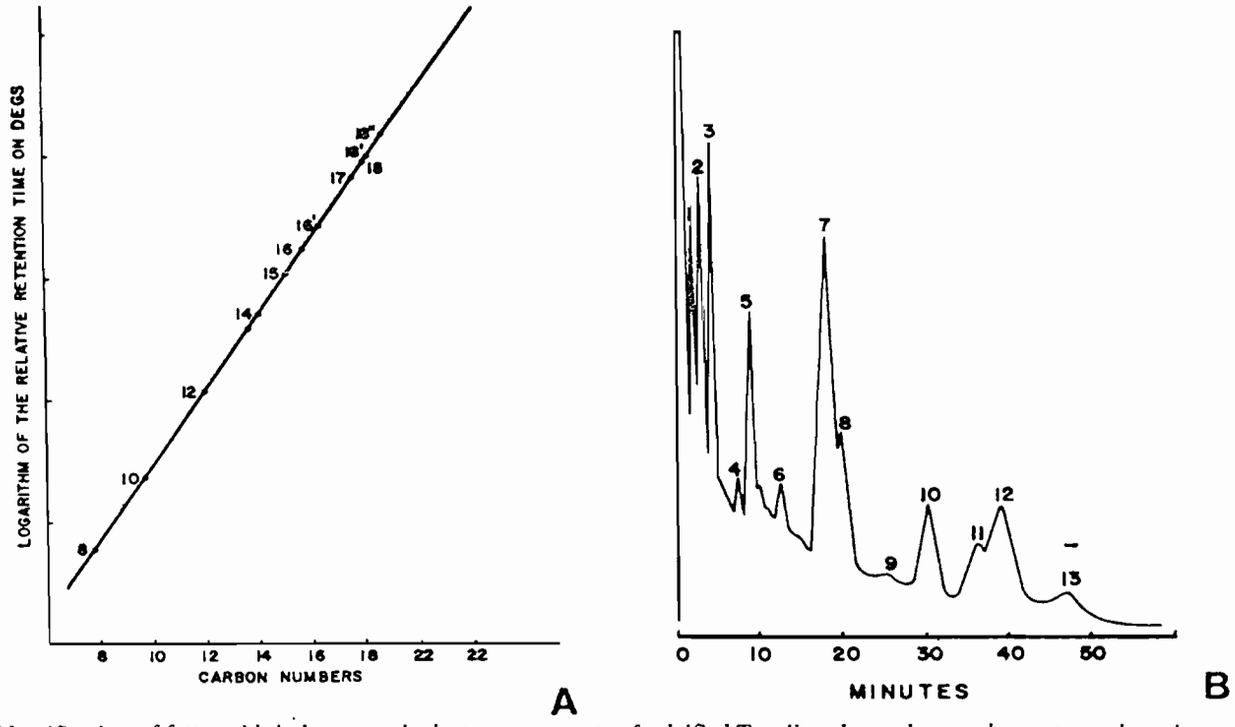


FIG. 7. Identification of fatty acids in brown and white components of calcified T. solium *larvae by gas chromatography using a stainless steel column (10' x 1/8") packed with 15% diethylene glycol succinate (DEGS; Varian) on chromosorb (white, silanized with hexamethyldisilazane, HMSD), 80/100 mesh. The column temperature was maintained at 170° C and argon was used as the carrier gas (flow rate, 25 ml/min).*
A. Standard lipid curve constructed with saturated and unsaturated (C-8 to C-22) fatty acids (continuous line). The dots indicate fatty acids identified in B. The number of carbon atoms in the fatty acids is indicated by the numbers above the line (18' = 18:1; 18'' = 18:2). B. Graph showing methyl esters of the following fatty acids: caprylic (1), capric (2), lauric (3), unidentified (4), myrisitic (5), pentadecylic (6), palmitic (7), palmitoleic (8), margaric (9), unidentified (10), stearic (11), oleic (12), and linoleic (13).

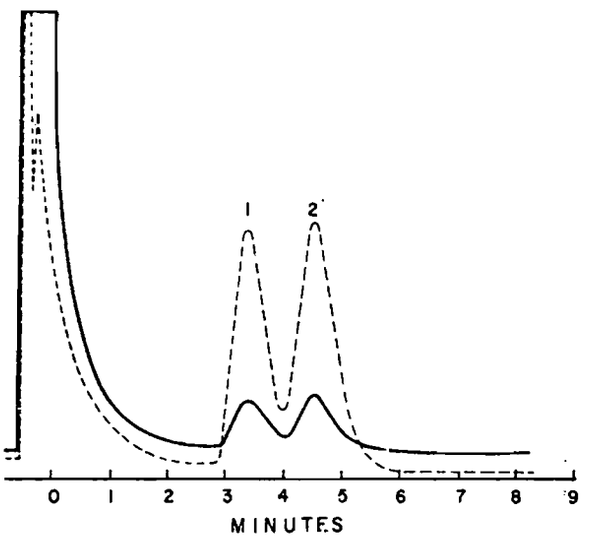


FIG. 8. Identification of glucose in the brown-white component (including transparent crystals) of calcified T. solium *larvae by gas chromatography using a stainless steel column (6' x 1/8'') packed with 5% methyl silicone gum rubber (SE-30: Varian) on chromosorb (white, acid-washed), 60/80 mesh. The temperature of the column was programmed to rise from 170–230 °C over 15 min and argon was used as the carrier gas (flow rate, 25 ml/min). The conditions of trimethylsilylation produced anomerization in pyridine during the preparation of the silyl derivative, generating α (1) and β (2) glucose which are easily identifiable. The test sample is indicated with a solid line, and the standards with a dotted line.*

IV. DISCUSSION

Degeneration of the parasite tissue was associated with the preservation of the extracellular fibers [29]. These changes were closely correlated with the transformation of amorphous calcium phosphate to hydroxyapatite and whitlockite. Additionally, protein was found to be the major organic component in calcified *T. solium* larvae, with a small quantity of lipids – a composition that has been described for other hard tissues at the beginning of calcification [32]. Glucose, the sole carbohydrate, may be derived from glycogen normally present in large amounts in the bladder wall of viable cysts.

Since calcium and small quantities of phosphorus have been described in the calcareous corpuscles of *T. solium* metacestodes [33], the possibility exists that these structures are, in fact, nucleation centers for the calcification process. The following evidence argues against this idea. First, cysts in early stages of calcifi-

cation stained with von Kossa's reagent [8] were examined for the presence of calcium. Our observations showed that calcium deposition initially occurred in structures other than the scolex [33]. Second, the racemose larvae of *T. solium* lack a scolex and, therefore, calcareous corpuscles. Their bladder wall is structurally very similar to that of *T. solium* metacestodes (*C. cellulosae*). Cañedo *et al.* have shown that during the final degenerative stages, the deposits of calcium are increased until the racemose larvae are transformed into calcified material [29]. Finally, in our studies only calcium carbonates were found in calcareous corpuscles. These data are compatible with the hypothesis that the calcification of *T. solium* cysticerci in the human brain is a process similar to other instances of ectopic calcification [3, 4], with the characteristic that the parasite extracellular fibers constitute part of the organic matrix of the calcified tissue.

ACKNOWLEDGMENTS

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REFERENCES

1. Howell, D.S. Current concepts of calcification. *J. Bone Joint Surg.* [*Am.*] 53: 250, 1971.
2. Schiffman, E., Martin, G.R. and Miller, E.J. Matrices that calcify. *In* "Biological Calcification" (H. Schraer, ed.), pp. 27–67. Appleton-Century-Crofts, New York, 1970.
3. Eisenstein, R., Trueheart, R.E. and Hass, G.M. Pathogenesis of abnormal tissue calcifications. *In* "Calcification in Biological Systems" (R.F. Sognnaes, ed.), pp. 281–305. American Association for the Advancement of Science, Washington, D.C., 1960.
4. Gabbiani, G., Tuchweber, B. and Selye, H. Experimental ectopic calcification. *In* "Biological Mineralization" (I. Zipkin, ed.), pp. 547–586. Wiley-Interscience, New York, 1973.
5. Von Brand, T. "Biochemistry of Parasites," 2nd ed. Academic Press, New York, 1973.
6. Escobar, A. and Nieto, D. Parasitic diseases. *In* "Pathology of the Nervous System" (J. Minkler, ed.), vol. III, pp. 2503–2521. McGraw-Hill, New York, 1972.
7. Von Brand, T., Mercado, T.I., Nylen, M.U. and Scott, D.B. Observations on function, composition, and structure of cestode calcareous corpuscles. *Exp. Parasitol.* 9: 205, 1960.
8. Lillie, R.D. "Histopathologic Technic and Practical Histochemistry," 3rd ed., pp. 545–547. McGraw-Hill, New York, 1965.
9. Wollman, M. On the use of polarized light in pathology. *In* "Pathology Annual" (S. Sommers, ed.), pp. 381–416. Appleton-Century-Crofts, New York, 1971.
10. Luft, J.H. Improvement in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* 9: 409, 1961.

11. Echlin, P. The application of scanning electron microscopy to biological research. *In* "New Developments in Electron Microscopy" (H.F. Huxley and A. Klug, eds.), pp. 135–172. The Royal Society, London, 1971.
12. Pastrana, L. and Cano, O. Identificación de substancias cristalinas por método de polvos. *Revista de la Sociedad Química de México* 3: 27, 1953.
13. American Society for Testing and Materials. "Alphabetical and Grouped Numerical Index of X-Ray Diffraction Data." American Society for Testing and Materials, Special Technical Publication No. 48-B, 1950.
14. Parrish, W. and Mack, C. Data for x-ray analysis, charts for the solution of Bragg's equation. Phillips Corporation, Centrex Eindhoven, 1963.
15. Pierce, A.E. "Silylation of Organic Compounds," pp. 332–418. Pierce Chemical Company, Rockford, Illinois, 1968.
16. Sweeley, C.C., Bentley, R., Makita, M. and Wells, W.W. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. *J. Am. Chem. Soc.* 85: 2501, 1963.
17. Folch, J. A single method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226: 497, 1957.
18. Metcalfe, L.D. and Schmitz, A.A. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. *Anal. Chem.* 38: 514, 1966.
19. Hartree, E.F. Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* 48: 422, 1972.
20. Miller, L. and Houghton, J.A. The microkjeldahl determination of the nitrogen content of amino acids and proteins. *J. Biol. Chem.* 159: 373, 1945.
21. Spirin, A.S. Spectrophotometric determination of total nucleic acid content. *Biokhimiia* 23: 617, 1958.
22. Loewus, F.A. Improvement in anthrone method for determination of carbohydrates. *Anal. Chem.* 24: 219, 1952.
23. Saifer, A. and Gerstenfeld, S. The photometric microdetermination of blood glucose with glucose oxidase. *J. Lab. Clin. Med.* 51: 448, 1958.
24. Zak, B. Simple rapid microtechnique for serum total cholesterol. *Am. J. Clin. Pathol.* 27: 583, 1957.
25. Fieser, L. and Fieser, M. "Steroids," pp. 53–60. Reinhold Publishing Company, New York, 1959.
26. Varian Associates. "Nuclear Magnetic Resonance Spectra Catalog," vol. 1. Varian Associates, Palto Alto, California, 1962.
27. Woessner, J.F., Jr. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch. Biochem.* 93: 440, 1961.
28. Termine, J.D. and Eanes, E.D. Comparative chemistry of amorphous and apatitic calcium phosphate preparations. *Calcif. Tissue Res.* 10: 171, 1972.
29. Cañedo, L., Garrido, F. and González, M. Host parasite relationship of cysticercosis in man. *In* "Biochemistry of Parasites and Host-Parasite Relationships" (H. Van den Bossche, ed.), pp. 385–392. North-Holland, Amsterdam, 1976.
30. Guerra, G., Cañedo, L. and Rojkind, M. Proteins of *Cysticercus cellulosae*. *In* "Biochemistry of Parasites and Host-Parasite Relationships" (H. Van den Bossche, ed.), pp. 109–115. North-Holland, Amsterdam, 1976.
31. Termine, J.D. Infrared and electron spin resonance spectroscopy of hard tissues. *In* "Biological Mineralization" (I. Zipkin, ed.), pp. 397–411. John Wiley & Sons, New York, 1973.

32. Von Brand, T., Scott, D.B., Nylen, M.U. and Pugh, M.H. Variations in the mineralogical composition of cestode calcareous corpuscles. *Exp. Parasitol.* 16: 382, 1965.
33. Grau, E.M., Cano, O. and Cañedo, L. A crystalline calcium carbonate in unheated calcareous corpuscles from *Cysticercus cellulosae*. *Proceedings of the Third International Congress of Parasitology, Munich* 3: 396, 1974.

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PROTEINS OF Cysticercus cellulosae

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INTRODUCTION

In human cysticerciasis, it is known that C. cellulosae calcifies spontaneously within the human brain. A study oriented toward the understanding of the phenomenon will promote better comprehension of the host-parasite relationship in this disease. As a partial approach toward this objective, we made a chemical analysis of human cysticerci in different stages of calcification. A prominent finding was the fact that a protein forms the major organic chemical component of the calcified cysticercus. To determine which type of protein this was, we started with a preliminary analysis of the C. cellulosae proteins.

Since very little is known about the protein composition of these parasites, a characterization of the major protein fractions of C. cellulosae was initiated, with emphasis in the extracellular protein. To make this analysis possible, the study was initiated with cysticerci obtained from pig muscle, with the idea of subsequently investigating their presence in the human calcified cysticercus, and then determining if it plays a role in the calcification process.

MATERIAL AND METHODS

Biological Samples: Cysticerci from naturally infested pigs were obtained from the Mexico City abbatoir. The infested meat was kept at 4°C. The muscle larvae were dissected within the first 24 hrs after death of the animal. No detectable changes were observed within the above period in larvae obtained from different samples. Since the collagen capsule encircling the cyst is not fixed to the larva, it was easily removed by cutting one end of the capsule and pressing on the opposite side, thereby forcing the cysticerci to pass through the opening. The bladder wall was separated from the scolex of the dissected larvae and kept at 4°C.

Chemicals: Pepsin, E.C. 3.4.4.1 (Sigma, twice recrystallized. 2 500 - 3 000 units per milligram), adenosin 5'-triphosphate disodium salt (Sigma), 2-mercaptoethanol (Merck), Sephadex G-100 and 2 000 blue dextran (Pharmacia). All other

reagents were of analytical grade.

Chemical Methods: Carbohydrate was determined by the anthrone method of Loewus (1) with D-glucose as a standard. Hartree's method (2) was used for determination of protein concentration, after removal of 2-mercaptoethanol. The nitrogen content of the samples was measured using the microkjeldahl analysis and the conversion factor of 6.224 (3).

Gel Chromatography: The protein fraction was chromatographed on a column (1.5 x 90 cm) of Sephadex G-100 superfine equilibrated with the elution buffer 0.05M tris (hydroximethyl) aminomethane, 1.0M calcium chloride, 0.001M 2-mercaptoethanol, and 0.02% sodium azide, pH 7.5. The relative molecular masses of the protein components were determined in SDS-acrylamide gel electrophoresis with a series of molecular weight standards (4).

Electron microscopy: Grids used for electron microscopy were 400 mesh copper screen (Ernest Fullam) on which a perforated carbon film had been deposited. Specimens were prepared by negative staining with 1% uranyl acetate ($\sim$pH 4.0) and 1% phosphtunstate at pH 6.5. A drop of sample was applied to a carbon-coated grid and washed off after 1 minute with two drops of distilled water. The grid was then washed with two drops of stain, and the excess liquid blotted with filter paper. The specimens were observed in a Jeol 100B electron microscope. Magnifications were calculated with the aid of a replica of cross-ruled grating 54 860 lines/inch (Polaron). Micrographs were taken on Kodak thick-base film.

Protein Extration: Several protein fractions were extracted from the bladder wall, using a solution of 0.45M NaCl with 0.02M 2-mercaptoethanol. The suspension was stirred at 4°C for 24 hours. The insoluble tissue (P_1) was removed by centrifuging for 30 min at 27 000 x g, and used for subsequent extractions. All other centrifuging was performed with the same relative centrifugal force. The supernatants (S_1) were dyalized against 0.45M NaCl to remove the remaining 2-mercaptoethanol, and a protein fraction was precipitated by dyalizing the sample against 0.5M acetic acid at 4°C for 24 hours. The precipitate (P_2) was removed by centrifuging for 30 min and resuspended in 0.45M NaCl with 0.02M 2-mercaptoethanol The carbohydrate content was determined and a sample was used for SDS-acrylamide gel electrophoresis, on which a Fuchsin reaction for carbohydrates was performed (5). The supernatant (S_2) was lyophylized and dissolved in a small volume of 0.25M acetic acid. The solution was dyalized against acetic acid at the same concentration containing 0.4% ATP. A sample was taken from the precipitate (P_3), for electron microscope examination and SDS-acrylamide gel electrophoresis. The remaining precipitated protein (P_3) was removed by centrifuging for 30 min in a solution of 0.25M acetic acid, and prepared for Sephadex chromatography by dyalizing the solution against the elution buffer. The solution was clarified

y centrifuging for 15 min, and then chromatographed on a Sephadex column at room emperature at a flow rate of 5 ml per hour. 1.5 ml fractions of the effluent ere collected and read at 230 and 255 nm by means of a Zeiss PMQ II Spectrophoto eter. Fractions with high protein content were chromatographed in SDS-acrylamide el, and dyalized against 0.25M acetic acid. This solution was subsequently yalized against acetic acid at the same concentration containing 0.4% ATP, and a recipitate was obtained (P_4).

Proteolytic Fragmentation Studies: Fifty milligrams of pepsin were dissolved ı 25 ml of 0.4M acetic acid at 4°C; to this was added 0.5 g of the bladder wall, ıd the suspension was stirred at 4°C for 24 hours, subsequent manipulations ɜing performed at 4°C. Undigested tissue was removed by centrifuging for 1 hour t 16 000 x g, and discharged. The supernatant (S_1') was dyalized against 0.02M a_2HPO_4 (6). No precipitate was observed. A fraction of the protein in the mple was then precipitated by dyalisis against 0.5M acetic acid (P_2'). The ɜecipitate was removed by centrifuging for 15 min at 27 000 x g, and lyophilized. ıe supernatant (S_2') was also lyophilized and dissolved in 0.25M acetic acid. ıis solution was dyalized against acetic acid at the same concentration ntaining 0.4% of ATP and a precipitate was formed (P_3'). A sample of the ɜecipitate was taken for electron microscopy examination and SDS-acrylamide gel ectrophoresis.

SULTS

During the characterization of the major protein fractions of C. cellulosae, e following preliminary results were found:

The insoluble protein fraction (P_1) obtained from the extraction with 45M NaCl, 0.02M 2-mercaptoethanol it is mainly composed of structures which ɜ morphologically identical to the extracellular fibers found in vivo in the adder wall of C. cellulosae (Plate 1 micrographs 1 and 2).

The soluble protein (S_1 - S_1') has two major fractions: a) 60% of the protein this supernatant is insoluble in acetic acid and has a molecular weight lower ɯn 68 000 daltons (Fig 1c); 90% of the total carbohydrate content of the ɬtract is contained in this fraction. b) When the acid soluble protein fraction ! - S_2') is dyalized against ATP it forms a precipitate (P_3 - P_3'); in SDS-ylamide gel electrophoresis, this fraction produces two major and four minor ıds whose molecular weight is between 70 000 - 90 000 daltons (Fig 1e). When ɜ fraction P_3 is chromatographied in Sephadex G-100 superfine, the several ɬecular species were eluted as a single peak (Fig 1 center). The effluent from ɜ column was dyalized against ATP and a precipitate was obtained (P_4). Said ɜcipitate produces the same migration pattern of P_3 and P_3' in SDS-acrylamide electrophoresis (Fig 1e).

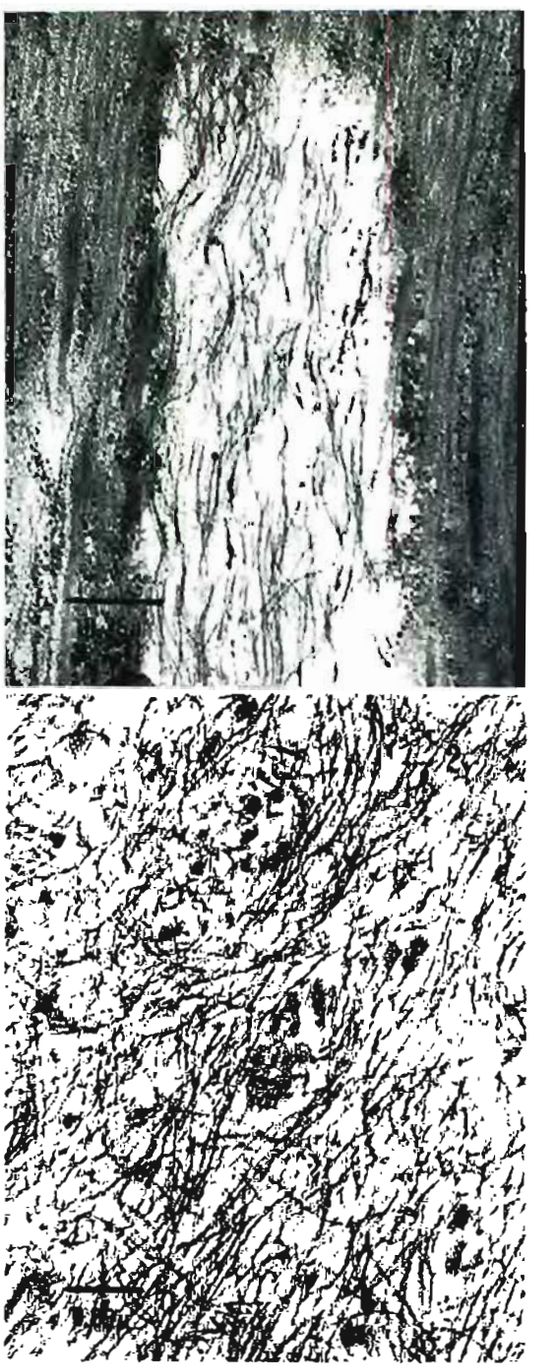
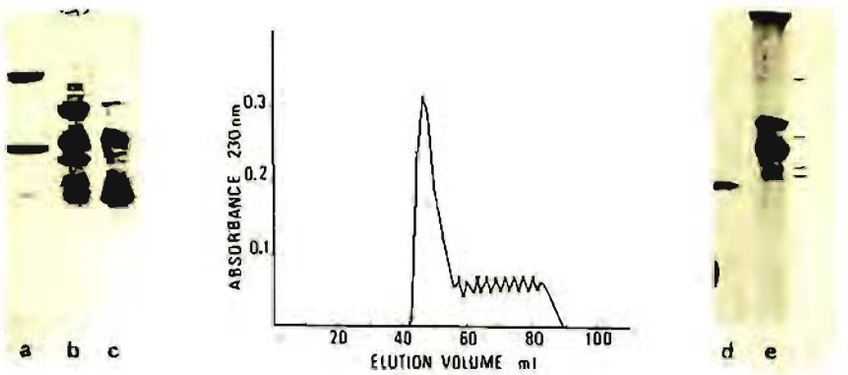


Plate 1. (1) Section of the bladder wall of C. cellulosae obtained from pig muscle and fixed with glutaraldehyde and osmium and stained with uranyl acetate and lead citrate (7). A portion of a muscle cell and the extracellular fibers are seen. (2) Insoluble material (P_1) after the second extraction with 0.4SM NaCl, 0.02M 2-mercaptoethanol.



ig. 1 Characterization of major protein fractions of C. cellulosae. Left: olyacrylamide gel electrophoresis in SDS. Gradient gels from 30% to 7.5%.) Albumin 68 000 m.w. and trypsin 23 300 m.w. b) Supernatant of extraction ith 0.45M NaCl, 0.02M 2-mercaptoethanol (S_1). c) Insoluble fraction in acetic cid 0.5M with high carbohydrate content (P_2). Center: Chromatography in ephadex G-100 superfine of fraction P_3. Right: 10% SDS-acrylamide gels. d) lbumin 68 000 m.w. e) Migration pattern of the fraction precipitated against yalisis with ATP 0.4%. (P_3) and subsequently eluted from Sephadex G-100 superine (P_4), two strong bands are seen, lines indicate four faint bands.

When the acid-soluble protein S_2, S_2' and the fraction containing the recipitate P_3 are resuspended in acetic acid, they provide an ill-defined lobular image on examination with electron microscope (Plate 2-1). When these olutions are incubated with ATP 0.2% (final concentration) a polymerization rocess takes place producing a protein net pattern P_3, P_3' and P_4 (Plate 2-2).

ISCUSSION

When the fraction obtained after treatment of the bladder wall with pepsin was alized against Na_2HPO_4, no precipitate was observed. However, preliminary data ow that when insoluble tissue (P_1) obtained after extraction of the bladder wall ith 0.45M NaCl, 0.02M 2-mercaptoethanol is treated in the same manner, a ecipitate is obtained; electron microscope observation of the sample of the ecipitate showed fibers having a periodic band structure (6). This finding, and e SDS-acrylamide gel electrophoresis pattern obtained, suggest that a collagen-ke material may be present in the bladder wall.

The fact that the P_1 fraction has less components than the complete bladder ll indicates the possibility that the protein fragments liberated by the enzyme P_1 could assemble much easier without interference from the other components esent in the bladder wall preparation. An alternative possibility could be at these fragments cannot be liberated until the bladder wall has been treated

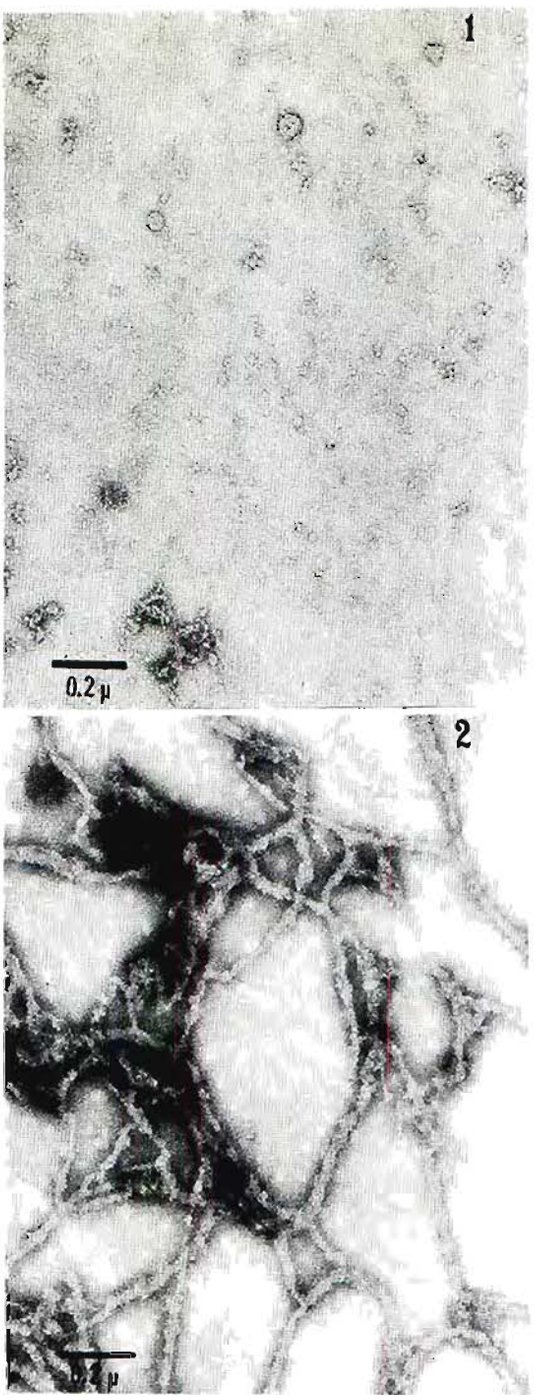


Plate 2. Negatively stained samples of P_3 fraction eluted from Sephadex G-10(superfine column and dyalized against acetic acid 0.25M. (1) 0.05 ml of the sample incubated with 0.05 ml of acetic acid 0.25M for one minute and stained with uranyl acetate 1%. (2) 0.05 ml of the sample incubated with 0.05 ml of acetic acid with 0.4% of ATP for one minute and stained with PTA 1%.

with the reducing agent. Experiments are in progress to show which, if any, of the above possibilities is correct.

The external coat of most cells is rich in carbohydrates, and unpublished experiments made in our laboratory have shown that the same condition obtains for the external surface of the bladder wall of C. cellulosae. This finding and the following observations support the hypothesis that the fraction P_2 contains part of the external surface of the bladder wall: a) When the insoluble tissue (P_1) obtained after the first extraction with 0.45M NaCl, 0.02M 2-mercaptoethanol is observed with the electron microscope, fragments from different bladder wall tissues can be recognized, including glycogen residues; the only exceptions are the cuticular elements. b) The acid insoluble fraction (P_2) contains 90% of the carbohydrates extracted with 0.45M NaCl, 0.02M 2-mercaptoethanol. c) Most of P_2 band pattern observed in SDS-acrylamide electrophoresis is also observed in the solution obtained after the bladder wall is washed with NaCl 0.15M for removal of the remaining cyst fluid.

Experiments are in progress to show if the protein which changes to a polymer structure in the presence of ATP corresponds to similar molecules previously described (8, 9) or constitutes a new class characteristic of C. cellulosae.

REFERENCES

1. Loewus, F.A. (1952) Improvement in Anthrone Method for Determination of Carbohydrates. Anal. Chem. 24, 219.
2. Hartree, F.E. (1972) Determination of Protein. Anal. Biochem. 48, 422-427.
3. O'Brien, D. et al (1968) Laboratory Manual of Pediatric Microbiochemical Techniques. 4th. Edition. Harper and Row, N.Y. 238-240.
4. Weber, K. and Osborn, M. (1975) In the Proteins. Vol. 1. Neurath, H. and Hill, R.L. (eds.). Academic Press, N.Y. Chap. 3. 192.
5. Glossmann, H. and Neville, D.M. (1971) Glycoproteins of Cell Surfaces. J. Biol. Chem. 246, 6339-6346.
6. Ervin, E.H. (1974) $[\alpha 1\ (III)]_3$ Human Skin Collagen. J. Biol. Chem. 249, 3225-3230.
7. Reynolds, E.S. (1963) The Use of Lead Citrate at High pH as an Electron Opaque Stain in Electron Microscopy. J. Cell Biol. 17, 208.
8. Oosawa, F. et al (1966) Polymerization of Actin and Flagellin. In Principles of Biomolecular Organization. Wolstenholme G.E.W. and O. Connor M. (eds.) Churchill, London 273-307.
9. Stephens, R.E. (1971) Microtubules. In Subunits in Biological Systems, part A. Timashef, S.N. and Fasman, G.D. (eds.) Marcel Decker, N.Y. 335-391.

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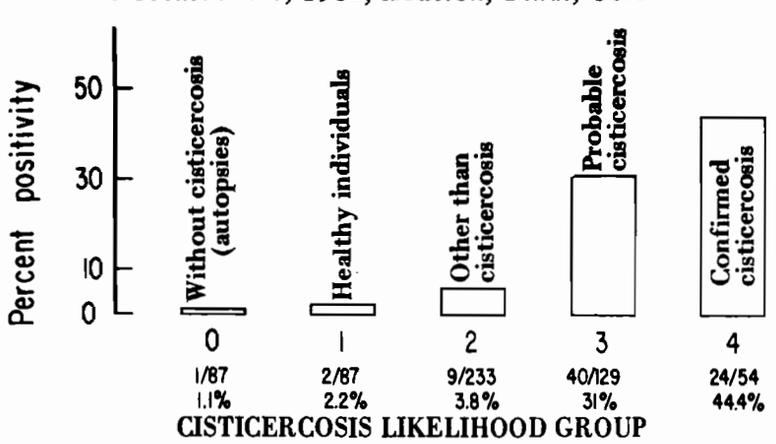

Flisser A, Plancarte A, Espinoza B, Woodhouse E, Rivera L, Trueba J, Yakoleff-Greenhouse V, Sierra A, Laclette JP, Guerra C, Cañedo L & Larralde C *(Mexico, MEX):* The immune status of patients with cisticercosis

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(continued on page 3 of cover)

Afr J Clin Exp Immunol 3[1982]183-190

A SUMMARY

THE IMMUNE STATUS OF PATIENTS WITH CYSTICERCOSIS

FLISSER A*, PLANCARTE A*, ESPINOZA B*, WOODHOUSE E*, RIVERA L**, TRUEBA J**, YAKOLEFF-GREENHOUSE V*, SIERRA A*, LACLETTE JP***, GUERRA C***, CAÑEDO L*** & LARRALDE C*

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(Received for publication 05 August 1981)

INTRODUCTION

Neural cysticercosis is found in an average of 2 percent of the autopsies carried out n Mexico, a figure that has varied little for the last 30 years (Flisser 1980). The high prevalence of this disease as well as its diverse symptomatology has motivated us to analyze the immune response of patients with brain cysticercosis in the hope of improving diagnostic procedures, of defining factors involved in this seemingly stalemated host-parasite relationship, and of identifying an effective immunizing procedure to prevent this disease. Our findings related to these aims have been summarized here.

IMMUNODIAGNOSIS

A crude cysticercus extract can be separated into its charged components by immunoelectrophoresis, prior to reaction with antisera. Since this procedure is easy to perform nd the results are reasonably unambiguous, we selected this method for use both as a iagnostic test and as a means of studying the humoral immune response of cysticercotic patients. The classes of antibodies that participate in the antigen-antibody reaction can also be identified by immunoelectrophoresis using monospecific fluoresceinted antihuman immunoglobulins after the antigen-antibody precipitates are formed i the gel. Antigens were extracted with 3 M KCl from the walls and scoleces of *Cysti-*

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FIGURE 1

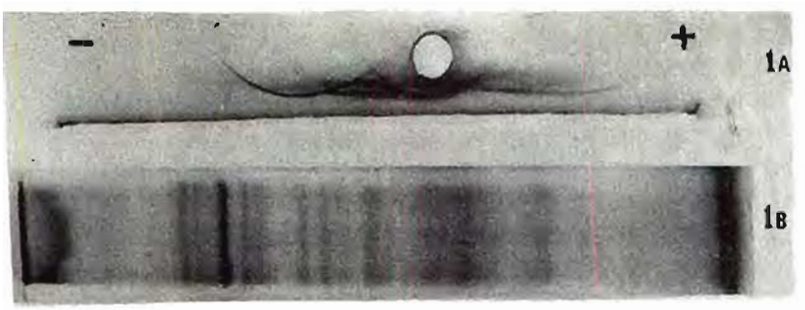

FIGURE 2

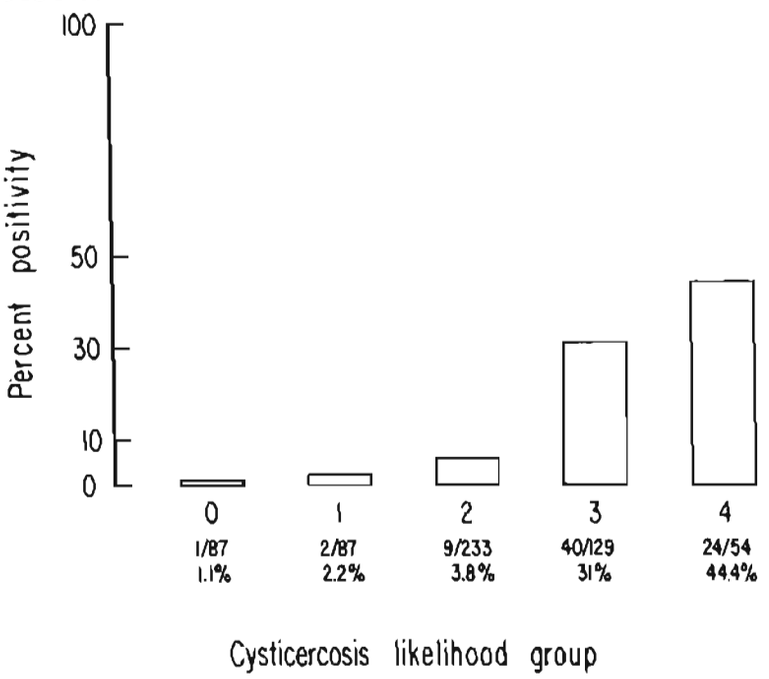

Figure 1: *C. cellulosae* antigen extract analyzed by immunoelectrophoresis against hyperimmune sheep serum (A) and polyacrylamide gel electrophoresis with sodium duodecyl sulfate and 2-mercaptoethanol (B). The crude extract obtained with 3 M KCl, which contained 25 mg protein/g wet weight and 60 mg carbohydrate/g wet weight, has at least 20 protein bands and 11 precipitating antigens. Figure 2: Histogram correlating the percent of positive sera, determined by immunoelectrophoresis with the likelihood of having cysticercosis: group 0, autopsies without brain or visceral cysticercosis; 1, healthy people; 2, neurological patients with a diagnosis other than cysticercosis; 3, neurological patients with probable cysticercosis; 4, neurological patients with confirmed cysticercosis. Percent indicating positivity increases with the likelihood group, indicating that the diagnostic procedure is suitable. Nevertheless, approximately 50 percent of the cysticercotic patients have very little or no precipitating antibodies in circulation.

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ercus cellulosae excised from pig muscle. This crude extract contained 25 mg of protein and 60 mg of carbohydrate/g wet weight and showed 20 protein bands on polyacrylamide gel electrophoresis with 2-mercaptoethanol and sodium dodecyl sulfate (Flisser et al 1980). At least 11 different antigens against hyperimmune serum could be detected in the extract by immunoelectrophoresis (Flisser et al 1980)(Figure 1).

The human sera used to test the diagnostic ability of immunoelectrophoresis were obtained from the following sources : autopsy cases without neurocisticercosis (N=87); apparently healthy individuals (N= 87); patients with cysticercosis confirmed during surgery or by autopsy (N=54); neurological patients with a clinical diagnosis of cysticercosis (N=129), and neurological patients with a diagnosis other than cysticercosis (N=233) (Flisser et al 1980). The results are shown in figure 2. Very few "false positives" to the antigenic extract were found and these are explicable. Thus, the figure of 1·1 percent obtained from the first group could be due to the presence of extracerebral cysticercosis, a condition not systematically searched for during an autopsy, while the rate of 2·2 percent found in the healthy group, consistent with the frequency of cysticercosis infections reported in autopsy studies, can be explained by the fact that a large proportion of cysticercotic individuals (43 percent) are asymptomatic (Briceño et al 1961). The patients with brain cysticercosis belong to two immunologically distinct groups: one (44·6 percent) with and the other (55·4 percent) without demonstrable anti-cysticercus antibodies "false negatives". A high incidence of seronegativity in cysticercotic individuals has also been reported by other research groups using different serological tests (see Flisser et al 1979). The possibility of different serotypes in cysticerci has been discarded since a thorough immunological taxonomy of the parasite revealed extensive sharing of antigens among different *T. solium* cysticerci (Yakoleff-Greenhouse et al, submitted for publication).Thus, immunoelectrophoresis of a patient's serum against a crude extract is practically diagnostic of cysticercosis when there is a positive response in a neurological patient, but has no value when negative. In fact, we believe this is a major finding of our work: a large fraction of cysticercotic patients do not appear to respond with an energetic immune response to the parasite.

THE IMMUNE RESPONSE OF THE HOST

Cellular Immune Response

Studies aimed at evaluating the state of the cellular immune response of cysticercotic patients are still not thorough enough. What follows are preliminary findings and tentative conclusions. Since around 60 percent of the Mexican population gives a positive response to *Mycobacterium tuberculosis* antigens (Basurto-Ortega 1976), the skin response to purified protein derivative (PPD) was chosen as one of the methods to monitor the cellular immune responses of cysticercotic patients. PPD reactivity was found to be significantly depressed in the infected population (17 per cent positive response in cysticercotic patients vs. 64 per cent in healthy individuals, $P<0.01$). Leukocyte and lymphocyte counts were also made after separation on Ficoll-Hypaque gradients. The formation of T-rosettes was measured by the direct technique after 24 h of incubation (Waller & MacLennan 1977), while the number of B-rosettes was determined

using sheep red blood cells sensitized with hemolysin and diluted complement after incubation for 30 min. A subgroup of cysticercotic patients was identified that had an increased number of lymphocytes (whole counts, and T and B cells).

Blastoid transformation was measured in leukocytes from normal and infected individuals that were cultured in complete RPMI medium containing 5 percent fetal calf serum following well-established procedures (Lopez et al 1980). Stimulation was induced with 50 μg/ml of concanavalin A (Con A) or 450 μg/ml of crude *C.cellulosae* extract. Cultures were incubated for 48 h before tritiated thymidine was added, and the cells were harvested 18 h later. Stimulation indices were obtained by dividing the counts per min obtained in the Con A- or antigen-treated cultures by those measured in the control cultures. The large experimental variation which we have not yet been able to control does not permit a definite conclusion concerning the ability of lymphocytes to respond to mitogenic stimulation in vitro. However, a cursory examination of the data suggests that while cisticercotic patients have a lower response to both Con A and the cysticercus exstract than do healthy people, they show a similar response to that of the other neurologic patients included in this study.

Since the responses of leukocytes from cysticercotic and neurological patients to Con A and the cysticercus extract were similar, and considering the results of the PPD reaction and the cell counts, we have tentatively concluded that while there are alterations in the cellular immune response in cysticercotic patients, they are more a reflection of the poor health of these patients than a specific effect of the parasite.

Humoral Immune Response

The immune response found in patients with precipitating antibodies was heterogeneous, both in the number and nature of the antigens recognized and in the classes of immunoglobulins produced. Approximately 77 percent (89/116) of the patients synthesized antibodies against 1 to 3 antigens. More rarely, the sera of infected individuals reacted with 4 or more antigens, while only one reacted with 8 antigens. Antigen B, an isoelectric component at pH 8·6, was by far the most frequently recognized (84 percent)(Flisser et al 1980).

The classes of precipitating antibodies were identified using an indirect immunofluorescence technique in conjunction with immunoelectrophoresis. The antigen extract, placed in wells, was subjected to electrophoresis. Aliquots of the sera from cysticercotic patients that had anticysticercus antibodies were placed in the troughs and allowed to diffuse. After thorough washing of the gels, troughs parallel to the central ones were cut and filled with fluoresceinated rabbit monospecific antihuman IgG, IgM, IgA, IgE or IgD. Following diffusion and thorough washing, the gels were viewed with UV light to determine which precipitation bands fluoresced, an indication of the presence of a specific class of immunoglobulin in the antibody-antigen reaction. The results showed that all classes of immunoglobulins were found in the antibodies produced by parasitized patients. Not surprisingly, the most prevalent was IgG, found in 98 percent of the cases. A notable finding was the presence of specific IgE in 37 percent of the 55 sera analyzed (Flisser et al 1980), a result similar to that reported in

Table 1
Frequency and Rank of Immunoglobulin Classes in each Precipitation Band Formed Between Human Antisera and the Antigenic Cysticercus Extract

	Precipitation Band																								
	B			A			E			C			H			D			G			F			
Ig Class	P/T*	%**	Rank***	P/T	%	Rank	P/T	%	Rank	P/T	%	Rank	P/T	%	Rank	P/T	%	Rank	P/T	%	Rank	P/T	%	Rank	Sum of Ranks
IgG	50/51	98	1	25/30	83	1	16/23	69	1	19/19	100	1	7/19	37	1	5/17	29	1	5/11	45	1	3/10	30	1	8
IgM	35/50	70	2	12/29	41	2	5/22	23	2	6/19	31	2	3/19	16	2	2/17	12	2	2/10	20	2	2/10	20	2	16
IgE	12/48	25	3	5/28	18	3	4/21	19	3	3/17	18	3	2/18	11	3	0/18	0	4	0/11	0	4	0/8	0	3	26
IgA	11/49	22	4	3/29	10	4	4/21	19	3	3/18	17	4	1/18	5	5	1/16	6	3	2/11	18	3	0/8	0	3	29
IgD	9/44	20	5	0/19	0	5	2/18	11	5	1/16	6	5	1/16	6	4	0/10	0	4	0/12	0	4	0/4	0	3	35

*,Cases with positive fluorescence to the corresponding antihuman immunoglobulin (P) over the total number of cases tested in which the precipitation band was present (T);**Frequency expressed as percent of positive cases;***,Ordered frequency of the immunoglobulin class in each precipitation band; S = 462·8, W = 0·035625, P<0·001.

individuals infected by other helminths (Johansson et al 1968; Hogarth-Scott et al 1969; Rosenberg et al 1970; Kojima et al 1972; Nepote et al 1974; Dessaint et al 1975a,b, Vervloet et al 1976; Somorin et al 1977). The majority of the antigens in the extract induced the different classes of antibodies in the following order: IgG>IgM>IgE >IgA>IgD (Flisser et al 1980)(Table 1).

PROTECTIVE IMMUNITY

The protective effect of immunization against cysticercosis in six host-taenia relationships was evaluated by statistically analyzing those experiments reported in the literature which could be reduced to a single response variable; namely, the efficiency of *Taenia* egg establishment in normal and immune hosts. A two-way analysis of variance was performed on data for which both the number of *Taenia* eggs administered to an animal as well as the number of cysticerci established were reported. The results indicate with a 100 percent level of confidence that immunization reduces the risk of contracting cysticercosis, although factors such as the species of *Taenia* were found to affect the magnitude of this effect (Flisser et al 1979). Regression analysis of the data revealed significant differences between the immune and normal states, and confirmed the lower efficiency of cysticercus establishment in the immune host: 1 percent versus 0·3 percent (Flisser et al 1979). Furthermore, the experiments reported in the literature show that IgG is mainly responsible for the immunity conferred in animal cysticercosis (Leid & Williams 1974; Musoke & Williams 1975a,b,1976; Musoke et al 1975) but is effective only against the early developmental stages of the parasite (one week) (Heath 1974, 1976; Rickard & Outteridge 1974; Heath & Pavloff 1975; Musoke & Williams 1975a) and is completely ineffective against fully developed cysticerci (Nemeth 1970; Heath 1973, 1976d; Musoke et al 1975). From these results, it can be concluded that immunization imparts some degree of protection.

PERSPECTIVES

Isoelectric antigen B is likely to be a good choice for an immunizing agent since it is recognized with the highest frequency by the sera of cysticercotic patients and has been shown to induce mainly IgG. Antigen B, purified by solubilization at low pH and precipitation with salts, is composed of two proteins with molecular weights of 95,000 and 110,000 daltons (Guerra et al, submitted for publication). A notable characteristic of antigen B is its great affinity for connective tissue, a property that could divert the host's anti-B response from the parasite. This interesting mechanism of evading the immune response (a smoke screen) is presently being analyzed. An enzyme-linked immunosorbent assay (ELISA) using antihuman IgG coupled to alkaline phosphatase and purified antigen B has recently been developed in our laboratory and is being used for immunodiagnosis. Preliminary results indicate that the ELISA discriminates between sera of the cysticercotic and healthy individuals better than immunoelectrophoresis,

a large fraction (20 percent) still does not have circulating antibodies demonstrable by this method. This finding further strengthens our impression that cysticerci display low antigenecity in a large number of patients. HLA typing currently being carried out to test for the presence of genetic factor possibly contributing to the risk of contracting cysticercosis. The ELISA method is also being employed to analyze the humoral immune response in the cerebrospinal fluid (CSF) of cysticercotic and other neurological patients. Preliminary findings indicate a significant difference in the response of the two populations. The data show that antigen B is also recognized in CSF and that IgG anti-antigen B is induced in practically all cysticercotic individuals. Finally, we have succeeded in isolating the eggs of *Taenia solium* after considerable difficulty in obtaining live specimens. Experiments are now being carried out in pigs to determine whether antigen B can induce protection in this only alternative host for *C.cellulosae*.

ACKNOWLEDGEMENT

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REFERENCES

Basurto-Ortega S (1976) La reaccion tuberculinica. *Salud Publica Mex 18:* 981-988.

Briceño CE, Biagi F & Martinez B (1961) Cisticercosis. Observaciones sobre 97 casos de autopsia.*Prensa Med Mex 26:* 193-197.

Dessaint JP, Bout D, Watre P & Capron A (1975a) Quantitative determination of specific IgE antibodies to *Echinococcus granulosus* and IgE levels in sera from patients with hydatid disease. *Immunology 29:* 813-823.

Dessaint JP, Capron M, Bout D & Capron A (1975b) Quantitative determination of specific IgE antibodies to schistosome antigen and serum IgE levels in patients with schistosomiasis *(S. mansoni* or *S. haematobium). Clin Exp Immunol 20:* 427-436.

Flisser A, Perez-Montfort R & Larralde C (1979) The immunology of human and animal cysticercosis: A review. *Bull WHO 57:* 839-856.

Flisser A (1980) The immunology of human cysticercosis. In: Larralde C, Willms K, Ortiz-Ortiz L & Sela M (Eds): Molecules, Cells, and Parasites in Immunology. New York, Academic Press, pp. 125-143.

Flisser A, Woodhouse E & Larralde C (1980) Human cysticercosis: Antigens, antibodies, and non-responders. *Clin Exp Immunol 39:* 27-37.

Heath DD (1973) Resistance to *Taenia pisiformis larvae* in rabbits. II.Temporal relationships and the development phase affected. *Int J Parasitol 3:* 491-498.

Heath DD (1976) Resistance to *Taenia pisiformis* larvae in rabbits: Immunization against infection using non-living antigens from in vitro culture. *Int J Parasitol 6:* 19-24.

Heath DD & Pavloff P (1975) The fate of *Taenia taeniaeformis* oncospheres in normal and passively protected rats. *Int J Parasitol 5:* 83-88.

Hogarth-Scott RS, Johansson SGO & Bennich H (1969) Antibodies to *Toxocara* in the sera of visceral larva migrans patients: The significance of raised levels of IgE. *Clin Exp Immunol 5:* 619-625.

Johansson SGO, Mellbin T & Vahlquist B (1968) Immunoglobulin levels in Ethiopian preschool chi dren with special reference to high concentrations of immunoglobulin E (IgND). *Lancet 1:* 1118-112

Kojima S, Yokogawa M & Tada T (1972) Raised levels of serum IgE in human helminthiasis. *Am Trop Med Hyg 21:* 913-918.

Leid RW & Williams JF (1974) The immunological response of the rat to infection with *Taenia taenia formis.* I.Immunoglobulin classes involved in passive transfer of resistance. *Immunology 27:* 195-20

Lopez JS, Scaly M, Ramos C, Melendro EI, Willms K & Ortiz-Ortiz L (1980) B lymphocyte stimula ion by parasitic organisms. In: Larralde C, Willms K, Ortiz-Ortiz L & Sela M (Eds): Molecules, Cel and Parasites in Immunology. New York, Academic Press, pp. 113-124.

Musoke AJ & Williams JF (1975a) Immunoglobulins associated with passive transfer of resistance *Taenia taeniaeformis* in the mouse. *Immunology 28:* 97-101.

Musoke AJ & Williams JF (1975b) The immunological response of the rat to infection with *Tae taeniaeformis.* V.Sequence of appearance of protective immunoglobulins and the mechanism action of 7Sγ2a antibodies. *Immunology 29:* 855-866.

Musoke AJ & Williams JF (1976) Immunological response of the rat to infection with *Taenia taeni formis* : Protective antibody response to implanted parasites. *Int J Parasitol 6:* 265-269.

Musoke AJ, Williams JF, Leid RW & Williams CSF (1975) The immunological response of the rat infection with *Taenia taeniaeformis* . IV.Immunoglobulins involved in passive transfer of resistar from mother to offspring. *Immunology 29:* 845-853.

Nemeth I (1970) Immunological study of rabbit cysticercosis. II.Transfer of immunity to *Cysticer pisiformis* (Bloch, 1780) with parenterally administered immune serum or lymphoid cells. *Acta Acad Sci Hung 20:* 69-79.

Nepote KH, Pawlowski ZS & Soulsby EJL (1974) Immunoglobulin levels in patients infected w *Taenia saginata.* In: Soulsby EJL (Ed): Parasitic Zoonoses. Clinical and Experimental Stud New York, Academic Press, pp. 241-247.

Rickard MD & Outteridge PM (1974) Antibody and cell-mediated immunity in rabbits infected v the larval stages of *Taenia pisiformis. Z Parasitenkd 44:* 187-201.

Rosenberg EB, Whalen GE, Bennich H & Johansson SGO (1970) Increased circulating IgE in a parasitic disease - human intestinal capillariasis. *N Engl J Med 283:* 1148-1149.

Somorin AO, Heiner DC & Ajugwo RE (1977) Immunoglobulin E in Nigerian onchocerciasis. *A Trop Med Hyg 26:* 872-876.

Vervloet D, Dumon H, Quilici M & Charpin J (1976) Les IgE specifiques dans l'hydatidose. Mis evidence et etude comparative avec les autres parametres sero-immunologiques. *Rev Fr Allergo* 73-78.

Waller CA & MacLennan ICM (1977) Analysis of lymphocytes in blood and tissues. In: Thompson (Ed): Techniques in Clinical Immunology. Oxford, Blackwell, pp.183-184.

(continued from page *a*)

3 LAYOUT

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The second innovation in this regard, that will not be published but will be used for administrative purposes, is that every referee will express the quality of the article by allocating from 1 to 30 points (directly proportional) for each of: Originality, Clarity and Priority for publication. The total score of one referee may vary from 3 to 90 points. The mean values of both referees are taken. The Editor-in-Chief will give every article 1 to 10 additional points, allocated for the *renomme* of the author and author's institution in the field, the quality of the answer to the referees' criticism and the number of articles from the author's institution published in this journal. The total number of points will indicate, arbitrarily, the value of the article and it will influence the minimum number of reprints and the purchasing price, as follows: a) 91 to 100 points, authors may order up to 1000 reprints at 20 percent reduced price b) 81 to 90 points, authors may buy reprints at nominal costs c) 61 to 80 points, authors have to buy not less than 150 reprints at nominal costs d) 50 to 60 points, authors have to buy not less than 250 reprints.

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BIOCHEMICAL AND IMMUNOLOGICAL CHARACTERIZATION OF ANTIGEN B PURIFIED FROM CYSTICERCI OF *TAENIA SOLIUM*

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I. INTRODUCTION

ıe high incidence of cysticercosis among Mexicans due to the metacestode *ıenia solium* is indicated by the fact that cerebral cysticercosis is found in ɔximately 2 o/o of the autopsies performed on hospitalized patients [1] and ɔ–3.8 o/o of the population surveyed in seroepidemiological studies [2–4]. ɔugh immunodiagnosis of human cysticercosis is currently carried out by ılement fixation [5] or immunoelectrophoresis [6], these methods, as well hers that have been employed or tested [7], show a lack of sensitivity and/ ecificity. It has been argued that the use of crude extracts in immunodiag- is the principal cause of these deficiencies [8].

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The humoral immune response of individuals with brain cysticercosis is heterogeneous. When tested against a crude extract of cysticerci in immuno trophoresis carried out at pH 8.6, the sera of such patients display diffe numbers and types of antigens. A total of eight precipitation bands have l detected, with the isoelectric band (antigen B) being by far the most freque recognized [6]. Protection by IgG antibodies has been demonstrated in se experimental models of cysticercosis [9–11]. As this is the most frequent of human anti-B antibodies [6], the availability of purified antigen B would mit an evaluation of its immunizing properties, as well as of its use in the provement of methods currently employed for the immunodiagnosis of this asitosis. This communication presents the purification and the biochemical immunological characterization of antigen B from cysticerci of *T. solium.*

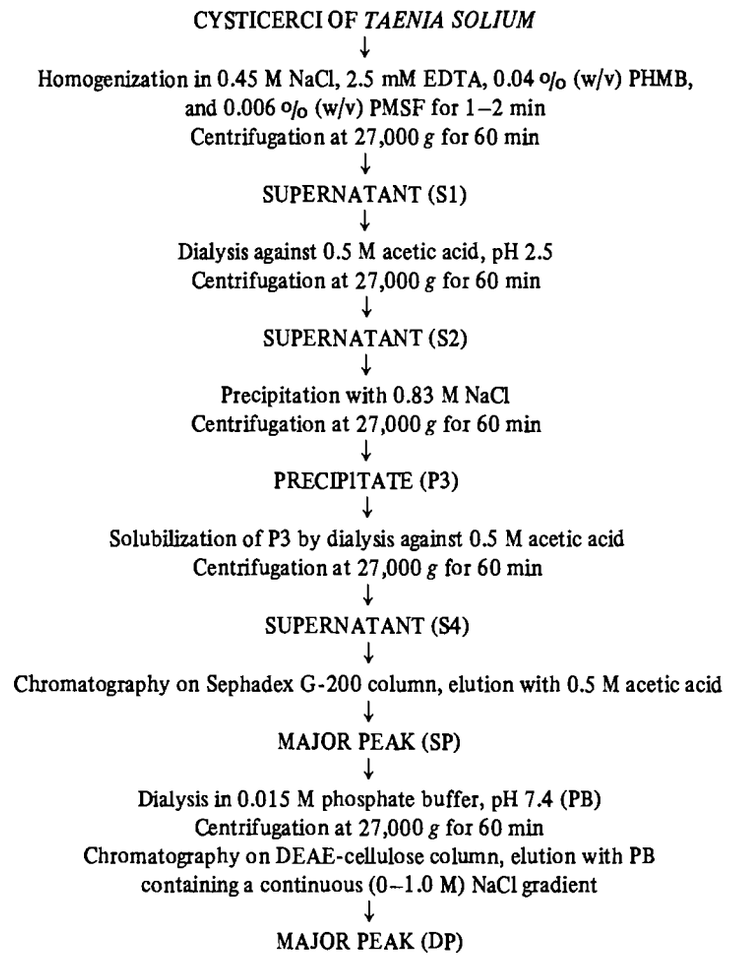
II. MATERIALS AND METHODS

Source of metacestodes. Cysticerci were dissected from the skeletal mu of highly infected pigs within 6 h after slaughter. The vesicular fluid wa moved by puncture and the larvae were stored at −70 °C until use.

Antigen preparation. A crude extract was obtained as previously desc [6]. Briefly, the larvae were homogenized in 3 M KCl, centrifuged at 1000 30 min at 4 °C, and the supernatant was dialyzed against 0.15 M NaCl in 0.0 phosphate buffer, pH 7.4 (PBS).

A purified protein fraction was obtained by a substantial modification (marized in Fig. 1) of a previously published procedure [12]. The cysticerci homogenized in 0.45 M NaCl (5 ml/g of humid larvae) containing 2.5 mM EI 0.04 % (w/v) *p*-hydroxymercuribenzoate (PHMB; Sigma), and 0.006 % phenylmethylsulfonylfluoride (PMSF; Sigma) in a Polytron homogenizer (E mann Instruments) at maximal speed for 1–2 min at 4 °C. The homogenat a whitish emulsion which was cleared by centrifugation at 27,000 *g* for 6(at 4 °C. The precipitate was discarded and the supernatant (S1) was imme ly dialyzed against 0.5 M acetic acid, pH 2.5, at 4 °C using standard cellulo alysis tubing (molecular weight cut-off: 12,000–14,000). The turbid emu obtained from dialysis was centrifuged at 27,000 *g* for 60 min at 4 °C, an clear supernatant (S2) was diluted to a protein concentration of 0.5 mg/ml 0.5 M acetic acid.

A salting-out precipitation was carried out on S2 by slowly adding a co trated solution of NaCl while stirring, until a final concentration of 0.83 M was reached. This mixture was stirred for 2 h at 4 °C and centrifuged at 27, for 60 min at 4 °C. The supernatant was discarded and the pellet (P3) was bilized by dialysis against 0.5 M acetic acid. The unsolubilized materia eliminated by centrifugation as above, and the clear supernatant (S4) was matographed on Sephadex G-200 (40 x 2.6-cm column) and eluted with



. *Summary of the purification protocol for the isolation of antigen B from cysticerci* :nia solium. *All procedures were carried out at 4 °C. For a detailed explanation, see n II.*

acid (flow rate: 5 ml/cm^2/h). Fractions of 2.5 ml were collected and their ɔance at 230 nm was determined using a PMQII Zeiss spectrophotometer. bance at 230 nm instead of at 280 nm was selected in order to enhance the vity of the detection. In a typical chromatographic run, 10 ml of S4 with ein concentration of 0.5 mg/ml were applied to the column. The proteins :red in the major peak (Fig. 2) accounted for 45–50 % of the applied ɜ, and the proteins recovered in the other peaks accounted for 15–25 %. fore, the quantitative recovery from this column was approximately 70 %. ɜ fractions corresponding to the major peak were pooled and concentrated imes using immersible CX-10 ultrafiltration units (Millipore Corp.). This

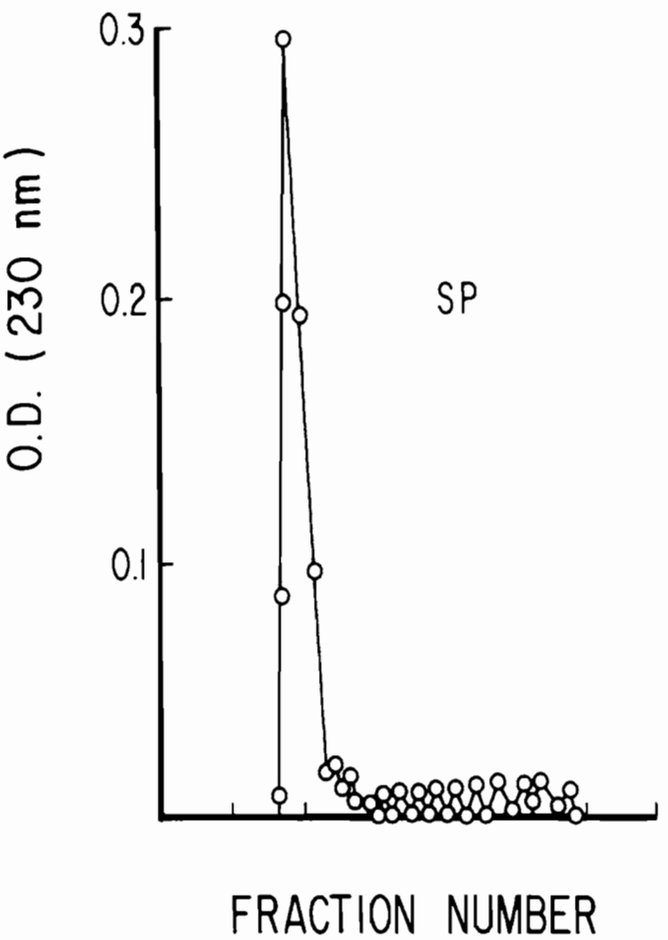
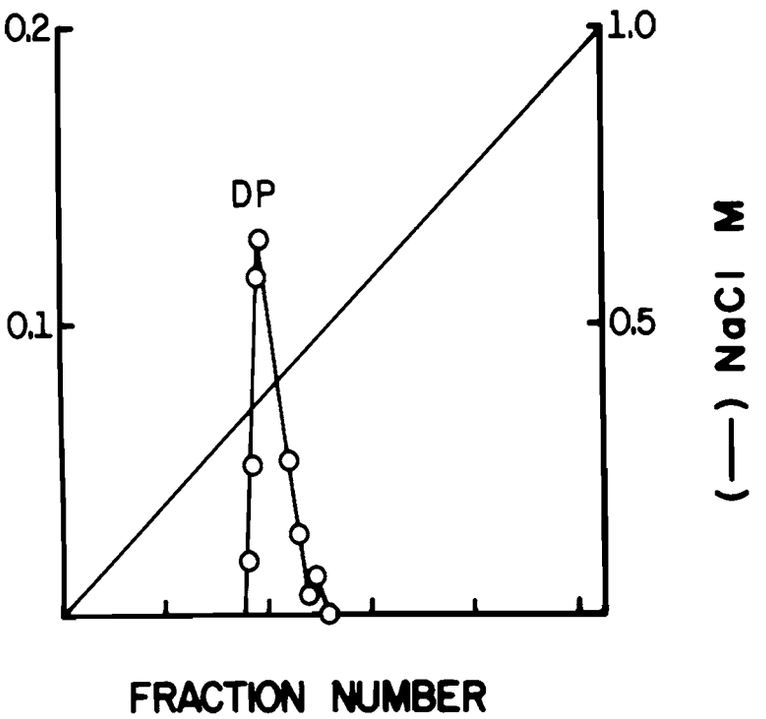


FIG. 2. Profile of S4 eluted from a Sephadex G-200 column with 0.5 M acetic acid protein present in the major peak (SP) was used in all experiments with the except amino acid analysis and molecular weight determination.

fraction, designated as SP, was dialyzed against 0.015 M phosphate buffe 7.4. After centrifugation at 27,000 *g* for 60 min at 4 °C, this fraction was to a DEAE-cellulose (Sigma) column (10–15 ml bed volume) and was with 0.015 M phosphate buffer, pH 7.4. Usually, 5–15 ml of SP (0.5 m tein/ml) were applied to the column with a flow rate of 25 ml/h. After lo the column, the unbound proteins were washed out from the resin with 1– ml of phosphate buffer. The bound proteins were eluted from the colum– phosphate buffer containing a continuous (0–1.0 M) NaCl gradient. Fra of 2.5 ml were collected and their absorbance at 230 nm was determined. tions included in the only peak obtained with the NaCl gradient (Fig. 3



. *Profile of SP eluted from a DEAE-cellulose column. The bound protein was eluted phosphate buffer containing a continuous (0–1.0 M) NaCl gradient. The major peak 'as used for amino acid analysis and molecular weight determination.*

l and concentrated 2–3 times. The resulting material was called the DP ɔn. The protein and carbohydrate content of samples from each step of ırification procedure was determined using the Lowry method as modified ⁻rtree [13] and the phenol-sulfuric method [14], respectively.

yacrylamide gel electrophoresis (PAGE). Sodium dodecyl sulfate (SDS)- was carried out in 7.5 o/o (w/v) cylindrical polyacrylamide gels (70 x 6 –vith 1 o/o SDS according to Payne [15]. For the molecular weight deter- ons, globular proteins were used as standards. The protein bands were =l with 0.06 o/o (w/v) Coomassie blue in 10 o/o (v/v) acetic acid, and de- -l in 10 o/o acetic acid. The relative amount of protein in each band was –ıined by densitometry of the gels.

ɔlectric-focusing. Gel isoelectric-focusing was carried out in 5 o/o (w/v) –rical polyacrylamide gels (70 x 6 mm) containing 2 o/o (w/v) carrier Bio- mpholytes (Bio-Rad) with a pH range of 3–10 [16]. The pH gradient was ıined at the end of the run by cutting 5-mm discs along the gels; each disc

was suspended in 1 ml of distilled water for 2 h and the pH was measured v microelectrode. The gels were stained as described above.

Amino acid analysis. A sample of the major peak obtained from DEAE- lose chromatography (Fig. 1, DP fraction) was hydrolyzed in duplicate ir HCl at 105 °C for 24 h. The amino acids were resolved in a Beckman S model 120C analyzer. Tryptophan and cysteine were not determined, and onine and serine values were not corrected.

Hyperimmune sera. After PAGE of the Sephadex G-200 peak (SP), tl was frozen. The sections corresponding to the upper and lower bands (U LB, respectively), which appeared as dense white discs, were carefully cu minced, diluted with 1 ml of PBS, and mixed with adjuvant. New Zealand rabbits were immunized subcutaneously according to the following schedul initial sensitizing dose in complete Freund's adjuvant was administered at (followed by three booster doses in incomplete Freund's adjuvant at days 1 and 45. One rabbit was injected each time with 330 μg of the proteins p in SP, while two others received doses of approximately 120 μg protein- either the UB or LB. All animals were bled 7 days after each booster ar sera (anti-SP, anti-UB, and anti-LB) were separated by centrifugatio stored at –20 °C until use.

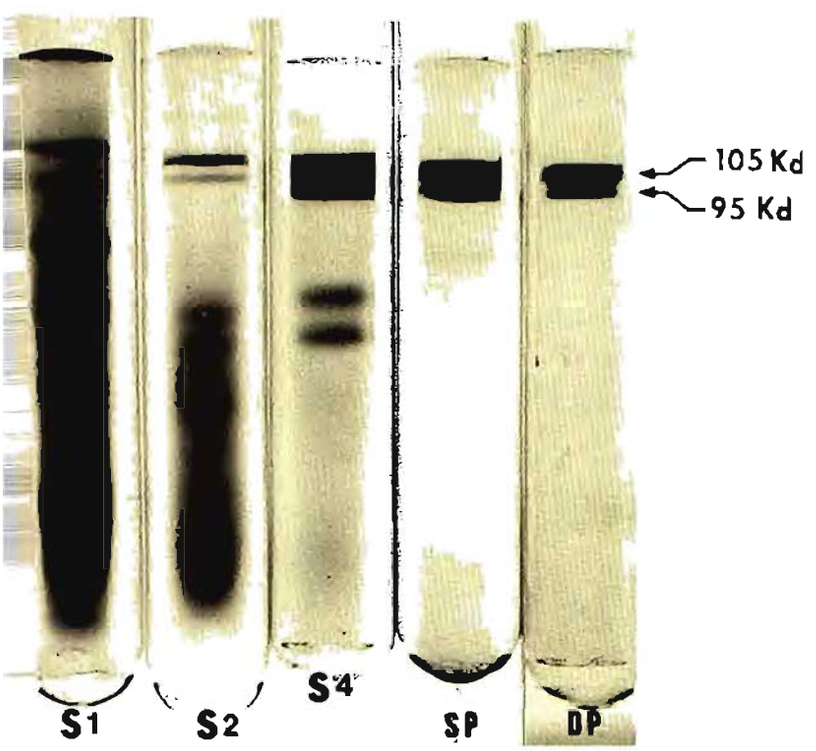
Human sera. Sera from patients with confirmed brain cysticercosis we tained from various hospitals in Mexico City and kept at –20 °C until use.

Double immunodiffusion and immunoelectrophoresis. Double immun sion (DID) was carried out in 1 % (w/v) agarose in 0.05 M barbital buff 8.6, according to the method of Ouchterlony [17]. For immunoelectroph (IEP) [17], microscope slides were layered with 1 % agarose in barbital and 25 μl of the crude extract (CE) or SP were applied to wells cut in t After electrophoresis at 1.5–2.0 mA per slide for 90 min, the sera were ad the troughs and allowed to diffuse in a humid chamber for 48 h. The slide then washed thoroughly with 0.5 M NaCl and dried. Finally, 0.1 % (w/v) black in 10 % acetic acid was employed for staining the gels, and 10 % acid for destaining. The crude extract (CE) and purified fraction (SP) wei at protein concentrations of 15 and 4 mg/ml, respectively.

Electron microscopy. One drop (50 μl) of SP (0.22 mg protein/ml) v plied to a double layered (Formvar-carbon) grid, followed by a second c 60 μM ATP in 0.25 M acetic acid (final concentration, 30 μM ATP) and 15–30 min at room temperature. After exhaustively washing the grid uranyl acetate was added to the sample and left for 2–3 min. The grid wa- washed with distilled water, dried, and examined in a JEOL 100B electi- croscope. Control samples were treated similarly except that no ATP was

III. RESULTS

Figure 4 illustrates the band patterns obtained in SDS-PAGE of fractions m each step of the purification procedure. The material extracted in the first ernatant (S1) obtained after homogenization of the cysticerci was very het- geneous, as evidenced by the number of protein bands found in SDS-PAGE. -lysis against 0.5 M acetic acid eliminated several of the medium molecular ght proteins from the S2 fraction. The low molecular weight proteins were ctively excluded from the S4 supernatant obtained by the salting-out of S2 dialysis of P3, producing a sizeable enrichment in two high molecular weight -teins and a less marked enrichment in two proteins of medium molecular ght. One major peak (SP) obtained following Sephadex G-200 chromatogra- , which eluted in the void volume of the column, included the two proteins



. *SDS-PAGE of the fractions obtained during purification of* T. solium *cysticerci (see 1). The arrows indicate the position of the upper (105 kd) and lower (95 kd) bands id LB, respectively). By densitometry of the gels, the combined UB and LB proteins he SP and DP fractions were determined to represent 85 o/o and 95 o/o of the total i, respectively.*

TABLE I. Protein Recovered During Purification of Antigen B

Fraction	*mg Protein/10 g Dry Tissue Weight*[a]
S1	*1100*
S2	*630*
S4	*30*
SP	*21*
DP	*7.2*

[a] *The dry weight of tissues from cysticerci was determined by desiccation of the l to a constant weight. Ten grams of dry tissue were obtained from approximately 37 fresh larvae.*

of high molecular weight as shown in the SDS-PAGE of this fraction. These protein bands comprised 85 % of the proteins in the SP fraction, as determ by densitometry of the gel. The amount of protein recovered in each step o purification procedure is shown in Table I.

Both the CE and SP, when reacted in DID against anti-SP serum, gave precipitation arc (Fig. 5), indicating that no antibodies against other ant were formed when the rabbit was immunized using the SP fraction. In IEP, antigens gave one isoelectric band when reacted against either anti-SP (Fig or anti-CE (Fig. 6b) serum. This result indicated that none of the antigen

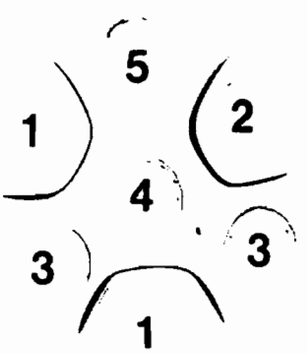


FIG. 5. Double immunodiffusion patterns of SP (1) and the crude extract (CE, 2) i reaction against anti-SP (3), anti-upper band (UB, 4) and anti-lower band (LB, 5) ser anti-SP serum recognized only one protein in both antigens, indicating its monospec The precipitation arc formed between SP and both the anti-UB and anti-LB sera s complete identity.

ON THE MECHANISM OF ACTION OF MEBENDAZOLE

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Mebendazole, a synthethic drug used as a potent and broad spectrum anthelmintic has been studied. Two approaches will be presented here, which can be considered as primary steps to obtain an adequate understanding of its mechanism of action.

The first approach is the characterization of the interaction of Mebendazole with partially purified tubulin, the monomeric subunit of microtubules. By turbidimetric measurements it was found an inhibitory effect of Mebendazole on the in vitro polymerization of bovine brain tubulin. Concentrations of 15 M produced a 50% of inhibition. Additionally, by equilibrium dialysis assays, it was determined that Mebendazole binds tubulin dimer at only one site, apparently the same as for colchicine.

The second approach is the study of Mebendazole on in vitro cultured cysticercus. These isolated cysts were used in order to dissect the direct effect of Mebendazole on the larvae from those produced indirectly through the host. The changes observed are: disappearance of microtubules and blocking of vesicular movements in subcuticular cells in the initial hours of treatment and a progressive degenerative damage in the whole structure during more prolongued incubations. An additional encountered effect is the occurrence, after 20 hours of treatment, of paracrystalline structures in the cytoplasm of the subcuticular cells. These structures are formed by tubular units with an external diameter of about 500, being arranged according to an hexagonal plan. This work provides a rational basis to explore the possibility of a chemical treatment in some cases of human cerebral cysticerciasis.

CLONAZEPAM AND PRAZIQUANTEL: MODE OF ANTISCHISTOSOMAL ACTION

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Clonazepam (CZ) and praziquantel (PZ) are potent antischistosomals. When male schistosomes (*S. mansoni*) are placed in a medium containing 10^{-6}M PZ or 10^{-5}M.CZ one observes a massive contraction of the parasite's musculature. We were unable to block this response with high concentrations of drugs (e.g., carbachol or pentobarbital) that relax parasite's musculature. Investigations have demonstrated that the membrane potential of muscle cells of a male schistosome is directly correlated to the tension developed by the parasite's musculature, i.e., depolarization produces an increase in tension, hyperpolari-

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With 3 plates

Paracrystalline bundles of large tubules, induced *in vitro* by Mebendazole in *Cysticercus cellulosae*

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SUMMARY

The effect of the anthelmintic Mebendazole on *Cysticercus cellulosae* maintained in culture medium was studied by transmission electron microscopy. In addition to the well-known morphological changes induced by Mebendazole in other cestode and nematode larvae, it also induced the cytoplasmic appearance of paracrystalline bundles in the secretory cells of the bladder wall. These bundles were formed by groups of large parallel tubules arranged in a hexagonal-like pattern. The tubules, which had an external diameter of about 50 nm and a length that might exceed 5 μm, were surrounded by a matrix and a distance between neighbouring tubules of 80–120 nm centre to centre was estimated. The tubules were stable to colchicine and low temperature. The temporary appearance of bundles is described and some alternative explanations on their origin are advanced.

INTRODUCTION

Mebendazole (methyl-5-benzoyl, benzimidazole-2-carbamate) is a synthetic drug used in the treatment of some helminthiasis (Van den Bossche, 1972). It has been proposed that Mebendazole exerts its action by interfering with the microtubular function of the parasite (Borgers, De Nollin, De Brabander & Thienpont, 1975; Borgers, De Nollin, Verheyen, De Brabander & Thienpont, 1975). Recently, it has been demonstrated that Mebendazole binds to purified bovine tubulin, the monomeric sub-unit of microtubules, inhibiting its polymerization *in vitro* (Friedman & Platzer, 1978; Ireland, Gull, Gutteridge & Pogson, 1979; Laclette, Guerra & Zetina, 1980). Because microtubules are involved in the movement of other cell organelles, an intracellular depolymerization of microtubules can explain the observed accumulation of secretory vesicles and the degenerative changes leading to necrosis of the parasite.

In addition to the already described effects, we report here the appearance of large tubules, organized as paracrystalline bundles in secretory cells of the bladder wall of *Cysticercus cellulosae* exposed to Mebendazole in culture.

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MATERIALS AND METHODS

Cysts were obtained by dissection from skeletal muscle of highly infected pigs. Mebendazole (99% pure) was kindly provided by Dr H. Van den Bossche from Janssen Pharmaceutica, Belgium. The culture medium RPMI 1640 and the foetal calf serum were from Difco. Colchicine was obtained from Merck. All other reagents were of analytical grade.

Maintenance of the larvae

After dissection the larvae were washed twice in RPMI medium (pH 7·3), containing 1% (w/v) foetal calf serum, 50 units/ml penicillin G, 50 μg/ml streptomycin sulphate and 30 μg/ml gentamycin. It will be referred to as culture medium. Groups of 2 larvae/tube were maintained in 2 ml of culture medium at 37 °C, under an atmosphere of 95% air, 5% CO_2 and a humidity at saturation. The first experimental group was incubated for varying periods from 2 to 156 h in culture medium containing Mebendazole at saturation concentration (0·01 % w/v). To solubilize Mebendazole, a saturating amount was added to the culture medium, energetically stirred for 2 h at room temperature and filtered through Millipore filters to remove the undissolved powder. The second group was first incubated for 36 h under the conditions described above. The larvae were then transferred to culture medium containing 0·5 or 1 mM colchicine and incubated for an additional 2, 4, 8 or 12 h. A third group of larvae was incubated for varying periods from 2 to 156 h in culture medium containing 0·5 or 1 mM colchicine. In the control group, the same conditions of incubation were maintained except that no Mebendazole or colchicine was added.

Electron microscopy

At the end of each incubation the bladder walls of the larvae were separated from the scolices. The bladder walls were fixed using 2·5% (v/v) glutaraldehyde in 0·1 M cacodylate buffer, pH 7·3, for 1 h at room temperature and then post-fixed in 1% (w/v) osmium tetroxide for 1 h and processed for electron microscopy. To study the effect of low temperature fixation on the pre-formed tubule bundles, some larvae from the first group, incubated for 36 h, were processed in the same conditions except that fixation was carried out at 4 °C. The tissue embedded in Epon 812 was sectioned on an LKB 8800 Ultratome. The sections were double stained with 5% (w/v) uranyl acetate for 15 min and 2·5% (w/v) lead citrate for 5 min. They were then examined in a JEOL 100B electron microscope. Calibration of the microscope for measurements was made using a carbon grating replica from Polaron.

RESULTS

Mebendazole induced several successive changes in the normal morphology of the bladder wall of *C. cellulosae*. The labile microtubules disappeared, producing the accumulation of secretory vesicles in the tegumental cells. The microvilli on the tegumental surface were lost. Generalized degeneration of the whole tegumental

structure occurred. An additional observation of the cytoplasm of tegumental cells, revealed the presence of large tubules grouped in bundles with a paracrystalline organization. A very similar pattern of damage was observed in colchicine-treated larvae except that no paracrystalline formations were found. In contrast, control larvae incubated without colchicine or Mebendazole, retained the normal morphology of the tegument, and microtubules measuring 25 nm in diameter could be observed. Tegumental cells showed no accumulation of vesicles or paracrystalline bundles (Pl. 1 A). Only after incubation for 72 h in culture medium were minimal morphological changes observed.

Temporary appearance of bundles

These bundles began to appear after incubating the larvae for 16 h in the presence of Mebendazole (Pl. 1 B). At this time, they were present in only a few cells and consisted of a large number of tubules. The portion of cytoplasm occupied by the bundles was relatively small. With prolonged incubation time, the number of bundles/cell increased as well as the number of altered tegumental cells. A typical tegumental cell after incubation for 36 h is illustrated in Pl. 2 A. A considerable portion of the cellular volume is occupied by several bundles and the normal cytoplasm is confined to areas along the plasma membrane. At this time, paracrystals were present in practically all cells (not shown). As the incubation period was extended, the bundles gradually became disordered without a decrease in the number of individual tubules. After incubation for 62 h the tubules were dispersed throughout the cytoplasm so that it was difficult to define organized bundles. After prolonged incubations, (between 62 and 108 h) the number of tubules gradually decreased until they could no longer be found.

Description of the paracrystalline bundles

As described above, the longitudinal arrangement of the tubules in bundles disappeared after incubation with Mebendazole during 62 h. Therefore, the description of the bundles is restricted to those found after incubation for between 16 and 62 h.

The tubules were parallel to the long axis of the bundle. Each tubule was surrounded by 6 more tubules in a hexagonal-like pattern (Pl. 2 B). The distance between neighbouring tubules was 100 ± 20 nm, centre to centre. The material surrounding the tubules was qualitatively different from the cytoplasm (Pls 1 B and 2 A), and at high magnification had a fibrilar appearance (Pl. 2 B). Because no connections were observed between tubules, this material could play a matrix-like function in positioning the tubules within a bundle. This idea was further supported by observation of regions where a loss of cytoplasm occurred in the vicinity of a bundle while the matrix remained adhered to the tubules (Pl. 3 A).

Description of the tubules

The tubules had an external diameter of 50 ± 5 nm. The thickness of the wall was 5 ± 1 nm and the internal diameter 40 ± 5 nm (Pl. 2 B). Pl. 3 B shows that the

length of the tubules could exceed 4 μm and that their ends appeared open. The tubules were stable to colchicine concentrations of 1 mM as observed in the second experimental group. In addition, they were resistant to fixation at low temperature (see Materials and Methods section).

DISCUSSION

The successive morphological changes produced by Mebendazole in *C. cellulosae* maintained in culture medium are in agreement with *in vivo* studies by Borgers & De Nollin (1975) on *Ascaris suum* intestine; by Borgers, De Nollin, De Brabander & Thienpont (1975) and Borgers, De Nollin, Verheyen, De Brabander & Thienpont (1975) on *Syngamus trachea* intestine; and by Borgers, De Nollin, Verheyen, Vanparijs & Thienpont (1975) on *Taenia taeniaeformis* cysticerci. It seems to be clear that the differences in experimental conditions, namely *in vitro* versus *in vivo* treatments did not significantly influence the degenerative changes exerted by Mebendazole.

The appearance of the bundles of tubules in secretory cells of the parasite, raises the question of their origin. Any explanation should take into account the mechanism of action of Mebendazole as an inhibitor of tubulin assembly.

Oaks & Lumsden (1971) have proposed that some materials produced in tegumental cells are enclosed in vesicles and transported to the outer surface. As Mebendazole produces an impairment in vesicular transport probably caused by *in vivo* depolymerization of microtubules, it is conceivable that proteins normally exported to the outer surface could be accumulated in the cytoplasm of tegumental cells. Under these conditions, a highly concentrated protein could aggregate in the form of the tubules here described. Catalase is an example of a protein that crystallizes in the form of tubules when subjected to abnormal conditions (Kiselev, Shpitzberg & Vainshtein, 1967).

An alternative and more attractive explanation arises from the mechanism of action of Mebendazole. If depolymerization of microtubules is occurring, the soluble tubulin under the action of Mebendazole could then be reassembled in an anomalous form, such as the paracrystalline bundles described here.

Schochet, Lambert & Earle (1968) and Bensch & Malawista (1968, 1969) have reported that vinblastine and vincristine, two inhibitors of tubulin assembly, induced the formation of microtubular crystals in mammalian cells. These crystals are formed by stacks of microtubules having an external diameter of 27–28 nm and the wall of a microtubule is shared with 6 surrounding units. Colchicine does not prevent the formation of the crystals and these are stable to low temperature fixation. In spite of the morphological differences between tubulin crystals in mammalian cells and the paracrystals in *C. cellulosae* cells, it is possible that both structures are the result of similar processes.

Colchicine also produced an accumulation of vesicles in the secretory cells of *C. cellulosae*, as observed in the third group of larvae. However, colchicine did not induce the appearance of bundles of tubules. This evidence suggests that the second explanation is more likely. However, direct evidence demonstrating the presence of tubulin in these paracrystals is needed to further support this possibility.

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REFERENCES

BENSCH, K. G. & MALAWISTA, S. E. (1968). Microtubule crystals: a new biophysical phenomenon, induced by Vinca alkaloids. *Nature, London* **218**, 1176.

BENSCH, K. G. & MALAWISTA, S. E. (1969). Microtubular crystals in mammalian cells. *Journal of Cell Biology* **40**, 95–107.

BORGERS, M. & DE NOLLIN, S. (1975). Ultrastructural changes in *Ascaris suum* intestine after mebendazole treatment *in vivo*. *Journal of Parasitology* **61**, 110–22.

BORGERS, M., DE NOLLIN, S., DE BRABANDER, M. & THIENPONT, D. (1975). Influence of the anthelmintic mebendazole on microtubules and intracellular organelle movement in nematode intestinal cells. *American Journal of Veterinary Research* **35**, 1153–66.

BORGERS, M., DE NOLLIN, S., VERHEYEN, A., DE BRABANDER, M. & THIENPONT, D. (1975). Effects of new anthelmintics on the microtubular system of parasites. In *Microtubules and Microtubule Inhibitors* (ed. M. Borgers and M. De Brabander), pp. 497–508. Amsterdam: North Holland Publishing Company.

BORGERS, M., DE NOLLIN, S., VERHEYEN, A., VANPARIJS, O. & THIENPONT, D. (1975). Morphological changes in cysticerci of *Taenia taeniaeformis* after mebendazole treatment. *Journal of Parasitology* **61**, 830–43.

FRIEDMAN, P. A. & PLATZER, E. G. (1978). Interaction of anthelmintic benzimidazoles and benzimidazole derivatives with bovine brain tubulin. *Biochimica et biophysica acta* **544**, 605–14.

IRELAND, C. M., GULL, K., GUTTERIDGE, W. E. & POGSON, C. I. (1979). The inhibition of mammalian brain microtubule assembly *in vitro* by benzimidazole carbamates. *Parasitology* **79**, xix.

KISELEV, N. A., SHPITZBERG, C. L. & VAINSHTEIN, B. K. (1967). Crystallization of catalase in the form of tubes with monomolecular walls. *Journal of Molecular Biology* **25**, 433–41.

LACLETTE, J. P., GUERRA, G. & ZETINA, C. (1980). Inhibition of tubulin polymerization by mebendazole. *Biochemical and Biophysical Research Communications* **92**, 417–23.

OAKS, J. A. & LUMSDEN, R. D. (1971). Cytological studies on the absorptive surface of cestodes. V. Incorporation of carbohydrate containing macromolecules into tegument membranes. *Journal of Parasitology* **57**, 1256–68.

SCHOCHET, S. S., LAMBERT, P. W. & EARLE, K. M. (1968). Neuronal changes induced by intrathecal vincristine sulphate. *Journal of Neuropathology and Experimental Neurology* **27**, 645–58.

VAN DEN BOSSCHE, H. (1972). Biochemical effects of the anthelminthic drug mebendazole. In *Comparative Biochemistry of Parasites* (ed. H. Van den Bossche), pp. 139–157. New York: Academic Press.

EXPLANATION OF PLATES

PLATE 1

Electron micrographs of sections from the bladder wall of *Cysticercus cellulosae*.

A. Control larva incubated for 48 h in culture medium without Mebendazole. No morphological alterations or paracrystalline bundles were observed. Microtubules measuring 25 nm in diameter were abundant in the neighbouring subtegumental space (insert, 50000 ×). *c*, Normal cytoplasm; *n*, nucleolus.

B. Tegumental cells of a larva incubated for 16 h in culture medium containing Mebendazole. Bundles of large tubules (arrow) are found in the cytoplasm of a few cells. *g*, Glycogen granules; *n*, nucleolus; *sv*, secretory vesicles; *tc*, tegumental cell.

PLATE 2

A. Tegumental cells of a larva incubated for 32 h in culture medium containing Mebendazole. The bundles of large tubules (arrow) and the secretory vesicles (*sv*) occupy significant areas of the cell and the normal cytoplasm is displaced to restricted regions along the plasma membrane. *g*, Glycogen granules; *m*, muscle cell.

B. Cross-section of a tubule bundle (arrow) in a tegumental cell after incubation for 32 h in Mebendazole. The large tubules are arranged in a hexagonal-like pattern. The distance between neighbouring tubules is 80–120 nm, centre to centre. The external diameter of the tubules is 50 ± 5 nm. *ma*, Fibrilar matrix; *sv*, secretory vesicles.

PLATE 3

A. Bundles of large tubules in the cytoplasm of tegumental cells after incubation for 36 h in Mebendazole. In areas where a loss of cytoplasm occurred in the vicinity of a bundle (arrows) the fibrilar matrix remained adhered. *g*. Glycogen granules; *ma*, fibrilar matrix; *sv*, secretory vesicles.

B. Longitudinal section of large tubules showing the maximal length observed (5 μm). Ends appear to be open. *g*, Glycogen granules.

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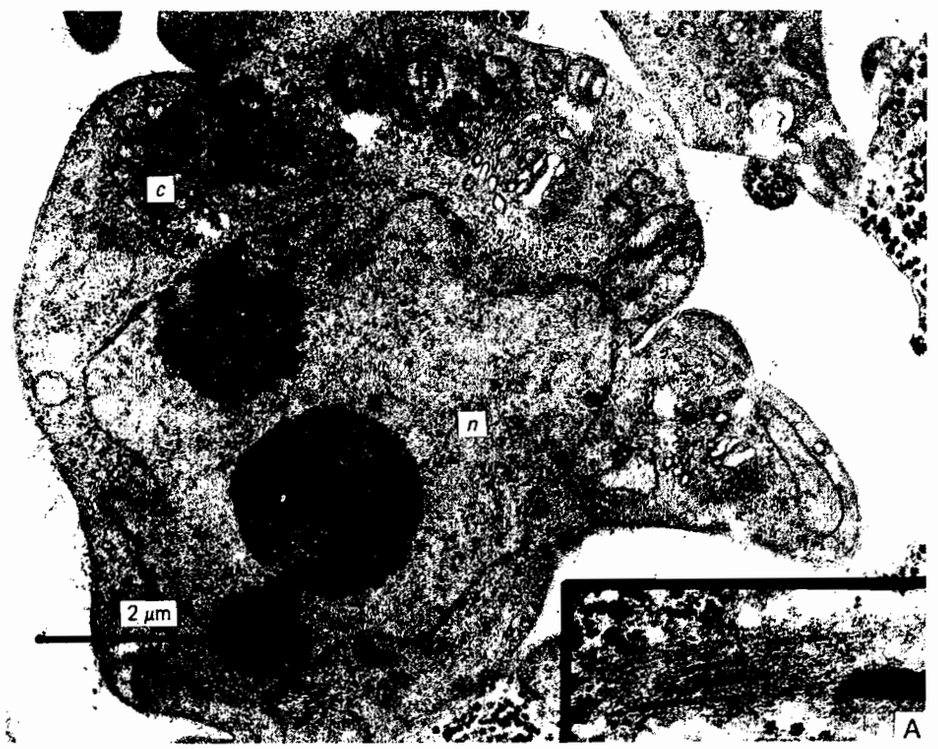

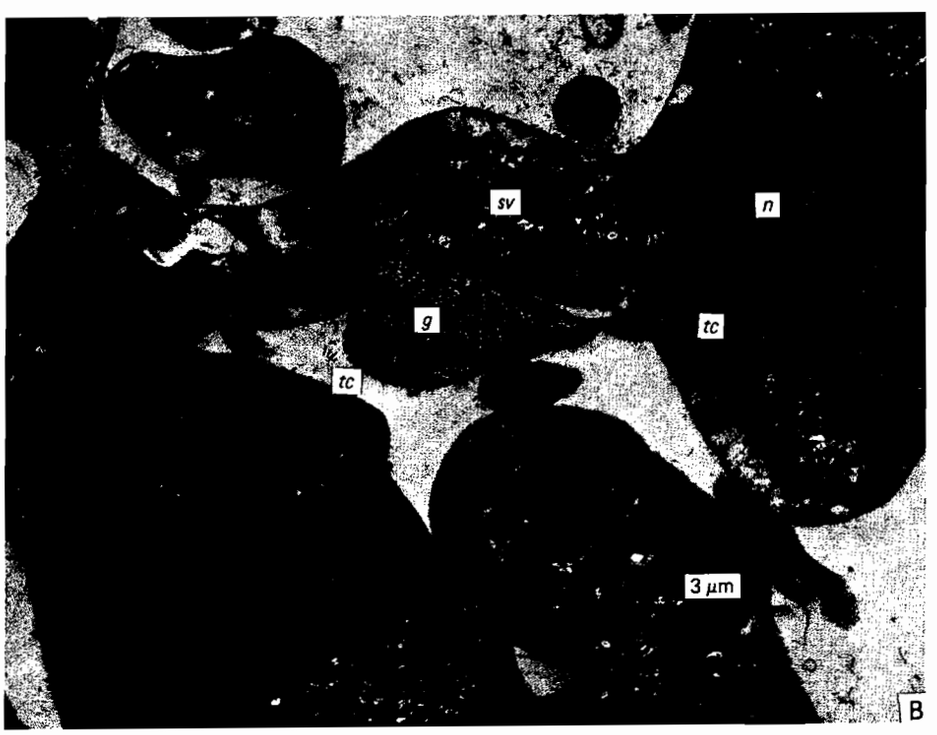

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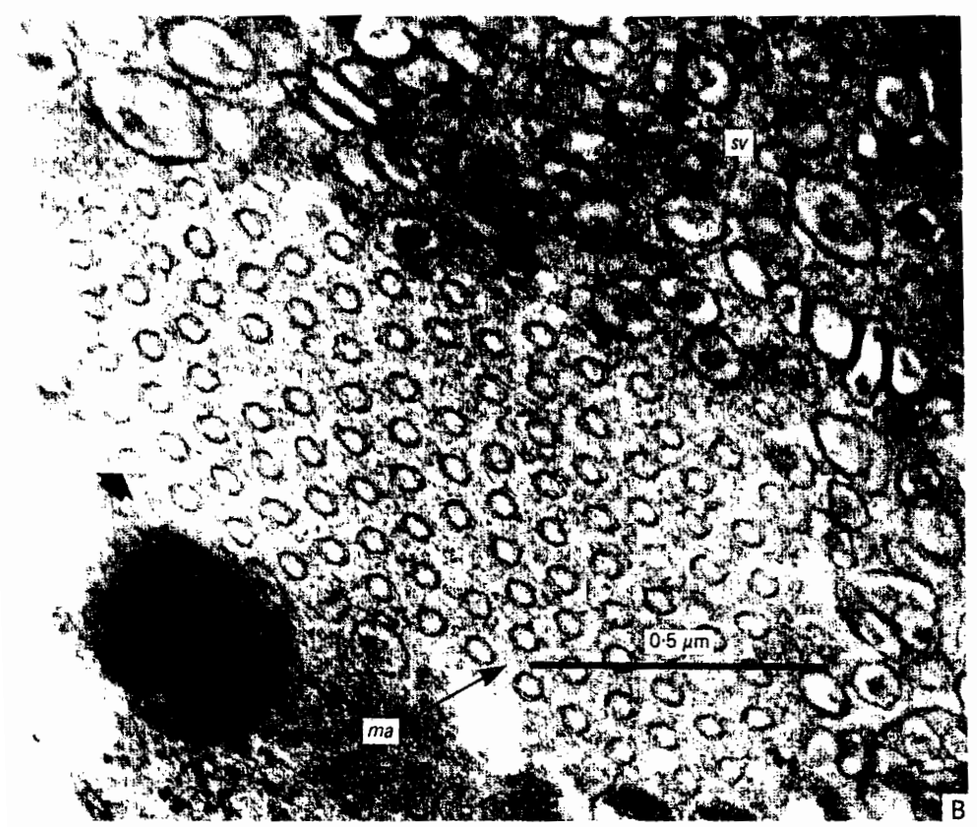
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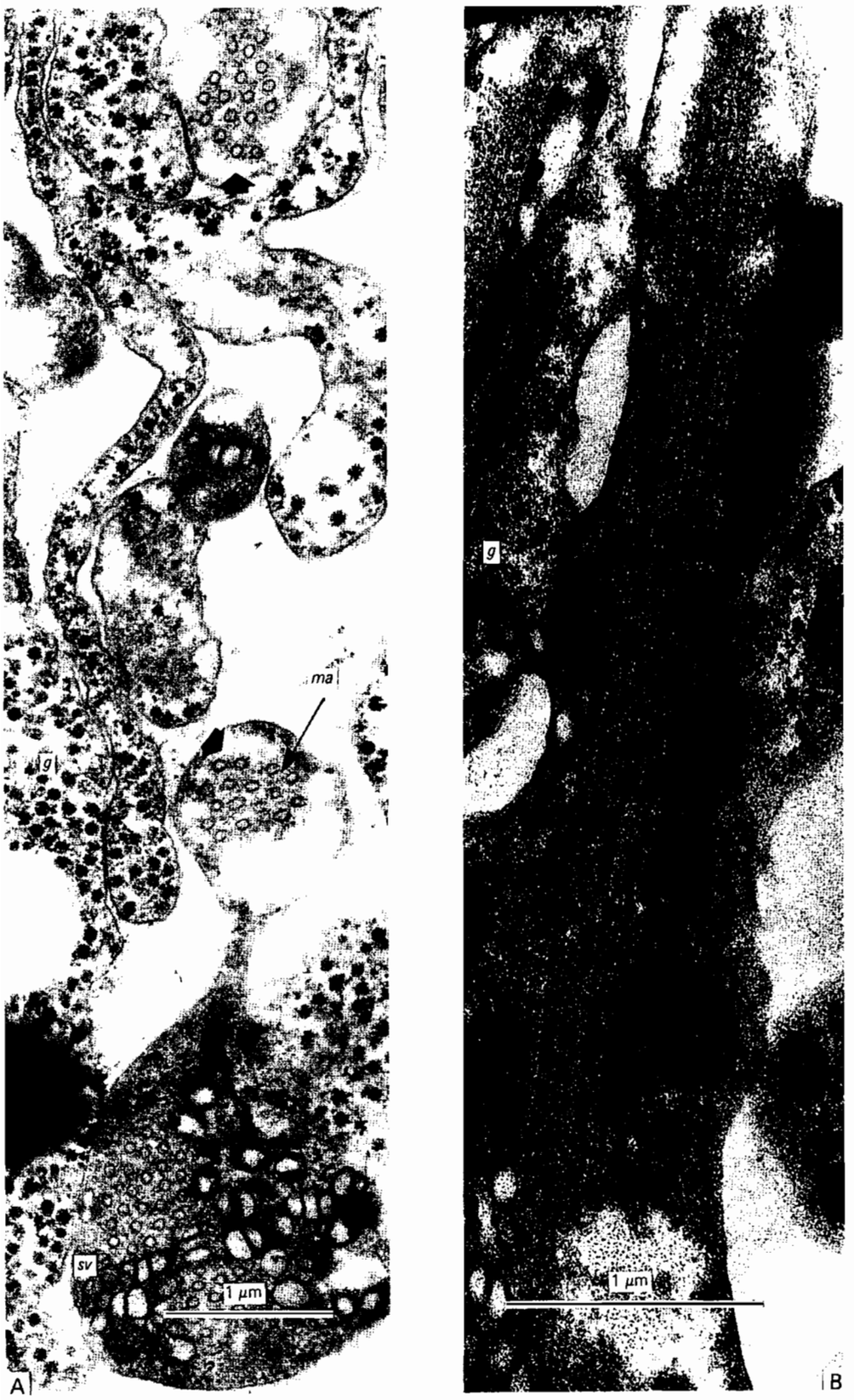
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