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Hydrophobic Attachment of *Trypanosoma cruzi* to the Rectal Cuticle of *Triatoma infestans* and its Influence on Metacyclogenesis - A Review

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Trypanosoma cruzi **colonizes mainly the rectum of the vector, especially the rectal glands. We investigated the basic architecture of the rectal cuticle of *Triatoma infestans* and the mode of attachment of *T. cruzi* in the small intestine and the rectum. In addition, we determined the capacity of culture-derived epimastigotes to attach to artificial substrates and the influence of attachment on metacyclogenesis. After incubation of the rectum with wheat germ lectin (WGA) coupled to gold particles, the procuticle contained chitin, but the two layers of the epicuticle and the superficial layer bordering the rectal lumen did not. The specific fluorochrom Nile Red stained the entire rectal cuticle green, indicating the waxy composition of the superficial layer. In electron microscopic analysis the parasites were attached to the hydrophobic superficial wax layer but not to the epithelium of the midgut. In vitro culture-derived epimastigotes attached with a high affinity to all hydrophobic substrates tested, whereas hydrophilic substrates did not permit attachment. Emulsified hexadecane localized the attachment molecules to the terminal part of the flagellum. Inhibition of attachment by coating the culture tubes with hydrophilic agarose and constant agitation decreased the rate of epimastigote to trypomastigote transformation, whereas wax coating enhanced metacyclogenesis.**

Key Words : rectal cuticle, superficial layer, chitin

INTRODUCTION

Trypanosoma cruzi, the causative agent of Chagas disease, develops mainly as multiplying epimastigote and vertebrate-infectious, metacyclic trypomastigote in the digestive tract of its reduviid bug vectors. The epimastigote stage colonizes the rectal wall, where the parasites attach to the cuticular lining via their flagella [18, 21]. Only non-attached epimastigotes and the mainly free-swimming trypomastigotes are excreted with the urine flow after a blood meal [20, 31]. The attachment of epimastigotes is not only a mechanism to prevent the excretion of this non-infectious stage, but it is also thought to be a necessary prerequisite for the transformation to metacyclic trypomastigotes [3]. Also, in other trypanosomes, i.e. *T. brucei* and *T. congolense*, attachment is necessary for the transformation to the vertebrate-infectious stages [10, 25]. In previous studies, chitin was investigated as the putative attachment substrate [4, 27], since several *Trypanosoma* and *Leishmania* species attach to the fore- and hindgut of their vectors, which are covered by cuticle [6, 15, 24, 26]. However, in the outer integument of insects, chitin is usually absent at the surface of the cuticle, and should therefore not be accessible for the attachment of trypanosomes to the luminal surface of the cuticular linings of the digestive tract. To clarify this and the conditions which are necessary for attachment we investigated the architecture of the rectal cuticle and the mode of attachment of *T. cruzi* epimastigotes in different parts of the digestive tract and to artificial substrates in vitro using electron and light microscopy [12, 13, 23].

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MATERIAL AND METHODS

Triatoma infestans strain "Chile" and the *T. cruzi* strain "Chile 5" originated from the same village and were maintained under standardized conditions [12, 19].

For electron microscopy recta and the small intestine of fifth instar larvae of *T. infestans* infected with *T. cruzi* were prepared following a conventional method using 2% glutaraldehyde in sodium cacodylate (pH 7.3) for fixation, 1% cacodylate-buffered osmium tetroxide for postfixing the specimens and ethanol or acetone for dehydration of tissues [13, 23]. In addition, recta of uninfected and infected bugs were prepared according to two modified fixation protocols which preserve the lipidic components of the cuticle well: 1. The samples were immersed with 3.3% glutaraldehyde and 0.1% ruthenium red in PIPES (105 mM 1,4-piperazinediethane-sulfonic acid buffer, pH 7.35) at 4℃, and then postfixed for 3h at 4℃ with 1% osmium tetroxide and 0.1% ruthenium red in PIPES. 2. After a first fixation with 2% glutaraldehyde in PIPES the samples were postfixed with 0.5% osmium tetroxide and 0.8% ferrocyanide in PIPES [22]. Samples were dehydrated in a graded series of dimethylformamide to avoid solution of lipidic components during dehydration with ethanol [23].

Samples were then embedded in Araldite or for the localization of chitin in LRGold after fixation in 2% glutaraldehyde. Chitin detection was performed in sections or in complete recta for the control using wheatgerm lectin (WGA) adsorbed to gold particles 12-nm in diameter. In controls N', N'', N'''-triacetylchitotriose, a competitive inhibitor for WGA-binding, was added to the lectin-gold solution prior to the incubation of the samples. For light microscopy the WGA-gold staining of chitin in sections of uninfected recta was enhanced with silver [23].

Lipidic components of the cuticle were detected with the fluorochrome Nile Red, which fluoresces green in contact with lipids [23].

To clarify the conditions which are necessary for the attachment of *T. cruzi* epimastigotes several artificial substrates and solutes (listed in results) were tested to permit or to inhibit the attachment of both epimastigotes in vitro and bug-derived flagellates. Therefore, glass microscope slides were coated with different lucent substrates. Opaque material was tested as small pieces applied on slides. Solutes with different chemical properties were tested in a concentration of 100 mM, or as stated in the results, for their capacity to inhibit the attachment of epimastigotes to silanized glass slides [12].

For the digestion of surface proteins possibly involved in attachment, epimastigotes were incubated for 10 min. in a solution of 0.25% trypsin (200 FIP-U/g; Merk, Darmstadt, Germany) in HEPES-saline (7 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 185 mM NaCl, 5 mM KCl and 5 mM glucose, pH 7.2, having an osmolarity of ca. 400 mosmol/kg).

Hydrophobic domains at the surface of the parasites were detected by incubation of epimastigotes in 0.1% hexadecane emulsified in HEPES-saline [12].

The influence of epimastigote attachment on the transformation to metacyclic trypomastigotes in the TAU3AAG medium [9] was investigated using either agarose-coated, uncoated or wax-coated polypropylene culture tubes, with or without constant agitation during cultivation [12].

RESULTS

In uninfected insects the extracellular membrane layers of the small intestine contained no chitin. In the rectum chitin was detectable by light microscopy using silver enhanced WGA-gold staining throughout the entire cuticle of sections of the rectum, but was absent or only present in low amounts at the rectal pads. Incubation of recta with WGA-gold before embedding showed no staining by light or electron microscopy; after incubation of sections for electron microscopy chitin was only detectable in the procuticle but was absent in the two epicuticular layers and the superficial layer. Inhibition of WGA-binding by adding N', N'', N'''-triacetylchitotriose indicates specific binding of this lectin to chitin.

The surface of the rectum was water repellent indicating hydrophobic properties of the outermost layers of the cuticle. Incubating the rectum with Nile Red resulted in a lipid specific green staining of the entire surface of the rectum including the

rectal pads, whereas the underlying tissue fluoresced red. Extraction of the lipids with hexane prior to the incubation with Nile Red abolished the specific green staining.

The outermost superficial layer of the rectal cuticle was retained as an electron dense layer (Fig. 1) by following the two modified fixation protocols to prepare the recta for electron microscopy. The superficial layer was sensitive to extraction with organic solvents indicating that it was composed of waxy material. This material was present as several lamina, whose thickness varied between 25 up to 85 nm. The superficial layer covered three more layers varying in electron density and thickness. At the main rectal sac the electron-lucent outer epicuticle (15 nm) was followed by the electron-dense inner epicuticle (30 nm) and the procuticle (150-190 nm) containing fibrilar structures. At the rectal pads the procuticle was only found as a thin layer whereas the epicuticle and the superficial layer did not differ in structure and thickness.

In the infected small intestine, the parasites accumulate at the border of the gut contents. Rarely an unmodified flagellum was inserted between the microvilli. Therefore, no firm attachment of *T. cruzi* to the wall of the small intestine seems to occur.

In infected recta the parasites attached to the superficial layer of the rectal cuticle with their flagella and formed dense carpets especially at the rectal pads. Using conventional fixation and rehydration methods a lower number of attached flagellates was observed and the superficial layer was only partially retained where it was protected against solvents by attached flagella (Fig. 1).

When artificial hydrophobic and hydrophilic substrates were tested for their ability to permit the attachment of culture-derived epimastigotes more than 90% of these flagellates attached to all hydrophobic substrates (Table 1), i.e. the coatings with paraffin wax, octacosane, malinol and dimethylsilane and the plastics polypropylene, polyethylene, polystyrene and polytetrafluorethylene (Teflon). Culture-derived epimastigotes bound mainly with a specialized area at the terminus of the flagellum in a similar fashion to that seen in vivo to the

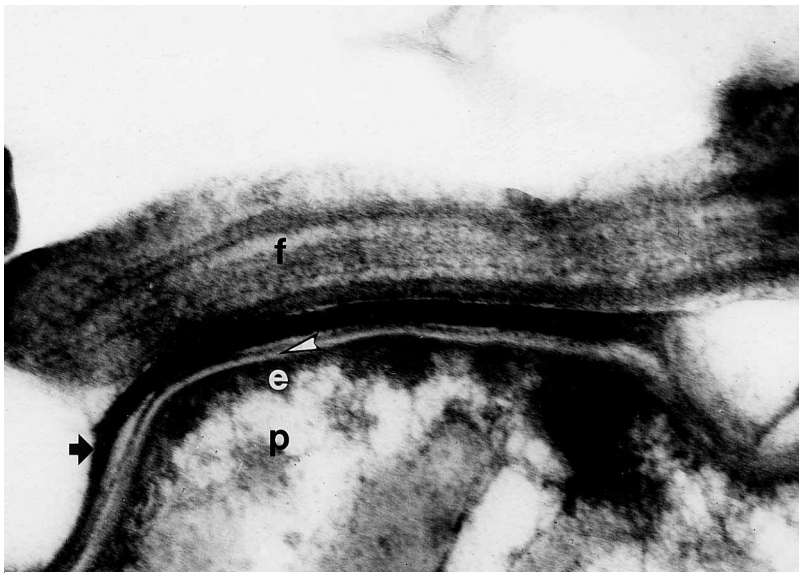

Fig. 1 Flagellum terminus of *Trypanosoma cruzi* epimastigote attached to the rectal cuticle of *Triatoma infestans*.
Note that the flagellum (f) is in a close contact to the electron dense superficial wax layer (arrow). This layer is only completely retained at the attachment site beneath the flagellum. Elsewhere the waxes are partially detached from the cuticle. Also visible are the layers of the outer epicuticle (arrowhead), the inner epicuticle (e) and the procuticle (p). × 100.000.

Table 1 Attachment of *Trypanosoma cruzi* epimastigotes to different hydrophobic and hydrophilic substrates.

Lucent substrates[a]	rate of attachment	opaque polymeric substrates [b]	score of attachment
hydrophobic substrates:		*hydrophobic polymers:*	
paraffin wax	99.3 ± 1.2	polypropylene	++
octacosane	95.2 ± 0.8	polyethylene	++
dimethylsilane	93.5 ± 3.5	polystyrene	++
malinol	95.1 ± 6.3	polytetrafluorethylene	++
amphiphilic substrates:		*hydrophobic polymers:*	
β-lactoglobulin	99.3 ± 1.2	polyacetate film	—
bovine-serum albumin	51.5 ± 14.1	Cellophan^{R} film	—
hydrophilic substrates:		*positively charged substrates:*	
agarose	3.3 ± 2.5	DEAE-Sephacel beads	c
alginate	6.1 ± 4.1	chitosan coating	c
		cuticle related polymers:	
		chitin coating	—
		chitosan flakes	+
		chitin flakes	+

a : These substrates which were lucent in light microscopy were coated on glass microscope slides and the percentage of attached flagellates was determined by counting 200 flagellates per slide (n = 4).
b : The opaque material was applied as small particles on glass microscope slides and the attachment was then estimated in subjective scores: ++ intense. + intermediate. — weak attachment.
c : cells were artificially bound by their entire surface to these substrates.

rectal cuticle. The hydrophilic coatings with agarose and alginate and the opaque hydrophilic materials i.e. acetate film and Cellophan^{R} film did not permit attachment of more than 6% (Table 1). On coatings with the amphiphilic proteins β-lactoglobulin and BSA (bovine serum albumin) ca. 90 and 50% of the flagellates respectively attached. Chitin and chitosan as cuticle related polysaccharides were also tested as coatings on glass slides or as flakes. All these preparations permitted only an intermediate attachment except the chitosan coatings, to which the flagellates were bound with their entire surface so that the cells were spread over the substrate (Table 1). The same effect was also observed using positively charged DEAE-cellulose beads as an attachment substrate. On all substrates only epimastigotes attached, whereas trypomastigotes were found mainly free swimming.

When different solutes were tested as possible inhibitors of attachment, Tween 20 [0.01%] and BSA [1%], both molecules with hydrophobic properties, reduced the ratio of attachment to about 7 and 49% respectively. A weak inhibition was observed with the positively charged molecules putrescine, glucosamine and galactosamine, whereas the other positively charged molecules i.e. L-lysine, betaine and ammonium salt and the related uncharged saccharides N-acetylglucosamine, glucose and galactose were ineffective as inhibitors of attachment.

A slight digestion of surface proteins by trypsin also inhibited the attachment by nearly 80%.

Small drops of the emulsified hydrocarbon hexadecane selectively bound to an area at the flagellum terminus which is similar to or the same as the attachment site. No drops bound to the remaining body except in some occasions in which they also bound near the flagellum pocket.

Inhibition of epimastigote attachment during metacyclogenesis by coating the culture tubes with agarose and by an additional constant agitation significantly reduced the transformation to trypomastigotes after 6 days to 26% and 19% respectively, compared to 47% in uncoated polypropylene culture tubes. In contrast the improvement of the attachment conditions by coating the tubes with wax enhanced the metacyclogenesis by up to 55%.

DISCUSSION

Colonisation of the intestinal tract of insects by trypanosomatids is enhanced if they can attach to the intestinal wall, thereby avoiding transport with the intestinal content and deposition of feces. In the first ultrastructural study of *T. cruzi* in the small intestine, no indications of strong attachment were evident [13]. This coincides with the fact that the rectum, and not the small intestine, is the main region for colonisation by *T. cruzi*. Although the attachment to this region has been investigated several times the attachment mechanisms were unknown.

The basic architecture of the outer integument of insects is well known [2, 14, 29, 30] but investigations of the cuticle of the digestive tract are rare or lacking, especially in insect vectors of parasites which attach to the cuticular lining. Using triatomines we demonstrated that the rectal cuticle has the same architecture as the outer integument of insects and is distinguishable into a hydrophobic waxy superficial layer, the outer and inner epicuticle and the procuticle [23]. Since chitin is one of the major components of the outer integument, this polysaccharide was proposed as a putative attachment substrate for several trypanosomatids colonizing the cuticular lining of the fore- and hindgut of their insect vectors [4, 27]. However, comparable to the outer integument of insects, chitin is a component of the procuticle and is absent in the layers of the epicuticle and the superficial wax layer of the rectal cuticle of *T. infestans*. Hence this polysaccharide is not accessible at the outermost surface for the attachment of *T. cruzi*. Since it is obvious that the basic architecture of the cuticle of the fore- and hindgut of other insects is similar to that of the rectum of *T. infestans*, chitin cannot be the native attachment substrate of other Trypanosomatids colonizing these parts of the digestive tract. Furthermore the luminal surface of the rectal cuticle is water repellent and was stained green after incubation with Nile Red, indicating waxes as the main components of the superficial layer. It seemed likely that the trypanosomes attach to this hydrophobic substrate. Indeed the in vitro attachment assays of culture-derived epimastigotes showed a clear preference for hydrophobic substrates in contrast to hydrophilic ones [12].

The binding of epimastigotes and trypomastigotes with the entire cell body to chitosan coatings and DEAE-Sephacel cellulose depends on interactions of the negatively charged surface with the positively charged substrates. This binding via electrostatic forces does not represent the natural mode of attachment and is absolutely artificial, since the flagellates perished rapidly due to spreading onto the substrate. The inability of *T. cruzi* to attach to hydrophilic substrates demonstrates the requirement of hydrophobic ligands for the binding of the flagellum terminus. Hydrophobic materials of very different chemical nature are suitable attachment substrates, hence the hydrophobic interaction of putative receptor molecules is designated as nonspecific, according to definitions for bacteria and fungi [2, 5, 7, 8, 16, 28].

This coincides with the strong inhibition of attachment by different solutes with hydrophobic properties. The weak inhibition by the charged molecules glucosamine, galactosamine and putrescine very likely depends on interactions of these molecules with the negatively charged surface of the flagellates and by sterically impeding the adjacent hydrophobic receptor molecules at the flagellum terminus. It seems evident that putative receptor molecules are localized at the terminus of the flagellum, since viable epimastigotes attach only with this area to suitable substrates, whereas the remaining flagellum and the body are kept in fast swinging movements. In addition small drops of emulsified hexadecane only bind to the terminus of the flagellum and occasionally to an area near the flagellum pocket, where other hydrophobic molecules like lipoprotein-binding proteins may occur [1]. Little is known about molecules which are involved in the attachment of trypanosomes. An amphiphilic 45-kDa protein eluted from glass-wool columns after the passage of epimastigotes was described as a putative attachment molecule. This protein was absent in non-adhering trypomastigotes [17]. Since the flagellates glided back and forth along the attachment site, suggesting a membrane mobility of the molecules, it seems likely that the receptor molecules are GPI-anchored. The protein nature of these molecules is indicated by the inhibition of attach-

ment after incubation with trypsin. In the attachment of fungi, amphiphilic peptides (designated as hydrophobins) mediate the interaction to the hydrophobic substrates by covering the surface of the organism and protruding their hydrophobic domains externally [28]. However, in *T. cruzi* attachment is only mediated by a small area at the flagellum terminus where attachment molecules obviously are membrane anchored.

Attachment is described as a necessary prerequisite for the transformation to the vertebrate infectious stages, not only in T cruzi, but also in other trypanosomes like *T. brucei* and *T. congolense* [3, 10, 25]. However, results differ after using siliconized tubes to prevent attachment and to inhibit the metacyclogenesis of *T. cruzi* [3, 11]. In our study the rate of transformation was reduced either by a hydrophilic coating of the culture tubes or an agitation during culturing. Hence, epimastigote attachment is one condition which positively influences metacyclogenesis. A hydrophobic coating with wax mimics the conditions existing in vivo at the rectal wall better and improves the attachment. This hydrophobic attachment seems to be a necessity for the transformation to the vertebrate-infectious metacyclic trypomastigotes.

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REFERENCES

1) Bastin P, Stephan A, Raper J, Saint-Remy J-M, Opperdoes FR, Courtoy PJ: An M_r 145000 low-density lipoprotein (LDL)-binding protein is conserved throughout the Kinetoplastida order. Mol Biochem Parasitol 76: 43-56, 1996.
2) Binnington KC: Ultrastructure of the attachment of the bacteria *Serratia entomophila* to foregut cuticle of *Costelytra zealandica* (Coleoptera: Scarabidae) and a review of nomenclature for insect epicuticular layers. Int J Insect Morphol Embryol 22: 145-155, 1993.
3) Bonaldo MC, Souto-Padron T, De Souza W, Goldenberg S: Cell-substrate adhesion during *T. cruzi* differentiation. J Cell Biol 106: 1349-1358, 1988.
4) Bonaldo MC, Silva CP, Petretski MD, De Souza W, Soares MJ: Attachment of *T. cruzi* epimastigotes to different substrates during metacyclogenesis. Mem Inst Oswaldo Cruz 90: 89, 1995.
5) Boucias DG, Pendland JC, Latge JP: Nonspecific factors involved in attachment of entomophagic *Deuteromyces* to host insect cuticle. Appl Environ Microbiol 54: 1795-1805, 1988.
6) Brooker BE: Flagellar attachment and detachment of *Crithidia fasciculata* to the gut wall of *Anopheles gambiae*. Protoplasma 73: 191-202, 1971.
7) Busscher HJ, Weerkamp AH: Specific and non-specific interactions in bacterial adhesion to solid substrata. FEMS Microbiol Rev 46:165-173, 1987.
8) Busscher HJ, Handley PS, Rouxhet PG, Hesketh LM, Van der Mei HC: Microbial Cell Surface Analysis. Structural and Physicochemical Methods. VCH Publishers, Weinheim, 1991, 317-338 pp.
9) Goldenberg S, Contreras VT, Bonaldo MC, Salles JM, Lima Franco MPA, Lafaille J, Gonzales-Perdomo M, Linss J, Morel CM: Molecular Strategies of Parasitic Invasion. Alan R Liss Inc, New York, 1987 203-212 pp.
10) Hendry KAK, Vickerman K: The requirement for epimastigote attachment during division and metacyclogenesis in *Trypanosoma congolense*. Parasitol Res 74: 403-408, 1988.
11) Homsy JJ, Granger B, Krassner SM: Some factors inducing formation of metacyclic stages of *Trypanosoma cruzi*. J Protozool 36: 150-153, 1989.
12) Kleffmann T, Schmidt J, Schaub GA: Attachment of *Trypanosoma cruzi* epimastigotes to hydrophobic substrates and use of this property to separate stages and promote metacyclogenesis. J Euk Microbiol 45: 548-555, 1998.
13) Kollien A, Schmidt J, Schaub GA (1997) Modes of association of *T. cruzi* with the intestinal tract of the vector *Triatoma infestans*. Acta Trop 70, 127-141.
14) Locke M: The structure and formation of the cuticulin layer in the epicuticle of an insect, *Calpodes ethlius* (Lepidoptera, Hesperiidae). J Morphol 118: 461-494, 1966.
15) Molyneux DH, Ashford RW: The biology of *Trypanosoma* and *Leishmania* parasites of man and domestic animals. Taylor and Francis, London, 1983.
16) Ofek I, Doyle RJ: Bacterial Adhesion to Cells and Tissues. Chapman & Hall, New York, 1994, 1-15 pp.
17) Pinho RT, Dutra HS, Giovanni-De-Simone S, Pontes de Carvalho LC: A glass wool-based method for purifying *Trypanosoma cruzi* trypomastigotes and identification of an epimastigote-specific glass-adherent surface peptide. Acta Trop 50: 29-38, 1992.
18) Schaub GA, Böker CA: Colonization of the rectum of *Triatoma infestans* by *Trypanosoma cruzi* studied by scanning electron microscopy: influence of blood uptake by the bug. Parasitol Res 73: 417-420, 1987.
19) Schaub GA: Developmental time and mortality of larvae of *Triatoma infestans* infected with *Trypanosoma cruzi*. Trans R Soc Trop Med Hyg 82: 94-96, 1988.
20) Schaub GA, Lösch P: *Trypanosoma cruzi* : Origin of metacyclic trypomastigotes in the urine of the vector *Triatoma infestans*. Exp Parasitol 65: 174-186, 1988.
21) Schaub GA: *T. cruzi*: Quantitative studies of development of two strains in small intestine and rectum of the vector *T. infestans*. Exp Parasitol 68: 260-273, 1989.
22) Schmidt J: Glycans with N-acetyllactosamine type 2 like residues covering adult *Schistosoma mansoni* and glycomimesis as a putative mechanism of immune

evasion. Parasitology 111: 325-336, 1995.
23) Schmidt J, Kleffmann T, Schaub GA: Hydrophobic attachment of *Trypanosoma cruzi* to a superficial layer of the rectal cuticle in the bug *Triatoma infestans*. Parasitol Res 84: 527-536, 1998.
24) Vickerman K: The mode of attachment of *Trypanosoma vivax* in the proboscis of the tsetse fly *Glossina fuscipes*: an ultrastructural study of the epimastigote stage of the trypanosome. J Protozool 20: 394-404, 1973.
25) Vickerman K, Tetley L, Hendry K, Turner C: Biology of African trypanosomes in the tsetse fly. Biol Cell 64: 109-119, 1988.
26) Vickerman K, Tetley L: Ciliary and flagellar membranes. Plenum Press, New York, 1990, 267-303 pp.
27) Wallbanks KR, Molyneux DH, Dirie MF: Chitin derivatives as novel substrates for *Trypanosoma brucei brucei* attachment in vitro. Acta Trop 46: 63-68, 1989.
28) Wessels J: Hydrophobins: Proteins that change the nature of the fungal surface. Adv Microbiol Physiol 38: 1-41, 1997.
29) Wigglesworth VB: Incorporation of lipid into the epicuticle of *Rhodnius* (Hemiptera). J Cell Sci 19: 459-485, 1975.
30) Wigglesworth VB: The transfer of lipids in insects from the epidermal cells to the cuticle. Tissue and Cell 17: 249-265, 1985.
31) Zeledón R, Bolaños R, Navarro MRE, Rojas M: Morphological evidence by scanning electron microscopy of excretion of metacyclic forms of *Trypanosoma cruzi* in vectors' urine. Mem Inst Oswaldo Cruz 83: 361365, 1988.