# Cytochemical localization of carbohydrate in the spermatid of Rhodnius prolixus (Hemiptera, Reduviidae)

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## Abstract

The present work analyses the presence of lectin binding sites in intracellular compartments of spermatids and spermatozoa of the blood sucking bug Rhodnius prolixus. Periodic acid-thiosemicarbazide-silver proteinate and direct lectin-gold labeling techniques were used on ultrathin sections. Carbohydrates were shown in association with the system of internal membranes and acrosome. The acrosome was the structure most intensely labeled by all lectins. This structure undergoes a significant increase in the labeling density for WGA (Triticum vulgaris), suggesting modification of acrosomal glycoproteins, at its differentiation. The cytoplasmic vesicles were labeled by HPA (Helix pomatia) and WFA (Wisteria floribunda). The nuclear compartment was weakly labeled in the spermatids and no labeling was observed by any lectin in the spermatozoa. The results obtained show that distinct glycoconjugate may be involved in the process of spermatid differentiation.

Keywords: blood sucking bug, carbohydrates, lectins, spermatids

## Introduction

Several evidences have suggested that carbohydrates are the primary markers for cell recognition. The cell surface carries a sugar coat which consists of the polysaccharide moieties of glycoproteins and glycolipids [1]. Carbohydrate interaction between cells is crucially important to the sperm, in order to distinguish eggs of different species from their own [2, 3, 4]. Lectins which are proteins or glycoproteins of non-immune origin have become valuable tools to identify, characterize and localize carbohydrate containing sites [5, 6, 7, 8]. Indeed, some studies have analysed the distribution of carbohydrates in vertebrates spermatozoa using lectin histochemistry [9, 10, 11, 12, 13, 14, 15, 16, 17]. However, few have been made showing the same arrangement in invertebrates spermatozoa [3, 18, 19, 20, 21]. In the present study we have used periodic acid-thiosemicarbazide-silver proteinate technique and gold-labeled lectins to identify specific glycoprotein conjugates in spermatid and spermatozoon of the blood sucking bug.

## Materials and Methods

Male adults of Rhodnius prolixus obtained from a colony maintained in the Multi-disciplinary Laboratory for Researches on Chagas Disease of the Department of Cell Biology of the University of Brasilia, DF, Brazil. The lectins used (Table 1) were obtained from E-Y Laboratories (San Mateo, Calif., USA) or Sigma Chemical Company. The various glycoproteins were labeled with colloidal gold particles (8-10 or 15 nm in diameter), according to Roth [7].

Table I - Lectins used as histochemical probes

| Source of Lectins | Abbreviations | Sugar specificity |
|---|---|---|
| Canavalia ensiformis | Con A | D-Mannose |
| Arachis hypogaea | PNA | D-galactose |
| Bandeira simplicifolia I | BS-I | D-galactose |
| Triticum vulgaris | WGA | N-acetyl-D-glucosamine/ N-acetylneuraminic acid |
| Wistaria floribunda | WFA | N-acetyl-D-galactosamine |
| Helix pomatia | HPA | N-acetyl-D-galactosamine |

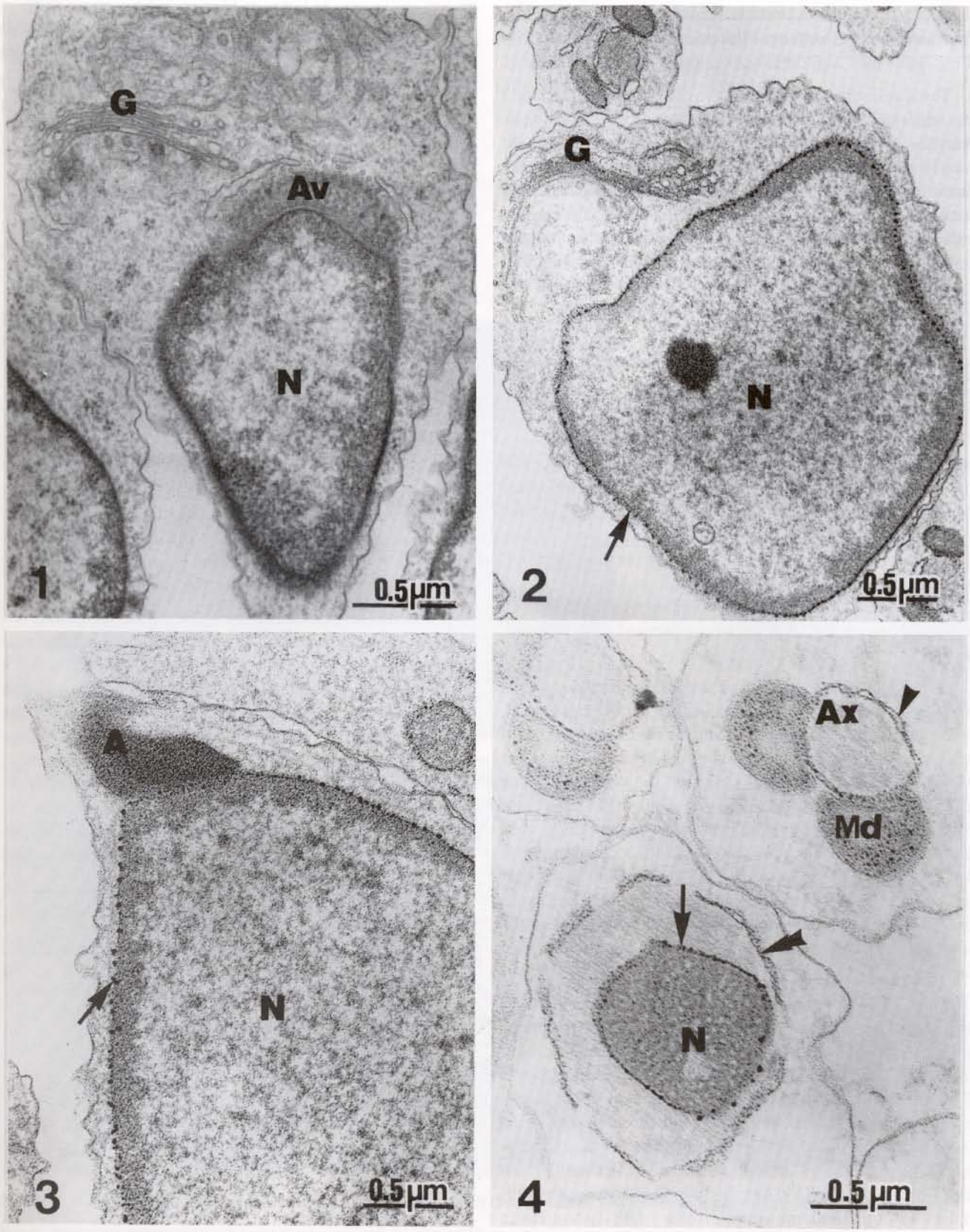



*Fig. 1 - Section of spermatid showing an acrosomal vesicle (Av), Golgi complex (G) and nucleus (N).*
***Figs. 2 to 4** - Localization of carbohydrate in thin sections submitted to the periodic acid-thiosemicarbazide-silver proteinate technique. Figure 2 shows a product of reaction in the Golgi complex (G) and nuclear envelope (arrow). In the figure 3 a sagittal section of the spermatid head shows reaction product in acrosome (A) and nuclear envelope (arrow). In the figure 4 a cross-section of spermatid shows reaction product on the membrane (arrowhead) that surround axoneme (Ax), nuclear envelope (arrow), system of internal membranes (double arrowhead) and layer of amorphous material of mitochondrial derivatives (Md). (N), nucleus.*

## Transmission Electron Microscopy

Testis and seminal vesicles were dissected and fixed for 3 h at room temperature in a solution containing 2% glutaraldehyde, 4% paraformaldehyde, 5% sucrose in 0.1 M cacodylate buffer, pH 7.2. After fixation, the specimes were rinsed in buffer, and postfixed in 1% osmium tetroxide, 0.8% potassium ferricyanide, 5 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.2. The tissue was dehydrated in acetone and embedded in Epon. After sectioning and staining with uranyl acetate and lead citrate the sections were examined in a JEOL JEM 100 C transmission electron microscope.

## Cytochemical Assays

A) For localization of carbohydrates ultrathin sections, processed as described above, were collected on gold grids and submitted to the periodic acid-thiosemicarbazide-silver proteinate technique [22]. Briefly the sections were treated with 1% periodic acid for 45 min and incubated for 72 h in 1% thiosemicarbazide. Subsequently, the sections were exposed to 1% silver proteinate for 30 min in dark conditions. Controls were done by omission of oxidation by periodic acid. In this technique the sections were observed without staining.

B) For lectins label, testis and seminal vesicles were fixed for 3 h at 4 °C in a mixture containing 4% paraformaldehyde, 1% glutaraldehyde, 0.2% picric acid, 3.5% sucrose and 5mM calcium chloride in 0.1 M cacodylate buffer, pH 7.2. After washing for 2 h with several changes of the same buffer, free aldehyde groups were quenched with 50 mM ammonium chloride in 0.1 M cacodylate buffer for 1 h at 4 °C, and then block-staining in 2% uranyl acetate in 15% acetone for 2 h at 4 °C [23]. Specimens were dehydrated in 30-90% acetone. Infiltration was performed in LR Gold resin (Polysciences, Inc, Warrington PA) for 48 h at -20 °C, and embedded in the same resin. Samples were polymerized in light ultra-violet for 48 h at -20 °C.

Ultrathin sections were collected in 400 mesh uncoated nickel grids. They were pre-incubated for 1 h at room temperature in PBS (phosphate buffer saline) solution, containing 1.5% bovine serum albumin (PBS-BSA) and 0.01% Tween 20, and subsequently incubated for 1 h at room temperature in the presence of different gold-labeled lectins in PBS-BSA. For Triticum vulgaris (wheat germ agglutinin - WGA), Canavalia ensiformis (Con A), Wistaria floribunda (WFA), Arachis hypogaea (peanut agglutinin - PNA), Bandeira simplicifolia I (BS-I) and Helix pomatia (HPA), incubation was carried out at pH 7.3 and at a 1:5 dilution. After incubation, the grids were washed with PBS and distilled water, stained with uranyl acetate and lead citrate, and observed in the transmission electron microscope.

Controls consisted of the addition to the incubation medium of 200-300 mM of the corresponding monosaccharides (Table 1).

## Results

The general structure of Rhodnius prolixus spermatid presents a conspicuous Golgi complex that was mainly associated with the formation of the acrosome. It also exhibits an elaborate system of internal membranes that participate actively in the process of spermatid differentiation (Fig. 1). Carbohydrates were localized in several cisternae of the Golgi complex using the periodic acid-thiosemicarbazide-silver proteinate technique applied to sections of Epon embedded cells (Fig. 2). Carbohydrates in the spermatids were also observed in the association with the system of internal membranes (Figs. 2 - 4).

The acrosome was localized in an anterior portion of the nucleus. A positive reaction for carbohydrates was uniformly distributed in the acrosome (Fig. 3). Reaction product, indicative of carbohydrates, was also seen in the layer of amorphous material that surrounded the paracrystalline structure of the mitochondrial derivatives of spermatids (Fig. 4).

Ultrathin section of LR Gold-embedded spermatids and spermatoozoa were used for the localization of binding sites specific for D-mannose (Con A), D-galactose (PNA and BS-I), N-acetyl-D-glucosamine and N-acetylneuraminic acid (WGA) and N-acetyl-D-galactosamine (WFA and HPA). All binding reactions could be inhibited by addition of the appropriate sugar to the incubation medium. Different labeling patterns were observed for the lectins tested. The result obtained are summarized in Table II. The Golgi complex was the structure most frequently and intensely labeled in the spermatids. The acrosome, was intensely labeled by some lectins.

Table II - Summary of the labeling pattern[a] in ultrathin sections of LR Gold-embedded spermatids and spermatozoa

| Lectins[b] | Spermatids | | | Spermatozoa | |
|---|---|---|---|---|---|
| | GC | N | CV | N | A |
| Con A | + | +/- | - | - | +/- |
| PNA | + | +/- | - | - | +/- |
| GSI B4 | + | +/- | - | - | +/- |
| WGA | + | +/- | - | - | ++ |
| WFA | + | +/- | ++ | - | +/- |
| HPA | + | +/- | ++ | - | +/- |

A, acrosome; CV, cytoplasmic vesicles; GC, Golgi complex; N, nucleus

[a] ++, high intensity labeling (more than 1000 gold particles/$\mu m^2$); +, moderate intensity labeling (10-1000 gold particles/$\mu m^2$); +/- low intensity labeling (less than 10 gold particles/$\mu m^2$); -, no labeling.

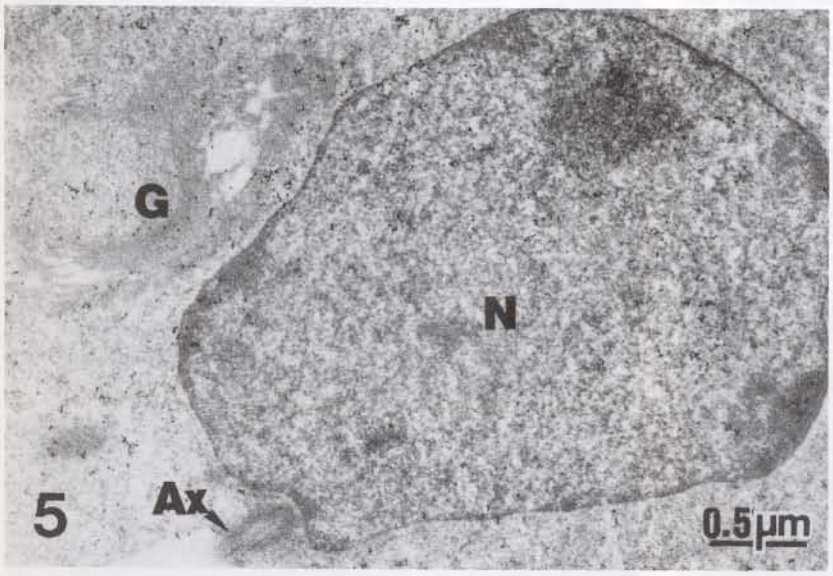
[b] The lectin derivations are given in Table I

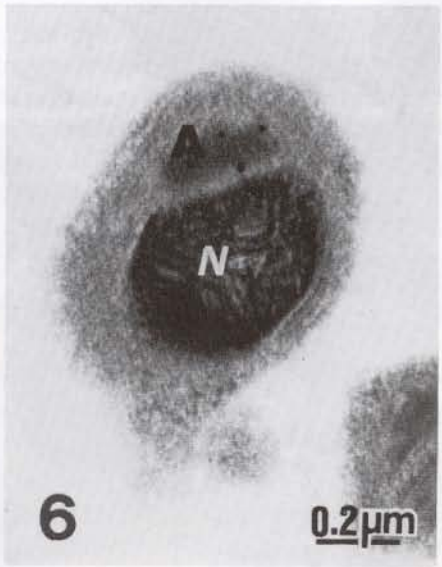
Thin sections of spermatids incubated in the presence of Con A-Au, revealed a moderate labeling in the Golgi complex (Fig. 5) and in the acrosome (Fig. 6) . The Golgi complex was labeled in ultrathin sections, which were incubated in the presence of PNA-Au (Fig. 7) and BS-I-Au (not shown). A moderate labeling pattern was observed on the acrosome with PNA (not shown) and BS-I (Fig. 8).

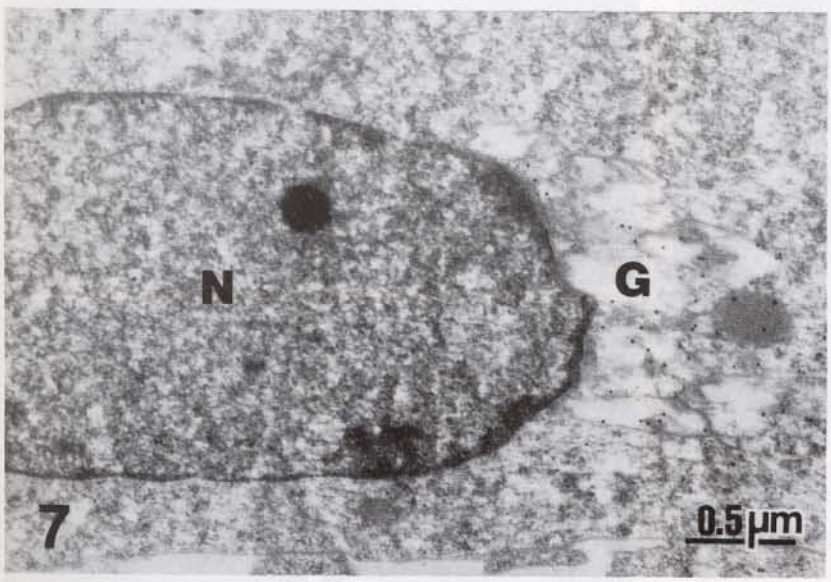
The lectin WGA, which bind to N-acetyl-D-glucosamine and N-acetylneuraminic acid residues, labeled the acrosome of the spermatid (Fig. 9). However, an intense labeling of the acrosome was observed in the last stages of the process of sperm maturation (Fig. 10).

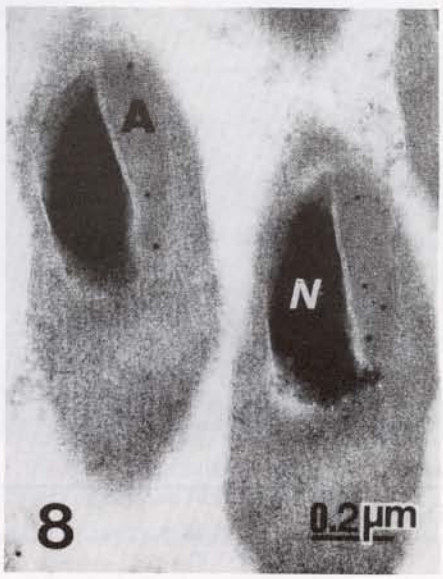
Helix pomatia and WFA showed a similar binding pattern. Cytoplasmic inclusions and vesicles in spermatids (Figs. 11 and 12) and spermatozoa acrosome (figs. 13 and 14) were also labeled with these lectins.

The nucleus of early spermatids was weakly labeled in the presence of Con A (Fig. 5) and PNA (Fig. 7), whereas gold particles were not seen on the compact nucleus of mature spermatids (Figs. 6, 8-10). In relation to the spermatozoal surface, very light labeling was observed when ultrathin sections were incubated in the presence of WGA (Fig. 10); HPA (Fig. 13) and WFA (Fig. 14).

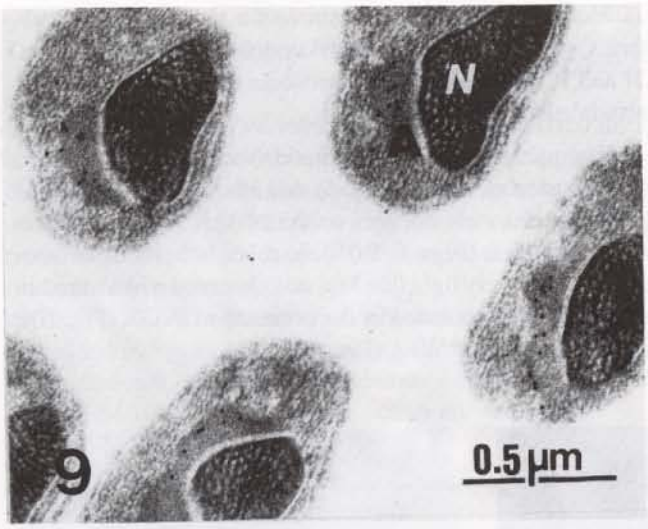


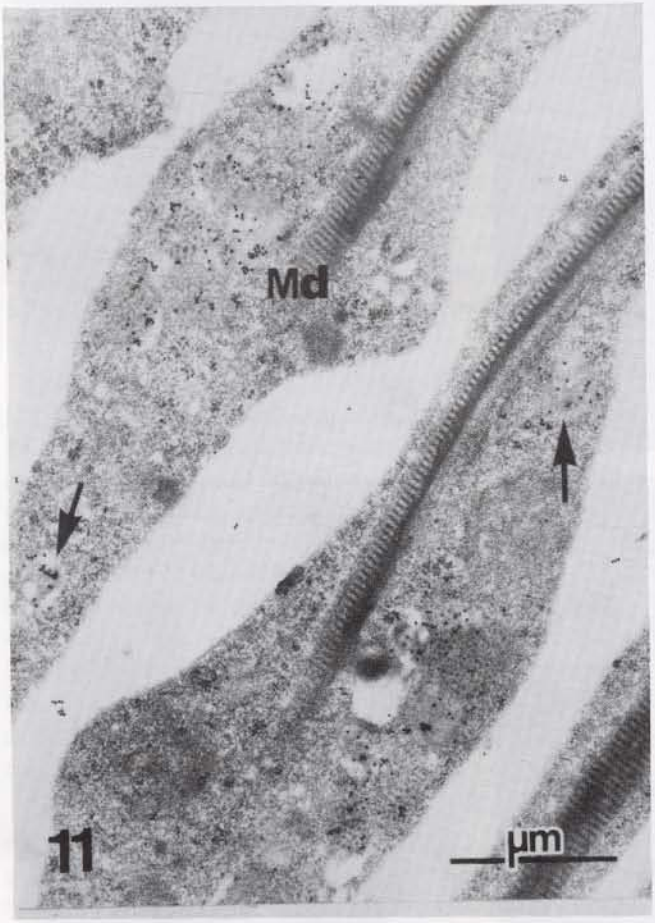



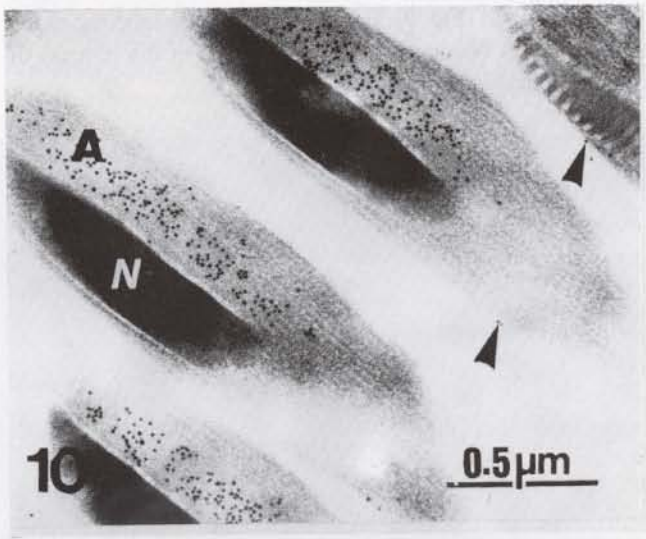



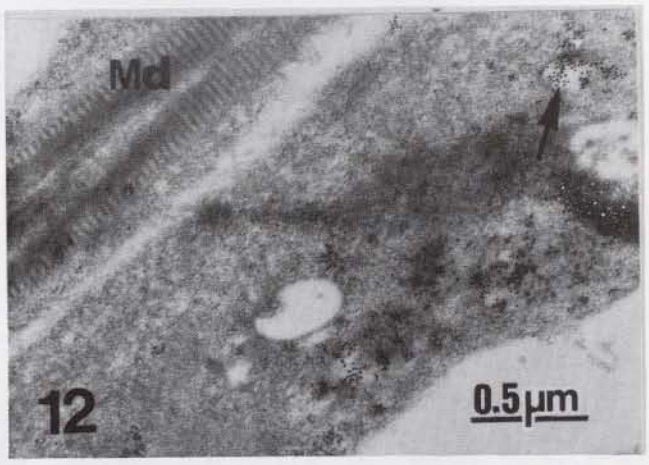

***Figs. 5 to 14** - Localization of carbohydrate residues in thin sections of LR Gold embedded spermatids and spermatozoa submitted to lectin-gold complexs.*
***Fig. 5** - Spermatid shows nucleus (N) and Golgi complex region (G) labeled by Cananalina ensiformis (Con A) lectin. (Ax), axoneme.*
***Fig. 6** - Canavalina ensiformis (Con A) lectin-labeled sperm acrosome (A). Note that neither gold particles were found in the nucleus (N).*
***Fig. 7** - Arachis hypogaea (PNA) labeled spermatid exhibiting gold particles over the Golgi complex region (G). (N), nucleus.*
***Fig. 8** - Saggital section of the head region of the sperm incubated with BS-I lectin, in which gold particles were distributed over the acrosome (A). The nucleus (N) was completely devoid of labeling.*

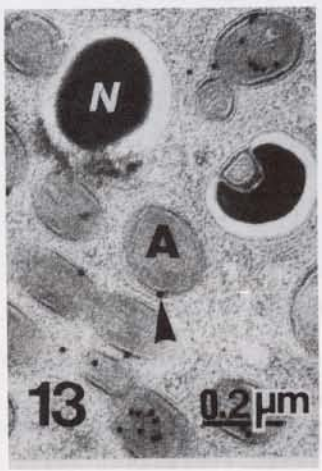


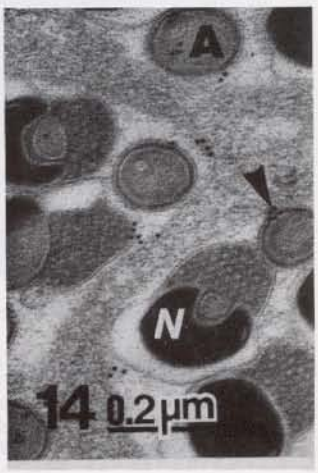




***Figs. 9 and 10*** *- Triticum vulgaris (WGA) labeled sperm acrosome (A). Note that had an increase in the labeling density with the advancement of the differentiation. Some particles can be seen in the cellular surface (arrowhead). (N), nucleus.*
***Figs. 11 and 12*** *- Spermatids cytoplasmic vesicles (arrow) labeled by HPA (Fig. 11), and WFA (Fig. 12). (Md), mitochondrial derivatives.*
***Figs. 13 and 14*** *- Spermatozoa acrosome (A) labeled by HPA (Fig. 13) and WFA (Fig. 14). Some particles can be seen in the cellular surface (arrowhead). (N), nucleus.*




## Discussion

The results of the study show that Rhodnius prolixus spermatid and spermatozoon contain glycoconjugates. Carbohydrates were shown in association mainly with the system of internal membranes when observer after the periodic acid-thiosemicarbazide-silver proteinate technique. These system of internal membranes were associated with the differentia-

tion of the spermatids. These morphofunctional events may involve the participation of the Golgi complex [24, 25] with the metabolism of glycoconjugates. The participation of Golgi complex in the synthesis of oligosaccharides chains at early steps of spermiogenesis was finally showed in spermatids of rat with a high resolution lectin cytochemical study [16].

In order to characterize further the nature of the carbohydrates present in the sperm, gold-labeled lectins were used. The main type of sugar residues detected by this technique were N-acetylglucosamine and/or N-acetylneuraminic acid, as indicated by the intense labeling with WGA. Most sugar residues were found in the intracellular compartments with the use of lectins; however, a more concentration of binding sites was observed in the acrosome region. Recent biochemical and cytochemical studies have shown the presence of sugar residues in intracellular compartments, with the nucleus [20, 21, 26, 27, 28], the nuclear envelope, endoplasmic reticulum and Golgi complex [11, 16, 29, 30].

The acrosome is a large secretory vesicle located in the anterior head region of the spermatozoon that carries a variety of hydrolytic enzyme stored in the form of proenzymes. In R. prolixus carbohydrates were showed in association with the acrosomal content using the periodic acid-thiosemicarbazide-silver proteinate technique.

Our present observations using lectins show that D-mannose, D-galactose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine and N-acetylneuraminic acid residues were uniformly distributed in the acrosomal region. However, the distribution of carbohydrate residues in the acrosomal content of mosquito spermatozoon, occupying the proximal acrosomal region principally, was observed using gold-labeled lectins [20]. In spermatozoon from beetles, galactose residues were observed only in the extra-acrosomal layer, but N-acetyl-glucosamine residues appear distributed in the different regions of the acrosomal complex [21]. In addition, numerous studies have shown glycoproteins in the vertebrates acrosome [12, 16, 17, 31].

The results reveal that the acrosome of blood sucking bug spermatid undergoes a significant increase in the labeling density for WGA-Au during this differentiation. Our present observations suggest that the glycosylated macromolecules with N-acetyl-D-glucosamine and N-acetylneuraminic acid, may play a key role in sperm-oocyte recognition and fertilization. This change may be associated with a selective enzymatic activation and hence carbohydrate modification of the acrosomal glycoproteins during sperm differentiation. Furthermore, it has been suggested that some of the carbohydrate-containing material in the acrosomal matrix may activate acrosomal enzymes [32].

The presence of sugar residues in cytoplasmic vesicles suggest that glycoproteins may be associated with the elimination of cytoplasm during the cellular elongation. The cytoplasmic droplets were labeled with lectins in boar and bovine spermatozoa, during the autolytic processes, which reduce the spermatid cytoplasm [10,11].

Our present observations show that all the lectins tested labeled the spermatid nucleus weakly. However, with the process of chromatin condensation, labeling of the nuclear compartment disappear so that no labeling was observed in the spermatozoon. These results were not in accordance with those obtained in mosquito [20] and beetles [21] spermatids when labeling of the nucleus during the process of chromatin condensation gradually increases and an intense labeling was observed in the spermatozoon. The result suggest that extensive changes in glycosylation occur as spermatozoa differentiate, and that the changes differ in detail between species.

In summary, the present data show that distinct glycoconjugates may be involved in the process of spermatid differentiation. These findings indicate that the characterization of the oligosaccharide side chains of the acrosomal glycoproteins may become an important factor for the complete knowledgement of sperm-egg interaction phenoma.

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