

Direct assay of bound sialic acids on rat spermatozoa from the caput and cauda epididymidis

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Summary. Bound sialic acids on rat spermatozoa were assayed by oxidation with 1 mM- NaIO_4 at 0°C, liberating C-9 as formaldehyde which was further quantitated using 3-methyl-2-benzothiazolinone. The mean \pm s.d. ($n = 20$) content of bound sialic acids of spermatozoa from the caput and cauda epididymidis was 50.9 ± 8.0 and 25.2 ± 3.8 nmol/ 10^8 spermatozoa respectively. About 85% of the former and 75% of the latter could be extracted by 1% Triton X-100 and 2 mM-dithiothreitol. About 70% of the former and 20% of the latter were released by neuraminidase from *Vibrio cholerae*. About 40% of the former and 30% of the latter were sensitive to trypsin. During sperm maturation, the decrease in the total bound sialic acids was due to the decrease in the neuraminidase-sensitive but not the neuraminidase-resistant sialic acids.

Introduction

The previous chemical method to assay sialic acids bound to spermatozoa (Gupta, Rajalakshmi, Prasad & Moudgal, 1974; Rajalakshmi *et al.*, 1976) requires prior liberation of the residues by acid hydrolysis, followed by quantitation of the free sialic acids using thiobarbiturate (Warren, 1959). One major drawback of this method is that the acid treatment of intact cells also releases other cellular components such as 2-deoxyribose (Warren, 1959), glycosides and fatty acids (Kuwahara, 1980) that can interfere with the subsequent thiobarbiturate reaction. To override the interference, a correction control is needed (Rajalakshmi & Prasad, 1968). To eliminate the interference, additional purification or extraction of the free sialic acids is required (Warren, 1963; Svennerholm, 1963; Roboz, Suttajit & Bekesi, 1981).

To avoid this problem, we have chosen a new and simple method of Massamiri, Durand, Richard, Feger & Agneray (1979) that can assay bound sialic acids without prior release of the residues. It involves a mild oxidation with periodate to liberate C-9 from bound sialic acids as formaldehyde which is quantitated colorimetrically by using 3-methyl-2-benzothiazolinone (MBTH). Using the MBTH-method, changes in surface-bound sialic acids of spermatozoa during epididymal transit have been studied.

Materials and Methods

Spermatozoa were extruded from the epididymis of adult albino rats into phosphate-buffered saline (PBS) containing 0.15 M-NaCl in 5 mM-phosphate, pH 7.4, at 4°C (see Chulavatnatol, Hasibuan, Yindepit & Eksittikul, 1977). The cells were washed 5 times by centrifugation at 600 g

for 15 min at 4°C and followed by resuspension in ice-cold PBS. The final sperm suspension was then inspected under a phase-contrast microscope and was found to be free from other cells and cell debris. A sperm count was taken after each washing by using a haemocytometer.

To determine bound sialic acids, spermatozoa ($15 \times 10^6/\text{ml}$) were incubated in PBS with freshly prepared NaIO_4 solution (1 mM) in darkness at 0°C (ice bath) with constant shaking for 15 min. The total volume was 4 ml. After the periodate oxidation, the cells were removed by centrifugation at 600 g for 15 min at 4°C. The supernatant fluid was quantitatively recovered and the formaldehyde content in the fluid was determined by using MBTH (Eastman Kodak, New York, U.S.A.) according to the method of Massamiri *et al.* (1979). A sperm-free sample incubated with NaIO_4 and treated identically was used as control. The control value was relatively small but was always subtracted from the experimental value. A standard curve was constructed using *N*-acetylneuraminic acid (Sigma, St Louis, U.S.A.). Between 10 and 50 nmol *N*-acetylneuraminic acid, the absorbance at 625 nm increased linearly with a slope of 0.016 optical density unit per nmol *N*-acetylneuraminic acid.

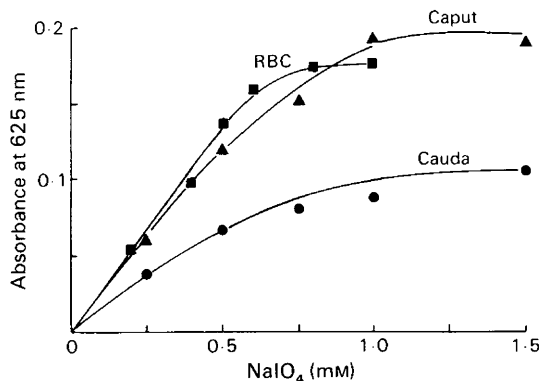
To remove plasma membrane and mitochondrial sheath, the washed spermatozoa were treated with 1% Triton X-100 and 2 mM-dithiothreitol in 50 mM-Tris-HCl, pH 6.8, at 25°C for 30 min with constant shaking according to the method of Olson & Sammons (1980). The treated spermatozoa were collected by centrifugation at 600 g at 4°C for 15 min and washed 3 times with PBS. The appearance of the treated spermatozoa was verified under a phase-contrast microscope. Sperm count was redetermined after the final washing.

For enzyme treatments, the washed spermatozoa were incubated in PBS with various amounts of neuraminidase (*Vibrio cholerae*; Calbiochem, La Jolla, U.S.A.) at 37°C for 30 min or trypsin (Sigma) at 25°C for 30 min. The total volume of the incubation was 2 ml. After the incubation, the enzyme-treated spermatozoa were collected by centrifugation at 600 g at 4°C for 15 min. They were washed 3 times with PBS. Under a phase-contrast microscope, the neuraminidase-treated spermatozoa appeared intact but the trypsin-treated sample showed heads detached from tails. Whole spermatozoa, heads or tails were re-counted after the final washing and no appreciable loss was encountered.

Results

To assay the bound sialic acids of rat epididymal spermatozoa by the MBTH-method which was developed for red blood cells (Massamiri *et al.*, 1979), two conditions had to be established. (1) It was necessary to determine the minimal concentration of NaIO_4 for complete but specific peroxidation of the C-9 of the bound sialic acids into formaldehyde. Under the assay condition, the minimal concentration of NaIO_4 for the spermatozoa was 1 mM while that for human red blood cells was 0.6 mM (Text-fig. 1). The latter compared favourably with the literature value of 0.5 mM (Massamiri *et al.*, 1979). Therefore, 1 mM- NaIO_4 was used for the assay of sperm-bound sialic acids. (2) The epididymal spermatozoa had to be washed free from the sialic acid-rich epididymal fluid. It was found necessary to wash the spermatozoa with PBS 3–5 times to remove progressively the loosely bound sialic acids in order to obtain a constant value for the sperm-bound sialic acids (Table 1). When the spermatozoa were first washed by centrifugation through a layer of 10% Ficoll in PBS, the assay was not satisfactory due to some unknown interfering factor from the Ficoll solution. If the spermatozoa were then washed another 4 times with PBS, values similar to those in Table 1 were obtained. Therefore 5 times-washed spermatozoa (without Ficoll) were always used in this study.

Although there were variations among individual rats, the mean \pm s.d. (for 20 rats) content of sialic acids bound to the spermatozoa from the cauda epididymidis (25.2 ± 3.8 (range 19.6–30.8) nmol/ 10^8 spermatozoa) was about half of that found in spermatozoa from the caput (50.9 ± 8.0 (range 40.3–63.7) nmol/ 10^8 spermatozoa). Suspecting protease action on the cells during



Text-fig. 1. Effect of increasing NaIO_4 concentration on formaldehyde released from sialic acids of rat spermatozoa from the caput and cauda epididymidis and of human red blood cells. Values are means of duplicate determinations.

Table 1. Effect of washing of rat epididymal spermatozoa on the bound sialic acids ($\text{nmol}/10^8$ spermatozoa)

Washing	Rat epididymal spermatozoa	
	Caput	Cauda
Unwashed	106.2	51.9
1st washing	93.3	33.4
2nd washing	84.0	21.5
3rd washing	60.1	23.3
4th washing	61.3	22.7
5th washing	63.7	23.3

Each value was an average of duplicate assays.

preparation and assay, 1 mM-phenylmethylsulphonyl fluoride in 1 mM-isopropanol was included in control and experimental tubes, but no change in the values of sialic acids was observed. When freshly extruded spermatozoa were immediately fixed with 2% glutaraldehyde in PBS and then washed 5 times, the cells formed small aggregates as verified under a phase-contrast microscope but the amounts of the bound sialic acids were found to be 51.8 ± 4.0 and 24.3 ± 4.2 $\text{nmol}/10^8$ spermatozoa (mean \pm s.d., $n = 4$) for caput and cauda spermatozoa respectively. These values were similar to those of unfixed spermatozoa. To verify the assay condition, human red cells prepared from heparinized blood from a volunteer donor were included in the assay: the sialic acid content was 50.3 ± 6.2 $\text{nmol}/10^9$ cells ($n = 10$) which was in good agreement with the reported value (Massamiri *et al.*, 1979).

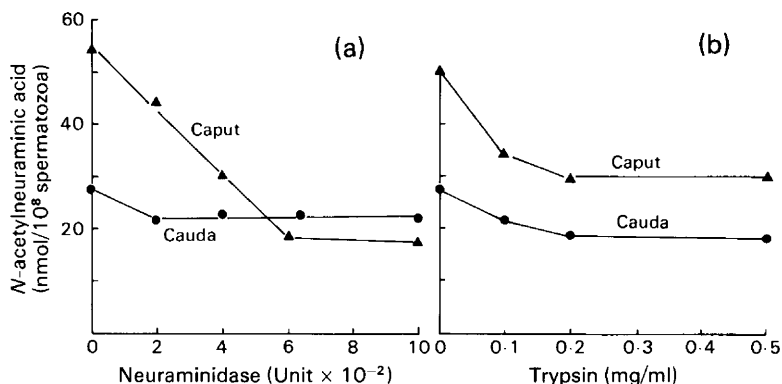
When spermatozoa were treated with Triton X-100 and dithiothreitol which are known to remove the plasma membrane and mitochondrial sheath (Olson & Sammons, 1980), about 85 and 75% of the bound sialic acids were removed from the caput and the cauda epididymal spermatozoa respectively (Table 2).

To test whether the bound sialic acids of the spermatozoa were sensitive to enzymic digestion, neuraminidase and trypsin were selected for pre-treatment of the spermatozoa. When added in excess, neuraminidase can release about 70% of the bound sialic acids from the caput epididymal spermatozoa. However, slightly less than 20% of the sialic acids bound to the cauda spermatozoa was found to be neuraminidase-sensitive. Furthermore, the amount of neuraminidase-resistant

Table 2. Effect of Triton X-100/dithiothreitol treatment on the bound sialic acids of rat epididymal spermatozoa (nmol/10⁸ spermatozoa)

Treatment	Rat epididymal spermatozoa	
	Caput	Cauda
Control	56.1 ± 6.6	26.2 ± 3.8
Triton X-100/dithiothreitol	7.9 ± 3.9	6.4 ± 1.3

Values are mean ± s.d. for 3 rats.



Text-fig. 2. Effect of increasing the amount of (a) neuraminidase and (b) trypsin on the sialic acids of rat spermatozoa from the caput and cauda epididymidis. Values are means of duplicate determinations.

sialic acids of the spermatozoa from the cauda epididymidis was slightly higher than that of the cells from the caput region (Text-fig. 2a).

The bound sialic acids were also sensitive to tryptic digestion (Text-fig. 2b). Again, a larger amount of sialic acids was released from spermatozoa from the caput (40%) than the cauda (30%) epididymidis when an excess of trypsin was used.

Under the conditions of the enzyme treatments, the protein profile of the trypsin-treated spermatozoa analysed by SDS-gel electrophoresis was different from that of untreated control spermatozoa which was identical with that of the neuraminidase-treated spermatozoa, suggesting that there was no protease contamination in the neuraminidase used.

Discussion

After treatment with periodate, compounds containing two or more hydroxyl or keto groups attached to adjacent carbon atoms undergo oxidation with cleavage of C-C bonds. The C-C bonds of the polyhydroxy side chain of sialic acids are more easily cleaved than are the bonds in the pyranose ring. Therefore, specifically and completely to liberate C-9 of sialic acids as formaldehyde, it is essential to use very mild conditions, i.e. low temperature, low NaIO₄ concentration, short incubation time and neutral pH. The minimal concentration (1 mM) of NaIO₄ to achieve maximal liberation of formaldehyde from rat epididymal spermatozoa is higher than that (0.6 mM) for human red blood cells (Text-fig. 1), reflecting the different microenvironments around the sialyl residues of the two cell types.

The sialic acids assayed by this technique should represent both sialoglycoproteins and sialo-

glycolipids of the spermatozoa. These two classes of compounds are mainly found associated with the plasma membrane or cell surface but some is associated with intracellular membranes or organelles (Hughes, 1976). That the majority of the assayed sialic acids is bound to the sperm surface is supported by two facts. Firstly, the low temperature of the NaIO_4 oxidation (0°C) will prevent the passage of NaIO_4 through the plasma membrane, minimize membrane damage and allow only externally exposed sialic acids to be oxidized (Gahmberg & Andersson, 1977). Secondly, the fraction of the sialic acids sensitive to protease-free neuraminidase attack (Text-fig. 2a) should be orientated externally. However, the presence of a large amount of sialic acids in the epididymal fluid has necessitated washing of the spermatozoa before the sperm-bound sialic acids can be assayed. One way to ensure the complete removal of the fluid content and of sperm-coating material from the sperm surface is to wash until a constant value of the sialic acids is obtained (Table 1). Since this requires 3–5 washings for rat epididymal spermatozoa, it is possible that the plasma membrane is damaged by the extensive washings and hence the intracellular components become accessible to NaIO_4 and neuraminidase. This possibility remains to be proven. However, since the glutaraldehyde-stabilized spermatozoa subjected to the washings do not exhibit any higher amount of bound sialic acids, the damage, if any, does not cause any loss of sialic acid-carrying components.

The trypsin-sensitive sialic acids (Text-fig. 26) are likely to be those attached to sialoglycoproteins while the trypsin-resistant ones may be sialoglycolipids and sialoglycoproteins inaccessible to or non-hydrolysable by the enzyme. The neuraminidase-sensitive sialic acids (Text-fig. 2a) must have an α -ketosidic linkage (Yu & Ledeen, 1969) and a free carboxyl group at C-1 (Karkas & Chargaff, 1964). *O*-Acetylation on C-4, C-7 or C-8 (Pepper, 1968; Schauer & Faillard, 1968; Schauer, 1970; Neuberger & Ratcliffe, 1972), bulky *N*-substitution (Hakomori & Saito, 1969), a shortened polyhydroxy side chain (Suttajit & Winzler, 1971) or masking will make the sialic acids resistant to neuraminidase. Our data suggest that the multiple forms of sialic acids may be bound to rat epididymal spermatozoa.

The sialic acid contents of the unwashed rat spermatozoa (Table 1) compare well with those obtained by Gupta *et al.* (1974) using thiobarbiturate method of Warren (1959). The observed decrease in total sperm-bound sialic acids during sperm maturation agrees with that of Gupta *et al.* (1974). The decrease is not an artefact of washing since it has been observed for unwashed (presumably intact) spermatozoa or at any step of washing (Table 1). It is also unlikely to be due to a permeability difference because NaIO_4 oxidation has been performed at 0°C to prevent the passage of NaIO_4 across the plasma membrane. However, our finding on the decrease in the neuraminidase-sensitive sialic acids (Text-fig. 2a) differs from that of Holt (1980) who has shown an increase in the neuraminidase-sensitive sialic acids of ram spermatozoa during maturation. This could be a genuine species difference, but the bound sialic acids on ram spermatozoa have been studied by cytochemistry using colloidal iron hydroxide at pH 1.8 in conjunction with automatic image analysis: as the *pK*_a of C-1 carboxylic group of free sialic acid is 2.75, only about 10% of the sialic acids will be negatively charged at pH 1.8 and hence detected by binding of colloidal iron hydroxide (Hughes, 1976). Since 90% of the sperm-bound sialic acids can be missed by the colloidal iron binding, the technique seems less satisfactory than our direct chemical assay. Furthermore, neuraminidase-resistant sialic acids, if also present in ram spermatozoa, will also be missed by the technique used by Holt (1980).

Among the complex changes in the sperm membrane during epididymal transit (for reviews see Bedford & Cooper, 1978; Koehler, 1981), an increase in negative charge has long been known (Bedford, 1963). An increase in bound sialic acids has been suggested as responsible for the enhanced negative charge in mature spermatozoa (Nicolson, Usui, Yanagimachi, Yanagimachi & Smith, 1977). To evaluate this suggestion, a sensitive and specific assay for total sialic acids, both neuraminidase-sensitive and neuraminidase-resistant, bound to epididymal spermatozoa is needed. The MBTH-method appears suitable for this purpose. However, our data cannot support the suggestion by Nicolson *et al.* (1977). In rat spermatozoa, at least, the bound sialic acids decrease

rather than increase during maturation. This is true for both unwashed (presumably intact) and washed rat spermatozoa (Table 1). It remains to be shown in other species.

P.T. was supported by a WHO-scholarship. We thank Thitika Vajrodaya and Urai Sajjharutai for skilful secretarial assistance.

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Received 27 May 1982