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V. Kopečný, Dana Čechová, Blanka Železná, J.-E. Fléchon, J. Motlik, et al.. Studies on acrosome labelling of mammalian spermatozoa by radioactive sugars. *Reproduction Nutrition Développement*, 1984, 24 (4), pp.419-429. <hal-00898163>

HAL Id: hal-00898163

<https://hal.science/hal-00898163v1>

Submitted on 11 May 2020

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Studies on acrosome labelling of mammalian spermatozoa by radioactive sugars

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Summary. The localization of glycoprotein synthesis and storage was studied during acrosome formation in guinea-pig using fine-structure autoradiography after (³H)-fucose incorporation. Three days after (³H)-fucose injection, labelling in spermatids was concentrated in the matrix of developing acrosomes, and it was evident that the fucosylation of acrosomal glycoproteins largely overshadowed the fucosylation of other spermatid glycoproteins.

Acrosin labelling and its quantitative relation to labelling of other glycoproteins was examined in mature rabbit spermatozoa after incorporation of (¹⁴C)-fucose or (¹⁴C)-glucosamine during spermatogenesis. Cauda epididymis spermatozoa recovered 21 days after intratesticular application of (¹⁴C)-fucose or (¹⁴C)-glucosamine were analysed for acrosin specific labelling after acid extraction and gel filtration. In all the material examined, radioactivity was detected in the proacrosin fractions; radioactivity in purified proacrosin amounted to at least 2 % of the total radioactivity in the epididymal sperm population. In addition to the peak with radioactive proacrosin, another radioactive peak in (¹⁴C)-glucosamine-labelled material was attributed to a glycoprotein intraacrosomal inhibitor of acrosin.

It is concluded that (pro)acrosin (acrosin-inhibitor) complexes seem to contribute significantly to acrosomal glycoprotein labelling by radioactive sugars and that the distribution of these complexes may at least partly correspond to their cytochemically detectable component, acrosin. The superposition of the distribution of acrosin and of other acrosomal glycoproteins during acrosome reaction can be explained by the fact that the dispersal of most of the acrosomal content is linked to proacrosin activation.

Introduction.

Sperm acrosomal contents can be labelled by radioactive sugars (Kopečný and Fléchon, 1981). Because of the chemical specificity of the precursors used, particularly fucose, it was thought that different acrosomal glycoproteins were labelled in the spermatid during acrosome formation and that most of them

« survived » in the acrosomal material until sperm maturity. In fact, Grootegoed *et al.*, (1982) showed the fucosylation of several glycoproteins in rat spermatids. Consequently, our previous study (Kopečný and Fléchon, 1981) is only of statistical significance, revealing the general behavior of all fucose or glucosamine-containing glycoproteins. However, it is surprising that the subcellular distribution of acrosomal proteases or of the trypsin-like serine proteinase, acrosin (Gaddum and Blandau, 1970 ; Sakai and Yasuda, 1981 ; Green and Hockaday, 1978 ; Harrison *et al.*, 1982 ; Huneau *et al.*, 1983), before, and especially during, acrosome reaction, is very similar to our findings for all labelled acrosomal glycoproteins (Kopečný *et al.*, 1980 ; Kopečný and Fléchon, 1981). The overlapping results include the localization of both radioactive proteins and enzymatic activities in the acrosomal matrix, their dispersal during acrosome reaction and the formation of diffusion « halos » around the sperm head, heavy labelling on the fenestrated acrosomal cap versus minimal or negative labelling on the denuded sperm head. As acrosin is a glycoprotein (for references see Anderson *et al.*, 1981), it may be assumed from these observations that acrosin reflects the behavior of all or most of the other glycosylated acrosomal proteins.

The aim of the present work was to study the distribution of spermatid glycoproteins fucosylated already at the beginning of acrosome formation and to test the hypothesis of acrosin glycosylation at that time as well as to evaluate approximately the amount of acrosin in the glycosylated proteins of the mature acrosome. For the fine structure autoradiographic study, guinea-pig was chosen for the exceptional size of the acrosome of its spermatozoa, in order to increase the labelling resolution. For biochemical studies, rabbit was used as acrosin has already been amply analysed in this species (Meizel and Mukerji, 1979). A preliminary account of our results was presented at the Histochemical Symposium at Nedvedice, Czechoslovakia (1983).

Material and methods.

1. — Autoradiography of guinea-pig testicular tissue.

A mature male guinea-pig was injected intratesticularly with L(6-³H)-fucose (specific activity : 740 GBq/mmol ; CEA, Service des Molécules marquées, Saclay, France) per testis ; the testes were recovered three days later and prepared for EM autoradiography using, as an additive to glutaraldehyde fixative, 0.5 % (W/V) of cetyltrimethylammonium bromide for improved glycoprotein preservation (Kopečný and Fléchon, 1981). Thin sections showing gold interference colour were coated with Ilford L.4 nuclear liquid emulsion and exposed for one month. After staining, they were observed in either a Tesla 500 or Jeol JEM CX II electron microscope. The method of relative grain concentration, used for autoradiogram analysis, indicated the level of labelling in different spermatid cell compartments.

2. — *Specificity of acrosin labelling.*

a) *Sperm labelling and collection in rabbit.* Eleven mature buck rabbits were injected intratesticularly with either L-(1-¹⁴C) fucose (Amersham ; specific activity : 2.19 GBq/nmol at a dose of about 1 MBq per testis) or D-(L-¹⁴C) glucosamine (Institute for Production, Research and Utilization of Radiotopes, Prague ; specific activity : 7.75 GBq/nmol at a dose of about 4 MBq per testis) in a volume of 0.5 ml of saline. Twenty-one days later, the caudae epididymides were isolated and the spermatozoa released through an incision in the epididymal tissue. As epididymal transit in rabbit is around 9 days (± 1.5) according to Amann, Koefoed-Johnsen and Levi (1965), the duration of the testicular phase of sperm cell development from the stage of incorporation in acrosomal glycoproteins (round spermatids) was about 12 days (± 1.5).

b) *Acid extraction of epididymal spermatozoa.* The extraction method using 0.25 M H₂SO₄ at 0 °C according to Meizel and Mukerji (1975) was applied with a modification : the luminal contents were extracted immediately after isolation. Acrosome destruction was monitored by electron microscopy. Dialyzed extracts were applied on Sephadex G-100 columns equilibrated and eluted with 0.001 M HCl. We collected 3.3 ml fractions each in 15 min per 2.7 × 95 cm column (¹⁴C-fucose) and 1.2 ml fractions each in 30 min per 1 × 48 column (¹⁴C-glucosamine). Proacrosin content and radioactivity were assayed in the fractions, and those having proacrosin activity were pooled. The pooled fractions of (¹⁴C)-glucosamine-labelled material were further purified by hydrophobic chromatography on a Spheron P-300 1 × 4.8 cm column equilibrated with 0.3 M acetic acid, 0.05 M NaCl, pH 3.0 (Železná and Čechová, 1982). After dialysis against the equilibration buffer in a volume of 6.8 ml, the sample was applied on the column, washed with the same buffer, and acrosin was eluted with a linear gradient of 1-50 % tertiarybutanol in the buffer (40 ml total).

c) *Acrosin, proacrosin and acrosin inhibitor assays.* As described elsewhere (Železná and Čechová, 1982), acrosin activity and acrosin inhibitor activity were determined spectrophotometrically using N-alfa-benzoyl-D, L-arginine-p-nitroanilide (BAPNA) as a substrate with the modification that the substrate was dissolved in di-methyl-formamide (1 mg/0.1 ml). Proacrosin was assayed as acrosin after 2 hours of autoactivation (Mukerji and Meizel, 1979). One mU of enzyme (acrosin) activity hydrolyzed one nanomole of BAPNA per min. One proacrosin unit was equal to one acrosin unit after autoactivation. One mIU of inhibitor activity reduced the acrosin activity of one mU.

d) *Measurement of radioactivity.* An aliquot (0.5 ml) of each fraction was mixed with 7 ml of Bray's solution (Spolana, Czechoslovakia) and checked for radioactivity by scintillation counting (Isocap II, Nuclear, Chicago).

e) *Electrophoretic methods.* Disc electrophoresis, protein staining and active enzyme staining were performed as described elsewhere (Železná and Čechová, 1982). Glycoproteins were assayed by a standard method using Schiff's reagent.

Results.

1. — *Fine-structure autoradiography of testicular tissue.*

In guinea-pig seminiferous epithelium at 3 days postinjection, label was found in Golgi apparatuses of spermatocytes and spermatids ; the prevailing part of the label was connected with the forming acrosome (fig. 1) : heavy labelling was seen in proacrosomal granules, the acrosomal granule and the acrosomal vesicle. Some labelling could also be attributed to postacrosomal vesicles (Courten, 1979), (fig. 2) and/or to material around the newly formed manchette. The label persisted in the acrosomal matrix in succeeding steps of acrosome development (figs. 3, 4) up to late spermatid stages with condensed nuclear chromatin. The analysis of relative grain concentration in the autoradiograms revealed that the label concentration in the spermatid acrosomal matrix was two orders of magnitude higher than that in other spermatid cell compartments.

2. — *Purification and quantification of radioactively labelled acrosin.*

The acid extracts applied on the columns contained approximately 20 % of the total radioactivity present in the luminal contents of the rabbit caudae epididymides. About 30 % of the radioactivity remained in the pellet after centrifugation, although the acrosomes had been entirely destroyed by the extraction procedure, as verified by electron microscopy. Quantitative data on further labelled acrosin purification are given on tables 1 and 2.

TABLE 1
Specific activities and purification factors after particular isolation steps of both ¹⁴C-glucosamine and ¹⁴C-fucose-labelled proacrosins.

Isolation step	¹⁴ C-Glucosamine-labelled proacrosin		¹⁴ C-Fucose-labelled proacrosin	
	Acid extract	Sephadex G-100	G-Spheron P-300	Sephadex G-100
Specific activity mU/mg . . .	2.6	90.0	400.0	280.0
Purification factor	1	35	154	108

TABLE 2
Amount of radioactivity obtained after chromatography of fractions with proacrosin activity.

Purification of proacrosin labelled by	Column bed	Acid extract applied on the column	Pooled proacrosin-rich fractions after column chromatography		
		cpm	cpm	%	(% *)
¹⁴ C-glucosamine	Sephadex G-100	8 000	4 460	43	(8.6)
	Spheron P-300	1 600	400	25	(2.1)
¹⁴ C-fucose	Sephadex G-100	3 300	1 220	37	(7.4)

(*) Percent of radioactivity in epididymal sperm sample.

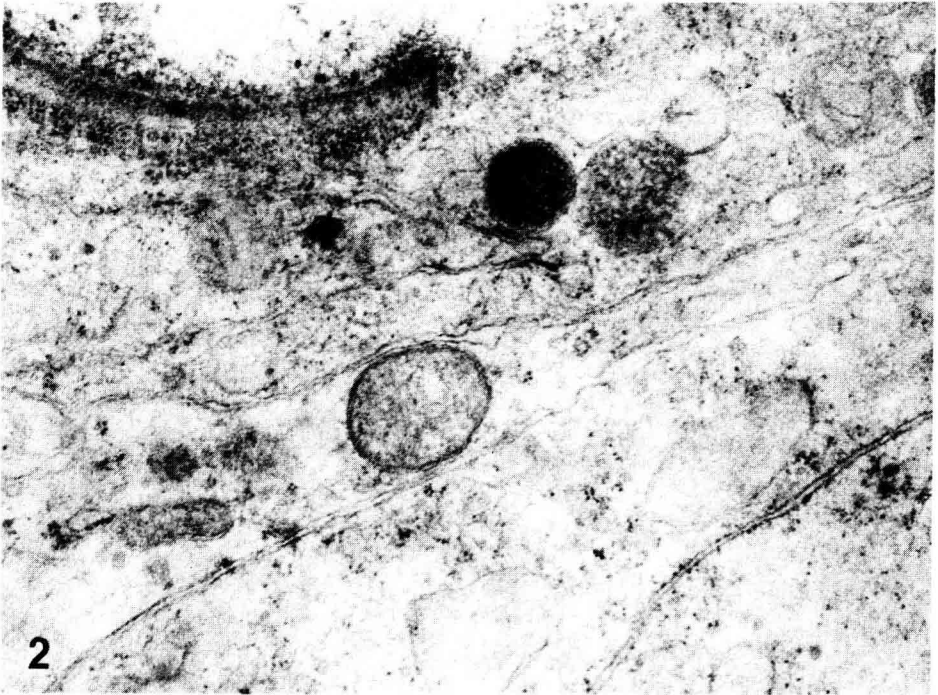
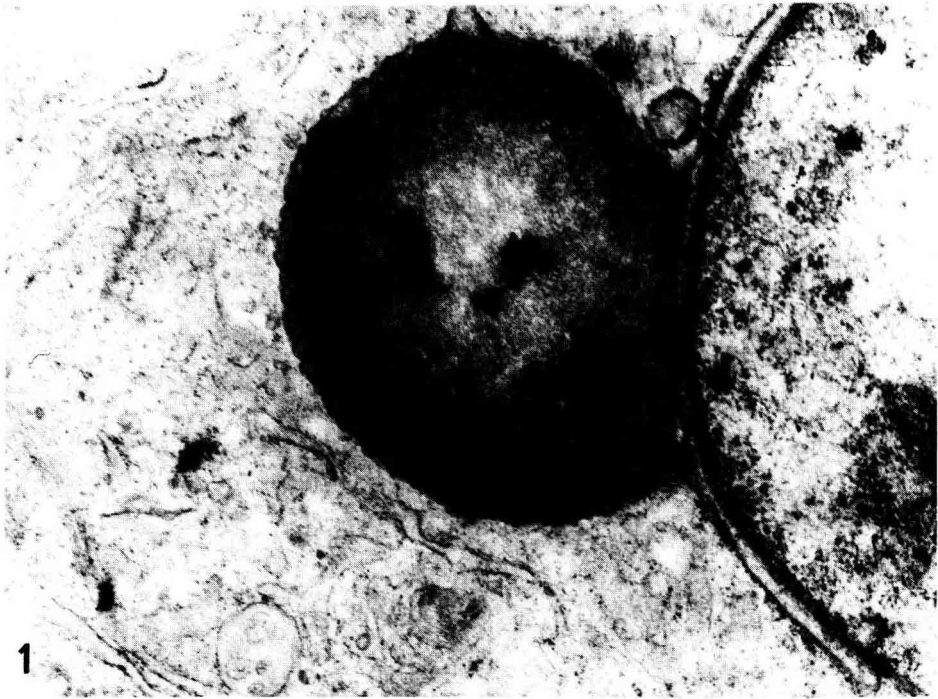


PLATE I. — Fine structure autoradiograms of guinea-pig spermatids three days after an intratesticular injection of (^3H) -fucose. Patterns of labelling observed in the acrosomal granule (FIG. 1 - $\times 17\ 000$) and a postacrosomal granule (FIG. 2 - $\times 25\ 000$) of round spermatids.

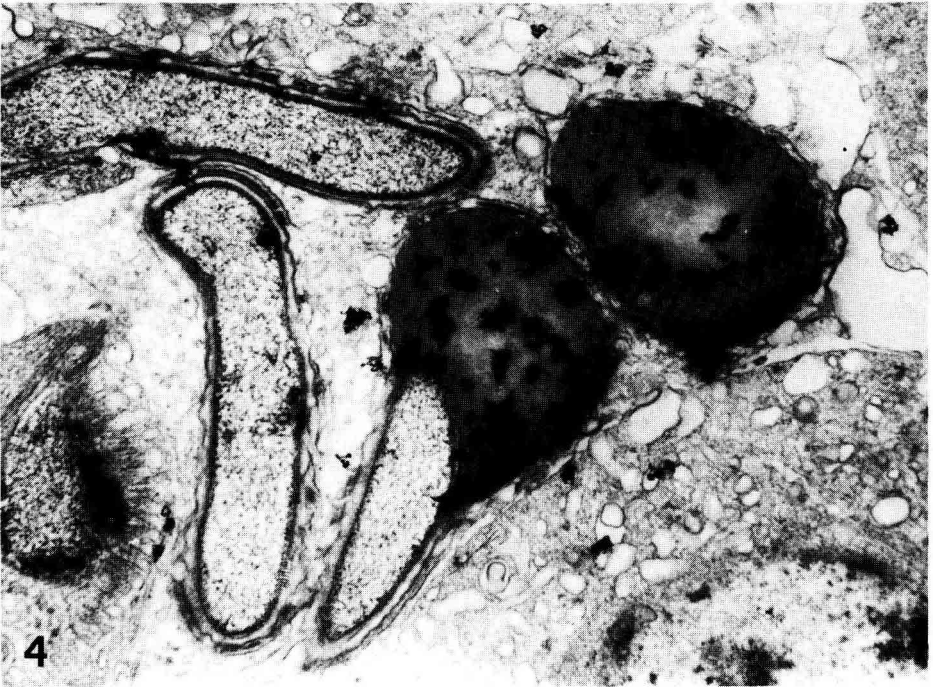


PLATE II. — Same material as for Plate I. Label accumulated in the matrix of the developing acrosome of round (FIG. 3 - $\times 7\ 000$) and elongating spermatids (FIG. 4 - $\times 9\ 000$).

The gel filtration elution profile of the acid extract from ^{14}C -fucose-labelled spermatozoa (fig. 5) shows clearly that the radioactivity peak was superimposed to the acrosin activity peak (after autoactivation); the absorbance peak was positioned just next to it, except for a small amount of radioactive material in the void volume of the column. The lyophilized material, obtained by pooling fractions with acrosin activity, showed a high acrosin activity (after autoactivation) compared to other attempts to isolate rabbit proacrosin (Meizel and Mukerji, 1975; Mukerji and Meizel, 1979).

To obtain highly purified radioactive proacrosin we used (^{14}C)-glucosamine labelling. In this second experiment, we applied column affinity chromatography after gel filtration. Spheron P-300 was chosen as a support since it was possible to use chromatography at pH 3.0, thus avoiding proacrosin autoactivation. It is seen from the elution profile that two asymmetric peaks of (A280) absorbancy and radioactivity were eluted. The peak of proacrosin activity was exactly superimposed to the second absorbancy and radioactivity peaks (fig. 6).

We obtained the highest degree of proacrosin purification (400 mU/mg) from the hydrophobic step of the chromatography (fig. 6). Proacrosin homogeneity was determined by disc electrophoresis. A single protein band showing acrosin activity after activation was also stained with Schiff's reagent. The first peak of high radioactivity (fig. 6) corresponded to strong acrosin inhibitor activity.

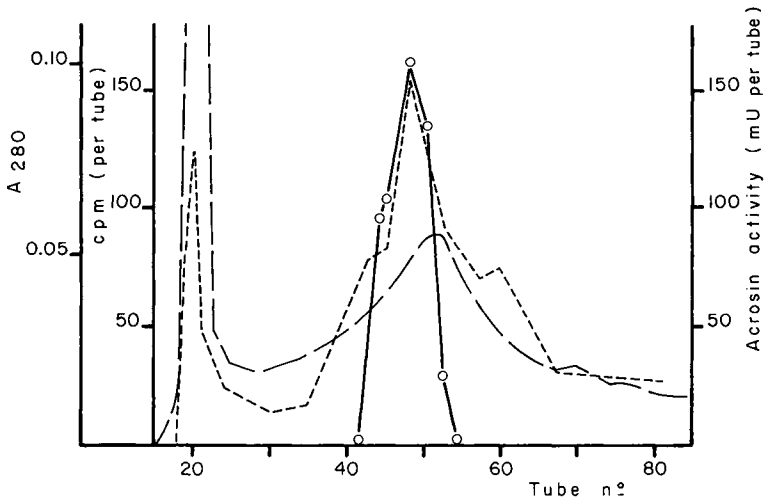


FIG. 5. — Gel filtration of acidic extracts of ^{14}C -fucose labelled spermatozoa. ——— Absorbance at 280 nm; ---- Radioactivity (minus background); ○—○ Acrosin activity after 1 hr activation. Conditions: see Material and Methods.

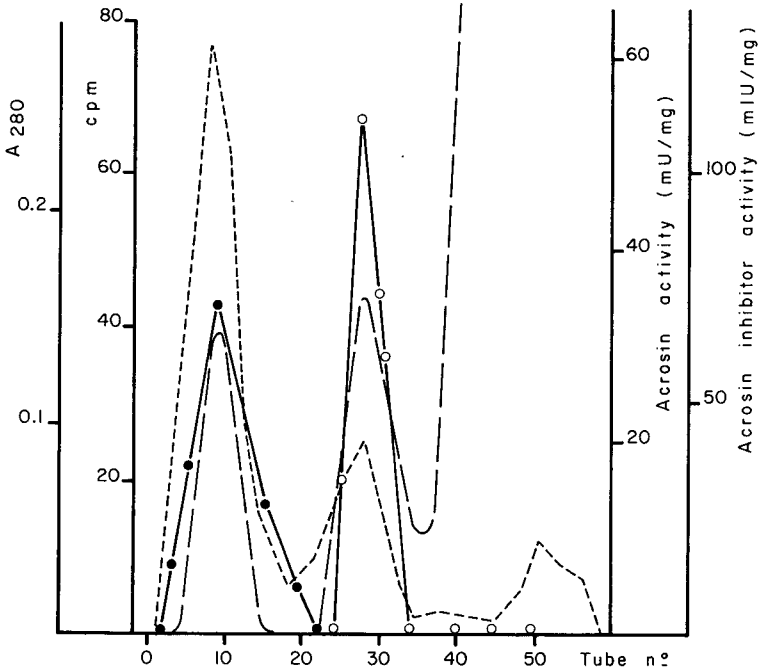


FIG. 6. — Hydrophobic affinity chromatography on Spheron P-300 of pooled proacrosin-rich fractions after gel filtration of acidic extracts of ^{14}C -glucosamine labelled rabbit spermatozoa. — Absorbance at 280 nm; --- Radioactivity (minus background); ○—○ Acrosin activity after autoactivation; ●—● Acrosin inhibition. Conditions: see Material and Methods.

Discussion.

Several stage-specific proteins are synthesized in mouse spermatocytes and spermatids at the time when protein synthesis rate decreases in the differentiating gamete (Kramer and Erickson, 1982). In pachytene spermatocytes and round spermatids of the same species more than 16 different glycoproteins are fucosylated within the molecular weight range of 20 000 to 100 000, as shown by (^3H)-fucose incorporation *in vitro* (Grootegoed *et al.*, 1982). Since (^3H)-fucose acrosome label is retained in ejaculated spermatozoa (Kopečný, 1976), it is supposed that at least some of the labelled glycoproteins take part in the formation of the mature acrosome, as rather complex cytological and cytochemical research has shown (reviewed in Tang *et al.*, 1982).

Labelled sugars appear to be relatively more specific precursors of enzymatically active material of acrosomes than amino acids: after similar (^3H)-fucose labelling in rabbit (Kopečný *et al.*, 1980) and boar (Kopečný, Sedláková and Čechová, unpublished results) a proteolytic activity is correlated with radioactivity in fractions of sperm extracts, but this correlation does not occur if

labelled amino acids, like tryptophane or glycine, are used (Kopečný, Sedlaková and Čechová, unpublished).

Our present fine-structure autoradiographic detection of newly fucosylated proteins, shortly (3 days) after their formation in spermatids, shows that their distribution is already very similar to that in mature spermatozoa analyzed 3 weeks after labelled precursor injection (Kopečný and Fléchon, 1981). It may be concluded that the evident prevailing part of newly formed glycoproteins in spermatids is specifically concentrated very soon in the acrosome. The broad spectrum of labelling of all round stages testifies that glycoprotein was continuously added to the material of the forming acrosome. Acrosin was first detected immunohistochemically in early round spermatids (Phi-Van *et al.*, 1983), and an increase in total acrosin was reported in late spermatids in comparison to early spermatids (Mansouri *et al.*, 1983).

As explained in the introduction, we chose proacrosin as a test for determining the nature of labelled acrosomal glycoproteins. In the first experiment, we labelled spermatozoa with (^{14}C)-fucose. The results obtained (fig. 5) show that proacrosin was most probably labelled.

As it has been reported that the sugar component of the rabbit proacrosin molecule contains more glucosamine than fucose (Mukerji and Meizel, 1979), the proacrosin isolation procedure was repeated (second experiment) with (^{14}C)-glucosamine-labelled spermatozoa. After isolation by two-step column chromatography, highly purified radioactive proacrosin was obtained. Without doubt the proacrosin was labelled with ^{14}C -glucosamine. So our autoradiographic and biochemical studies suggest that proacrosin is already synthesized in spermatids, as described by Mansouri *et al.*, (1983).

Another finding of the present study is that at least 2 % of the radioactivity, introduced by (^{14}C)-glucosamine and recovered in the epididymal sperm, is located in the proacrosin molecule. Most of the luminal radioactivity, as shown by autoradiography (Kopečný and Fléchon, 1981), is located solely in the acrosome, the only exception being the cytoplasmic droplet. In fact, it is supposed that the yield of radioactive proacrosin may be actually higher since Schill *et al.* (1982) reported that different physiochemical treatments, as opposed to acid extraction alone, increased total acrosin activity in sperm acid extracts by 50-200 %. Furthermore, proacrosin can be adsorbed on disrupted sperm cells (Harrison *et al.*, 1982), thus decreasing the radioactivity of the extracts used for proacrosin isolation.

Even if a higher rate of incorporation into proacrosin actually occurred, it would not be enough to equate our ultrastructural localization of labelled glycoproteins with that of this enzyme. However, the striking similarity between label and acrosin distributions may be considered from another point of view, if our present finding of the labelling of intraacrosomal glycoprotein acrosin inhibitors is confirmed. An intracellular acrosin inhibitor from boar spermatozoa was characterized as a glycoprotein probably forming a complex with proacrosin within the acrosome (Tschesche *et al.*, 1982). If so, an autoradiographic approach might permit the detection of labelled proacrosin/acrosin inhibitor complexes which, in turn, behave similarly to their cytochemically detectable component,

acrosin. Also, in the guinea-pig, acrosin and acrosin inhibitor may be associated in the acrosome and released together upon acrosome reaction (Goodpasture *et al.*, 1981).

Finally, we know that acrosomal contents are lost in two steps after acrosome reaction : readily soluble substances like hyaluronidase may diffuse instantaneously, while most of the acrosomal matrix is dispersed only when proacrosin is activated (Shams-Borhan and Harrison, 1981 ; Perreault *et al.*, 1982 ; Harrison *et al.*, 1982). This may ultimately explain why most glycoproteins — including (pro)-acrosin whose distribution seems homogeneous in the anterior segment (Huneau *et al.*, 1983) — have the same location in intact acrosomes and are solubilized according to the same pattern after acrosome reaction.

Reçu en septembre 1983.

Accepté en mars 1984.

Résumé. *Etudes sur le marquage de l'acrosome des spermatozoïdes de Mammifères par des sucres radioactifs.*

La localisation de la synthèse et du stockage de glycoprotéines pendant la formation de l'acrosome chez le cobaye a été étudiée par autoradiographie en microscopie électronique après incorporation de (³H)-fucose. Trois jours après l'injection intratesticulaire du précurseur, le marquage dans les spermatozoïdes est concentré dans la matrice des acrosomes en développement ; il apparaît que la plus grande partie des glycoprotéines fucosylées dans les spermatozoïdes sont stockées dans l'acrosome.

La contribution relative de l'acrosine au marquage des glycoprotéines a été évaluée par voie biochimique. La recherche de l'activité acrosine a été effectuée dans des spermatozoïdes de la queue de l'épididyme de lapins obtenus 21 jours après injection intratesticulaire de (¹⁴C)-fucose ou (¹⁴C)-glucosamine. Après extraction acide et filtration sur gel, une incorporation a été obtenue dans les fractions proacrosine de tous les échantillons étudiés ; la radioactivité dans la proacrosine purifiée représente au moins 2 % de la radioactivité totale du matériel. L'autre pic majeur de radioactivité après incorporation de (¹⁴C)-glucosamine pourrait correspondre à l'inhibiteur d'acrosine intraacrosomique.

Les complexes (pro)-acrosine (inhibiteur d'acrosine) semblent donc représenter une part notable des glycoprotéines acrosomiques marquées par des sucres radioactifs ; la distribution des complexes pourrait correspondre au moins partiellement à celle de leur composant cytochimiquement détectable, l'acrosine. La superposition de la localisation de l'acrosine et des autres glycoprotéines acrosomiques au cours de la réaction acrosomique peut être expliquée par le fait que la dispersion de la grande masse du contenu acrosomique suit l'activation de la proacrosine.

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