Labelling of membrane glycoproteins on rat spermatozoa collected from different regions of the epididymis

Roy JONES,*† Chumpol PHOLPRAMOOL,‡ Brian P. SETCHELL‡ and Colin R. BROWN*

*A.R.C. Institute of Animal Physiology, Animal Research Station, 307 Huntingdon Road, Cambridge CB3 0JQ, and ‡Department of Physiology, Babraham, Cambridge CB3 4AT, U.K.

(Received 27 July 1981/Accepted 7 September 1981)

The major glycoprotein on the plasma membrane of testicular spermatozoa labelled with the galactose oxidase/ NaB^3H_4 technique has mol.wt. 110 000. As spermatozoa pass through the epididymis, labelling of this glycoprotein disappears and is replaced by labelling of a 32 000-mol.wt. protein. The latter protein is a major component of epididymal secretions. The evidence suggests that it is inserted into or adsorbed on to the plasma membrane, and since its appearance on spermatozoa correlates with the acquisition of fertilizing capacity it should serve as a good marker for assessing maturation *in vitro*.

Spermatozoa acquire their motility and fertilizing capacity during passage through the epididymis. Current evidence indicates that changes in membrane glycoproteins play an important role in this process. Olson & Hamilton (1978) used the galactose oxidase/ NaB^3H_4 technique to label membrane glycoproteins and observed uptake of radiolabel into a 37 000-mol.wt. protein on spermatozoa from the cauda but not the caput epididymidis. As a prelude to examining the spermatozoal plasma membrane during maturation, we have investigated the hormonal regulation of protein synthesis in the rat epididymis (D'Agostino *et al.*, 1980; Jones *et al.*, 1980). We found that the synthesis of four proteins of mol.wts. 18 500, 19 000, 32 000 and 47 000 was androgen-dependent. These proteins are also major components of the secreted luminal plasma. In the present investigation we have used the galactose oxidase technique to label glycoproteins of spermatozoa collected by micropuncture from different levels of the epididymal duct, and compared the glycoproteins so labelled with those shown previously to be synthesized and secreted into the epididymal lumen.

Materials and methods

Materials

All chemicals were of the highest purity available commercially and were obtained from sources

† To whom reprint requests should be addressed.

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

described previously (Jones *et al.*, 1980). [^{14}C]-Methylated molecular-weight marker proteins and NaB^3H_4 (450–500 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Galactose oxidase (EC 1.1.3.9) from *Dactylium dendroides* was obtained from Worthington Enzymes (Millipore Ltd., Abbey Road, London NW10 7SP, U.K.).

Collection of spermatozoa

Spermatozoa were collected from four sites in the epididymis of anaesthetized Wistar rats by using micropuncture techniques described previously (Hinton *et al.*, 1979). The sampling sites are shown in Fig. 1. Approx. 1 μl of luminal contents was collected over a period of 1–2 h, diluted into 300 μl of iso-osmotic buffered sucrose (0.264 M-sucrose/5 mM-Hepes, pH 7.4), and the spermatozoa were washed free of surrounding secretions by layering over 1 ml of 0.3 M-sucrose/5 mM-Hepes, pH 7.4, and centrifuging at 600 g for 10 min. The first supernatant, representing dilute epididymal plasma, was removed and stored frozen at -20°C , and the infranatant or wash fluid was discarded. The washed spermatozoal pellet was resuspended in iso-osmotic buffered sucrose and labelled as described below. Testicular spermatozoa were collected by puncturing the extratesticular rete testis 16–18 h after ligation of the efferent ductules. Spermatozoa were flushed from the cauda epididymidis of normal rats, and of rats castrated 3 days earlier, by retrograde injection of iso-osmotic buffered sucrose via the ductus deferens (Jones *et al.*, 1980); all spermatozoa were washed as described above.

Fig. 1. Sketch of rat epididymis (testis removed) illustrating the sites from which spermatozoa were removed by micropuncture for labelling experiments

Labelling of spermatozoal plasma-membrane glycoproteins and epididymal secretions

Galactose and *N*-acetyl-D-galactosamine residues on glycoproteins were labelled with galactose oxidase and NaB^3H_4 (Gahmberg & Hakomori, 1973; Olson & Hamilton, 1978). To $200\mu\text{l}$ of washed spermatozoa (1×10^7 – 2×10^7 cells total) in iso-osmotic buffered sucrose was added approx. $250\mu\text{Ci}$ of NaB^3H_4 dissolved in the same buffer and 2 Sigma units of galactose oxidase (1 unit will produce a ΔA_{425} of 1.0/min at pH 6.0 and 25°C in a peroxidase/*o*-toluidine system). The final volume was adjusted to $220\mu\text{l}$ with buffered sucrose. Spermatozoal suspensions were incubated at 25°C for 10 min, and then washed through 1 ml of 0.3 M-sucrose to remove free label and enzyme. Labelled spermatozoa were then incubated with $100\mu\text{l}$ of 0.2% (w/v) sodium deoxycholate in iso-osmotic buffered sucrose, pH 8.0, for 1 h at 4°C , centrifuged at $10000g$ for 5 min, and the extracted proteins stored frozen at -20°C . Total incorporation of NaB^3H_4 into protein was measured by mixing $20\mu\text{l}$ portions of spermatozoal suspensions with excess ice-cold 10% (w/v) trichloroacetic acid and collecting precipitated protein on GF/C filters. Controls containing no enzyme, enzyme + NaB^3H_4 , or NaB^3H_4 alone were also included to assess non-specific uptake of radiolabel. Radioactivity was measured in 5 ml of Packard Scintillator 299 with a Nuclear-Chicago liquid-scintillation counter. A time-course of total incorporation of radiolabel

showed that, under the conditions described above, maximum uptake was reached within 10 min after the addition of galactose oxidase.

Proteins in testicular fluids and in epididymal plasma collected from the different sampling sites were dialysed against several changes of distilled water and freeze-dried. A sample, containing $5\mu\text{g}$ of protein, was incubated with 3 units of galactose oxidase for 30 min at 25°C in $100\mu\text{l}$ of iso-osmotic buffered sucrose. Approx. $200\mu\text{Ci}$ of NaB^3H_4 was added, and after 10 min at 25°C the protein was precipitated with 9 vol. of ethanol. Centrifuged protein pellets were dissolved in Tris/SDS/ β -mercaptoethanol buffer (see below) and analysed on denaturing polyacrylamide gels. Preliminary experiments showed that with plasma from the cauda epididymidis, incorporation of NaB^3H_4 into acid-insoluble material was linear between 1 and $10\mu\text{g}$ of total protein incubated.

Electrophoresis

Proteins extracted with detergent were precipitated with 9 vol. (v/v) of ethanol, then dissolved in 62.5 mM-Tris/HCl, pH 7.4, containing 1% (w/v) SDS and 1% (v/v) β -mercaptoethanol, and analysed on 15% (w/v) denaturing polyacrylamide slab gels (Jones *et al.*, 1980). After electrophoresis proteins were stained with Coomassie Brilliant Blue and radioactive proteins detected by fluorography (Bonner & Laskey, 1974).

Other procedures

Total protein was measured by the Hartree (1972) modification of the Lowry procedure. Bovine serum albumin was used as a standard. Fluorographs were quantified by densitometry on pre-flashed film.

Results

Labelling of membrane-bound glycoproteins

The amount of NaB^3H_4 incorporated into membrane glycoproteins depended principally on the region of the epididymis from which the spermatozoa were collected (Table 1). Acid-insoluble radioactivity on spermatozoa collected directly from the testis and incubated in the presence of galactose oxidase and NaB^3H_4 was about 150% higher than for controls. However, this fell to approx. 90% for spermatozoa from the proximal caput epididymidis (site 1) and to only 25% for spermatozoa collected from the distal caput epididymidis (site 2). A 75% increase was found with spermatozoa from the distal corpus epididymidis (site 3), and with spermatozoa from the cauda epididymidis (site 4) the percentage increase above controls (250%) was greater than that found with testicular spermatozoa.

Analysis of deoxycholate extracts prepared from labelled spermatozoa on SDS/polyacrylamide gels

showed that over 80% of the radioactivity detected in detergent extracts of testicular spermatozoa was associated with a 110000-mol.wt. polypeptide (Fig. 2a). However, labelling of this 110000-mol.wt. polypeptide decreased sharply in spermatozoa taken

Table 1. *Total incorporation of NaB³H₄ into acid-insoluble protein on spermatozoa*

Spermatozoa were collected from the testis and sites 1–4 in the epididymis, washed, and labelled with galactose oxidase and NaB³H₄. Total incorporation of radiolabel was measured by precipitation with trichloroacetic acid, and results are expressed as the percentage stimulation caused by the addition of galactose oxidase. The values shown are calculated from the value for spermatozoa + galactose oxidase + NaB³H₄ minus the value for NaB³H₄ alone, compared with the sum of the values for spermatozoa + NaB³H₄ and for galactose oxidase + NaB³H₄, both corrected by subtracting the value for NaB³H₄ alone. These controls were necessary, as some NaB³H₄ was bound non-specifically to glass-fibre filters, to spermatozoa, and to galactose oxidase itself. Values are means $\pm$ S.E.M. for three experiments.

Spermatozoa collected from:	Stimulation above controls (%)
Testis	148 $\pm$ 31
Epididymal site 1	91 $\pm$ 28
Epididymal site 2	25 $\pm$ 8
Epididymal site 3	75 $\pm$ 17
Epididymal site 4	250 $\pm$ 52

from the proximal caput epididymidis (site 1), and it was not detectable thereafter on spermatozoa removed from more distal regions of the epididymal duct. Instead, an increasing amount of incorporated NaB³H₄ was associated with a 32000-mol.wt. polypeptide. Labelling of this polypeptide was first detected on spermatozoa from the distal caput epididymidis (site 2) and reached a maximum on spermatozoa from the cauda epididymidis (site 4), where it constituted approx. 85% of the total label. It was also noticeable that there was labelling of a 13500-mol.wt. polypeptide on spermatozoa from the distal corpus epididymidis, but this disappeared when spermatozoa reached the distal cauda epididymidis.

Thus the intensity of labelling of the 32000-mol.wt. protein on epididymal spermatozoa correlates with the acquisition of their fertilizing capacity (Dyson & Orgebin-Crist, 1973). Dyson & Orgebin-Crist (1973) have also shown that the fertilizing capacity of spermatozoa removed from the cauda epididymidis of rats castrated 3 days previously has decreased to approx. 15% of those from normal animals. As shown in Fig. 2(b), labelling of the 32000-mol.wt. polypeptide on spermatozoa from rats castrated 3 days previously was approx. 12% of that on normal spermatozoa.

Labelling of secreted glycoproteins

In all samples of spermatozoa-free epididymal plasma, a considerable amount of labelled material

Fig. 2. *Fluorographs of galactose oxidase/NaB³H₄-labelled spermatozoa and epididymal secretions*
(a) and (b), Proteins extracted with detergent from labelled spermatozoa were analysed in SDS/polyacrylamide gels and radioactivity was detected by fluorography. (a): (i) Testicular spermatozoa; (ii), (iii), (iv) and (v), epididymal spermatozoa removed by micropuncture from sites 1, 2, 3 and 4 respectively. (b): (vi) Epididymal spermatozoa flushed from site 4 of normal animals. (vii) Epididymal spermatozoa flushed from site 4 of 3-day-castrated animals. (c), Labelled proteins in testicular fluid (viii), and epididymal secretions from site 1 (ix), site 2 (x), site 3 (xi) and site 4 (xii) of normal rats. Each track received approx. 10000 c.p.m.

either remained in the stacking gel or was associated with high-molecular-weight proteins (Fig. 2c). However, in plasma from the distal corpus and cauda epididymidis an increasing amount of label was also found in a 32 000-mol.wt. polypeptide. Less radioactivity was associated with high-molecular-weight components in testicular fluid than in epididymal plasma (Fig. 2c), but a significant, albeit small, amount of label was detected on a polypeptide of approx. 110 000 mol.wt., which co-migrated with the major labelled polypeptide on testicular spermatozoa.

Discussion

In this investigation it was important that spermatozoa collected from different regions of the epididymis should be free from blood, lymph and epididymal epithelial cells, since even a small amount of extraneous tissue could give spurious results in subsequent labelling experiments. Micropuncture procedures, although technically demanding, are probably the best methods currently available to meet this requirement. Using these techniques we have found that as spermatozoa pass through the epididymis there is a considerable alteration in the pattern of glycoproteins on the plasma membrane that are labelled with galactose oxidase and NaB^3H_4 . The change from labelling of a 110 000-mol.wt. glycoprotein on testicular spermatozoa to a 32 000-mol.wt. glycoprotein on cauda-epididymal spermatozoa takes place in a sequential and ordered fashion in the caput region. Labelling of the 110 000-mol.wt. polypeptide decreases to very low values after spermatozoa have passed through the initial segment and disappears altogether before the 32 000-mol.wt. protein can be detected in the distal caput. Since spermatozoa normally pass through the initial segment within 1–2 days, this suggests that there is a rapid and controlled interaction between the spermatozoal plasma membrane and the luminal secretions. A decrease in labelling of a glycoprotein by this technique may be caused by one of the following. (1) Complete removal of the glycoprotein from the membrane, e.g. by proteinases. (2) Removal of galactose and galactosamine residues from the oligosaccharide chain by glycosidases. In this context it is worth noting that the epididymis is the richest source of glycosidases in the animal body and that secretion of these enzymes into the luminal plasma is androgen-dependent (Conchie & Mann, 1957; Jones, 1974). (3) Masking of existing membrane proteins by the addition of new surface

proteins. At present it is not possible to distinguish between these three possibilities, although circumstantial evidence favours the second one. Similar arguments may be invoked for the appearance and disappearance of the 13 500-mol.wt. protein on cauda-epididymal spermatozoa.

The observation that labelling of the 32 000-mol.wt. protein on spermatozoa takes place concomitantly with its appearance in the luminal secretion suggests that this protein is inserted into or adsorbed on to the plasma membrane. Our own results, and those of others, indicate that it is synthesized in the distal caput epididymidis and is androgen-dependent (Cameo & Blaquier, 1976; Lea *et al.*, 1978; Jones *et al.*, 1980). It is the most acidic protein in the epididymal secretions and may account for the increase in electronegativity of spermatozoa as they pass down the epididymis (Moore, 1979). Furthermore, the correlation between the intensity of labelling of the 32 000-mol.wt. polypeptide and the fertilizing capacity of spermatozoa suggests that it may play a role in fertilization, either in cellular recognition or in the acrosome reaction. Whatever its function, it should provide a good marker for investigating spermatozoal maturation *in vitro*.

We thank Mr. K. I. von Glos for technical assistance and the World Health Organisation for the award of a grant to C.P.

References

- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Cameo, M. S. & Blaquier, J. A. (1976) *J. Endocrinol.* **69**, 47–55
- Conchie, J. & Mann, T. (1957) *Nature (London)* **179**, 1190–1191
- D'Agostino, A., Jones, R., White, R. & Parker, M. G. (1980) *Biochem. J.* **190**, 505–512
- Dyson, A. L. M. B. & Orgebin-Crist, M. C. (1973) *Endocrinology* **93**, 391–402
- Gahmberg, C. G. & Hakomori, S. (1973) *J. Biol. Chem.* **248**, 4311–4317
- Hartree, E. F. (1972) *Anal. Biochem.* **48**, 422–427
- Hinton, B. T., Dott, H. M. & Setchell, B. P. (1979) *J. Reprod. Fertil.* **55**, 167–172
- Jones, R. (1974) *J. Endocrinol.* **63**, 157–165
- Jones, R., Brown, C. R., von Glos, K. I. & Parker, M. G. (1980) *Biochem. J.* **188**, 667–676
- Lea, O. A., Petrusz, P. & French, F. S. (1978) *Int. J. Androl. Suppl.* **2**, 592–607
- Moore, H. M. D. (1979) *Int. J. Androl.* **2**, 449–462
- Olson, G. E. & Hamilton, D. W. (1978) *Biol. Reprod.* **19**, 26–35