TANNIC ACID FIXATION OF HUMAN SPERMATOZOA

GODFRIED M. ROOMANS

*Wenner-Gren Institute, University of Stockholm, Norrtullsgatan 16,
S-113 45 Stockholm, Sweden*

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A new fixation technique using tannic acid in combination with glutaraldehyde has recently been developed (Mizuhira & Futaesaku, 1972). Tannic acid is reported to precipitate with polypeptides and proteins, and to make stable chelate compounds with osmium and uranyl acetate. As a result, the precipitate gives a high electron density corresponding to the site of proteins. These properties make the method suitable for the fixation of biological structures which, like cytoplasmic membranes, contain protein.

The membranes in the mammalian sperm head play an important rôle in the fertilization process. The acrosome reaction involves fusion of the plasma membrane and outer acrosomal membrane with subsequent release of the acrosomal contents (Bedford, 1970). Other changes in the sperm head involving membrane processes have also been demonstrated (Jones, 1973). The ultrastructure of the human spermatozoon has been scrutinized by Pedersen (1974) who used normal glutaraldehyde fixation followed by osmium post-fixation. The aim of the present study was to provide additional data on the properties of the sperm membranes, by means of the tannic acid fixation technique.

Human spermatozoa were obtained from healthy volunteers. To the liquefied semen, 2 vols of veronal acetate buffer, pH 7.4, containing 8% sucrose, were added. The sample was then centrifuged in small plastic tubes at 1000 *g* in the cold, and the ends of the tubes containing the pellets were cut off and placed in the fixative. This procedure removed most of the seminal plasma, which otherwise precipitates with the tannic acid. The fixative contained 2% tannic acid, 2.5% glutaraldehyde, and 1.7% sodium chloride in veronal acetate buffer, pH 7.4. Fixation was carried out for 1 hr at 4°C. The material was then rinsed for 1 hr in veronal acetate buffer containing 8% sucrose, post-fixed in 1.5% osmium tetroxide, dehydrated in a graded ethanol series and embedded in Epon (Luft, 1961). During dehydration, the material was gently removed from the centrifuge tubes. Thin sections were cut with a diamond knife on an LKB Ultratome ultramicrotome set at about 600 Å section thickness, stained with uranyl acetate and lead citrate (Reynolds, 1963), and examined in a JEOL 100C electron microscope fitted with a high resolution goniometer.

The ultrastructure of the sperm head was seen to correspond basically to that described by Pedersen (1974). The membranes stained heavily. The plasma membrane was generally well preserved, and its three-layered structure could be clearly seen, both opaque layers having approximately the same density

(Pl. 1, Fig. 1). A striking asymmetry, however, was observed in the membranes of the acrosome (Pl. 1, Fig. 2). Both in the inner and the outer acrosomal membranes, the outer opaque layer (i.e. the layers facing the nuclear and the plasma membrane, respectively) was more densely stained than the inner layer, which was sometimes hardly discernible from the acrosomal contents. The distance between the plasma membrane and the outer acrosomal membrane was small and in some places the membranes seemed to touch. The sub-acrosomal and peri-acrosomal cytoplasm was clearly visible. The nuclear membrane could be clearly seen and presented a five-layered appearance (Pl. 1, Figs 2 and 4). This appearance did not change, when, by using the goniometer stage, the angle of viewing was altered. In the middle piece, the mitochondrial membranes were symmetrically stained, as was also the plasma membrane (Pl. 1, Fig. 5).

Though the results obtained with the tannic acid-glutaraldehyde fixation method were in general agreement with those based on glutaraldehyde fixation only, there were also striking differences.

The asymmetrical appearance of the membrane limiting the acrosome, contrary to the symmetrical structures of the other membranes, was not expected on the basis of the Danielli-Davson (1935) or the Singer (1972) model. As the acrosomal membrane appears symmetrical in other fixatives, a major difference in protein content between the outer and the inner opaque layer is unlikely. The explanation of the asymmetry may lie in the permeability properties of the acrosomal membrane. Tannic acid penetrates the plasma membrane and also the mitochondrial membrane, so that the precipitates of tannic acid with the membrane proteins are formed on both sides of the membrane. In the case of the acrosomal membrane, little or no tannic acid appears to penetrate the membrane, and it does not stain the acrosomal contents. The tannic acid-protein precipitate is formed on the outside of the membrane only, and as a result it gives an asymmetrical appearance. The distance between the plasma membrane and the outer acrosomal membrane after glutaraldehyde-tannic acid fixation is smaller than after fixation with glutaraldehyde only. With the latter fixative, the thickness of the membranes was about 120 Å (Pl. 1, Fig. 3), which is in good agreement with the value given by Austin & Bavister (1974). Jones (1971) demonstrated that one of the factors affecting the distance between the plasma

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EXPLANATION OF PLATE 1

Abbreviations: a = acrosome; n = nucleus.

FIG. 1. Cross-section through the head of a human spermatozoon fixed with tannic acid. The distance between the plasma membrane and the outer acrosomal membrane is small, and sometimes these membranes seem to join (arrows). $\times 94,000$.

FIG. 2. High magnification of the acrosome. The cross-sectioned acrosomal membranes (arrows) are asymmetrically stained. The five-layered appearance of the nuclear membrane is clearly visible. $\times 280,000$.

FIG. 3. Cross-section of acrosome fixed with glutaraldehyde only and post-fixed with osmium. The distance between the plasma membrane and the acrosome is about 120 Å, and the acrosomal membranes are symmetrically contrasted. $\times 280,000$.

FIG. 4. Close union between the plasma membrane and the nuclear membrane posterior to the acrosome limiting the post-acrosomal sheath. The nuclear membrane has a five-layered structure. $\times 190,000$.

FIG. 5. Longitudinal section through the mid-piece of a human spermatozoon. The membranes are clearly and symmetrically stained. $\times 125,000$.

PLATE 1

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membrane and the acrosome was the concentration of the buffer; a reduction of the buffer concentration resulted in a separation of the plasma membrane from the underlying structures. As an increase of the osmotic value of the glutaraldehyde fixative by the addition of 200 mM-NaCl did not decrease the distance between the plasma membrane and the acrosome, osmotic forces are unlikely to have caused the decreased distance in the tannic acid fixative.

Due to the precipitation of membrane proteins with tannic acid, the surface charges of the two membranes facing each other could be masked, promoting apposition. At places where the distance is small enough, a linkage of the membranes by precipitation with tannic acid might occur. Lateral fusion of membranes is a common phenomenon that can be produced by treatment with basic proteins or divalent cations (Green, 1972). Tannic acid might have a similar effect on two apposed membranes. Austin & Bavister (1974) suggested that this type of mechanism, also involving divalent cations, might be responsible for the fusion between the plasma membrane and the outer acrosomal membrane which is the first step in the acrosome reaction. An attempt was therefore made to increase the incidence of the membrane fusions by exchanging the sodium ions in the fixative for calcium and magnesium ions, but this did not appear to result in any increase in the number or extension of the membrane fusions.

The nuclear membrane is more densely stained after tannic acid fixation than after normal glutaraldehyde fixation and stands out more clearly against the chromatin, which is left virtually unstained. This reveals more details about the structure of the nuclear membrane. As the five-layered appearance of the nuclear membrane does not change with the angle of viewing, it can be concluded that the human sperm nucleus is surrounded by a double membrane (Pl. 1, Fig. 2), presumably formed by lateral fusion. The membrane possesses the characteristics of a three-layered membrane (Green, 1972). The middle layer is more electron-dense and wider than the outer layers. A double nuclear membrane has been demonstrated by the freeze-etching technique in the spermatozoa of *Periplaneta americana* (Liu, 1973). It can also be seen that the post-acrosomal sheath is limited posteriorly by a close union of the plasma membrane and the nuclear membrane (Pl. 1, Fig. 4) as reported by Pedersen (1974).

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