

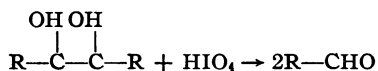
*THE CHEMICAL NATURE OF THE ACROSOME
IN THE MALE GERM CELLS*

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The close connection of the acrosome of the animal sperm with the Golgi apparatus was first suggested by Bowen¹ in 1923, who stated that the acrosomal material might be "secreted" from the Golgi apparatus. While his cytological studies seem to support the concept that the acrosome is derived from the Golgi apparatus, the staining methods employed do not allow any conclusions as to the chemical nature of either structure. The recent development of cytochemical procedures, using specific staining reactions for the chemical characterization of cellular constituents *in situ* now makes possible an analysis of some of the chemical components of the dictyosomal material²² and the acrosome of the male germ cells. *Arvelius albopunctatus*, an hemipteran insect, provides an especially favorable material for the cytochemical studies, since the different lobes of the testis are characterized by a constant and marked difference in the size of their spermatocytes, spermatids and sperms.^{2, 19, 20} In the present paper evidence is presented that the acrosome of the sperm is derived from the dictyosomes of the primary spermatocytes, and that both contain polysaccharides in a 1,2 glycol grouping. The amount of polysaccharides in the dictyosomal material and in the acrosome of spermatocytes, spermatids and sperms is strikingly higher in the large sized cells of the third and fifth lobes than in the normal and small sized cells of the remaining four lobes. For the identification of the polysaccharides in the cells of the testis we used the microchemical periodic acid Schiff (PAS) reaction resulting in the staining of polysaccharide structures in tissues as described by Hotchkiss⁵ in 1948. According to Hotchkiss⁵ and McManus,¹² the reaction of periodic acid with carbohydrates, when present as 1,2 glycol grouping, is considered to be the following:



The aldehydes which are formed after periodic acid oxidation from 1,2 glycols in sections form a colored complex with the Schiff reagent. In order to characterize the type of polysaccharides in our tissues, we used the Hotchkiss reaction in combination with the acetylation technique of McManus and Cason¹³ and with enzymes such as amylase, diastase and various hyaluronidases (derived from bull testis and from bacteria).²³

Furthermore additional specific staining for desoxyribosenucleic acid (DNA) by means of methyl-green,¹⁴ and for basic proteins by means of fast-green²⁰ was secured simultaneously in the same sections. The detailed technique of these procedures as well as a standardization of the Hotchkiss reaction for quantitative estimation of polysaccharides in tissues will be described in another publication (in collaboration with Orbison and Lieb).

The testes of Arvelius²⁴ were fixed in Carnoy's acetic-alcohol and sectioned at 6, 10 and 16 microns. Staining was always performed in the same way under standardized conditions, and enzyme experiments, such as for instance the treatment with hyaluronidase, were made with sections directly adjacent to the control section (without periodic acid) and to the test slide (with periodic acid but without the enzyme). The amount of polysaccharides was judged on the basis of the intensity of the Schiff color after periodic acid oxidation in individual cells by the photometric microscopic method, as described by Schrader and Leuchtenberger²⁰ and with an apparatus of the type designed by Pollister and Moses.¹⁵ Control sections, without periodic acid oxidation, did not show any development of color after exposure to the Schiff reagent. For the absorption measurements of the Schiff color in the sections, acrosomal material of spherical shape was selected in large and in normal sized cells which were in the same stage of development and were present in the same slide. Photometric measurements of the acrosome in the small sized cells were not possible, due to their small dimensions. The amounts of polysaccharides are expressed in arbitrary PAS units (periodic acid Schiff) and are obtained by multiplying the extinctions by the square of the radius of the sphere of the acrosome.

The third and fifth lobes of the testis, which contain the large sized cells, show the same picture in regard to size, as well as the PAS color of the dictyosomes and their behavior in the formation of the acrosome. The lobes carrying the normal and small sized cells show a markedly smaller amount of acrosomal material, though essentially the same steps of development can be observed.

The formation of the acrosomal material from the dictyosomes in the large sized cells, as seen in slides treated with PAS and counterstained with methyl-green may be outlined as follows:

(a) In the confused stage of the first spermatocytes, the dictyosomes tend to form larger aggregates in the vicinity of the nuclear membrane. This dictyosomal material shows the characteristic red stain resulting from the PAS treatment, a stain not shown by the nucleus, nucleolus and the cytoplasm.

(b) In the course of the two spermatocyte divisions, the dictyosomal material is distributed approximately equally to the resulting spermatids, and is present in the latter as a granular mass. This constitutes the so-

called acroblast. The large Nebenkern does not stain after PAS at this or any later stage.

(c) The acroblast gives rise to the acrosome which at first is applied to one side of the spherical nucleus as a round cap, staining an intense red after PAS treatment.

(d) In the succeeding stage, this cap appears to become more liquid and extends over half or more of the still spherical nucleus.

(e) When the nucleus elongates, the acrosomal material elongates simultaneously. But the long, pointed acrosome of the finished sperm is not molded solely by the lengthening of the sperm nucleus, since it extends far beyond the anterior tip of the latter.

While all the earlier stages (a-d) show the characteristic methyl-green staining of the DNA in the nucleus, no such color is discernible with certainty in the final stages. Apparently this is due to the fact that the disproportionately large amount of PAS positive material in the large cells completely covers the nucleus of the elongated phase. That DNA is present in such spermatids in a normal quantity has already been demonstrated by Schrader and Leuchtenberger.²⁰ Indeed, in the small and normal sized spermatids, in which the quantity of DNA is the same as in the large cells just described, there is no difficulty in observing the methyl-green stain of the DNA in the nucleus, for the relatively much smaller amount of acrosomal material does not obscure it.

TABLE 1

COMPARISON OF AMOUNTS OF POLYSACCHARIDES (PERIODIC ACID SCHIFF REACTION) IN THE ACROSOME OF LARGE AND NORMAL SIZED SPERMATIDS OF ARVELIUS ALBOPUNCTATUS, BY MICROSCOPIC PHOTOMETRIC MEASUREMENTS

LOBE OF TESTIS	TYPE OF CELL	NUMBER MEASURED	ACROSOME		POLY-SACCHARIDES, MEAN AMOUNT IN ARBITRARY PAS UNITS PER ACROSOME
			MEAN DIAMETER IN MICRONS	MEAN EXTINCTION	
Third (large sized cells)	Early spermatid	10	4.75	1.0 ± 0.02	5.76 ± 0.16
Sixth (normal sized cells)	Early spermatid	10	0.76	1.0 ± 0.03	0.14 ± 0.04

From the cytological studies there seems to be no doubt that the PAS positive material of the acrosome in the spermatids and sperms is derived from the PAS positive material in the dictyosomes of the primary spermatocytes (confused stage). A similar "gradual transformation of the Golgi material of the young spermatid into the sperm cap and acrosome," also by the use of the Hotchkiss reaction, has been observed by Leblond⁸ in the rat testis.

The striking increase of the amount of PAS positive material in the acrosome of the large sized cells as compared with the amount in the

normal sized cells is demonstrated in table 1. On the basis of the measurements tabulated in table 1, it appears that the acrosome of the large-sized spermatids contains considerably more polysaccharides (about 40 times more) than the acrosome of the normal sized spermatids in the same meiotic stage. This increase in carbohydrates in the large sized cells is in accordance with the previously reported increase in proteins and ribonucleic acid of these cells²⁰ and supports the concept of Schrader and Leuchtenberger that in Arvelius the increase in volumes of the nucleus, nucleolus and cytoplasm in the large sized cells, as compared with those of the normal and small sized cells, represents a true growth.

TABLE 2

EFFECTS OF VARIOUS REAGENTS AND ENZYMES ON THE POLYSACCHARIDES (AS DEMONSTRATED BY THE PERIODIC ACID SCHIFF REACTION) OF THE DICTYOSOMAL AND ACROSOMAL MATERIAL OF THE GERM CELLS OF THE TESTIS OF ARVELIUS ALBOPUNCTATUS

REAGENT	CONCENTRATION	TIME OF EXPOSURE	TEMPERATURE	PAS REACTION
Acetic anhydride	13 cc. }	45 min.	Room	Negative
+	+ }			
Pyridine	20 cc. }			
Acetic anhydride	13 cc. }	45 min.	Room	...
+	+ }			
Pyridine	20 cc. }			
followed by KOH	0.1 N	45 min.	Room	Positive
Methanol chloroform	1:1	24 hrs.	60°C.	Positive
Saliva	Conc.	30 min.	Room	Positive
Amylase (Fisher Scientific "Amylopsin")	1%	60 min.	37°C.	Positive
Diastase (Merck U. S. P. IX)	1%	60 min.	37°C.	Positive
Schering hyaluronidase bull testis A	4 T.R.U. per 1 cc.	24 hrs.	37°C.	Positive
Schering hyaluronidase bull testis B	3.3 T.R.U. per 1 cc.	24 hrs.	37°C.	Positive
Wyeth hyaluronidase bull testis	140 T.R.U. } 70 T.R.U. } per 1 cc.	24 hrs.	37°C.	Positive
<i>Clostridium welchii</i> hyaluronidase	24 hrs.	37°C.	Positive

Some further analysis as to the chemical nature of this differential growth was attempted and from the results presented in the first two rows of table 2 it is evident that the chemical groups of the acrosomal material reacting with the Schiff reagent after periodic acid oxidation are aldehydes derived from 1,2 glycol grouping of carbohydrates. Using the reversible acetylation technique in tissue sections, as described by McManus and Cason,¹³ the acetylation of the 1,2 glycols by acetic anhydride prevents the

formation of aldehydes after periodic acid oxidation and thus gives a negative PAS reaction, as seen in the first row of table 2. The removal of the acetyl groups from the acetylated 1,2 glycols by 0.1 *N* KOH restores the 1,2 glycol linkage and thus allows the formation of aldehydes after periodic acid oxidation, which color with the Schiff reagent, as seen in row 2 of table 2. That the positive PAS reaction is actually due to 1,2 glycols of carbohydrates and not to similar groups of glycolipids is shown by the experiments recorded in the third row of table 2; here the method devised by Gersh⁴ showed that an extraction of glycolipids with hot methanol chloroform resulted in no effect on the positive PAS reaction. Furthermore it is obvious from table 2 that the polysaccharide content of the dictyosomal and acrosomal material is not due to the presence of starch or glycogen, because pretreatment of the cells with amylase, diastase or saliva did not change the positive PAS reaction. Control slides containing glycogen in liver cells, fixed and treated in the same manner as the testis slides of Arvelius, showed a negative PAS reaction of the glycogen granules after diastase and saliva treatment. It is further evident that different types of hyaluronidases, even in concentrations as high as 140 Turbidity Units (T.R.U.) per 1 cc., which readily digested the hyaluronic acid of umbilical cord, did not affect the PAS reaction of the acrosomal carbohydrates. These results more or less exclude the presence of hyaluronic acid in the acrosome and dictyosomal material of the germ cells of the Arvelius testis; although the possibility must be admitted that, due to a species specificity, the bull testis and bacterial hyaluronidases might not act on insect hyaluronic acid—or the substrate after fixation with Carnoy might be present in a form in which the enzyme is unable to attack it. It may be of interest to note that the treatment of the Arvelius testis slides with the enzyme solution of *Clostridium welchii* abolished the methyl-green stainability of the desoxyribose-nucleic acid in the nuclei of the cells and thus indicated the presence of a desoxydepolymerase in this enzyme solution. The cytochemical detection of a desoxydepolymerase in bacterial filtrates of *Clostridium welchii* is in good agreement with the observation of Warrack and coworkers,²¹ who have obtained independently the same results by chemical means (personal communication).

Since the presence of polysaccharides with 1,2 glycol grouping in the dictyosomal material and in the acrosome of the male germ cells cannot be explained by its content of starch, glycogen or hyaluronic acid, the question arises as to the possible chemical nature of this substance. It is known that male germ cells of all species examined contain an enzyme hyaluronidase, which dissolves the cementing material surrounding the female germ cells and thus makes fertilization possible.^{3, 11, 17, 18} Exceptions are the male germ cells of reptiles and birds, in which hyaluronidase has not been found and where accordingly the ova are not surrounded by

follicle cell cumuli.^{6, 7} Moreover, the acrosome of the sperm has already been regarded by early workers, such as Lillie⁹ and Bowen,¹ as being of great importance for the fertilization process, especially in connection with the penetration of the sperm and the activation of the egg. Bowen,¹ who pointed out the close analogy between the formation of the acrosome and that of a "secretory granule," has suggested that in the case of the acrosome the Golgi apparatus may be a center for the formation of enzymes which may play a part in the activation of the egg. The existence of enzymes in the sperm and their possible importance for the process of fertilization has already been stressed by Lillie⁹ and Loeb.¹⁰ The presence and possible role of the enzyme hyaluronidase in the acrosome and its elaboration by the dictyosomal material of the spermatocytes therefore demands consideration. The studies of Riisfeldt¹⁶ demonstrating that during rat spermatogenesis the hyaluronidase is first found in the spermatocytes may be a corollary to our findings of the appearance of the dictyosomal material in the primary spermatocytes and suggest a possible relationship between dictyosomal material and hyaluronidase. Whether the 1,2 glycol grouping of the polysaccharides in the dictyosomal material and the acrosome might be indicative of the presence of the enzyme hyaluronidase itself must await further chemical characterization of the enzyme. Studies in our laboratory, in which bull testis hyaluronidase was tested *in vitro* for 1,2 glycol groups, gave a positive PAS reaction. Moreover, a series of preparations of this enzyme, containing respectively 220, 550, 900 and 1400 T.R.U. per mg., showed a corresponding increase in the intensity of the PAS reaction. Since, according to Hotchkiss, the amount of dye fixed is dependent upon the actual weight of glycol structure present, it seems that the more purified the enzyme preparation (for instance 1400 T.R.U. per mg. as compared with 220 T.R.U. per mg.), the more 1,2 glycol groups can be demonstrated by means of the PAS reaction. Whether the 1,2 glycol groups are actually a part of the chemical constitution of the enzyme hyaluronidase, or whether they happen to be an "impurity" which became more concentrated during the purification process of the enzyme, must await the testing of enzyme preparations with higher T.R.U. per mg., which are not yet available. The speculation that the enzyme hyaluronidase contains 1,2 glycol linkage and thus gives a positive PAS reaction if present in tissues, led us to investigate the snake sperm, in which, as previously mentioned, no hyaluronidase has been found, and to compare it with the bull sperm, which serves as a good source for the extraction of hyaluronidase. While both sperms show a distinct acrosome, the snake sperm showed only a very slight amount of PAS positive material at the extreme tip of the acrosome, in contrast to the bull sperm where the whole acrosome, which consists of a thin hull covering two-thirds of the sperm head, was stained by the PAS reaction. That the PAS positive reaction in

the acrosome of the bull sperm is not due to starch, glycogen or hyaluronic acid was shown by pretreating sections with amylase, diastase and bull testis hyaluronidases without any effect on the intensity of the PAS reaction.

Summary.—Evidence is presented that the acrosome of the sperm in *Arvelius albopunctatus* is derived from the dictyosomal material of the primary spermatocytes and that the dictyosomal material and the acrosome contain a polysaccharide with a 1,2 glycol grouping which is neither starch, glycogen nor hyaluronic acid. The amount of polysaccharides is approximately 40 times larger in the acrosome of the sperms derived from the large sized cells than in that derived from the normal sized cells. The possibility of the relationship between acrosome and the enzyme hyaluronidase is discussed.

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²² We prefer to call the Golgi apparatus in the germ cells dictyosomal material since the dictyosomes have been seen frequently in living germ cells, while such structures as the Golgi network now appear to be fixation artifacts.

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