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STUDIES ON THE DNA CONTENT, DRY MASS AND OPTICAL AREA OF BULL SPERMATOZOAL HEADS DURING EPIDIDYMAL MATURATION*)

By

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In the past, the epididymis was viewed as merely a storage organ in which spermatozoa underwent, with the passage of time, an intrinsic developmental aging process (*Young* 1929, 1931). In general, however, the current opinion is that the epididymis actively promotes the maturation of spermatozoa (*Crabo* 1965) as well as acting as a storage reservoir.

A number of morphological and physiological alterations of spermatozoa have been reported as taking place during epididymal passage (see reviews in *Bishop & Walton* 1960; *Bishop* 1961; *Mann* 1964). Recently, *Bedford* (1965) presented results which indicate that, in the normal rabbit epididymis, virtually all spermatozoa are functionally immature before they reach the middle of the corpus epididymidis; complete functional integrity of most spermatozoa is finally achieved at some point in the lower half of the corpus region.

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Table 1. Relative Feulgen reactivity of spermatozoa from individual bulls.

Bull		Relative Feulgen reactivity	
No.	Age (yrs.)	Caput epididymidal spermatozoa/ Round spermatids	Ejaculated spermatozoa/ Round spermatids
Lowered fertility			
1	4½	0.69	0.60
2	4½	0.69	0.76
3	3½	0.71	0.55
4	2½	0.73	0.71
5	2½	0.66	0.65
6[a])	2	0.55	0.41
Normal fertility			
7	15	0.32	0.26
8	2	0.57	0.44
9	1½	0.42	0.26

[a]) Testicular degeneration

The present study was undertaken to investigate whether the maturation of bull spermatozoa is associated with any detectable changes in the major chemical components of the spermatozoal head. To this end, cytophotometric techniques were utilized.

MATERIALS AND METHODS

Nine Swedish Red and White breed bulls of varying ages (Table 1) were used in this study. Six of the bulls had been admitted to the clinic of the Obstetrics and Gynecology Department, Royal Veterinary College, Stockholm because of lowered fertility. In this group with lowered fertility, one bull has been included with consistently high percentages of morphologically abnormal head forms in his ejaculates, presumably due to testicular degeneration. The other five animals had overall semen pictures that were morphologically normal, but at times ejaculates from these bulls contained lower than normal numbers of motile spermatozoa. The reasons for the reduced fertility of these five bulls were not clear; these animals were judged to be free from infectious diseases which are known to cause infertility. Of the bulls forming the normal group, one (Table 1, no. 7) had proven fertility while the other two, approved for use in artificial insemination, were assumed to be normally fertile. All had good semen quality. The bulls were kept on a routine of moderate

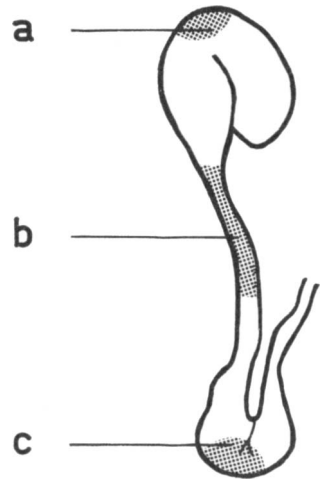

Figure 1. Schematic drawing of a left epididymis indicating (stippling) where samples were obtained from the caput epididymidis, a; corpus epididymidis, b; and cauda epididymidis, c.

serving frequency (2—4 ejaculates per week) for approximately two weeks before slaughter or castration.

Semen was collected, using an artificial vagina, not more than 15 minutes prior to the slaughter or castration of each animal. The methods used to prepare *ejaculated spermatozoa* for cytophotometric analyses were the same as those described earlier (*Gledhill* 1966) where the spermatozoa were first washed three times in a buffered balanced salt solution (*Mann* 1964) and then fixed in a neutral buffered formaldehyde solution (*Lillie* 1948). After dissecting either the right or the left epididymis free of the ipsilateral testicle, the fibrous capsule and connective tissue which surround the ductus epididymidis were incised and the exposed area was blotted. Underlying sections of the duct were carefully opened with the point of a scalpel blade. The several drops of epididymal content which oozed out were collected with a drawn glass pipette; epididymal spermatozoa were prepared for cytophotometric analyses in the same manner as used for ejaculated spermatozoa.

Epididymal spermatozoa were obtained from the caput epididymidis, the corpus epididymidis and the cauda epididymidis (Fig. 1). In the terminology of *Nicander*'s (1958) regional classification of the ductus epididymidis in bulls, the caput area referred to in this study would encompass most of region 2 and

overlap somewhat into regions 1 and 3; the corpus area would correspond to the distal portion of region 4 and the proximal portion of region 5; the cauda area would be located completely within region 6. Since *Nicander*'s classification is based on the regional histology and cytochemistry of the ductus epididymidis, the relationship between these regions and the present macroscopically located areas can be only approximative.

Only morphologically normal spermatozoal heads were chosen for measurement in each of the cytophotometric parameters investigated (*Gledhill*). Measurement of ultraviolet light (u.v.) absorbing substances in nearly 2000 glycerol immersed spermatozoal heads was performed at both 2650Å and 2800Å on a rapid high-resolution scanning and integrating cytophotometer (*Lomakka* 1965a). All 2650Å and 2800Å total extinction values are uncorrected for non-specific light loss as represented by 3150Å total extinction (*Caspersson* 1940, 1950) since these values were always so small as to be negligible. Absorption of light at 5460Å by slightly more than 2500 Feulgen stained spermatozoal nuclei and nearly 300 similarly stained human polymorphonuclear leukocytes was measured on the rapid high-resolution scanning and integrating ultramicrospectrophotometer described by *Caspersson & Lomakka* (1962). The polymorphonuclear leukocytes, which were simultaneously Feulgen stained with the spermatozoa, were used as a stain reference system and all mean light absorption values for the Feulgen stained spermatozoal nuclei herein reported have been adjusted through the use of this reference system.

Microinterferometric determination of values proportional to the total dry mass of slightly more than 2200 spermatozoal heads was performed on a rapid scanning and integrating microinterferometer (*Lomakka* 1965b). The preparations used for microinterferometry were also used for the measurement of the optical area of about 700 spermatozoal heads. These measurements were obtained on the recording microplanimeter described by *Caspersson et al.* (1960).

Testicular cells were obtained by scraping freshly cut surfaces of the testicles and were thrice washed, fixed in formaldehyde solution and Feulgen stained in the same manner as the spermatozoa. *Round spermatids* were identified principally by the shape of their nuclei and were taken as being representative of spermatids in the Golgi, cap and earliest stage of the acrosome phase of

spermateliosis (*Leblond & Clermont* 1952). Microspectrophotometry (*Caspersson & Lomakka*) of 300 round spermatids was performed at 5460Å. Approximately the same number of cells was measured in each parameter for each individual bull.

RESULTS AND DISCUSSION

Because the interpretation of each of the cytophotometric parameters investigated in this study has been discussed earlier in relation to bull spermatozoa (*Gledhill* 1966), only a short synopsis is presented here. Microspectrophotometrically determined cellular absorption at 2650Å is a measure of total nucleotides (*Caspersson* 1936, 1950); in the case of bull spermatozoa, *total extinction at 2650Å* has been assumed to be practically all due to DNA. Nearly all of the *2800Å total extinction* has been credited to the greatly dominating amounts of DNA in bull spermatozoal heads and not to the proteins. Light absorption measurements on Feulgen stained bull spermatozoa have been used for the estimation of DNA content (*Leuchtenberger* 1960; *Welch* 1960 and *de la Torre & Salisbury* 1964). However, evidence has been presented which shows that, in bull spermatozoa, the amount of Feulgen stain (*total extinction at 5460Å*) seems to be influenced by, in addition to the amount of DNA, factors which, in all probability, are intimately connected with the spermatozoal basic nuclear protein (*Gledhill et al.* 1966). The microinterferometrically determined *surface integral of the optical path difference* (o.p.d.) between the head and its background may be converted to total dry mass in grams by dividing the integrated o.p.d. by 0.12 cm^3/g (*Carlson & Gledhill* 1966). Although this factor has been experimentally determined only on ejaculated spermatozoa, there seems to be no indication that it can not be applied with reasonable accuracy to epididymal spermatozoa. *Optical area,* as determined by the convenient technique of microplanimetry (*Caspersson et al.* 1960), relates in this investigation the surface area of one broad, flat aspect of the entire spermatozoal head in water.

When the mean values within each parameter for caput epididymidal spermatozoa from bulls of lowered fertility were compared by the analysis-of-variance method (*Bonnier & Tedin* 1940) to corresponding mean values from bulls of normal fertility, a significant difference ($0.01 > P > 0.001$) was found only

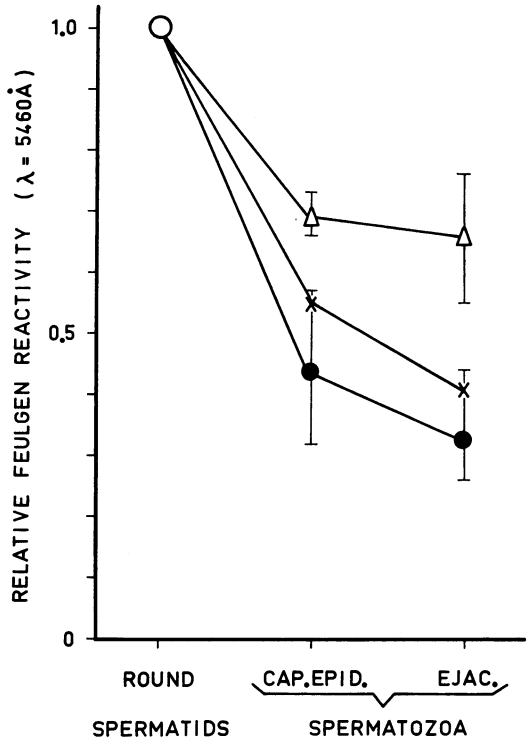

Figure 2. Mean relative Feulgen reactivity values (cf. Table 1) for five bulls with lowered fertility of unknown cause (△), one bull with testicular degeneration (×) and three bulls with normal fertility (•) are shown. Each respective range of individual bull means is indicated.

between the 5460Å total extinction mean values for Feulgen stained nuclei. When the mean values for ejaculated spermatozoa were tested, again only the mean 5460Å total extinction values differed with any significance ($0.01 > P > 0.001$). Corpus and cauda epididymidal spermatozoa likewise showed little difference in mean values between fertility groups with the exception of mean 5460Å total extinction values.

In an attempt to evaluate why Feulgen light absorption measurements differed between fertility groups, the relative Feulgen reactivity (Table 1) of caput epididymidal and ejaculated spermatozoa was obtained by comparing, within each bull, the spermatozoal mean 5460Å total extinction value to the mean value for round spermatids. During spermiogenesis the relative Feulgen reactivity of the spermatozoa from the bulls with normal

fertility was reduced significantly ($P < 0.001$) more than it was in the spermatozoa from the bulls with lowered fertility (Table 1 and Fig. 2). The reduced Feulgen reactivity of the spermatozoa was not due to a decrease in the amount of DNA present. It has been shown (*Gledhill et al.*) that qualitative changes in the composition and binding of basic nuclear protein to DNA take place as bull spermatids transform into spermatozoa and that these changes occur concomitantly with a reduction in Feulgen reactivity. Such qualitative changes in spermatozoal basic nuclear protein would not likely have an effect on the other cytophotometric parameters investigated in this study. On the basis of the clear difference in relative Feulgen reactivity of spermatozoa between the normal and five of the six low fertile bulls one could suspect that in some way the spermatozoal nuclear protein may be related to the fertility status of the bull.

In Fig. 2, the values for the bull with testicular degeneration have been separated from the mean values for the group of bulls with lowered fertility of unknown cause. The reduction in Feulgen reactivity of spermatozoa from this bull was decidedly greater than that for spermatozoa from the five other bulls with reduced fertility; in fact, the values for this bull fell in the ranges for the normally fertile bulls (Table 1). A reduction such as this suggests that testicular degeneration probably does not alter to any great extent the normal pattern of basic nuclear protein transition during spermiogenesis. The possible existence of a relationship between infertility and qualitative alterations of spermatozoal basic nuclear protein in men and bulls will be the subject of future investigations in these laboratories.

Within each of the parameters investigated, there was no significant difference, when tested with the analysis-of-variance method, between mean values for caput, corpus and cauda epididymidal and ejaculated spermatozoa (Table 2). Therefore, it is apparent that the maturation of bull spermatozoa during their approximately 11 day transit through the epididymis (*Orgebin-Crist* 1962), does not involve a change in the DNA content or total dry mass of the spermatozoal heads. The fact that the total dry mass of the spermatozoal heads was unchanged between the areas sampled does not disagree with the findings of *Lindahl & Kihlström* (1952). The increase in specific gravity during spermatozoal "ripening" found by these authors was due to progressive dehydration; the o.p.d. values reported herein are propor-

Table 2. Summary of cytophotometric results[a]).

Parameter		Caput epididymidal spermatozoa			Corpus epididymidal spermatozoa			Cauda epididymidal spermatozoa			Ejaculated spermatozoa		
		Mean	± s.e.m.	No. bulls	Mean	± s.e.m.	No. bulls	Mean	± s.e.m.	No. bulls	Mean	± s.e.m.	No. bulls
Total ext. at 2650Å		6.68	0.01	(9)	6.69	0.07	(5)	6.73	0.07	(5)	6.67	0.03	(9)
Total ext. at 2800Å		4.72	0.04	(9)	4.61	0.04	(5)	4.59	0.02	(5)	4.65	0.04	(9)
Total ext. at 5460Å	l[b])	4.66	0.11	(6)	4.45	0.13	(4)	4.48	0.16	(4)	4.12	0.23	(6)
	n	3.26	0.33	(3)	2.86		(1)	3.22		(1)	2.43	0.25	(3)
Surface integr. o.p.d.		1.52	0.01	(9)	1.52	0.01	(5)	1.52	0.01	(5)	1.51	0.01	(9)
Optical area		40.7	1.2	(6)	42.2	1.9	(2)	42.8	2.3	(2)	39.6	1.0	(6)

[a]) Mean per head ± standard error of the mean: total extinction and optical area values given in μ^2; surface integr. o.p.d. values given in $10^{-12}cm^3$

[b]) l = lowered fertility; n = normal fertility. For parameters other than total ext. at 5460Å, there was no significant difference between the mean values for the fertility groups; consequently, the means have been pooled

tional to the total *dry* mass and thus are not affected by differing degrees of dehydration. Although a reduction in the dimensions of the acrosome of rabbit spermatozoa has been shown to be associated with the epididymal maturation of these cells (*Bedford* 1963), no significant reduction in optical area of bull spermatozoal heads was found between areas sampled in the present study.

Because the total dry mass of the spermatozoal heads does not change during epididymal passage, it is most likely that the continued decrease in Feulgen reactivity as spermatozoa pass from the caput through the corpus and cauda epididymidis and into the ejaculate (Table 1 and Fig. 2), albeit statistically non-significant, is the result of even further qualitative changes in the spermatozoal deoxyribonucleoprotein complex. Whether this continued decrease in Feulgen reactivity is indicative of a factor or factors which play a role in the spermatozoon's acquisition of complete functional integrity, and therefore fertilizing ability (*Bedford* 1963, 1965), remains to be investigated.

CONCLUSIONS

With respect to caput, corpus and cauda epididymidal and ejaculated bull spermatozoa as cytophotometrically analyzed in this study, the following conclusions are made.

1. In spermatozoal heads, there is no *quantitative* change in DNA, in total dry mass or in optical area that is associated with the maturation of the spermatozoa as they pass between these areas of the epididymis.

2. There is a marked reduction in relative Feulgen reactivity during spermiogenesis that is significantly more pronounced in spermatozoa from bulls of normal fertility than in spermatozoa from bulls of lowered fertility. This reduction is interpreted as signifying *qualitative* alterations of the spermatozoal deoxyribonucleoprotein complex and may be related to the fertility status of the bull.

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SUMMARY

Microspectrophotometric, microinterferometric and microplanimetric techniques were used to investigate whether the maturation of bull spermatozoa is associated with any detectable changes in the major chemical components of the spermatozoal head. Round spermatids, caput, corpus, cauda epididymidal and ejaculated spermatozoa were obtained from six bulls with lowered fertility and three bulls with normal fertility. There was no quantitative change in DNA, in total dry mass or in optical area associated with the passage of spermatozoa between these areas of the epididymis. A marked reduction in relative Feulgen reactivity during spermiogenesis was found and was significantly more pronounced in spermatozoa from bulls with normal fertility than in spermatozoa from bulls of lowered fertility. It was suggested that this reduction signifies qualitative alterations of the spermatozoal deoxyribonucleoprotein complex and may be related to the fertility status of the bull.

ZUSAMMENFASSUNG

Untersuchungen über DNA-Gehalt, Trockengewicht und Fläche von Bullen-Spermatozoenköpfen während ihrer epididymale Reifung.

Mikrospektrophotometrische, mikrointerferometrische und mikroplanimetrische Methoden wurden angewandt, um festzustellen, ob die Reifung von Bullen-Spermatozoen mit nachweisbaren Veränderungen in der makromolekülären Zusammensetzung des Spermatozoenkopfes verbunden ist. Runde Spermatiden, Spermatozoen von Caput, Corpus

und Cauda epididymidis, sowie ejakulierte Spermatozoen, wurden von sechs Bullen mit herabgesetzter Fertilität und drei Bullen mit normaler Fertilität erhalten. Es konnten keine quantitativen Unterschiede im Gehalt an DNA, im Totaltrockengewicht und in der Zelloberfläche des Spermatozoenkopfes im Zusammenhang mit ihrer Passage unter den untersuchten Gebieten des Nebenhodens gefunden werden. Eine auffallende Reduktion in der Feulgenreaktivität während der Spermiogenese wurde festgestellt und trat offensichtlich stärker in Spermatozoen von Bullen mit normaler Fertilität hervor als in Spermatozoen von Bullen mit herabgesetzter Fertilität. Es wird angenommen, dass diese Reduktion qualitative Veränderungen des Deoxyribonukleoprotein-Komplexes der Spermatozoen zum Ausdruck bringt und möglicherweise in Beziehung zur Fertilität des Bullen steht.

SAMMANFATTNING

Undersökningar över DNA-mängd, torrvikt och cellyta av spermiehuvuden hos tjur under bitestikelmognaden.

Mikrospektrofotometriska, mikrointerferometriska och mikroplanimetriska metoder har använts för att undersöka om tjurspermiens mognad är förenad med några påvisbara förändringar i den makromolekylära sammansättningen i spermiehuvudet. Runda spermatider, spermier från caput, corpus och cauda epididymidis samt ejakulerade spermier undersöktes från sex tjurar med nedsatt fertilitet samt från tre tjurar med normal fertilitet. Inga kvantitativa förändringar i spermiehuvudets halt av DNA, torrvikt eller i dess cellyta befanns vara förbundna med passagen mellan de olika bitestikelregionerna. En kraftig minskning i relativ Feulgenreaktivitet kunde påvisas under spermiogenesen. Denna minskning visade sig vara mer uttalad hos spermier från tjurar med normal fertilitet än hos spermier från tjurar med nedsatt fertilitet. Dessa resultat tyder på kvalitativa förändringar i deoxyribonukleoprotein-komplexet som kan tänkas vara relaterade till tjurens fertilitet.

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