REACTIVATION OF THE FEULGEN REACTION OF RAM SPERMATOZOA BY DITHIOTHREITOL

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Reduction in the intensity of staining by the Feulgen reaction for DNA has been demonstrated in mammalian spermatids during spermiogenesis (Gledhill, Gledhill, Rigler & Ringertz, 1966) and in spermatozoa during transit through the epididymis (Gledhill, 1966, 1971; Bouters, Esnault, Ortavant & Salisbury, 1967; Bouters, Esnault, Salisbury & Ortavant, 1967). On the other hand, it has been reported that there is a reactivation of the Feulgen reaction for spermatozoa in the female genital tract (Chang, 1959; Esnault, Orgebin-Crist & Ortavant, cited by Orgebin-Crist, 1969). As these variations in the intensity of the Feulgen reaction are not related to changes in DNA concentration as measured by ultraviolet absorption, it is likely that they are due to chemical alteration of deoxyribonucleoprotein (Gledhill *et al.*, 1966).

This paper gives the results of measurements of Feulgen staining in germinal cells during spermatogenesis and transit through the epididymis in the ram and reports a reactivation of the Feulgen reaction in spermatozoa after treatment with dithiothreitol, a reagent which reduces disulphide bonds (Cleland, 1964).

Specimens from fourteen Île-de-France rams were used. Smears were made by touching pieces of testis against a slide. This gave a single layer of well separated nuclei. The preparations were immediately fixed in a mixture of three vols 95% ethanol and one vol. acetic acid for 1 hr, or by exposure to formaldehyde vapour for 15 min. Smears of spermatozoa from the head, body and tail of the epididymis, and ejaculated spermatozoa collected into an artificial vagina before slaughter were fixed in the same way. Ejaculated spermatozoa were treated for 15 to 45 min with millimolar dithiothreitol (DTT) buffered to pH 9 with sodium borate before preparing smears (Calvin & Bedford, 1971). Absorption measurements were made on a Zeiss ultraviolet microspectrophotometer (USMP 1) by scanning the nuclei and integrating the optical densities (Caspersson, 1936). The preparations were mounted in glycerol on Quartz slides. Measurements were made at 265 nm and 280 nm on germinal cells from the testis treated with 1 mg RNase/ml distilled water at 40° C for 4 hr (Amano, 1962) and on spermatozoa. The total extinction values have been corrected for non-specific light losses measured at 315 nm. Measurements were performed at 560 nm on material prepared on glass slides and stained by the Feulgen reaction (Bouters *et al.*, 1967).

Ultraviolet absorption measurements of DNA showed the differences in ploidy which are found in primary and secondary spermatocytes and in round

Table 1. Microspectrophotometric measurements on ram germinal cells during spermatogenesis and transit through the epididymis

	Primary spermatocytes	Secondary spermatocytes	Round spermatids	Spermatids stages 3, 4 and 5	Spermatids stages 6 and 7	Testicular spermatozoa	Spermatozoa from the cauda epididymidis
UV-DNA (265 nm)	29.05* ± 0.564	15.18 ± 0.293	8.03 ± 0.144	6.78 ± 0.093	—†	6.30 ± 0.103	6.43 ± 0.071
Feulgen-DNA (560 nm)	29.50 ± 0.289	15.41 ± 0.250	7.83 ± 0.070	8.25 ± 0.134	8.02 ± 0.137	4.46 ± 0.116	3.93 ± 0.068

* Results are expressed as mean total extinction values ± S.E. in arbitrary units for sixty cells of each class.

† No measurements were made because the cells were observed unstained under ultraviolet light and it was therefore not possible to differentiate elongated spermatids from spermatozoa.

spermatids (Table 1). At the start of the phase of spermatid elongation during stages 2 and 3 of the cycle of the seminiferous epithelium (Ortavant, 1958), there was a slight reduction in total extinction. The reason for this is not known. Ultraviolet measurements in elongated spermatids during the remainder of spermiogenesis, and in spermatozoa during passage through the epididymis showed no differences in DNA concentration. The curves of measurements made at 265 nm and at 280 nm were parallel throughout spermatogenesis and in the spermatozoa.

TEXT-FIG. 1. Influence of the acid-hydrolysis period (N-HCl at 60° C) on the staining of spermatids and spermatozoa by the Feulgen reaction. Mean of thirty nuclei for each class (total extinction at 560 nm in arbitrary units). ●, Round spermatids; ▲, spermatids at stages 3, 4 and 5; ○, spermatids at stages 6 and 7; ■, testicular spermatozoa; □, spermatozoa from the cauda epididymidis.

In the spermatocytes and in the round spermatids, measurements of the total extinction of the Feulgen-stained nuclei were practically identical to the ultraviolet absorption measurements. However, at stage 8 of the cycle, and the moment of release of spermatozoa in the seminiferous tubules, there was a 45% reduction in the concentration of Feulgen-staining material (Table 1). This phenomenon could not have been due to a change in sensitivity to acid hydrolysis during the Feulgen reaction, because the optimum time for hydrolysis was the same for each type of cell (Text-fig. 1). During the passage of spermatozoa through the epididymis, there was a further, but less marked, reduction in Feulgen staining.

After treatment by DTT and staining by the Feulgen reaction, 52% to 84% of the ejaculated spermatozoa, depending on the specimen, stained very densely with a total extinction identical to that of the nuclei of round or elongating spermatids. The nuclear chromatin of this group of spermatozoa was slightly granular. The other spermatozoa showed nuclear staining comparable to that in control spermatozoa (Table 2). This treatment did not have a significant effect on nuclear size.

As there was no change in ultraviolet absorption during the completion of spermiogenesis, the sudden reduction in the intensity of Feulgen staining accompanying the differentiation of elongated spermatids into spermatozoa could not have been due to a reduction in the quantity of DNA. Just before this reduction in Feulgen staining, there is an increase in the intensity of staining by alkaline Fast Green (Alfert & Geschwind, 1953) and by Sakaguchi's test (Liebman, 1951) suggesting changes in the basic nuclear proteins. The reactivation of the Feulgen reaction in DTT-treated spermatozoa implies that disulphide groups play an

Table 2. Effect of dithiothreitol on total extinction at 560 nm of ram spermatozoa stained by the Feulgen reaction

Treatment	Time of incubation		
	15 min	30 min	45 min
Borate buffer alone	3.56* ± 0.037	3.67 ± 0.040	3.36 ± 0.053
Dithiothreitol with borate buffer			
Class 1	3.64 ± 0.035	4.39 ± 0.045	4.90 ± 0.063
Class 2	7.91 ± 0.074	8.21 ± 0.039	8.08 ± 0.069

Untreated spermatozoa had a total extinction of 3.43 ± 0.016 .

* Results expressed as mean total extinction values $\pm$ S.E. in arbitrary units for thirty cells of each class.

important rôle in the reaction. Loir (1970) has shown that although there is practically no change in the intensity of staining of SH groups during the course of spermiogenesis, the staining of disulphide groups increases up to stage 7. Furthermore, spermatozoa are known to be very rich in the basic disulphide-containing amino acid, cystine (Bril-Petersen & Westenbrink, 1963; Henricks, 1965; Chamberlain & Walker, 1965; Coeling, Rozijn & Monfoort, 1969).

In the female genital tract, there is a 25% increase in the intensity of Feulgen staining in ejaculated rabbit spermatozoa without any variation in the amount of DNA as measured by ultraviolet absorption (Esnault *et al.*, cited by Orgebin-Crist, 1969). Perhaps this phenomenon is related to the reactivation of the Feulgen reaction which occurs when spermatozoa are treated with dithiothreitol.

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