DIFFERENTIAL RESPONSE OF DNA OF FRESH AND STORED BOVINE SPERMATOZOA TO FEULGEN (HCl) HYDROLYSIS

W. J. BIRGE and G. ANKLESARIA
University of Illinois, Urbana

F. D. TIBBITTS
University of Nevada, Reno

In recent considerations (Birge, *et al,* 1960; Salisbury, *et al,* 1961) it was shown that the deoxyribonucleic acid of bovine spermatozoa undergoes a significant change in stability during the *in vitro* storage of semen in a yolk-citrate medium. Cytophotometric analysis of Feulgen-stained spermatozoa indicated a substantial loss of Feulgen-positive material (DNA) during *in vitro* storage. However, in this study fresh and stored spermatozoa were given identical treatment regarding Feulgen hydrolysis (12 min.; Carnoy fixation) and staining. Therefore, it was not shown whether the diminished response of stored spermatozoa to Feulgen staining resulted solely from a loss of DNA, or whether it was due in part to increased lability of sperm DNA to Feulgen (HCl) hydrolysis. In the latter event, a substantial fraction of DNA could be removed from stored spermatozoa by prolonged Feulgen hydrolysis (*N* HCl at 60°C. for 12 min.)

The initial concern of the present study is to determine whether fresh and stored bovine spermatozoa give a differential response to Feulgen hydrolysis. In addition, sperm samples characterized by the retention of high and low motility potential during storage will be compared regarding DNA lability.

Materials and Methods

Semen samples were obtained from each of two Hereford bulls and immediately diluted 1:4 with a storage medium consisting of 1 part egg yolk to 3 parts of 2.9 per cent sodium citrate dihydrate. Subsamples from each bull were cooled slowly to a storage temperature of 5°C. and held for periods of 5 and 10 days.

Smears of fresh sperm (also diluted with yolk-citrate) and spermatozoa from the 5 and 10-day samples were prepared on standard microscope slides, fixed for 7 minutes in Carnoy solution (1 part glacial acetic acid to 3 parts absolute ethanol) and stained by the Feulgen procedure as previously outlined by Salisbury, Birge, de la Torre and Lodge (1961; *c.f.* Leuchtenberger, 1958). The Feulgen reagent was prepared from a solution of 0.5 per cent basic fuchsin (National Aniline, dye content 93 per cent, C.I. No. 677).

Hydrolysis was carried out in *N* HCl which was carefully maintained at a temperature of 60°C. A continuous record was kept of the temperature of the hydrolyzing medium. In order to minimize dilution, an excess (1 liter) of freshly prepared HCl was used for each set of slides. In order to maintain maximum uniformity of treatment, the semen sam-

ples from bulls 1 and 2 were processed together. Each of the three sets of slides was handled in a single glass rack. For control purposes, non-hydrolyzed and TCA-treated (5% for 10 minutes at 90°C.) preparations of spermatozoa from each bull were included in each of the 3 sets of slides. In addition, sections from a single block of rat liver tissue were processed with each set of materials. Photometric measurements of the Feulgen stained liver tissue served as a reference base in determining the uniformity of the staining reaction for the three separate sets of slides.

In order to determine the response of fresh and stored spermatozoa to Feulgen hydrolysis, treatment with *N* HCl was administered for several different time intervals. Slides of unstored spermatozoa from each bull were hydrolyzed for 6, 10, 11, 12, 13 and 14 minutes. Preparations of day 5 and day 10 material were hydrolyzed for 4, 5, 6, 7, 8, 9, 10 and 12 minutes. Slides were mounted in Clarite-X.

In order to gain an accurate measurement of the extent of dye binding (optical density) in each of the preparations, photometric measurements were taken for a 2.5 μ plug through the sperm nucleus in 30 different cells. Transmission values, reflecting the degree of Feulgen dye absorptivity per unit area, were taken with light isolated from the mercury green line (546 mμ). In each case, extinction (E) values were computed from the per cent transmission (T).

$$\text{Extinction values } (E = \log_{10}\frac{1}{T})$$

reflect the optical density *per unit area* only if a constant light path is insured. Otherwise such values should be expressed as E/t, where *t* equals thickness of the plug area. As noted earlier by Salisbury, *et al* (1961), the bovine sperm nucleus is in the form of a distinct platelet. Cell thickness is highly uniform for a given animal, as indicated by measurements made in the present study. A sample of 300 sperm cells from animal 1 were measured for thickness. Only cells oriented "on edge" were considered. This sample included 3 subsamples consisting of fresh, day 5 and day 10 semen. For each subsample, measurements were made on cells which received optimum hydrolysis and cells treated for 2 minutes above and below the optimum point. No statistically significant interactions were noted regarding the various subsamples. Over-all mean thickness was found to be 0.54 $\pm$ 0.03 μ. It seems apparent, therefore, that plug thickness was quite uniform from cell to cell. Cells with the flat parallel sides orientated normal to the optical axis were selected for absorption measurements. Also, the absorbing dye was found to be distributed quite evenly throughout the sperm nucleus, particularly is the anterior half.

The plugs were taken at a point approximately 1/3 the length of the sperm head from the anterior end. In a number of instances, a second plug was also taken through the center of the posterior half of the sperm nucleus (*c.f.* Table 1).

The cytophotometer used in making the absorption measurements has been described previously, (Salisbury, *et al*, 1961). It consists essen-

tially of a Leitz Aristophot photomicrographic stand; a photometer head assembly (containing a 1 P 21 photomultiplier tube) previously described by Birge (1959); a Farrand power unit; a Rubicon galvanometer; a Leitz type B microscope stand equipped with a binocular-monocular head, and a Bausch and Lomb grating monochromator. The monochromator is provided with a 1200-groove per millimeter grating and gives a linear dispersion of 3.3 mμ/mm. Second order overlap was eliminated by the use of filters. Slits were set at 1.5 mm. A mercury Hanovia lamp served as the light (line) source. The condenser of the microscope was set to give a N.A. of 0.35. A field diaphram was used to limit the microscope field to a small central area 31 μ in diameter.

Results

The results of this study are summarized in Table 1. It should be noted that the semen samples collected from the two bulls differed markedly with respect to motility potential. Not only were spermatozoa much less concentrated in the semen collected from bull no. 2, but the motility of samples warmed to room temperature was exceedingly low after the 3rd day of storage. On the other hand, bull no. 1 produced excellent semen which retained high motility up to 15 days of storage, at which point the samples were discarded. Visual inspection of motility was made on alternate days during this 15-day period. It is evident that maximum Feulgen staining of the fresh semen samples was achieved after hydrolysis (*N* HCl at 60°C.) for 12 minutes. This is the usual response given by most Carnoy-fixed animal tissues. The progressive increase in Feulgen intensity with hydrolysis time, up to the optimum point of hydrolysis, is generally associated with the removal of base units from the DNA, thus exposing sugar aldehyde groups to bind with leucofuchsin (reduced basic fuchsin). After peak hydrolysis has been reached, Feulgen intensity begins to diminish as continued acid hydrolysis results in the progressive breakdown and removal of the DNA. Optimum (peak) hydrolysis time is defined, therefore, as the point at which the maximum number of aldehyde groups are exposed without effecting the removal of DNA by acid hydrolysis.

Considering the data for day 5 semen, it is obvious that a decided shift occurs in the hydrolysis peak for both animals. Optimum Feulgen hydrolysis is attained at 7 to 8 minutes for animal 1 and 8 minutes for animal 2. In bull no. 1, a significant amount of DNA is not lost until 10 minutes of hydrolysis, 2 or 3 minutes after peak hydrolysis is reached. However in bull no. 2, DNA removal begins immediately after the peak is reached, perhaps indicating increased lability to acid extraction in this animal.

This shift in the response to Feulgen hydrolysis is also obvious in the 10-day samples. Regarding bull no. 1, the peak occurs at 8 minutes. A significant amount of DNA is lost by 9 minutes of hydrolysis, possibly indicating increased lability of the DNA to acid extraction in comparison with the 5-day material of the same animal. Regarding animal no. 2, the peak has shifted to 7 minutes,

TABLE 1.—Extinction Values are Given for Sample Plug Readings for Semen Hydrolized From 4 to 14 Minutes. These Values Reflect the Relative Intensity of Feulgen Dye Per Unit Area. The Areas Covered by Anterior and Posterior Plugs are Described Above.

Hydrolysis Time in Minutes	BULL NO. 1						BULL NO. 2			
	Fresh Sperm		5-Day Sperm		10-Day Sperm		Fresh Sperm	5-Day Sperm	10-Day Sperm	
	Post. Plugs	Ant. Plugs	Post. Plugs	Ant. Plugs	Post. Plugs	Ant. Plugs	Ant. Plugs	Ant. Plugs	Ant. Plugs	
4	...	...	...	.146	...	.138	...	.150	.145	
5	...	...	...	.165	...	.152	...	.170	.159	
6	...	.131	...	.204	...	.177	.122	.187	.181	
7	...	...	.200	.218	.183	.193	...	.198	.217	
8	...	...	.203	.220	.218	.228	...	.215	.209	
9	...	...	.202	.217	.193	.214	...	.180	.197	
10	...	.184	.183	.198	...	.197	.171	.158	.176	
11	.192	.204	...	...	...		.188	...	...	
12	.213	.223	...	.154	...	.168	.223	.146	.151	
13	.202	.216	...	...	...		.208	...		
14	...	.201	...	...	...		.190	...		

also indicating increased lability of the DNA to Feulgen hydrolysis with additional storage time. In comparison with bull no. 1, the DNA of spermatozoa from bull no. 2 becomes somewhat more labile to acid hydrolysis upon storage.

On the basis of these data it may be concluded that *in vitro* storage of bovine spermatozoa in a yolk-citrate medium results in a distinct change in the response of the deoxyribonucleic acid to Feulgen hydrolysis. Not only are base units more easily split off to expose aldehyde groups for dye binding, but also the DNA is more easily extracted by hydrolysis with HCl. This response may indicate a partial depolymerization of the deoxyribonucleic acid during storage.

Regarding the results given by Salisbury, Birge, de la Torre and Lodge, (1961; *c.f.* Birge, *et al*, 1960.) it seems that this increased lability may be accompanied by the actual loss of DNA during storage. However, the figures previously reported by Salisbury, *et al* (1961) and Birge, *et al* (1960) would seem to be somewhat high, as the stored spermatozoa were hydrolyzed for 12 minutes. Undoubtedly, a substantial fraction of the DNA regarded as *lost during storage* was removed during hydrolysis. At the moment, a study is underway to determine whether DNA is actually lost during the storage of semen from bulls nos. 1 and 2. Preliminary results indicate a downward trend in Feulgen-positive material with storage for bull no. 2. However, this result may not hold true for bull no. 1. It should be noted, however, that the semen samples from these bulls did not display *typical* motility potentiality during storage. Semen from animal no. 1 exhibited greater motility over a longer period of storage than normally found in the majority of bulls heretofore considered by the present investigators (7 in all). On the other hand, semen from animal no. 2 exhibited much less motility potential than normally encountered.

Summary

During the *in vitro* storage of bovine spermatozoa in a yolk-citrate medium, the deoxyribonucleic acid becomes more labile to Feulgen (HCl) hydrolysis. Base units are more easily split off, exposing aldehyde groups for dye binding. In addition, the DNA is more readily removed from the cells by acid hydrolysis. Comments are given regarding the possible nature of these storage-induced changes in the DNA of bovine spermatozoa.

References Cited

Birge, W. J., G. W. Salisbury, L. de la Torre, and J. R. Lodge. 1960. Diminution of the deoxyribonucleic acid content of bovine spermatozoa during *in vitro* storage. Anat. Rec. 137:340.

Leuchtenberger, C. 1958. Quantitative determination of DNA in cells by Feulgen microspectrophotometry, In: *General Cytochemical Methods*, Vol. 1. Ed. by J. F. Danielli. Academic Press, Inc. New York, pp. 219-278.

Salisbury, G. W., W. J. Birge, L. de la Torre, and J. R. Lodge. 1961. Decrease in nuclear Feulgen-positive material (DNA) upon aging in *in vitro* storage of bovine spermatozoa. J. Biophysical and Biochemical Cytology, 10:353-359.

Manuscript received June 20, 1961.