

DECREASE IN NUCLEAR FEULGEN-POSITIVE  
MATERIAL (DNA) UPON AGING IN  
*IN VITRO* STORAGE OF BOVINE SPERMATOZOA

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ABSTRACT

The Feulgen-DNA content of sperm cells from 5 bulls was studied by means of microspectrophotometry after storage at 5°C for 2, 3, 5, and 10 days in a yolk-citrate diluent permitting slow aerobic metabolism. A subsample of sperm cells from each bull was subjected to the Feulgen technique on each of the storage days selected. The cells sampled on each of these days received a standard 12 minute, 60°C hydrolysis. Absorption measurements at 546 m $\mu$  of the individual cells indicated a marked progressive decrease in the Feulgen-DNA content of the stored spermatozoa. The loss of 30 per cent of the initial DNA at the end of 5 days' storage was highly significant statistically. This decrease approximately parallels the known decrease in fertility of stored sperm cells, as well as the increase in apparent embryonic mortality resulting from the use of similarly aged spermatozoa for artificial insemination.

The relative constancy of the deoxyribonucleic acid (DNA) in the resting cell nucleus is a basic tenet of modern biology (8). This tenet for the male gametes is implicit in the recommendation that Feulgen-stained bull spermatozoa could provide a standard reference material for the microphotometric quantitation of DNA in other cell nuclei (14).

The assumption of constancy in spermatozoa arises from a number of observations. Among these is the correlation, first established in the bovine (34), between the DNA content of haploid spermatozoan nuclei and the DNA content and the number of chromosome sets to be found in spermatogenic and somatic tissues (10, 17, 21, 34).

A second supporting fact is that in the absence of chromosome change the DNA content of cell nuclei appears to be relatively stable (1), for, except in the case of some secretory cells (16), it does not become metabolically so involved in

the activity of cells as do other cellular components. This fact has lent support to the commonly held belief that the spermatozoon serves primarily as a convenient mechanism for transport of genetic information via DNA. During this transport it has been assumed that the DNA is wholly passive and is not involved in energy exchange mechanisms.

No one seems to have seriously challenged the concept of constancy of the DNA in living spermatozoa, except Salisbury, who did so recently (22) on the basis of an increase in apparent early embryonic mortality in cattle resulting from the aging of spermatozoa by storage at 5°C before insemination (23). The evidence involving the aging of spermatozoa in the incidence of early embryonic mortality in cattle is supported by evidence on aging of spermatozoa in the female reproductive tract for other mammalian species—the rat and the guinea pig (28, 29)—and for poultry (9, 20),

and by the evidence that similar aging of *Drosophila* spermatozoa leads to a higher than normal rate of mutation (6).

There is a general belief that DNA alone or in combination with proteins is the material of which the gene is composed (2, 19) and that this must be produced in an exact amount and be carefully conserved in the interest of the veracity of reproduction of multicellular species. Part of the supporting evidence is the finding of Leuchtenberger and her associates, using the Feulgen reaction for DNA and the microspectrophotometer for determination of the Feulgen-positive material in the individual spermatozoan heads, that infertile men (18) and bulls (15) produced spermatozoa having a lower and more variable DNA content than was found in the spermatozoa of fertile males of each species. These observations can be interpreted as being due to the vagaries of abnormal chromosome assortment in spermatogenesis (22). However, the possibility suggested itself that they and the aging effects mentioned above might be due to breakdown of the Feulgen-positive material, following completion of spermiogenesis and the initiation of chemical activities coincident with the existence of the sperm cell independent of its own parental tissue. The results of the first test of such an hypothesis, preliminary abstracts of which have been published (4, 27), are reported here.

#### MATERIALS AND METHODS

A cattle-breeding organization<sup>1</sup> provided a single normal ejaculum from each of 5 bulls representing the various levels of fertility found for its population of about 40 bulls. The samples were diluted, immediately after collection, 1:4 with egg yolk (25 per cent) and a solution of 2.9 per cent sodium citrate dihydrate (75 per cent) (25), a medium isosmotic with semen which is widely used in commercial artificial insemination. A portion of each of the diluted samples was then smeared on glass slides for immediate fixation and processing for the Feulgen staining procedure. These smears were to provide evidence on the initial DNA level in a representative sample of the individual cells.

2½ ml of the diluted semen samples were poured into 4 separate centrifuge tubes (1 for each day of subsampling except for days 5 and 10, where only 1 subsample vial was used), stoppered, slowly cooled to 5°C, and then stored for 10 days in a refrigerator

<sup>1</sup>The Southern Illinois Breeding Association, Breese, Illinois.

held at that temperature. These samples were stored under conditions permitting slow oxidative metabolism (5, 26) and similar to the conditions obtaining in commercial artificial insemination of cattle. On the 2nd, 3rd, 4th, 5th, and 10th days of storage, after careful mixing, a subsample was removed from the stored samples of each bull for staining and determination of Feulgen-DNA.

The sperm cell smears were made on standard microscope slides, and were immediately fixed (without being allowed to dry) in acetic alcohol (1:3 glacial acetic acid: absolute alcohol) for 7 minutes. They were washed in 3 changes of absolute alcohol for a total of 30 minutes and were carried through the usual graded alcohols (95, 70, 50, and 30 per cent) to distilled water. All slides prepared for each day of storage were handled in two staining racks from the postfixation wash to the final xylene clearing bath previous to mounting. Each rack of slides contained a control slide of rat liver which had been fixed in acetic alcohol. Control slides of spermatozoa which received no hydrolysis, and others which were subjected to a 5 per cent trichloroacetic acid (TCA) treatment for 10 minutes at 90°C to extract the DNA, were used as additional controls.

The Feulgen staining technique and solutions were used as outlined by Leuchtenberger (13). Because of the importance of the hydrolysis procedure in the Feulgen reaction (35), it was designed to insure a constant 60°C hydrolysis temperature with a minimum of HCl dilution. A thermostatically controlled water bath was used and the temperature of the hydrolyzing (N HCl) solution was precisely maintained for 12 minutes.

The Feulgen reagent was made to contain 0.5 per cent basic fuchsin based on the indicated actual dye content of the basic fuchsin used (National Aniline, dye content 93 per cent, C.I. no. 677). The slides were mounted in Clarite X. The refractive index of this medium matched that of the unstained elements so as to render them completely invisible, thus minimizing light scatter.

The cytophotometer used in making the Feulgen absorption measurements was assembled on a Leitz Aristophot photomicrographic stand. The photometer unit consisted of a head assembly (housing a 1 P 21 photomultiplier tube) previously described by Birge (3), a Farrand electron multiplier unit, and a Rubicon galvanometer. The optical equipment consisted of a Leitz binocular-monocular microscope, × 10 Periplane oculars, a Leitz × 90 achromatic oil immersion objective (corrected for spherical aberration in the green), and an aplanatic, achromatic Leitz condenser (NA 1.40). The substage condenser diaphragm was stopped down to give a numerical aperture of 0.37. A Bausch and Lomb filar ocular micrometer was used

for all the size determinations, which were made at a magnification of 1350.

The wavelength used for the Feulgen absorption measurements was the mercury green line (546 m $\mu$ ) isolated from a Hanovia mercury lamp by a Bausch and Lomb grating monochromator. This instrument, equipped with a 1200 grooves per millimeter grating, has a focal length of 250 mm and gives a linear dispersion of 3.3 m $\mu$ /mm. Entrance and exit slits were set at 2 mm, giving a full band width of 13.2 m $\mu$ . A field diaphragm was placed in front of the exit lens of the monochromator to reduce the microscope field to a small central portion, 31  $\mu$  in diameter.

The amount of absorbing material in the liver nuclei was calculated using the equation  $M = E\pi C^2/F$  (30), where  $M$  = the amount of absorbing material in arbitrary units,  $E$  = extinction,  $C$  = radius of the nuclear plug, and  $F$  = the fraction of total nuclear volume included in the plug. The plug diameter used for liver nuclei was 4.10  $\mu$ . The absorbing material in the spermatozoan nucleus was calculated by the formula

$$M = \frac{E(\text{plug area})}{F^1},$$

where

$$F^1 = \frac{(\text{plug area})(\text{plug thickness})}{(\text{area of sperm nucleus})(\text{thickness of sperm nucleus})}.$$

The geometry of bull sperm heads is such that the thickness of the plug cannot be demonstrated to be numerically different from the average thickness of the sperm nucleus. Thus the above formula simplifies to  $M = E \times \text{area of sperm head}$ . The area of the sperm heads was determined from the formula published by vanDuijn (33). Extinction measurements made along a transect from side to side of the sperm nucleus, using a plug diameter of 1.3  $\mu$ , showed no variation. However, transmission values of several spermatozoa measured averaged 12 per cent higher near the anterior end than at the base. Therefore, all the plug measurements in the storage experiments were taken from the geometrical center of each sperm head. At first, 3.30  $\mu$  plugs were used, but because of the decrease in size of the aging spermatozoa this value was reduced to 2.64  $\mu$ . The quantitative data presented herein are based on measurements of 24 cells per daily sample per bull.

## RESULTS

### *Spermatozoa Controls*

The spermatozoa not subjected to hydrolysis did not show a positive Feulgen reaction. Similarly,

other sperm heads treated with 5 per cent TCA at 90°C for 10 minutes, for the removal of nucleic acid, also failed to show a staining reaction.

### *Liver Controls*

The rat liver control slides were subjected to precisely the same hydrolysis and staining procedure as were the stained spermatozoa. Photometric determinations from the liver nuclei served as a reference base in aiding the interpretation of sperm cell values. Mean values for the liver nuclei, within the limits of statistical variation found by analysis of variance, were constant throughout the duration of the experiment. As the Feulgen-DNA in liver nuclei showed no downward trend during the experiment, the trend of decreasing Feulgen-DNA shown below to occur during the aging of the sperm cells cannot be due to changes in the quantitative technique employed.

### *Experimental Spermatozoa*

The results of the experiment, expressed as absolute values for the area of the spermatozoan nuclei and as arbitrary units of Feulgen-positive material (DNA) per sperm head for each of the bulls for each storage interval, are given in Table I. The DNA content of the fresh spermatozoa did not vary significantly from one bull to another. The over-all means and their standard deviations for the spermatozoa of all the bulls are summarized in Table I.

The DNA values for spermatozoa decreased markedly during the first 2 days after collection. The difference is statistically significant ( $P \leq 0.01$ ). The apparent mean increase on the 3rd day is probably an artifact, for the over-all mean value on that day as compared with the mean for the 2nd day is not significantly different on analysis of variance. However, the over-all trend downward in DNA content of spermatozoa with aging is highly significant statistically ( $P \leq 0.001$ ). A similarly highly significant interaction was found for the bull  $\times$  age of the sperm cells, indicating that the Feulgen-DNA in the spermatozoa of some bulls decreased faster on aging than did those of others.

When the slides prepared for this experiment were scanned, three were found, all from the same bull, which contained at least one cell that stained much darker than the normal sperm-

TABLE I  
 Mean and Standard Deviation of Spermatozoan Head Area and Feulgen-Positive Material (DNA) of Spermatozoa Stored in Yolk-Citrate at 5°C for 0, 2, 3, 5, and 10 Days (n = 24)

Bull	Length of storage									
	Fresh		2 days		3 days		5 days		10 days	
	Area μ <sup>2</sup>	DNA A.U.*	Area μ <sup>2</sup>	DNA A.U.	Area μ <sup>2</sup>	DNA A.U.	Area μ <sup>2</sup>	DNA A.U.	Area μ <sup>2</sup>	DNA A.U.
1	34.46 ± 2.51	9.70 ± 1.18	34.03 ± 2.34	7.83 ± 0.60	30.85 ± 2.14	8.77 ± 0.80	31.99 ± 2.52	6.88 ± 0.73	28.05 ± 2.19	5.42 ± 0.80
2	36.47 ± 2.47	8.73 ± 0.64	35.17 ± 1.85	6.56 ± 0.36	26.38 ± 1.43	8.62 ± 0.74	30.31 ± 1.81	6.66 ± 0.60	26.07 ± 1.76	6.47 ± 0.67
3	33.18 ± 1.92	9.05 ± 0.64	31.21 ± 1.97	6.72 ± 0.44	25.69 ± 1.57	7.58 ± 0.54	29.76 ± 2.23	6.86 ± 0.54	25.38 ± 1.84	6.57 ± 0.65
4	34.41 ± 2.35	10.92 ± 0.69	33.42 ± 1.88	8.36 ± 0.44	25.80 ± 1.43	7.78 ± 0.47	28.16 ± 2.24	6.60 ± 0.67	24.62 ± 1.22	6.49 ± 0.72
5	35.22 ± 1.85	11.09 ± 0.59	33.60 ± 2.11	8.25 ± 0.48	27.32 ± 1.18	7.88 ± 0.43	34.27 ± 3.16	7.16 ± 0.84	28.98 ± 1.44	7.06 ± 0.48
Over-	34.8 ± 2.45	9.90 ± 1.23	33.5 ± 2.31	7.54 ± 0.89	27.2 ± 2.47	8.13 ± 0.77	30.9 ± 3.18	6.83 ± 0.70	26.6 ± 2.36	6.40 ± 0.85
all										

\* A.U. = Arbitrary units.

matozoa. Microspectrophotometric measurements of these cells yielded values twice the normal level of absorbing material.

It will be noted that there was a marked decrease in calculated area of the sperm heads from the 2nd to the 3rd day of storage. The area increased to the 5th day and was again lower on the 10th day of storage. The mean area of sperm heads on the 5th day was  $30.9 \mu^2$  as compared with a mean of  $34.8 \mu^2$  for the samples taken on the day of collection. This difference of 11.3 per cent of the initial value is statistically significant ( $P \leq 0.01$ ).

This contraction might be considered only as a storage-induced response to fixation and the treatments attendant on staining, were it not that individual living spermatozoa showed a similar degree of contraction (13 per cent) by light reflection values during the same period of storage (32). Thus the present interpretation is that the head area change is a property of the living cell and not due solely to the differential response of fresh and stored spermatozoa to fixation and treatment.

By analyses of variance the 5 bulls providing the semen did not vary significantly in fertility or in the DNA content of fresh spermatozoa. The range of fertility was from a mean of 67.0 for the most fertile to a mean of 58.2 per cent of the total artificial inseminations for the least fertile bulls that were successful during the previous year of service. The number of inseminations varied from 2400 to 233 per bull during the year prior to the sampling for this study.

It was not possible to obtain a fertility test on the actual ejacula from which the slides were prepared, nor do we now have available evidence on the fertility of semen samples from these bulls for each of several days after collection. On the other hand, while the fertility of the bulls was not directly tested with the same ejacula, the curve showing depletion of Feulgen-positive material during aging approximately paralleled the decrease in fertility from about 67 per cent to 52 per cent of successful inseminations experienced by the breeding organization from insemination of semen aged by storage for 5 days under the same conditions (24). Throughout the same period of storage there was a corresponding increase in apparent early embryonic mortality as the age of the semen used increased (23, 24).

## DISCUSSION

The corollary of the concept of constancy of the DNA is the implicit assumption of the economy of the genetic material in the male gametes. This view can be stated as the assumption that the genetic material in the haploid spermatozoon, being in transport between its genesis and its ultimate function, is then inert metabolically and is strictly rationed in the sperm cell nucleus to the exact molecular arrangement and amount that will include all the genetic information required for the continuance of the species.

The decreased fertility and the increase in embryonic mortality mentioned above, using spermatozoa handled in a manner similar to that used in these experiments, suggest an increasing loss of fidelity in the transmission of genetic information by the aging spermatozoa.

However, in the light of the concept of economy of the genetic material in male gametes, it is difficult to reconcile the evidence showing a consistent decrease in Feulgen-positive material on aging of spermatozoa with the maintenance of relatively high fertility of semen routinely stored for 4 to 5 days in artificial insemination practice (approximately 50 per cent successful inseminations).

It might be argued that, from semen inseminated after 4 to 5 days of storage, none of the cells measured here could have been successful in fertilization and that, therefore, our measurements are not made on the kind of spermatozoa providing the evidence on fertility. This argument cannot, of course, be refuted by the evidence at hand. That is, one might assume that a decrease in DNA was to be found only in those sperm cells which died during storage, and that those spermatozoa continuing to live, and presumably capable of initiating fertilization, would maintain a constant level of measurable DNA. Nevertheless, the data indicate that these speculations are not very likely explanations of the observations. The statistics of dispersion of apparent DNA values did not increase in size as aging proceeded, and the mean decrease in DNA content was apparent in the sperm cells of all bulls sampled, suggesting that all sperm cells reacted similarly by a loss of Feulgen-DNA on storage.

Analyses of the data indicate that the number of cells measured per slide was wholly adequate to test statistically such propositions. Based on the

size of the error variance with 24 cells measured per slide, doubling of the number of cells per slide would have increased the precision of estimate approximately 0.5 per cent. Halving the number would have decreased the precision only 1.0 per cent.

The concept of economy of the DNA appears to hold for nuclei of ova as well as of spermatozoa, at least in some species. Although the cytoplasm of frog ova contains excess deoxyribosides, considered to be DNA (11), England and Mayer (10) have found that nuclei of frog ova before reduction division contain only 2 times the DNA found in frog spermatozoa. The cytoplasmic deoxyribosides of ova may represent only a nucleoside reserve for rapid nuclear multiplication after fertilization. On the other hand, the exact correspondence of the amount of DNA in the haploid spermatozoa and the haploid ova does not necessarily prove DNA in these cell nuclei to be limited solely to that involved in transfer of genetic information. If both contained a minimum nuclear reserve of DNA to insure the stability of the genetic DNA, the reserve DNA might follow chromosome number exactly. No one has ventured to suggest that spermatozoa might contain a limited reserve of DNA for this purpose. However, this is one hypothesis which follows from the observations recorded herein.

It was earlier speculated (23) that the damage to spermatozoa by aging might result from the production of  $H_2O_2$  by the sperm cells in the oxidative deamination of certain amino acids found in egg yolk (31). It was then thought that  $H_2O_2$  was itself directly mutagenic for bacteria. Present concepts indicate that  $H_2O_2$  may be involved primarily in changing the level of the

interionic attraction of bonds between DNA and protein. In the oxidized state certain other mutagens are more effective in disrupting that bonding than when the cation bonds are reduced (12). It is possible that other metabolic channels, opened to the spermatozoa for maintenance of independent existence in the highly artificial conditions of *in vitro* handling, also may involve chromatin and its genetic veracity.

Although the identity with DNA of the Feulgen-positive material in the nucleus has been established for some cells (21) and has been accepted as the case for fresh bovine spermatozoa (15), the possibility exists that this perfect positive relationship might not be the case for the *aged* sperm cells. For example, some changes in the sperm cell membrane during aging might interfere progressively with the entrance of the leucofuchsin, as iron ions have been shown to interfere in *Tetrahymena* and *Acanthamoeba* (7), or might shift the point of optimum hydrolysis, thus reducing the intensity of the staining reaction. This possibility, among others, is being tested, and independent techniques such as ultraviolet microspectrophotometry (14) and chemical determinations (8) are to be employed to determine the identity of the material which is depleted on aging of spermatozoa.

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