The Chemical Composition of Bull Semen with Special Reference to Nucleic Acids, Free Nucleotides and Free Amino Acids

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It has recently been shown that normal bull spermatozoa are able to incorporate amino acids into their proteins (Bhargava, 1957). As a preliminary to further work we have examined the nucleic acid, free nucleotide and free amino acid content of bull spermatozoa and seminal plasma, together with the general chemical composition of these spermatozoa. The nucleic acids of bull spermatozoa have received considerable attention in the past (Walker & Yates, 1952; Leuchtenberger, Leuchtenberger, Vendrely & Vendrely, 1952; Vendrely & Vendrely, 1953; Mann, 1954; Leuchtenberger, Murmanis, Murmanis, Ito & Weir, 1956), but no previous work seems to have been done on the free nucleotide content of these cells and there is only one earlier investigation (Gassner & Hopwood, 1952, 1953) on the free amino acids in bull semen. Other aspects of the chemistry of spermatozoa and seminal fluid have been extensively reviewed by Mann (1954).

Our results show that bull spermatozoa contain no ribonucleic acid, a large free nucleotide pool and a very small free amino acid pool. Bull seminal plasma was found to contain a higher concentration of free amino acids, and very low concentrations, if any, of free nucleotides and nucleic acids. This work has been carried out mainly on semen from only two bulls; this limitation was imposed partly by the necessity of using freshly collected semen from animals housed near a laboratory. Since the animals were of normal fertility, there is no reason to suppose that the results are not valid for such bulls generally.

MATERIALS AND METHODS

Semen. Bull semen was collected by the artificial-vagina technique (Walton, 1945) with precautions to avoid contamination of the sample with talc from the rubberware. The semen was collected, on the average, once a week. Each of the several ejaculates was examined separately under the microscope, and only those were mixed and used which appeared uncontaminated and did not contain an excessive number of dead spermatozoa.

The four bulls used are referred to as N₁, N₂, N₃ and N₄. Bull N₁ was a 3-years-old Friesian maintained at the National Institute for Medical Research. He had earlier been used for artificial-insemination purposes and was then shown to be of high fertility. Bulls N₂, N₃ and N₄ were in use at an artificial insemination centre (Cambridge) and were all of high fertility. The semen of bulls N₁, N₂, N₃ and N₄ is judged to be normal in all respects and it is for this reason that the prefix N has been used.

The majority of the observations recorded below on the chemical composition of semen were made on material from bulls N₁ and N₂. Unless otherwise stated, semen from bull N₁ was used within 2 hr. of collection. Semen from the remaining bulls was used about 3–4 hr. after collection. In all cases semen was kept at room temperature during the period between collection and use.

Concentration of spermatozoa. The concentration of spermatozoa in whole semen, or in suspensions of washed cells, was determined with a Fuchs-Rosenthal cytometer and a phase-contrast microscope, by the method of Bishop, Campbell, Hancock & Walton (1954). In experiments where analyses were made on washed spermatozoa, cell counts were also made on the rejected washes (these washes contained up to 10% of the cells originally present in the semen).

Buffers. The diluent hereafter referred to as bicarbonate buffer is Krebs-Ringer bicarbonate (Umbreit, Burris & Stauffer, 1957). The diluent referred to as fructose-bicarbonate buffer is Krebs-Ringer bicarbonate containing 5% (w/v) of fructose.

Dry weight of spermatozoa. Semen (2 ml.) was diluted with 4 ml. of fructose-bicarbonate buffer in weighed 15 ml. centrifuge tubes and centrifuged for 25 min. at 2500 rev./min. in an MSE Major centrifuge. The spermatozoa were washed once with 5 ml. of fructose-bicarbonate buffer and twice with 5 ml. of water. The cells were then dried to constant weight at 105° (4–5 hr.) in air.

General chemical composition of spermatozoa. Semen (4 ml.) was diluted and the cells were washed with buffer as described above. The cells were then suspended in 4.8 ml. of bicarbonate buffer and cooled to 0°. Ice-cold trichloroacetic acid [30% (w/v); 2.5 ml.] was added and the precipitate was washed once with 5 ml. of 5% (w/v) trichloroacetic acid, twice with 5 ml. lots of 5% NaCl and then twice with water (10 ml. lots). The residue was dried to constant weight *in vacuo* over CaCl₂ at room temperature. Subtraction of this weight from the dry weight of the cells gave the weight of the acid-soluble material. The residue was dissolved in 5 ml. of N-NaOH; the solution was rid of the small amount of insoluble residue by centrifuging and then mixed with 2.5 ml. of 60% (w/v) trichloroacetic acid. The precipitate was washed once with 5 ml. of 5% (w/v) trichloroacetic acid and then thrice with 10 ml. lots of water. The

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lipids were removed from the precipitate by successive washings (10 ml. of solvent each time), with ethanol (twice), acetone (twice), ethanol-ether (3:1, v/v; heating at 60° for 10 min.; twice) and finally once with ether. The lipid-free residue, consisting of proteins and nucleic acids, was then dried to constant weight. Nucleic acids were removed by heating the lipid-free precipitate with 8 ml. of 5% (w/v) trichloroacetic acid at 90° for 20 min. The residue was washed twice with 5% trichloroacetic acid, and once each with ethanol and with ether (10 ml. each). It was then dried at 105° to constant weight. Usually, 12–13% loss of weight occurred when proteins dried over CaCl_2 at room temperature were subjected to further drying at 105°. All values have been adjusted to dry weight at 105°.

Free nucleotides. Semen (1 ml.) was diluted with 5 ml. of fructose-bicarbonate buffer. The cells were separated by centrifuging, resuspended in fresh buffer (5 ml.) and centrifuged again. After wiping the inside of the tube, the cells were cooled to 0° and mixed with 5 ml. of ice-cold 0.5M- HClO_4 . In some experiments, a measured volume of the fructose-bicarbonate buffer washings, containing the diluted seminal plasma, was treated with an equal volume of m- HClO_4 at 0°. The precipitate in each case was removed as soon as possible by centrifuging at 0° (10 min. at 1000 g. for washed cells, and 20 min. at 20 000 g. for seminal plasma) and the ultraviolet spectrum of the clear supernatant, containing the nucleotides, was determined with a Unicam spectrophotometer. Adjustment to pH 7.4 with NaOH did not significantly affect the spectrum obtained or the absorption at 260 m μ . With the extract from washed cells, the form of the absorption curve indicated that nucleotides were present in significant amounts and the nucleotide content was calculated assuming a molar-extinction coefficient of $E_{1\text{cm}}^{260}$ (P) 10 000 (average mol. wt. taken to be 320, cf. Beaven, Holiday & Johnson, 1955). Duplicate runs on the same batch of semen gave identical results.

In a series of experiments with semen from bull N_1 , the nucleotide estimations were made after storing the undiluted semen at room temperature for periods of 1, 3 and 4½ hr. After the appropriate time interval the semen was diluted with fructose-bicarbonate buffer, and centrifuged and the cells were washed as described above. The interval between first addition of buffer and addition of HClO_4 was 1 hr.

Nucleic acids. Nucleic acids were estimated in whole semen, in washed cells and in seminal plasma, from bulls N_1 and N_2 .

Before treatment with trichloroacetic acid, the different samples were prepared as follows. Whole semen (1 ml.) was diluted with 3 ml. of fructose-bicarbonate buffer. Washed cells (from 1–5 ml. of semen) were suspended in 4 ml. of fructose-bicarbonate buffer. The seminal plasma examined was that material left in the supernatant after centrifuging samples of semen (1–5 ml.) previously diluted with an equal volume of fructose-bicarbonate buffer. Each sample was treated at 0° with half its volume of 30% (w/v) trichloroacetic acid, centrifuged, and the residue washed once with 5% (w/v) trichloroacetic acid and then twice with 5% NaCl at 0°. Lipids were removed as already described and the protein-nucleic acid residue was suspended in 5 ml. of m- HClO_4 at 4° for 18 hr. (cf. Ogur & Rosen, 1950). The mixture was then centrifuged at 0°. Ribose was estimated in 3 ml. of the supernatant by the method of Mejsbaum (1939); 1 ml. was brought to pH 7.4 with NaOH, made up

to 4 ml. with water and the ultraviolet spectrum was determined against a similarly treated HClO_4 blank. The ribonucleic acid (RNA) content was calculated assuming a statistical equimolarity in the tetranucleotide composition of RNA.

For estimation of deoxyribonucleic acid (DNA), the residue left after cold HClO_4 digestion was washed with two 5 ml. lots of m- HClO_4 at 0° and then extracted at 90° with 5 ml. and 3 ml. of m- HClO_4 (20 min. extractions). The combined hot HClO_4 extracts were brought to pH 7.4 with NaOH, made up to 10 ml. and the ultraviolet spectrum was determined. The DNA content was calculated from the absorption at 260 m μ , assuming that DNA has $E_{1\text{cm}}^{260}$ (P) 8780 (Ogur & Rosen, 1950).

Free amino acids. Semen (2 ml.) was diluted with an equal volume of fructose-bicarbonate buffer and centrifuged. The supernatant was removed and the cells were resuspended in fructose-bicarbonate buffer and re-centrifuged. The free amino acids were estimated in the residue (spermatozoa) by paper chromatography and in the combined supernatants (seminal plasma) by column chromatography, as described below.

Cells. The washed spermatozoa were suspended in water (2 ml.) and 30% (w/v) trichloroacetic acid was added (1.2 ml.). After 1 hr. at room temperature the precipitate was collected and washed twice with 2 ml. portions of 5% (w/v) trichloroacetic acid, the washings being added to the first extract. The extract was freed of trichloroacetic acid by repeated extraction with ether. The aqueous phase was added to a 3 cm. $\times$ 0.9 cm. column of Zeo-Karb 225 (150–200 mesh; hydrogen form) and the column was washed with about 20 ml. of water; during washing, no ninhydrin-positive material was eluted. The amino acids were then displaced with aq. 0.5N- NH_3 soln., taken to dryness over H_2SO_4 and estimated by paper chromatography. The amino acid mixture was chromatographed on Whatman no. 1 paper, with phenol as solvent, in an atmosphere of NH_3 . Serial dilutions of a standard mixture of amino acids were chromatographed on the same sheet of paper and the amount of each amino acid in the cell extract was estimated by visual matching with a corresponding spot from the standard mixture. Estimations made in this way are subject to a fairly large error (20%) but, in view of the small quantity of amino acids present in the cell extract, this method was considered the most appropriate.

Seminal plasma. The combined supernatants from the washed cells (diluted seminal plasma) were treated with 10% (w/v) trichloroacetic acid to give a final acid concentration of 5%. The residue was centrifuged, washed with trichloroacetic acid and the trichloroacetic acid removed from the combined washings with ether. Amino acids were isolated from the aqueous phase with Zeo-Karb 225 as described above. This material, from 2 ml. of semen, was then fractionated on a column of Zeo-Karb 225 WR 1-55, as described by Campbell, Jacobs, Work & Kressman (1955), and the individual amino acids were estimated with ninhydrin (Moore & Stein, 1948).

Breakdown of intracellular proteins. Spermatozoa were isolated from semen (4.5 ml.; bulls N_2 and N_4), after dilution with an equal volume of bicarbonate buffer, by centrifuging; they were then washed twice with buffer and finally suspended in a volume of buffer about equal to the volume of the original semen sample. Samples (1 ml.) of each suspension were incubated aerobically at 37° in 15 ml. centrifuge

tubes, one for each time point. At appropriate intervals (0, 30, 60, 120 and 240 min.), 0.3 ml. of 30% trichloroacetic acid was added. The precipitate was removed by centrifuging and washed with 1 ml. of 5% (w/v) trichloroacetic acid. Trichloroacetic acid was removed from the combined supernatants as already described, and the resulting aqueous phase was taken to dryness *in vacuo* over H_2SO_4 and made up to 1 ml. with water. Half (0.5 ml.) of this solution was used for direct estimation of total free amino nitrogen, with ninhydrin. On 0.3 ml. of the remainder, ninhydrin estimations were done after hydrolysis with 6N-HCl at 105° for 18 hr.

RESULTS AND DISCUSSION

General chemical composition of bull spermatozoa

The composition of bull spermatozoa, as found by us with the procedures described in the text, is given in Table 1. The dry weight recorded here (16.5×10^{-9} mg./cell) is somewhat lower than that reported earlier (Zittle & O'Dell, 1941), but this may be due to differences in the drying technique. Our value is for cells dried at 105°. One observation requires special comment, namely the large loss of weight produced by treating the trichloroacetic acid-precipitable material with organic solvents. This procedure would normally be expected to extract only lipid and phospholipid, but, in the present work, appreciable quantities of protein were also extracted by the organic solvents. These proteins became progressively more insoluble on repeated dissolution in, and evaporation

from, alcoholic solutions. In later experiments on protein metabolism in spermatozoa (Bhargava, Bishop & Work, 1959), extraction with organic solvent was carried out after removal of DNA with hot trichloroacetic acid. Even under these conditions, some protein was extracted by organic solvents. It seems likely that this is due to the presence of much protamine-like material and phospholipid which form complexes soluble in organic solvents (cf. Chargaff, 1938).

Deoxyribonucleic acid of bull spermatozoa and seminal plasma

The DNA content of bull spermatozoa was found to be 3.4×10^{-9} mg./cell (Table 2). This figure agrees with that reported by Vendrely & Vendrely (1953), but values as low as 2.8×10^{-9} mg./cell (Mirsky & Ris, 1949) and as high as 4.5×10^{-9} mg./cell (Zittle & O'Dell, 1941) have been reported. In the present work, the same DNA value per cell was obtained when either whole semen or washed cells were used.

Ribonucleic acid of bull spermatozoa and seminal plasma

A small amount of ribose-containing material was detected in spermatozoa from bull N_1 and a similar amount in cells from bull N_2 (Table 2). Calculated as a percentage of the DNA present it is less than 1%. The RNA content of nuclei from other cells is invariably much higher, though it varies over wide limits among cell types, and probably lies between 10 and 30% of the DNA content for most cells (Dounce, 1955). It is likely that the true figure for bull spermatozoa is substantially below 1%, since, owing to the enormous excess of DNA, estimations of ribose will be somewhat high owing to interference by deoxyribose.

The seminal plasma of both bulls contained small and variable amounts of ribose-containing material. This may be RNA derived from cytoplasmic droplets (Bishop & Austin, 1957) and its presence accounts for the slightly higher RNA values obtained when whole semen is used instead of washed cells (Table 2).

The ultraviolet spectrum of the cold perchloric acid digests derived from washed spermatozoa

Table 1. *Composition of normal bull spermatozoa*

Estimations were made on cells derived from 3 ml. of semen, as described in the text. The results quoted are mean values of three determinations on bull N_1 .

	10 ⁹ × Amount per cell (mg.)	Percentage of dry weight
Dry weight	16.5	100
Trichloroacetic acid- soluble material	3.3	20
Alkali-insoluble material	0.17	1.3
Lipid and lipoprotein*	6.4	39
Nucleic acid	3.4	21
Protein other than lipoprotein	3.2	19

* The term 'lipoprotein' is used for protein extractable into organic solvent under conditions defined in the text.

Table 2. *Nucleic acid content of bull spermatozoa*

All DNA and RNA estimations were on material from the same batch of semen.

Bull	Fraction	Vol. of semen used (ml.)	No. of cells used (millions)	DNA (mg./10 ⁹ cells)	RNA† (mg./10 ⁹ cells)	RNA (as per- centage of DNA)
N_1	Semen	1.0	2350	3.30	0.050	1.5
	Cells	1.8*	6680	3.34	0.025	0.75
N_2	Cells	2.0*	1880	3.43	0.007	0.2

* Amount of semen from which the cells were derived.

† Based on ribose estimations.

resembles that of nucleic acid (Ogur & Rosen, 1950). Comparison of the RNA values calculated from extinction at $260\text{ m}\mu$ with those obtained by the ribose method indicates that, in addition to the small amount of RNA, some DNA also had been extracted. This DNA, however, represented only a small fraction (less than 5%) of the DNA extracted by hot perchloric acid. The RNA content as estimated by the ribose method was about 0.015×10^{-9} mg./cell, but the apparent RNA value as determined by ultraviolet absorption on the perchloric acid extract was 0.26×10^{-9} mg./cell. Our results show therefore that spectrophotometric estimation of RNA by the method of Ogur & Rosen (1950) is likely to give erroneous results when the RNA content of the material under examination is low.

Table 3. *Free nucleotide content of spermatozoa*

Experiments bearing the same numbers were done on a single batch of semen.

Expt. no.	Bull	No. of cells used (millions)	Amount of nucleotide (mg./ 10^9 cells)	Period of storage of semen after collection (min.)
1	N ₁	1600	0.184	120
2	N ₁	1440	0.158	150
3	N ₁	1130	0.090	60
3	N ₁	1130	0.071	180
3	N ₁	1130	0.078	270
4	N ₂	1580	0.120	120

Fig. 1. Ultraviolet spectra of the acid-soluble fractions derived from the spermatozoa ($\square$) and from the seminal plasma ($\circ$) of bull N₁.

The present conclusion that bull spermatozoa contain, at most, only traces of RNA is in agreement with other work in which different kinds of spermatozoa have been examined by a variety of methods. Mauritzen, Roy & Stedman (1952) could not detect any RNA in fish sperm heads; White, Leslie & Davidson (1953) found no RNA in human spermatozoa and Mann (1954) found none in ram spermatozoa. The value for RNA in bull spermatozoa reported by Vendrely & Vendrely (1948) is probably attributable to contamination with non-RNA phosphorus, as is suggested by the later work of Mauritzen *et al.* (1952).

Intracellular free nucleotides

When washed spermatozoa from bull N₁ or N₂ were extracted with ice-cold perchloric acid, an extract was obtained which had the typical absorption spectrum of nucleotides (Fig. 1). It is unusual to obtain by direct extraction of cells with cold perchloric acid an extract which is clearly mainly nucleotide. We have found, however, that spermatozoa have a particularly low free amino acid pool together with an exceptionally large free nucleotide pool. The nucleotide values obtained are given in Table 3. Since the free nucleotides were not purified, these values must be regarded as approximations. The size of the nucleotide pool varied somewhat with different batches of semen (range 0.4–1.1% of dry weight). No appreciable loss of nucleotide from the cells was observed during storage of semen for periods of up to $4\frac{1}{2}$ hr. at room temperature. The ultraviolet-absorption spectrum (Fig. 1) of the acid-soluble fraction derived from seminal plasma indicated that free nucleotides are present, at most, in traces.

Free amino acids of seminal plasma and spermatozoa

The values for free amino acids found in the seminal plasma of bull N₁ are given in Table 4. Since the estimations were made by the method of Moore & Stein (1951) after chromatographic separation on ion-exchange resin, the values for the major components can be regarded as fairly accurate ($\pm 5\%$). In addition, a number of minor components with ninhydrin-positive reactions were detected. Some of these may have been peptides but no attempt was made to identify them. The free amino acid content of the washed cells was too low to permit accurate estimation, but approximate values were obtained by paper chromatography (Table 4).

Gassner & Hopwood (1952, 1953), using quantitative paper chromatography, determined the free amino acids in whole semen and in seminal plasma. They found no difference between whole semen and seminal plasma, which suggested, in agreement

with our results, that bull spermatozoa have a small amino acid pool. Their values for whole semen are considerably lower than our values for seminal plasma, and they were able to find only five amino acids (glutamic acid, aspartic acid, glycine, serine and alanine). A possible source of this discrepancy is that the column method detected components missed in paper chromatograms. It is also possible that Gassner & Hopwood analysed their specimens sooner after ejaculation than we have done. A fairly rapid increase in

amino nitrogen on storage of semen has been reported by Lundquist (1949, 1952) and by Jacobsson (1950). Since we were primarily interested in the size of the amino acid pool during periods of incubation of spermatozoa with isotopically labelled amino acids (Bhargava *et al.* 1959), it seemed preferable to estimate the amino acid pool at about 1 hr. after ejaculation, the time at which incubations were usually set up.

Our values for the intracellular amino acid pool of bull spermatozoa (1.07 m-moles/100 g. dry wt.) are low compared with those reported for other mammalian tissues, which seem to exceed 5 m-moles/100 g. dry weight (cf. Tallan, 1955; Smith & Nelson, 1957).

Table 4. *Free amino acid pool of spermatozoa and seminal plasma from bull N₁*

Where no amino acid was detected this is indicated as ND. The concentration of spermatozoa in the semen used was $1525 \times 10^6/\text{ml}$.

Amino acid	Amino acid ($\mu\text{mole}/\text{ml}$. of semen)	
	Cells	Seminal plasma
Aspartic acid	ND	0.32
Threonine	ND	0.15
Serine	0.04	0.62
Glutamic acid	0.08	2.79
Glycine	0.07	0.85
Alanine	0.04	0.49
Valine	0.02	0.15
Leucine	0.02	ND
Isoleucine	ND	ND
Tyrosine	ND	0.13
Lysine	ND	0.11
Histidine	ND	Trace
Arginine	ND	0.11
Total	0.27	5.72

Fig. 2. Release of amino nitrogen (Δ) and peptides ($\blacktriangle$) during incubation of spermatozoa in bicarbonate buffer. (For conditions of incubation see text.)

Proteolysis during incubation

In earlier experiments (Bhargava, 1957) it was noted that there was some loss of protein during incubation of spermatozoa at 37° . This observation prompted us to determine the rate of release of amino acids and peptides during incubation of washed spermatozoa in bicarbonate buffer. Free amino nitrogen was determined directly with ninhydrin, and trichloroacetic acid-soluble peptides were estimated with the same reagent after hydrolysis. The results, given in Fig. 2, show that there was a linear increase in the concentration of amino nitrogen during incubation. This increased amino nitrogen must be derived ultimately from cell peptides or cell proteins.

In these experiments, the values for free amino nitrogen at zero incubation time are higher than those in the experiment reported in Table 4, but this discrepancy is not unexpected since the values in Table 4 exclude ammonia, whereas ammonia would be estimated along with amino acids in the incubation experiments. Although there was a substantial increase in total amino nitrogen on incubation, there was no increase in total peptide, and subtraction of the free amino nitrogen values from the total amino nitrogen values results in a straight line parallel to the base line (Fig. 2). We conclude therefore that any peptides released during incubation undergo further proteolysis to free amino acids.

SUMMARY

1. Data are presented on the chemical composition of bull spermatozoa and seminal plasma.

2. The dry weight of the spermatozoon was found to be 16.5×10^{-9} mg., of which 3.38×10^{-9} mg. was deoxyribonucleic acid. Ribonucleic acid, if present at all, occurred only in trace amounts.

3. The free nucleotide content of bull spermatozoa is unexpectedly large and constitutes on average 0.75% of the dry weight of the cell.

4. The spermatozoa contain only traces of a few free amino acids. The seminal plasma contains 5.72 μ moles of total amino acids/ml.

5. The spermatozoon proteins were found to undergo slow degradation to free amino acids on incubation at 37°.

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Incorporation of [¹⁴C]Amino Acids into the Proteins of Bull Spermatozoa

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During the last two decades, a considerable amount of evidence has accumulated to suggest that ribonucleic acid plays an essential role in protein biosynthesis (see Brachet, 1955). Since bull spermatozoa are virtually devoid of ribonucleic acid (Bhargava, Bishop & Work, 1959), they would be expected to be incapable of protein synthesis. It has already been shown, however, that washed bull spermatozoa can incorporate radioactive amino acids into their proteins (Bhargava, 1957); these observations are extended and evaluated in the

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present paper. We have also studied amino acid incorporation by unwashed spermatozoa in seminal plasma since this system more closely resembles that met with *in vivo*. Marked differences were observed between the washed and the unwashed cells in both the rates and the patterns of incorporation of amino acid into protein. The effect of several metabolic inhibitors on incorporation by both washed and unwashed cells was also investigated. For comparative purposes, the rate of incorporation of amino acids into proteins of reticulocytes has been measured under similar conditions to those used for washed spermatozoa.