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Nucleic Acid Metabolism of Mammalian Spermatozoa

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The absence of ribonucleic acid from spermatozoa has been reported by several workers (Brachet, 1933; Mauritzen, Roy & Stedman, 1952; White, Leslie & Davidson, 1953; Mann, 1954; Daoust & Clermont, 1955; Bhargava, Bishop & Work, 1959a). RNA is considered to be an essential component of all protein-synthesizing systems. Therefore the demonstration that suspensions of spermatozoa can incorporate radioactive amino acids into their proteins (Bhargava, 1957; Bhargava, Bishop & Work, 1959b) was of interest. Martin & Brachet (1959), on the basis of micro-radioautographic studies, concluded that this incorporation did not actually take place in the spermatozoa but occurred in the cell debris or bacteria contained initially in the semen. Recent work (Abraham & Bhargava, 1963; P. T. Iype, K. A. Abraham & P. M. Bhargava, unpublished work) has, however, demonstrated that the above-mentioned incorporation occurs in the spermatozoa and represents synthesis of spermatozoal proteins; this work has also afforded an explanation for the negative findings of Martin & Brachet (1959). It therefore appeared to be of interest to re-investigate the reported absence of RNA from spermatozoa.

In most of the earlier experiments, the RNA estimations were made on small quantities of semen. In the investigation by Bhargava *et al.* (1959a) the lower limit of detection of RNA in bull spermatozoa was 0.1% of the deoxyribonucleic acid, or 0.033% of the dry weight of the cells. It therefore seemed possible that spermatozoa might contain a small amount of RNA, which could have escaped detection in earlier investigations with conventional analytical techniques. The presence of even traces of RNA in spermatozoa would be significant from the point of view of protein syn-

thesis by these cells. Recent investigations suggest that the code for the determination of the sequence of amino acids in a protein molecule is carried from the DNA in the form of a short-lived RNA, the 'messenger RNA' (Brenner, Jacob & Meselson, 1961; Gros *et al.* 1961). Both the 'messenger RNA' and the 'soluble RNA' (which is believed to carry the activated amino acids to the site of protein synthesis), are unlikely to represent more than a small fraction of the total RNA content of somatic mammalian cells.

This paper reports studies on the incorporation of radioactive precursors of nucleic acids into the total nucleic acid fraction of bovine spermatozoa; the effect of a few inhibitors on this incorporation has also been studied. These studies show the presence of minute quantities of a metabolically active RNA in mature bovine spermatozoa. A preliminary communication based on this work has already appeared (Abraham & Bhargava, 1961).

EXPERIMENTAL

Materials

Semen. The semen was obtained from two normal, fertile buffaloes (BB1 and BB2), and from five normal, fertile bulls (CB1, CB2, CB3, CB4 and CB5). Semen was collected by the artificial-vagina technique (Walton, 1945) under conditions which minimized bacterial contamination; precautions were also taken to avoid contamination of the semen with talc from the rubber. The semen was collected, on an average, once a week from each bull. The samples were checked, just before use, under a phase-contrast microscope for motility and for the presence of possible contaminants such as bacteria and cell debris. The semen of these animals appeared quite normal in all respects, except for the relative abundance of cytoplasmic droplets in the semen of bull CB3. Usually, the spermatozoa collected after an interval of more than 3 weeks from the date

of the previous collection were mostly immotile; such spermatozoa were not used. The pH of the semen at 1 hr. after collection varied between 6.7 and 7.0. Unless otherwise stated, the semen was used within 2 hr. of collection; it was kept at room temperature during this period. In some experiments, the semen from two animals of the same species was mixed before use.

Radioactive chemicals. [^{32}P]Phosphate was obtained from The Atomic Energy Establishment, Bombay, as orthophosphate in iso-osmotic salt solution buffered with phosphate buffer to give a concentration of 1 mg. of P/ml. (specific radioactivity 1 mc/mg.). [$8\text{-}^{14}\text{C}$]Adenine, [$6\text{-}^{14}\text{C}$]orotic acid, [$2\text{-}^{14}\text{C}$]thymine and [$2\text{-}^{14}\text{C}$]uracil were obtained from The Radiochemical Centre, Amersham, Bucks., and were dissolved in iso-osmotic salt solution to give a concentration of 1.8, 0.9, 0.95 and 0.9 $\mu\text{moles/ml.}$ respectively; each of these solutions contained 9.1 $\mu\text{C/ml.}$

Buffers. The spermatozoa were incubated in one of the following buffers, each of which contained 1% (w/v) of fructose in addition to its normal constituents (Dawson, Elliott & Jones, 1959): (1) Krebs-Ringer bicarbonate buffer; (2) Krebs-Ringer original phosphate buffer; (3) Krebs-Ringer improved phosphate buffer, without the glycolytic and tricarboxylic acid-cycle intermediates; (4) Tyrode's solution. The buffers were prepared in sterilized water. In experiments in which the effect of the addition of a mixture of 21 commonly occurring amino acids on the incorporation of the various precursors was studied, Krebs-Ringer original phosphate buffer containing 20 $\mu\text{g.}$ of each of the amino acids/ml., in addition to fructose, was used as the incubation medium.

Metabolic inhibitors. The following inhibitors were used: chloramphenicol (Chloromycetin, Parke, Davis and Co. Ltd.), a mixture of penicillin and streptomycin (Dierysticin-S, Sarabhai Chemicals), and ribonuclease (crystalline, C. F. Boehringer und Soehne, Mannheim, Germany).

Methods

Preparation of suspensions of spermatozoa. Suspensions of washed spermatozoa were prepared as described by Bhargava *et al.* (1959b). The suspensions used for incubation contained $3\text{--}9 \times 10^8$ spermatozoa/ml.

Motility of spermatozoa. Estimates of the motility of spermatozoa in fresh undiluted semen, in washed cell suspensions just before incubation, and, in a few experiments, in washed cell suspensions after incubation for various periods of time, were made by visual observation under a phase-contrast microscope.

Concentration of spermatozoa. The concentrations of spermatozoa were determined with a haemocytometer.

Bacterial count. In some experiments, the number of bacteria present in the suspension, before incubation and after incubation for various periods of time, was estimated as described by Bhargava *et al.* (1959b).

Preparation of rat-testes homogenate. An adult male rat was killed by a blow on the head. The testes were excised, weighed and quickly homogenized at 2° in the desired buffer (approx. 10 ml./g. wet wt. of the organ) in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 200g for 2 min. The residue was rejected and the supernatant was used immediately after it was obtained; the concentration of the material, derived from the testis, in the supernatant (referred to subsequently as the 'testes

homogenate') was determined by drying 1 ml. to constant weight and subtracting the weight of the solids derived from the buffer.

Incubations. The semen, or the suspension of washed spermatozoa in the appropriate buffer, was incubated with the radioactive precursor in 15 ml. centrifuge tubes at 37° in a water bath, with shaking at 40 oscillations/min. and an amplitude of 5 cm. In some experiments, designed to study the effect of the addition of testes homogenate on the rate of incorporation of nucleic acid precursors into spermatozoa, a portion of the homogenate, containing the required amount of the material derived from the tissue, was added to the suspension of spermatozoa before incubation. In experiments in which the effect of metabolic inhibitors on the incorporation was studied, the incubations were carried out in tubes in which the appropriate amount of an aqueous solution of the inhibitor had been evaporated to dryness at room temperature, under vacuum.

Isolation of nucleic acids. The five methods described in the following paragraphs were used for the isolation of the total nucleic acids from spermatozoa incubated with labelled precursors. These are referred to subsequently as methods 1A, 1B, 1C, 2A and 2B. The extraction of the total nucleic acids was also attempted with phenol in the presence of lauryl sulphate (Gros *et al.* 1961); the yield of the nucleic acids by this method was, however, very low, probably due to the inability of the above treatment to lyse the spermatozoa.

Method 1A. After the appropriate period of incubation, the samples were precipitated with ice-cold trichloroacetic acid to a final concentration of 10%. The precipitate was quickly washed twice with 5% trichloroacetic acid at 2° , and the pH was brought to about 6 by repeated washing in the cold with 5% NaCl. Lipids were removed (Bhargava, Simkin & Work, 1958) and the nucleic acids were extracted three times, each time with 1–2 ml. of 10% NaCl at 98° for 30 min. The nucleic acids were precipitated from the NaCl extract by adding 2 vol. of ethanol and allowing the mixture to stand for 24–48 hr. at -15° . The precipitate was removed by centrifuging at 5000 rev./min. for 15 min., washed twice with aq. 80% ethanol in the cold, dissolved in water, reprecipitated by 2 vol. of ethanol and washed again as above.

Method 1B. This method differed from the preceding one in the use of 4 vol. of ethanol for the precipitation of nucleic acids. This resulted in the precipitation of appreciable quantities of NaCl as well, which was removed by dissolving the washed precipitate in water and reprecipitating with 4 vol. of ethanol.

Method 1C. This method was the same as method 1A, except that the nucleic acids, precipitated from the NaCl extract by 2 vol. of ethanol, were not dissolved and reprecipitated by ethanol.

Method 2A. This method was the same as that employed by Schneider & Potter (1958) for rat liver. In this method the nucleic acids are extracted with hot 10% NaCl directly from the washed trichloroacetic acid precipitate without the prior removal of lipids, and are isolated by two precipitations with 2 vol. of ethanol each time.

Method 2B. This method differed from the preceding one in the use of 4 vol. of ethanol for precipitation of the nucleic acids, in place of 2 vol. The salt was removed by reprecipitation as in method 1B.

Treatment of the total nucleic acid fraction with alkali. RNA was removed from the DNA by treatment of the total (dry) nucleic acid fraction with 0.5 ml. of 0.3N-KOH at 37° for 18 hr., followed by neutralization with 0.4N-perchloric acid to pH 4 in the cold, and removal of the DNA-KClO₄ precipitate by centrifuging. The precipitate was washed twice in the cold with water adjusted to pH 4.

Treatment of the total nucleic acid fraction with ribonuclease. A portion (2 ml.) of a solution of the total nucleic acids (isolated by method 2B from spermatozoa labelled with [¹⁴C]orotic acid) in phosphate buffer, pH 6.7, was incubated with 100 µg. of ribonuclease at 37° for 1 hr. The solution was cooled to 0°, treated with 4 vol. of ice-cold ethanol, and the resulting precipitate of DNA removed by centrifuging. The DNA was further purified by dissolving in water and reprecipitating with 4 vol. of ethanol.

Separation of bases by chromatography. The total nucleic acid fraction isolated by method 1C from spermatozoa (buffalo BB1), incubated in 1 ml. of Krebs-Ringer bicarbonate buffer with the labelled orotic acid (0.082 µmole and 0.109 × 10⁶ counts/min.), was hydrolysed with 0.1 ml. of 72% perchloric acid/mg. of nucleic acid, at 100° for 1 hr. The hydrolysate was diluted with an equal volume of water and centrifuged. The bases present in the supernatant were separated by one-dimensional paper chromatography of a known volume of the solution (Smellie, Thomson & Davidson, 1958). The bases were located on the paper by ultraviolet light and eluted with 5 ml. of 0.02N-HCl. The identity of each base was established by measurement of the ratios $E_{250\text{ m}\mu} : E_{260\text{ m}\mu}$, $E_{280\text{ m}\mu} : E_{260\text{ m}\mu}$ and $E_{290\text{ m}\mu} : E_{260\text{ m}\mu}$.

Estimation of nucleic acids. The amount of nucleic acid in the various extracts was determined by the measurement of $E_{260\text{ m}\mu}$ in either a Beckmann or a Hilger Spekker ultraviolet spectrophotometer. An extinction of 1.0 was taken to correspond to 27 µg. of nucleic acid/ml. (Warburg & Christian, 1942).

Determination of the RNA content of spermatozoa. The total nucleic acid fraction was isolated by method 2B from a large batch of semen. A portion (10–20 mg.) of the nucleic acid was hydrolysed with 0.3N-KOH (2 ml.) and the DNA removed by acidification with perchloric acid as already described. The hydrolysate was made up to 4.5 ml. Ribose was estimated in 3 ml. of this solution by the method of Mejbaum (1939); deoxyribose was estimated in 1 ml. by Seibert's (1940) modification of Dische's method.

Measurement of radioactivity. A measured volume of a solution containing a known concentration of nucleic acid, or a known proportion of the total amount of base eluted from the chromatogram, was plated on aluminium planchets (2 cm.²) and dried in an air oven at 105°. The radioactivity was measured in either a Philips or an Ecko end-window Geiger-Müller counter, each with 6 and 10% counting efficiency for ¹⁴C and ³²P respectively for the counting geometry used. The radioactivity of the total nucleic acids is expressed as either the specific radioactivity (counts/min./mg. of the total nucleic acids) or as µm-moles of the radioactive precursor incorporated/mg. of the total nucleic acids; since more than 99% of the total nucleic acid fraction of spermatozoa consists of DNA (Bhargava *et al.* 1959b), the specific activities are sometimes expressed as counts/min./mg. of DNA. All the radioactivity data for ³²P-containing samples have been corrected for decay to the date on which the incubations were done.

RESULTS

Incorporation of [³²P]orthophosphate, [8-¹⁴C]-adenine and [6-¹⁴C]orotic acid. Washed bull and buffalo spermatozoa incorporated the isotopically labelled phosphate, adenine and orotic acid into their total nucleic acid fraction, usually linearly for 2–3 hr.; sometimes there was a slight initial lag. In most experiments, the incorporation fell after 2–3 hr., and in no experiment did it continue after 4 hr. The results of typical incorporation experiments, two for each of the above precursors, are illustrated in Figs. 1–3. The results of a series of

Fig. 1. Incorporation of [³²P]phosphate into the total nucleic acids of spermatozoa: Expt. 29 (○) and Expt. 48 (△) of Table 1.

Fig. 2. Incorporation of [¹⁴C]adenine into the total nucleic acids of spermatozoa: Expt. 6 (○) and Expt. 19 (△) of Table 1.

experiments in which the incorporation of [^{32}P]-phosphate, [^{14}C]adenine and [^{14}C]orotic acid into the total nucleic acids of washed spermatozoa derived from four different animals was studied, and in which the buffer used for the suspension of spermatozoa and the method used for the extraction and isolation of the nucleic acids were varied between experiments, are given in Table 1.

Considerable variation in the rate of incorporation of all the three precursors into the total nucleic acids of washed spermatozoa was observed from experiment to experiment (Table 1). This variation did not appear to be related to the use of different buffers or different animals or both in the various experiments. However, the method used for the extraction and isolation of the total nucleic acids had a marked effect on the final recovery of the radioactivity in the nucleic acids; the incorporation of all the precursors into the total nucleic acids was usually lower when method 1A was used. In one experiment (Table 1) in which, with the same batch of spermatozoa, the incorporation of [^{14}C]orotic acid was determined by four of the five methods described earlier for the isolation of nucleic acids, the incorporation at 3 hr. by methods 1A, 1B and 2A was 8, 64 and 79% of that obtained with method 2B. The range of incorporation obtained with methods 1B, 1C, 2A and 2B in various experiments, with the same precursor, was similar. Since this range was rather wide, minor differences in the relative efficiencies of these four methods for the extraction of the labelled nucleic acids would pass unnoticed.

Fig. 3. Incorporation of [^{14}C]orotic acid into the total nucleic acids of spermatozoa: Expt. 8 (Δ) and Expt. 20 (○) of Table 1.

In method 1A, the nucleic acids were isolated from a NaCl extract of lipid-free trichloroacetic acid precipitate of spermatozoa, by two precipitations with 2 vol. of ethanol each time. In method 1B there were two precipitations with 4 vol. of ethanol each time and in method 1C one precipitation with 2 vol. of ethanol. It would therefore appear that during the isolation by method 1A most of the labelled nucleic acid was lost in the aqueous ethanol supernatant from the second precipitation. Good recoveries of radioactivity were obtained in the total nucleic acid fraction in experiments employing method 2A, in which the nucleic acids were isolated by two precipitations with 2 vol. of ethanol each time as in method 1A, but in which, unlike in method 1A, the lipids were not removed before the extraction of nucleic acids by 10% NaCl. These results show that the process of extraction of lipids from the trichloroacetic acid precipitate results in an enhancement of the solubility of the nucleic acids that are labelled on incubation of spermatozoa with isotopic precursors; this may be due to a partial degradation of the labelled nucleic acids during the removal of lipids.

Incorporation of [2- ^{14}C]uracil and [2- ^{14}C]thymine. In two experiments, the incorporation of [2- ^{14}C]uracil into the total nucleic acids of washed spermatozoa was studied, the same concentration of the precursor in the incubation medium being used as that in the experiments with [^{14}C]orotic acid. No incorporation of the labelled precursor was obtained. [^{14}C]Thymine was also not incorporated into the nucleic acid of washed spermatozoa, under conditions that gave good incorporation of adenine and orotic acid.

Metabolic stability of the radioactivity incorporated into nucleic acids. In one experiment, after initial incubation of the suspension of spermatozoa with [^{32}P]phosphate for various time-intervals, the cells were washed twice and reincubated for 1-3 hr. in the same buffer in the presence of an excess of non-radioactive phosphate. There was no significant loss of radioactivity from the nucleic acids during the reincubation (Table 2).

Hydrolysis of the labelled nucleic acids. ^{32}P -labelled total nucleic acid fraction, isolated by method 2B, was hydrolysed with alkali in one experiment, under conditions which are known to hydrolyse only RNA to nucleotides, and the radioactivity in the DNA fraction was determined. Although all the nucleic acid present initially was quantitatively recovered in the DNA fraction, the recovery of radioactivity in this fraction was only 8%; 92% of the radioactivity was rendered soluble (Table 3). The total nucleic acid fraction (isolated by method 2B) labelled with [^{14}C]orotic acid, from one experiment, was treated with ribonuclease. All the radioactivity initially present

Table 1. *Incorporation of isotopically labelled nucleic acid precursors into the total nucleic acids of spermatozoa of several bulls and buffaloes under various experimental conditions*

The incubations were carried out, the total nucleic acid was isolated and the values in the last column were calculated, as described in the text. *Spermatozoa* motility was 50-85% in all the experiments. A separate batch of semen was used for each experiment. Solutions used for incubation: 1, Krebs-Ringer bicarbonate buffer; 2, Krebs-Ringer original phosphate buffer; 3, Krebs-Ringer improved phosphate buffer without the glycolytic and citric acid-cycle intermediates; 4, Tyrode's solution.

Expt. no.	Name	Radioactive precursor		Final concn. in incubation mixture	Sohn. used for incubation	Method of extraction of the total nucleic acids*	Precursor incorporated (μ moles/mg. of DNA)					Precursor incorporated in 1 hr. (μ moles/mg. of DNA)†	RNA newly synthesized from labelled precursor‡		
		μ moles/ml.	$10^4 \times$ Counts/min./ml.				1 hr.	1½ hr.	2 hr.	3 hr.	4 hr.		5 hr.	ang./mg. of DNA	% of initial RNA
31	Phosphate	3.0	14.1	4§	BB1	1A	—	350	—	598	—	—	233	70	35
10	Phosphate	4.8	22.1	1	BB1	1O	—	—	575	968	900	—	393	118	59
3	Phosphate	2.7	7.5	1	BB1+BB2	1O	273	—	633	665	—	—	360	108	54
4	Phosphate	3.2	11.1	1	BB1+BB2	1O	348	—	855	—	—	—	507	152	76
7	Phosphate	3.2	11.1	1	BB1+BB2	1O	66	—	155	—	—	—	89	27	14
29	Phosphate	3.2	14.3	1	BB1+BB2	1A	21	—	51.5	74	107	102	33	10	5
32	Phosphate	3.2	14.3	1	BB1+BB2	1A	—	73.5	—	104	—	—	49	15	8
30	Phosphate	3.0	14.3	4	BB1+BB2	1A	—	48	—	150	—	203	68	20	10
39	Phosphate	16.5	14.3	2	CB3	1A	—	98	—	151	—	—	65	20	10
48	Phosphate	16.4	5.2	2	CB4	1B	4550	—	7950	9800	—	—	4550	1365	683
6	Adenine	0.164	0.109	1	BB2	1O	37.5	—	153	307.5	—	—	155	186	93
36	Adenine	0.180	0.120	1	BB2	1A	0	—	0	55.5	—	—	56	67	34
11	Adenine	0.164	0.109	1	BB1+BB2	1O	—	135	—	264	—	—	90	108	54
45	Adenine	0.164	0.109	2	BB1+BB2	2A	237	—	—	111.5	—	—	439	527	264
19	Adenine	0.164	0.109	3	BB1+BB2	1A	328.5	—	680	685	—	—	329	395	198
49	Adenine	0.180	0.120	2	CB4	2B	—	118.5	—	237	—	—	79	95	48
14	Orotic acid	0.082	0.109	1	BB1	1O	—	718.5	—	4640	—	6600	2615	3140	1570
33	Orotic acid	0.082	0.109	1	BB1	1A	—	22.5	—	38.5	—	—	15	18	9
47	Orotic acid	0.082	0.109	2	BB1	2B	—	96.5	—	277.5	—	—	181	217	109
5	Orotic acid	0.082	0.109	1	BB1+BB2	1O	23	—	95	132.5	—	—	72	86	43
8	Orotic acid	0.082	0.109	1§	BB1+BB2	1O	47.5	—	88.5	145.5	168	165	57	68	34
24	Orotic acid	0.082	0.109	2	BB1+BB2	1A	—	25	—	120	—	—	63	76	38
45	Orotic acid	0.082	0.109	2	BB1+BB2	2A	150	—	193.5	—	—	—	150	180	90
20	Orotic acid	0.082	0.109	3	BB1+BB2	1A	148	—	399	755	820	865	356	427	214
41	Orotic acid	0.082	0.109	3	BB1+BB2	1A	—	13.5	—	97.5	—	182.5	57	68	34
37	Orotic acid	0.082	0.109	1	CB2	1A	—	41.5	—	69	—	—	28	34	17
34	Orotic acid	0.082	0.109	1	CB3	1A	—	6	—	10	—	14.5	4	5	3
49	Orotic acid	0.082	0.109	2	CB4	2B	—	51	—	121.5	—	—	47	56	28
46	Orotic acid	0.082	0.109	2	CB1+CB4	2A	7	—	15	16	—	—	8	10	5
		0.082	0.109	2	CB1+CB4	1B	28.5	—	116.5	115	—	—	88	106	53
		0.082	0.109	2	CB1+CB4	2A	93	—	143.5	157.5	—	—	93	112	56
		0.082	0.109	2	CB1+CB4	2B	127.5	—	181.5	295	—	—	128	154	77

* For identification see Experimental section.
 † The hour of maximum incorporation in the experiment.
 ‡ It is assumed that 1 μ mole of incorporated P corresponds to 0.3 μ g., and 1 μ mole of incorporated adenine or orotic acid corresponds to 1.2 μ g. of RNA, and that the amount of RNA initially present in spermatozoa represents 0.09% (by wt.) of their DNA content (see text).
 § Contained 5% of fructose and 1% of glucose, in place of 1% of fructose, in Expts. 31 and 8 respectively.
 || 44 hr.

was solubilized, without any detectable loss of nucleic acid (Table 3). These experiments strongly suggest that the radioactivity in the total nucleic acid fraction was entirely due to the presence of labelled RNA in small amounts.

In one experiment the bases in the total nucleic acid fraction, obtained from washed spermatozoa incubated with [14 C]orotic acid, were separated by hydrolysis and paper chromatography. Only four ultraviolet-absorbing spots, corresponding to adenine, guanine, cytosine and thymine, were obtained. A synthetic mixture of these four bases and uracil, spotted on the same chromatogram, gave five distinct spots. Of the radioactivity initially put on the paper 90 % was recovered in the portion of the paper where the uracil spot was to be expected; no radioactivity was detected in the guanine, adenine, cytosine and thymine fractions. The ratios $E_{250\text{ m}\mu} : E_{260\text{ m}\mu}$, $E_{280\text{ m}\mu} : E_{260\text{ m}\mu}$ and $E_{290\text{ m}\mu} : E_{260\text{ m}\mu}$ of the four eluted bases for which the spots were obtained were in close agreement with the values obtained by Beaven, Holiday & Johnson (1955), which showed that a complete separation of the bases had occurred.

Table 2. *Retention of radioactivity in the total nucleic acids of 32 P-labelled spermatozoa on reincubation in a non-radioactive medium*

A portion (1 ml.) of a suspension of washed spermatozoa (buffaloes BB1 + BB2) in Tyrode's solution was incubated with [32 P]phosphate (3 μ moles and 14.3×10^6 counts/min.). At the end of the period of incubation, one sample for each time-point was precipitated as usual; another sample was resuspended in a non-radioactive medium (Krebs-Ringer original phosphate buffer) and reincubated for $1\frac{1}{2}$ hr. as described in the text; nucleic acids were isolated by method 1A.

Time of incubation (hr.)		Specific activity of total nucleic acids (counts/min./mg.)
In radioactive medium	In non-radioactive medium	
$1\frac{1}{2}$	Nil	209
$1\frac{1}{2}$	$1\frac{1}{2}$	189
3	Nil	713
3	$1\frac{1}{2}$	698

Table 3. *Loss of radioactivity from the total nucleic acids on removal of ribonucleic acid*

Total nucleic acids were isolated from a portion (1 ml.) of washed spermatozoa suspension incubated with [32 P]phosphate (16.4 μ moles and 5.2×10^6 counts/min.) or [14 C]orotic acid (0.082 μ mole and 0.109×10^6 counts/min.) for 3 hr.; RNA was removed by hydrolysis, either with KOH or with ribonuclease, as described in the text.

Animal	Radioactive precursor	Hydrolysing agent used for removal of RNA	Acid- or ethanol-precipitable nucleic acids			
			Before RNA removal		After RNA removal	
			Wt. (μ g.)	Radioactivity (counts/min.)	Wt. (μ g.)	Radioactivity (counts/min.)
CB4	Phosphate	KOH	219	331	217	27
CB5	Orotic acid	Ribonuclease	480	281	482	0

Effect of ribonuclease. Ribonuclease (100 μ g./ml.) had no effect on the incorporation of [32 P]-phosphate, [14 C]adenine and [14 C]orotic acid into the total nucleic acids of washed spermatozoa (Table 4).

Effect of chloramphenicol, penicillin and streptomycin. Chloramphenicol (100 μ g./ml.) stimulated the incorporation of [32 P]phosphate and [14 C]orotic acid into the total nucleic acids of washed spermatozoa by 30–120 % (Table 4); there was a considerable variation in this stimulation from experiment to experiment. In one experiment in which a lower concentration (50 μ g./ml.) of chloramphenicol was used, the stimulation was also lower (28 and 6 % at $1\frac{1}{2}$ and 3 hr. respectively). A mixture of penicillin (80 i.u./ml.) and streptomycin (100 μ g./ml.) had no significant effect on the incorporation of [14 C]orotic acid into the total nucleic acids (Table 4).

Effect of amino acids. Addition of a mixture of the 21 commonly occurring amino acids (unlabelled) to the incubation medium caused a two- to three-fold stimulation of the incorporation of the labelled phosphate, adenine and orotic acid, but not of the labelled uracil, into the total nucleic acids of washed spermatozoa (Table 5).

Effect of rat-testes homogenate. In one experiment, washed spermatozoa were incubated with [14 C]-orotic acid in the presence of known amounts of rat-testes homogenate; the spermatozoa and the testes homogenate were also incubated separately, as controls. The incorporation was higher in the total nucleic acids derived from a suspension of spermatozoa to which no testes homogenate had been added than in the total nucleic acids from mixtures of spermatozoa and testes homogenate, or from testes homogenate alone (Table 6).

Effect of seminal plasma. In two experiments in which the whole semen was incubated with [14 C]-adenine, no radioactivity was recovered in the total nucleic acid fraction, even after incubation for 3 hr. In several experiments, in which either the whole undiluted semen or a suspension of washed spermatozoa in undiluted seminal plasma (both derived from the same batch of semen) was incubated with [14 C]orotic acid, no incorporation of the precursor into the total nucleic acid of the system

Table 4. *Effect of inhibitors on the incorporation of nucleic acid precursors into the total nucleic acids of spermatozoa*

A portion (1 ml.) of a suspension of washed spermatozoa was incubated with the radioactive precursor in the presence of the inhibitor, and the nucleic acids were isolated as described in the text. The final concentration of [32 P]phosphate was 3 μ moles/ml. and 14.1×10^6 counts/min./ml., 3.2 μ moles/ml. and 14.3×10^6 counts/min./ml., and 16.5 μ moles/ml. and 14.3×10^6 counts/min./ml. in Expts. 31, 32 and 39 respectively. The final concentrations of [14 C]adenine and [14 C]orotic acid were 0.164 μ mole/ml. and 0.109×10^6 counts/min./ml., and 0.082 μ mole/ml. and 0.109×10^6 counts/min./ml. respectively. Chloramphenicol and ribonuclease were used at a concentration of 100 μ g./ml., except in Expt. 31, in which the chloramphenicol concentration was 50 μ g./ml. With the mixture of penicillin and streptomycin, the final concentrations were 80 i.u. and 100 μ g./ml. respectively. The results are expressed as a percentage increase or decrease over a control to which no inhibitor was added. Separate controls were run for each experiment.

Expt. no.	Animal	Buffer used for incubation*	Radioactive precursor	Inhibitor	Method of extraction of nucleic acids	Percentage increase (+) or decrease (-) in specific activity of total nucleic acids	
						1½ hr.	3 hr.
31	BB1	4	Phosphate	Chloramphenicol	1A	+28	+6
32	BB1 + BB2	1	Phosphate	Chloramphenicol	1A	+29	+42
39	CB3	2	Phosphate	Chloramphenicol	1A	+59	+31
				Ribonuclease	1A	-4	-8
49	CB4	2	Adenine	Ribonuclease	2B	+6	-4
37	CB2	1	Orotic acid	Chloramphenicol	1A	+38	+123
33	BB1	1	Orotic acid	Chloramphenicol	1A	+57	+20
				Ribonuclease	1A	+13	-8
47	BB1	2	Orotic acid	Ribonuclease	2B	-2	-4
				Streptomycin + penicillin	2B	-11	-4

* For identification see Table 1.

Table 5. *Effect of addition of amino acids on incorporation of nucleic acid precursors into total nucleic acids of spermatozoa*

A portion (1 ml.) of a suspension of washed spermatozoa (bull CB4) in Krebs-Ringer original phosphate buffer was incubated with the radioactive precursor ([32 P]phosphate, 16.4 μ moles and 5.15×10^6 counts/min.; [14 C]adenine, 0.18 μ mole and 0.12×10^6 counts/min.; [14 C]orotic acid, 0.082 μ mole and 0.109×10^6 counts/min.) in the presence of a mixture of 21 commonly occurring amino acids, and the nucleic acids were isolated by method 1B for [32 P]phosphate and by method 2B for [14 C]adenine and [14 C]orotic acid, as described in the text. The concentration of each amino acid (glycine, alanine, valine, leucine, isoleucine, serine, threonine, phenylalanine, tyrosine, tryptophan, proline, hydroxyproline, histidine, arginine, lysine, glutamic acid, aspartic acid, glutamine, asparagine, methionine and cysteine) in the incubation mixture was 20 μ moles/ml. Results are expressed as a percentage increase over a control to which no amino acid was added. Separate controls were run for each experiment.

Expt. no.	Radioactive precursor	Percentage increase in specific activity of total nucleic acids	
		1½ hr.	3 hr.
48	Phosphate	112	169
49	Orotic acid	116	148
49	Adenine	143	180

could be detected. These observations show that seminal plasma has an inhibitory effect on the incorporation of nucleic acid precursors into the nucleic acids of spermatozoa.

Bacterial contamination. Since bull semen usually contains a small number of bacteria (Dott & Glover, 1954), in one experiment in which [14 C]orotic acid was used as the precursor in the same concentration as in the experiments described above, the number of bacteria present in the suspension of washed spermatozoa, initially and after various periods of incubation, was determined. The initial bacterial count of the suspension was negligibly small, and there was no significant increase in the number of bacteria during incubation. The incorporation of orotic acid into the total nucleic acids of spermatozoa was higher in this experiment than in most others.

RNA content of spermatozoa. Previous work on the chemical determination of the RNA content of bull spermatozoa had shown that freshly ejaculated spermatozoa might not contain RNA in amounts exceeding 1% of their DNA content (Bhargava *et al.* 1959*b*). These workers estimated RNA as ribose in the cold perchloric acid hydrolysate of the total nucleic acids of the cells, and pointed out that the actual RNA content of spermatozoa is probably much lower than was ob-

Table 6. *Effect of addition of testes homogenate on incorporation of [¹⁴C]orotic acid into total nucleic acids of spermatozoa*

A portion (1 ml.) of a mixture of different proportions of a spermatozoa suspension (buffaloes BB1 + BB2) and rat-testes homogenate, both in Krebs-Ringer original phosphate buffer, was incubated with [¹⁴C]orotic acid (0.082 μ -mole and 0.109×10^6 counts/min.) as described in the text; nucleic acids were isolated by method 1A.

Dry wt. of the biological material used for incubation (% of total)		Specific activity of total nucleic acids (counts/min./mg.)	
Spermatozoa*	Testes homogenate†	1½ hr.	3 hr.
100	0	33	160
80	20	31	58
60	40	30	53
0	100	0	0

* Dry wt. of spermatozoa was calculated from the cell count, assuming the dry wt. of 10^6 cells to be 16.5 mg. (Bhargava, Bishop & Work, 1959a).

† Dry wt. was determined as described in the text.

served, in view of the interference by deoxyribose derived from partial hydrolysis of DNA; up to 5% of the DNA was found to be hydrolysed. The interference by deoxyribose has been reported to be 12.5% (Webb, 1956; Hutchison & Munro, 1961); this value was confirmed in this investigation with both deoxyribose and spermatozoa DNA, and would completely account for the RNA values of the order obtained by Bhargava *et al.* (1959b) for bull spermatozoa.

To gain further information on the upper limit of the content of RNA in bull spermatozoa, ribose and deoxyribose were estimated in the alkaline hydrolysate of nearly 20 mg. of the total nucleic acids obtained by method 2B from a large batch of bull semen. The amount of deoxyribose in the hydrolysate, derived from a partial hydrolysis of DNA, was always just sufficient to account for the colour in the orcinol reaction for ribose, on the basis of the assumption that 8 mg. of deoxyribose gives the same colour as 1 mg. of ribose in this reaction. In these experiments it should have been possible to detect as little as 2 μ g. of RNA in the total nucleic acids (10–20 mg.) taken initially for the estimation. It is therefore concluded that the RNA content of spermatozoa is lower than 0.02% of its DNA content.

DISCUSSION

The experiments described in this paper were designed to search for traces of metabolically active RNA in spermatozoa. The incorporation of [³²P]phosphate and of [¹⁴C]adenine shows an active nucleic acid metabolism in bovine spermatozoa. Malkin (1953) reported an incorporation of

[2-¹⁴C]glycine and of [4,6-¹⁴C₂]adenine into the DNA of mature sea-urchin spermatozoa. The observations reported in this paper, however, suggest that, at least in bovine spermatozoa, it is the cellular RNA and not the DNA which is metabolically active: (a) [³²P]phosphate is incorporated only into an alkali-labile material contained in the total nucleic acid fraction, and not into the DNA (Table 3); (b) there is a considerable incorporation of [¹⁴C]orotic acid into the total nucleic acid fraction (Table 1, Fig. 3), and this incorporation occurs entirely into a ribonuclease-sensitive material (Table 3); orotic acid is not converted into cytosine or into thymine, and is probably converted exclusively into uracil. The incorporation observed into DNA by Malkin (1953) could have been due to a contamination of the DNA with traces of labelled RNA.

Since the amount of RNA, if any, present in the suspensions of spermatozoa at the beginning of the incubation was so small as to make it undetectable by chemical methods, care must be exercised in the interpretation of the results of incorporation experiments described in this paper. Before it can be concluded that the nucleic acid precursors are incorporated into the RNA of spermatozoa, the possibility must be considered that the observed incorporation may be due to bacteria or somatic cell debris or both, present initially in the semen as contaminants, as suggested by Martin & Brachet (1959) for amino acid incorporation into the proteins of spermatozoa suspensions.

The possibility that the observed incorporation of nucleic acid precursors may be due to bacterial contamination can be ruled out for the following reasons.

(1) Labelled uracil and thymine were not incorporated, even in the presence of a mixture of the commonly occurring amino acids; this suggests a mammalian system, where orotic acid and thymidine are the precursors for nucleic acid uracil and thymine respectively.

(2) [³²P]Phosphate was incorporated only into the RNA, and not at all into the DNA, of the spermatozoa suspensions.

(3) Chloramphenicol, or a mixture of penicillin and streptomycin, even at 100 μ g./ml., did not inhibit the incorporation of any of the precursors used; on the contrary, the former stimulated the incorporation of all the precursors (Table 4).

(4) Ribonuclease (100 μ g./ml.) did not inhibit the incorporation of any of the precursors used (Table 4). Ribonuclease is known to inhibit RNA synthesis in bacteria (Kramer & Straub, 1956) and in other micro-organisms, but not in mammalian whole cells.

(5) The incorporation, in every experiment, declined after incubation for 2–4 hr. and did not continue to any significant extent beyond 4 hr. (Table 1; Figs. 1–3); bacterial growth in the incu-

bation mixture would be expected to be most significant after 4 hr. of incubation.

(6) The incorporation observed with every precursor used is too high to be accounted for by intracellular turnover of bacteria present in the system initially, without a rise in the bacterial population.

The possibility that the observed incorporation of RNA precursors may be in unidentified cell debris present as a contaminant in the semen is ruled out for the following reasons.

(1) The incorporation cannot be due to the presence of mitochondria or smaller subcellular particles in the semen, since they are not sedimented at the speed used for washing the spermatozoa. Bigger subcellular particles, such as nuclei or cell fragments, should be easily visible in a phase-contrast microscope at a high magnification. The spermatozoa suspensions used for incubation were always examined under phase contrast at a magnification nearly $\times 800$, and were invariably found to be clean and uncontaminated with any such particles, except 'cytoplasmic droplets' (Mann, 1954), which do not contain DNA and therefore are unlikely to synthesize RNA. No example of the continuance of RNA synthesis for 2-4 hr. in a DNA-free mammalian system is known; this, in fact, is also a strong argument against incorporation of the nucleic acid precursors into cytoplasmic particles such as mitochondria, even if they were to be present in the spermatozoa suspensions.

(2) Likely contaminants of somatic cell origin in semen are broken testicular cells and cell debris derived from these cells. The addition of rat-testes homogenate to the incubation medium in quantities that were such that their dry weight represented 20-40% of the dry weight of the spermatozoa in the suspension did not increase the rate of incorporation of [14 C]orotic acid into the total nucleic acids of the system; in fact, the testes homogenate caused an appreciable reduction in the incorporation rate (Table 6).

The results reported in this paper therefore indicate a truly intracellular metabolism of RNA in spermatozoa. The observed incorporation of RNA precursors would imply either (a) a turnover of the RNA initially present in the spermatozoa, without a net increase in the RNA content of the spermatozoa on incubation, or (b) a net synthesis of RNA, resulting in a progressive increase in the RNA content of the spermatozoa on incubation. Table 1 gives the net amount of RNA synthesized/unit wt. of DNA in 1 hr. (the hour of peak incorporation was chosen for this calculation), in the various experiments with one of the three isotopic precursors that were found to be incorporated into RNA. If it is assumed that the RNA content of bovine spermatozoa is 0.02% of their DNA content, that during the incubation no net increase

occurs in the amount of RNA present initially in these cells, and that no dilution of the labelled precursors (for example, by the intracellular pool of the precursor) occurs before its incorporation, the rate of turnover of the small amount of RNA initially present in the cells can be calculated; these calculated values for the various experiments are given in the last column of Table 1. Excluding the 14 experiments in which the total nucleic acid was isolated by method 1A, which, as stated earlier, gave a very low recovery of the labelled nucleic acid, the RNA turnover rate is found to be less than 40%/hr. in only two, and less than 50%/hr. in only four, out of 18 incorporation experiments (Table 1). If the probable dilution of the labelled precursor with the large intracellular free nucleotide pool (Bhargava *et al.* 1959a; Abraham & Bhargava, 1962) or with the phosphate pool of spermatozoa is taken into account, it can be safely concluded that, in most experiments, the actual turnover rate of the spermatozoal RNA, calculated as above, will exceed 100%/hr. Such a high turnover rate of the total cellular RNA does not seem possible, in view of the following considerations.

(1) No loss of radioactivity from isotopically labelled spermatozoa was observed on incubation in a non-radioactive medium (Table 2).

(2) In most experiments the incorporation proceeded linearly, at the maximum rate, for at least 2 hr. (Table 1; Figs. 1-3). A turnover rate of 100%/hr. will not allow the incorporation to proceed linearly for more than 1 hr.

It is therefore probable that there is a net increase in the amount of RNA per cell when washed spermatozoa are incubated with a nucleic acid precursor. The nature and function of the RNA synthesized by spermatozoa is not clear from this investigation. The stimulation of the incorporation of nucleic acid precursors into RNA by a mixture of amino acids, and the inhibitory effect of seminal plasma on both RNA and protein syntheses in spermatozoa, indicate a relationship between the synthesis of RNA and some aspect of amino acid metabolism, presumably protein synthesis, in spermatozoa.

Whereas undiluted seminal plasma inhibits completely the synthesis of RNA, the inhibition of protein synthesis occurs, rather abruptly, after 15-30 min. of incubation; in suspensions of washed spermatozoa protein synthesis continues for several hours, usually until the cells have lost their motility (Bhargava *et al.* 1959b; Abraham & Bhargava, 1963). Preincubation of whole bull or buffalo semen for 30 min. results in a complete loss of its ability to incorporate labelled amino acids into its proteins (K. A. Abraham & P. M. Bhargava, unpublished work). These observations suggest the

initial presence, in semen, of a small amount of a factor which is required for protein synthesis by these cells, which is lost or rendered functionally incompetent after 15–30 min. of incubation, and the further synthesis or re-formation of which is inhibited by seminal plasma. It is possible that this factor is a 'messenger RNA', which after performing its function is not fully degraded as in bacteria (Brenner *et al.* 1961; Gros *et al.* 1961), but is converted into a functionally incompetent form with a molecular weight large enough to make it trichloroacetic acid-precipitable. It is unlikely that the incorporation of adenine and phosphate represents only the terminal addition of an adenine nucleotide to pre-existing 'soluble RNA', as orotic acid is incorporated to the same extent as adenine and phosphate.

The variation in the rate of incorporation of nucleic acid precursors into spermatozoa from experiment to experiment (Table 1) may be partly due to a variation in the size of the intracellular phosphate and nucleotide pools from animal to animal, and from ejaculate to ejaculate for the same animal; such a variation has been demonstrated for the free nucleotide pool of bovine spermatozoa (Bhargava *et al.* 1959a; Abraham & Bhargava, 1962).

The cause of the stimulatory effect of chloramphenicol on the incorporation of RNA precursors in spermatozoa (Table 4) is not understood, particularly since chloramphenicol has very little inhibitory effect on the incorporation of amino acids into washed spermatozoa (Bhargava *et al.* 1959b). A similar stimulation of RNA synthesis by chloramphenicol has been recently observed in liver-cell suspensions (F. Jacob & P. M. Bhargava, unpublished work).

SUMMARY

1. The incorporation of [32 P]phosphate, [8- 14 C]-adenine, [6- 14 C]orotic acid, [2- 14 C]thymine and [2- 14 C]uracil in suspensions of washed buffalo and bull spermatozoa has been studied. The first three of these precursors, but not thymine and uracil, are incorporated into the total nucleic acids of these spermatozoa. This incorporation has been shown to be exclusively into RNA, which is present in these cells in amounts not exceeding 0.02 % of their DNA.

2. Chloramphenicol stimulates the incorporation of [32 P]phosphate, [14 C]adenine and [14 C]orotic acid into washed spermatozoa. Ribonuclease and a mixture of penicillin and streptomycin have no significant effect on this incorporation.

3. Addition of free amino acids to the incubation medium results in a two- to three-fold increase in the incorporation of [32 P]phosphate, [14 C]adenine and [14 C]orotic acid into washed spermatozoa.

4. There is no significant loss of radioactivity

from the nucleic acids on reincubation of washed 32 P-labelled spermatozoa in a medium containing a large excess of non-radioactive phosphate.

5. [14 C]Orotic acid is incorporated into washed spermatozoa at a much higher rate than in rat-testes homogenate.

6. Seminal plasma inhibits the incorporation of nucleic acid precursors into spermatozoa.

7. It is suggested that, during the incubation of washed bovine spermatozoa, there occurs a net synthesis of a functionally labile RNA.

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