

## RNA POLYMERASE ACTIVITY IN BOVINE SPERMATOZOA

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### ABSTRACT

Washed mature spermatozoa from bulls incorporate ribonucleoside triphosphates into RNA using an endogenous template. Maximum incorporation was observed at 31°C in the presence of MgCl<sub>2</sub>, all four ribonucleoside triphosphates, β-mercaptoethanol, and glycine sodium hydroxide buffer at pH 9.0. The amount of synthesis was linearly dependent upon the concentration of spermatozoa and continued for at least 4 h. Digestion studies revealed the RNA to be present in a protected (intracellular?) location in the spermatozoa. The RNA synthesis was inhibited by ethidium bromide, rifampicin, acriflavine, actinomycin D, and caffeine, but not by α-amanitine or rifamycin SV. Fractionation of the spermatozoa by sonication and separation of the heads and tails by centrifugation through a discontinuous gradient revealed that more than half of the total RNA polymerase activity was associated with the tail fraction.

KEY WORDS spermatozoa · bovine ·  
RNA · RNA polymerase

Autoradiographic studies have revealed that the incorporation of radioactive precursors into RNA occurs in a well-defined pattern during spermatogenesis in mammals (14–16, 26). It is generally agreed that high levels of RNA synthesis occur in spermatogonia, primary spermatocytes at the pachytene stage of meiotic prophase, and in Sertoli cells. By the time the differentiating germ cells have matured to the spermatid stage of spermatogenesis, the incorporation of precursors into RNA has declined appreciably and, with autoradiographic techniques, no RNA synthesis has been observed in spermatozoa.

The existence of RNA in mature mammalian spermatozoa has been demonstrated by several investigators (1, 5, 11, 17, 19, 20). In 1963, Abraham and Bhargava (1) discovered that a net synthesis of radioactively labeled RNA occurred when washed bovine spermatozoa were incubated with radioactive orthophosphate, adenine, or orotic acid. In later work, Premkumar and Bhargava

(19, 20) reported the incorporation of <sup>32</sup>P into RNA and a <sup>14</sup>C-labeled protein hydrolysate into protein in spermatozoa. Inhibitor studies and purification of the RNA and protein products led Premkumar and Bhargava to conclude that the transcription and translation found in bovine spermatozoa were likely to be of mitochondrial origin (19). MacLaughlin and Terner (11), using spermatozoa from the cauda of the epididymis of hamster and rat, were able to demonstrate incorporation of [<sup>3</sup>H]uridine into high molecular weight RNA that had an electrophoretic mobility intermediate between that of 28S and 23S RNA markers. The authors did not attempt to localize this RNA to any specific region of the spermatozoa. Recently, Betlach and Erickson (4) reported that a 90-min incubation of 10<sup>8</sup> spermatozoa with [<sup>3</sup>H]uridine incorporated 8.02 × 10<sup>5</sup> dpm into RNA. However, polyacrylamide gel electrophoresis of this labeled RNA disclosed no discernible radioactive peaks despite the fact that the authors were able to detect 28S and 18S RNA by a staining procedure.

In this communication, we present evidence for

RNA polymerase activity in ejaculated bovine spermatozoa. Inhibitor and sperm fractionation studies lead us to conclude that much of the enzyme activity is present in the mitochondria of the spermatozoa.

## MATERIALS AND METHODS

### Materials

[<sup>3</sup>H]UTP (sp act, 14–36.8 Ci/mmol), [<sup>3</sup>H]ATP (sp act, 25–40 Ci/mmol), and Protosol were obtained from New England Nuclear (Boston, Mass.). The unlabeled ribonucleoside triphosphates and enzyme grade sucrose were purchased from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, New York). Trizma base, calf thymus DNA, glycine,  $\beta$ -mercaptoethanol, ethidium bromide,  $\alpha$ -amanitine, actinomycin D, caffeine, acriflavine, pancreatic RNase A, and electrophoretically pure DNase were products of Sigma Chemical Co. (St. Louis, Mo.). Rifampicin and pronase were obtained from Calbiochem (La Jolla, Calif.). Bovine semen was obtained from the Eastern Artificial Insemination Cooperative (New York), and was stored under liquid nitrogen until use.

### Methods

**PREPARATION OF SPERMATOZOA:** Frozen bull semen (10–50 ml) was thawed and diluted in at least 3 vol of buffer A (0.25 M sucrose, 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.9). The suspension was centrifuged at 5,000 g for 10 min at room temperature. The pellet containing the spermatozoa was resuspended in buffer A and the wash was repeated. The final pellet was resuspended in buffer A and layered over 1.8 M sucrose (dissolved in buffer A) and centrifuged for 30 min at 13,000 g at 4°C. The pellet was resuspended in buffer B (0.05 M Tris-HCl, pH 7.9, 0.1 mM EDTA, 50  $\mu$ g/ml soybean trypsin inhibitor, 25% glycerol) at a final concentration of  $1 \times 10^9$  cells/ml. No cellular debris or bacterial contamination was detected by microscope examination. Initially, the preparation and assay of the spermatozoa were performed with sterile technique. Later, nonsterile experiments yielded results identical to those obtained with sterile procedures.

**ASSAY OF RNA POLYMERASE:** The RNA polymerase activity was measured by a modification of a method previously described (7). The standard assay mixture (0.2 ml total volume) contained 10  $\mu$ mol of glycine-NaOH buffer, pH 9.0; 1  $\mu$ mol of MgCl<sub>2</sub>; 0.4  $\mu$ mol of  $\beta$ -mercaptoethanol; 0.1  $\mu$ mol each of ATP, GTP, and CTP; 0.1 nmol of [<sup>3</sup>H]UTP (250–1,000 cpm/pmol); and an aliquot of sperm suspension. After incubation for 45 min at 31°C, the assay tubes were placed in ice and the reaction was stopped by adding to each assay tube 2 ml of cold 10% trichloroacetic acid (TCA) containing 10 mM sodium pyrophosphate. The precipitated product was collected by filtration on glass fiber filters (Whatman GF/A). The filters were washed with 10 ml of

cold 10% TCA followed by a 10-ml wash with 95% ethanol. After being dried, the filters were counted for radioactivity in a toluene-based scintillation fluid in a liquid scintillation spectrometer. All samples were assayed in duplicate. The values from samples incubated at 4°C (35–75 cpm) were subtracted from the values obtained from those incubated at 31°C. When the effects of antibiotics or inhibitors were tested, these substances were added to the assay mixture before the addition of the sperm suspension.

**ISOLATION OF RNA FROM SPERMATOZOA:** Spermatozoa ( $2.5 \times 10^9$ ) were incubated for 60 min at 31°C in a total volume of 10 ml of the standard assay mixture. To terminate the reaction, the spermatozoa were centrifuged at 13,000 g for 10 min at 4°C and resuspended in 0.01 M Tris, pH 7.4, containing 0.01 M UTP. After a second centrifugation at 13,000 g for 10 min, the pellet containing the spermatozoa was resuspended in 0.01 M Tris at pH 7.4 containing sodium dodecyl sulfate (SDS) (0.5%), proteinase K (100  $\mu$ g/ml) and 0.01 M EDTA. 10 ml of water-saturated phenol was added and the mixture was shaken for 2 min at room temperature. The emulsion was broken by centrifugation for 20 min at 13,000 g at 4°C and the aqueous (upper) layer was removed. The phenol fraction was reextracted as above with 5 ml of buffer C (0.01 M Tris HCl at pH 7.4 containing SDS (0.5%) and 0.01 M EDTA) and the aqueous phases were combined and reextracted with 10 ml of water-saturated phenol. Sodium acetate (final concentration 2%) and 2.5 vol of cold anhydrous ethanol were added to the final aqueous fraction and the preparation was stored at –4°C for 18 h. The precipitate was recovered by centrifugation at 4°C for 40 min at 13,000 g and the pellet was dissolved in buffer B for the digestion studies (Table II) or in E buffer (10) for the electrophoretic studies.

**POLYACRYLAMIDE GEL ELECTROPHORESIS OF RNA:** The procedure for the preparation of 2.4% polyacrylamide gels was essentially as described by Hecht and Woese (10) with the modification that the gels contained 0.5% agarose. RNA samples containing 30,000–38,000 cpm were electrophoresed on 2.4% polyacrylamide gels (9.0 cm long) for 2 h. Yeast 28S, 18S, and 4S RNAs were used as markers to calibrate the gels. At the termination of the run, gels were frozen with dry ice and sliced into fractions of 1 mm. Each fraction was incubated overnight at 37°C in toluene-based scintillation fluid containing 3.5% Protosol, cooled to room temperature, and counted in a liquid scintillation spectrometer.

**LOCALIZATION OF RNA POLYMERASE IN SPERMATOZOA:** Spermatozoa were disrupted and fractionated by a modification of a method previously described (9). Aliquots (2–3 ml) of washed sperm ( $1-2 \times 10^9$  cells/ml) were suspended in buffer B and sonicated for 15 s at a setting of 4 in a Branson sonifier (Branson Sonic Power Co., Danbury, Conn.) (model W-185E). The sonicate was layered over a discontinuous gradient consisting of 5 ml of 0.9 M sucrose (dissolved in buffer

A) and 5 ml of 2.0 M sucrose (dissolved in buffer A). The gradient was centrifuged for 30 min at 13,000 g at 4°C and fractionated. Under the conditions employed, sperm heads and intact sperm were pelleted, tails were found at the interface between the 0.9- and 2.0-M sucrose layers, and soluble enzyme was at the top of the gradient. Each fraction was collected, resuspended in buffer B, and assayed for RNA polymerase activity. To detect any RNA polymerase solubilized by the sonication, the assays of the soluble fraction contained 40 μg of calf thymus DNA (20 μg of native DNA and 20 μg of denatured DNA) in the reaction mixture. The addition of DNA to the assay mixtures for the fractions containing heads and tails did not significantly alter the activities.

## RESULTS

### Characteristics of RNA Synthesis in Spermatozoa

Washed bovine spermatozoa catalyze the incorporation of the RNA precursor, [<sup>3</sup>H]UTP, into a cold TCA-insoluble product. The incorporation of the radioactively labeled triphosphate was linear for up to 60 min and continued at a reduced rate for up to 4 h, the time at which the experiment was terminated (Fig. 1). No incorporation was observed in the absence of spermatozoa, and the amount of incorporation was linearly dependent upon the concentration of sperm present in the reaction mixture between  $0.5 \times 10^7$  and  $5 \times 10^7$  sperm/assay (Fig. 2).

The maximum level of incorporation was dependent on the presence of MgCl<sub>2</sub>, β-mercaptoethanol, and all four ribonucleoside triphosphates (Table I). Addition of exogenous calf thymus DNA did not affect the amount of incorporation observed, suggesting that the RNA polymerase

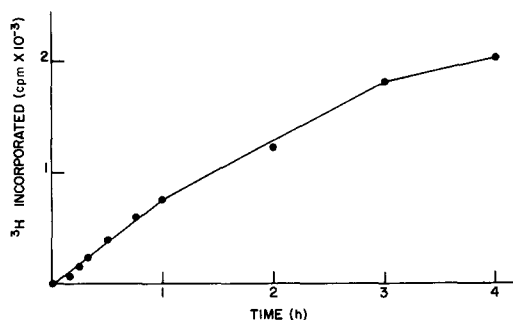


FIGURE 1 Time-course of [<sup>3</sup>H]UMP incorporation into spermatozoa. RNA polymerase activity was measured using the assay procedure described in Materials and Methods. The time of incubation was varied while all other reaction parameters were held constant.

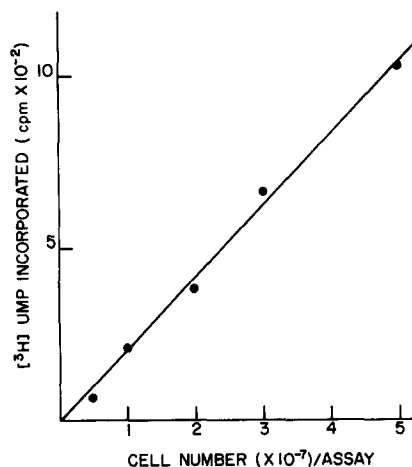


FIGURE 2 Concentration dependence of [<sup>3</sup>H]UMP incorporation into spermatozoa. RNA polymerase activity was measured using the assay procedure described in Materials and Methods. The number of spermatozoa assayed was varied while all other reaction parameters were held constant.

TABLE I  
Requirements for RNA Synthesis in Bovine Spermatozoa

Components	Relative activity %
Complete	100
+ DNA (20 μg native + 20 μg denatured)	98
- MgCl <sub>2</sub>	52
- MgCl <sub>2</sub> + MnCl <sub>2</sub> (5 mM)	11
- CTP, GTP, ATP	63
- β-Mercaptoethanol	94
- MgCl <sub>2</sub> + EDTA (10 mM)	10
+ (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (50 mM)	76
+ (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (200 mM)	48
+ Complete reaction (nonsterile conditions)	97
+ Preincubation of spermatozoa for 2 h at 37°C in the presence of penicillin (200 μg/ml) + streptomycin (300 μg/ml)	87

The complete system (0.2 ml) contained  $2 \times 10^7$  spermatozoa and the standard assay mixture is described in Materials and Methods. The complete reaction in the representative experiment described above incorporated 696 cpm.

was utilizing an endogenous template. Although a substantial incorporation was observed in the absence of any exogenous divalent cation, the addi-

tion of  $MgCl_2$  at concentrations between 1 and 10 mM enhanced the reaction approximately two-fold. Therefore, 5 mM  $MgCl_2$  was routinely added to the assay mixture. At the same concentration,  $MnCl_2$  depressed the level of incorporation below that observed when no cations were added (Table I). The addition of buffered EDTA to the reaction mixture decreased the incorporation to about 10% of the control value (Table I), suggesting the requirement of cations for RNA synthesis in spermatozoa. The amount of synthetic activity seen in the absence of added cations was variable and is likely due to residual levels of cations remaining in the spermatozoa. Mann has reported that declining motility is accompanied by an increased permeability of the sperm membranes leading to a leakage of intracellular constituents from the spermatozoa (12), and the washing procedures described in Materials and Methods drastically decrease the motility of the spermatozoa.

The high level of incorporation observed in the presence of only one nucleoside triphosphate was not specific for UTP but was also seen when  $[^3H]CTP$ ,  $[^3H]GTP$ , or  $[^3H]ATP$  was used as the sole RNA precursor. The reaction was slightly enhanced by the addition of  $\beta$ -mercaptoethanol but did not require it. The presence of  $(NH_4)_2SO_4$  at concentrations of 50 and 200 mM in the assay mixture reduced the amount of incorporation to 76 and 48%, respectively, of control values (Table I).

Identical results were obtained when the spermatozoa were washed and assayed under sterile or nonsterile conditions, suggesting that the incorporation of the nucleotide was not due to contamination in any of the wash or assay solutions (Table I). In addition, preincubation of the spermatozoa at 37°C for up to 2 h with streptomycin and penicillin failed to substantially decrease the amount of  $[^3H]UMP$  incorporated into acid-insoluble product.

The uptake of  $[^3H]UTP$  into an acid-insoluble product was temperature dependent. Maximum synthesis occurred at 31°C with 95 and 90% of this value at 28° and 37°C, respectively. No incorporation was observed above 41°C (Fig. 3).

The pH found to be optimal for the incorporation of  $[^3H]UMP$  into an acid-insoluble product was determined to be pH 9.0, using a glycine sodium hydroxide buffer (Fig. 4). When Tris maleate and Tris HCl buffers between pH 6 and 9 were tested, significantly less synthesis was observed.

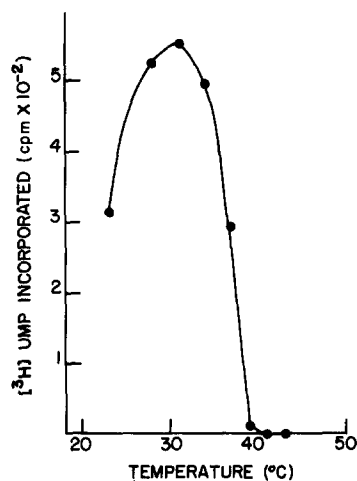


FIGURE 3 Temperature dependence of  $[^3H]UMP$  incorporation into spermatozoa. RNA polymerase activity was measured using the assay procedure described in Materials and Methods. The temperature of incubation was varied while all other reaction parameters were held constant.

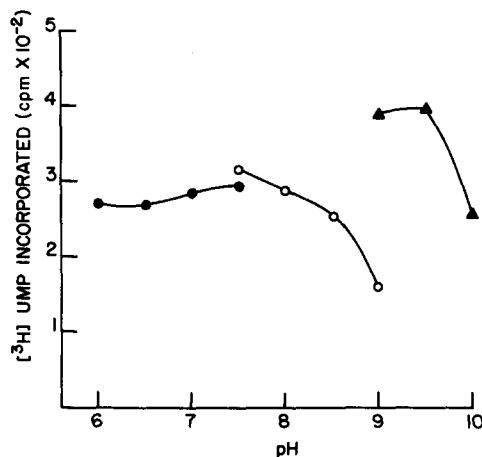


FIGURE 4 Effect of pH on the incorporation of  $[^3H]UMP$  into spermatozoa. RNA polymerase activity was measured using the assay procedure described in Materials and Methods. The pH of the assay mixture was varied while all other reaction parameters were held constant. Tris maleate buffer (●), Tris HCl buffer (○), Glycine sodium hydroxide buffer (▲).

#### Characterization of Product

The nature of the acid-insoluble radioactive product was characterized by its sensitivity to various treatments. Aliquots of sperm were incubated in an RNA polymerase assay mixture for 45 min, centrifuged, and resuspended in buffer B. The

recovered sperm, containing the incorporated radioactivity, were then incubated with NaOH, RNase A, or DNase. The radioactive product was totally hydrolyzed by NaOH but was not affected by incubation with DNase or RNase A (Table II). When the RNA was extracted from the spermatozoa by the phenol method described in Materials and Methods, the alcohol-precipitable product was hydrolyzed by NaOH and digested by RNase A (Table II). No decrease in radioactivity was observed after incubation with DNase. These digestion studies indicate that the product formed is indeed RNA and is present in the spermatozoa in a protected (intracellular?) location.

Polyacrylamide gel electrophoresis of this labeled RNA revealed a heterogeneous profile of

RNA with electrophoretic mobilities ranging from greater than 28S to 4S. Although a quantitative recovery of the radio-labeled RNA was routinely achieved, the predominant peak varied between 18S and 4S from experiment to experiment. These electrophoretic studies establish that the RNA products synthesized by spermatozoa have electrophoretic mobilities coincident with or larger than those of 4S RNA.

To determine whether the RNA synthesized was the product of an RNA polymerase or a homopolymerase, RNA was isolated from a standard reaction mixture in which [<sup>3</sup>H]ATP was substituted for [<sup>3</sup>H]UTP. RNase A, an enzyme that cleaves adjacent to pyrimidine residues, digested 61% of the total radio-labeled RNA, suggesting that the majority of the isolated molecules were RNA molecules and not the homopolymer, poly A.

An additional line of experimental evidence supporting the hypothesis of RNA synthesis in spermatozoa comes from the use of the RNA inhibitor, actinomycin D. The presence of 10, 50, and 100 μg/ml of actinomycin D in the sperm assay mixture led to levels of RNA synthesis of 84, 56, and 24%, respectively, of the control values.

#### Effect of Inhibitors

To help determine which of the multiple eucaryotic RNA polymerases is present in bovine spermatozoa, rifampicin, ethidium bromide, acriflavine, and α-amanitine, compounds that show selective inhibition of RNA synthesis, were tested. The incorporation of [<sup>3</sup>H]UMP was severely depressed by rifampicin and ethidium bromide, two compounds that have been demonstrated to inhibit RNA synthesis in intact mitochondria (Fig. 5) (8, 18). At concentrations of 50 μg/ml, rifampicin and ethidium bromide reduced the amount of RNA synthesized to 36 and 23%, respectively, of control values (Fig. 5). No significant inhibition was observed in the presence of concentrations up to 50 μg/ml of rifampicin SV, an analogue of rifampicin. Acriflavine, a compound also reported to inhibit mitochondrial but not nuclear RNA synthesis at low concentrations (18), decreased the incorporation of [<sup>3</sup>H]UMP into RNA to 22% of control values at a concentration of 3 μg/ml. In contrast, the mushroom toxin, α-amanitin, a compound that has been shown to inhibit nuclear RNA polymerases II and III from mammalian cells (7, 22, 24), failed to significantly inhibit spermatozoan RNA synthesis at concentrations

TABLE II  
Effect of NaOH, RNase, and DNase on Reaction Product

Components	Relative activity %
A. Intact spermatozoa	100
+ RNase (100 μg/ml) incubated 1 h at 37°C	97
+ DNase (100 μg/ml) incubated 1 h at 37°C	101
+ Buffer B incubated 1 h at 37°C	96
+ 0.2 N NaOH incubated 2 h at 37°C	3
B. Isolated product	100
+ RNase (100 μg/ml) incubated 1 h at 37°C	21
+ DNase (100 μg/ml) incubated 1 h at 37°C	105
+ 0.2 N NaOH incubated for 1 h at 37°C	13

For part A, spermatozoa ( $2 \times 10^7$ /assay) were incubated for 45 min at 31°C in the standard assay mixture and were collected by centrifugation at 13,000 g for 10 min at 4°C. The pellet was washed and resuspended in buffer B, and equal aliquots containing between 460 and 1,020 cpm were removed and treated as above. A final concentration of 0.05 M MgCl<sub>2</sub> was added to the sample when DNase was to be tested. The digestions were terminated by the addition of cold 10% TCA, and the precipitated product was collected by filtration on glass fiber filters.

The digestion studies of the isolated product in part B were performed on aliquots of RNA isolated as described in Materials and Methods. Aliquots to be tested contained between 544 and 4,036 cpm, and the digestions were terminated by the addition of cold 10% TCA. The precipitated product was collected by filtration on glass fiber filters.

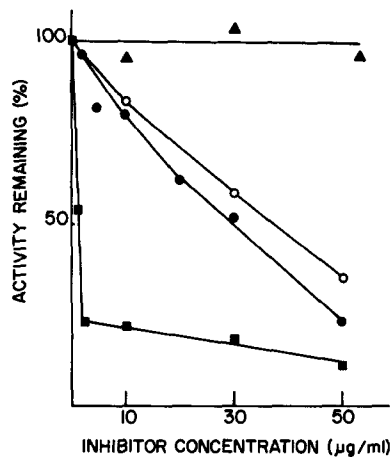


FIGURE 5 Effect of inhibitors on  $[^3\text{H}]\text{UMP}$  incorporation into spermatozoa. Freshly prepared stock solutions of rifampicin, ethidium bromide, acriflavine, and rifamycin SV were prepared and added to the RNA polymerase reaction mixture at  $4^\circ\text{C}$  before the addition of the spermatozoa. Spermatozoa ( $2 \times 10^7$  cells) were added, and RNA polymerase activity was measured using the assay procedure described in Materials and Methods. Ethidium bromide (●), rifampicin (○), rifamycin SV (▲), acriflavine (■).

between 0.02 and 10  $\mu\text{g}/\text{ml}$ . Care must be taken in interpreting these results since the lack of an effect by an inhibitor need not indicate a lack of inhibition of RNA synthesis but may reflect a permeability barrier to this compound in bovine spermatozoa. The ability of rifamycin SV and  $\alpha$ -aminin to enter intact spermatozoa was not determined.

Caffeine at a concentration of 6 mM has been reported to stimulate motility and prolong longevity of spermatozoa from humans (23). Since the increased motility could be due to a general stimulation of spermatozoan metabolism, the effect of caffeine on RNA synthesis was studied. At concentrations between 1 and 10 mM, spermatozoan RNA synthesis was markedly inhibited (Fig. 6).

#### Fractionation of Spermatozoa

Gentle sonication of a suspension of spermatozoa will detach the heads from the tails of many of the sperm cells (9, 13). Since the sperm heads contain the majority of the DNA of the sperm, the head and tail fractions can be readily separated by taking advantage of their vastly different densities. Intact sperm and sperm heads pass through 2.0 M sucrose when centrifuged at 13,000  $g$  for 30 min, while the less dense tail fraction is arrested at the interface between the 0.9- and 2.0-M sucrose so-

lutions (9). When the fractions are monitored by light microscopy, no heads or intact sperm are found in the interface fraction, and the pellet consists of an enriched fraction of heads containing appreciable numbers of intact sperm. (The number of intact sperm found in the pellet depends upon the time and conditions of the initial sonication.)

Aliquots of washed spermatozoa were sonicated for 15 s, layered over a discontinuous sucrose gradient and centrifuged as described in Materials and Methods. An assay for RNA polymerase activity in the soluble, tail, and head fractions revealed that 54% of the total RNA polymerase activity was found in the tail fraction, 39% in the pellet, and 7% in soluble form (Table III).

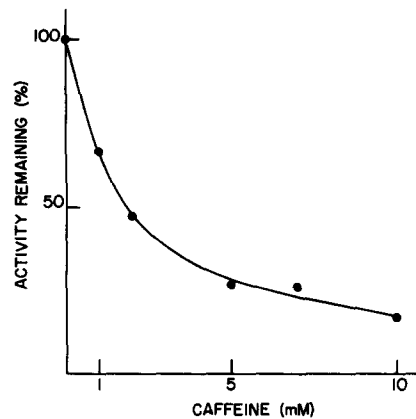


FIGURE 6 Effect of caffeine of  $[^3\text{H}]\text{UMP}$  incorporation into spermatozoa. Aliquots from a freshly prepared stock solution of caffeine were added to the RNA polymerase reaction mixture at  $4^\circ\text{C}$  before the addition of spermatozoa. Spermatozoa ( $2 \times 10^7$  cells) were added, and RNA polymerase activity was measured using the assay procedure described in Materials and Methods.

TABLE III  
Distribution of RNA Polymerase Activity after Disruption of Spermatozoa by Sonication

	$[^3\text{H}]\text{UMP}$ incorporated	% of Total
Soluble fraction	940	7
Interface fraction	7,270	54
Pellet	5,250	39
Total	13,460	

Fractionation of the spermatozoa was performed as described in Materials and Methods. An equivalent control sample of intact spermatozoa assayed before sonication incorporated 17,120 cpm; the RNA synthetic activity of the fractionated sperm representing a recovery of 79%.

## DISCUSSION

Washed mature spermatozoa from bulls incorporate ribonucleoside triphosphates into RNA for at least 4 h at 31°C. The maximum level of incorporation occurs in the presence of MgCl<sub>2</sub>, β-mercaptoethanol, all four ribonucleoside triphosphates and glycine sodium hydroxide buffer at pH 9.0. The RNA polymerase utilizes an endogenous template and requires the presence of divalent cations, since MgCl<sub>2</sub> markedly stimulates the synthesis of RNA and EDTA will depress the incorporation. High amounts (up to 59% of control values) of incorporation were observed when [<sup>3</sup>H]UTP or one of the other ribonucleoside triphosphates was the sole precursor added. These results may be due to (a) the intrinsic ability of the spermatozoan RNA polymerase to incorporate one nucleotide as has been shown for some DNA polymerases, including one isolated from bovine spermatozoa (9, 25), (b) the existence of a large, free nucleotide pool in spermatozoa as reported by Bhargava et al. to be present in the spermatozoa of bulls (5), or (c) the presence of multiple enzymes, e.g., homopolymerase and RNA polymerase activities in the spermatozoa. Solubilization and purification of the enzyme(s) from the spermatozoa will help differentiate between these alternatives.

We believe that the RNA synthetic activity is part of an endogenous system and is not due to a contaminant or a nuclear enzyme adventitiously bound to the sperm, for the following reasons: (a) the preparation of spermatozoa has been extensively washed and sedimented through 1.8 M sucrose, (b) identical results are obtained when we use the hypo-osmotic saline wash procedure of MacLaughlin and Turner (11), a technique designed to lyse cytoplasmic droplets, (c) nucleotides are not transported into bacteria (21), (d) the time-course of incorporation seen in Fig. 1 is not exponential as would be expected with bacteria, (e) the synthesis of RNA by suspensions of washed spermatozoa occurs in the absence of any added DNA, (f) preincubation of washed spermatozoa with DNase before assaying for RNA polymerase fails to decrease the RNA synthesis, suggesting a protected template, (g) RNase cannot digest the RNA until it is isolated from the spermatozoa, and (h) attempts to solubilize the RNA polymerase have indicated that it is a very tightly bound labile enzyme.

Internal synthesis of RNA requires that ribonucleoside triphosphates be able to enter bovine

spermatozoa. Berger and Johnson (3) have reported a procedure to make mouse L-cells permeable. They cold-shock the cells with a buffer containing 0.01 M Tris-HCl at pH 7.9, 0.25 M sucrose, 1 mM EDTA, 30 mM β-mercaptoethanol, and 4 mM MgCl<sub>2</sub>, a solution similar to our wash and suspension buffers. Furthermore, Chevallier and Philippe (6) have observed that sections of frozen but unfixed mouse testes incorporate all four deoxyribonucleoside triphosphates into spermatozoan DNA.

We have attempted to gain insight into the physiological function of transcription in spermatozoa through inhibitor and sperm fractionation studies. In agreement with the results obtained by Premkumar and Bhargava (19), we find that RNA synthesis in bovine spermatozoa is inhibited by rifampicin, ethidium bromide, and acriflavine, three compounds that preferentially inhibit mitochondrial RNA synthesis (18). Rifamycin SV and α-amanitine did not inhibit RNA synthesis in the intact spermatozoa. The effect of these inhibitors on the purified enzyme(s) found in bovine spermatozoa is not known.

When spermatozoa are subfractionated into heads (nuclear fraction) and tails (containing the mitochondrial fraction) (2) and then assayed for RNA polymerase activity, both fractions synthesize RNA. The RNA synthesis found in the head fraction may be evidence for an enzyme activity in the sperm heads or may reflect the synthesis occurring in the mitochondria of the intact sperm present as contaminants in this fraction. Since the tails can be isolated with no contaminating heads or intact sperm, it is likely that sperm mitochondria synthesize RNA and much of the RNA synthesis observed in intact sperm occurs in the mitochondria. Preliminary attempts to use differential assays to distinguish between the RNA polymerase activities in isolated head and tail fractions have revealed differences in the temperature optima and the effect of salt on these two fractions. Work is currently in progress to determine whether in intact sperm a small amount of an RNA polymerase activity present in the heads of sperm is being masked by the presence of a higher level of activity of mitochondrial RNA polymerase. The presence of a homopolymerase activity in one or both of the fractions also cannot be excluded.

Several investigators have reported RNA synthesis in mammalian spermatozoa. Premkumar and Bhargava have isolated <sup>32</sup>P-labeled RNA from

spermatozoa and, after characterization by chromatography on methylated albumin-kieselguhr, gel filtration, electrophoresis on polyacrylamide gels, and molecular hybridization, they have concluded that under sterile conditions bovine spermatozoa synthesize small amounts of 16S and 23S ribosomal RNA, transfer RNA, and a species of nonribosomal 16S RNA (19). Additional inhibitor and protein synthesis studies have led Premkumar and Bhargava to the conclusion that transcription and translation in bovine spermatozoa are solely mitochondrial (19). Using a wash procedure that avoided bacterial and other cellular contamination, MacLaughlin and Turner have synthesized radioactive spermatozoan RNA by incubating spermatozoa from the cauda of the epididymis of the hamster and rat with [<sup>3</sup>H]uridine (11). They isolated a high molecular weight RNA by gel filtration which when analyzed by polyacrylamide gel electrophoresis had a heterogeneous size distribution between 28S and 23S RNA markers. Paul and Duerksen have found that the head fraction of bull sperm contains a single low molecular weight species of RNA that migrates on polyacrylamide gels at a rate just slightly slower than that of chromatin-associated RNA (17). Using spermatozoa from the epididymis and ductus deferens of mice and from human ejaculates, Betlach and Erickson have reported that the majority of the RNA in spermatozoa appears to be 28S and 18S RNA (4). They conclude that this RNA is transcribed in the primary spermatocyte and that no discernible radioactive peaks are observed when the RNA synthesized in sperm is analyzed by polyacrylamide gel electrophoresis. We find that spermatozoa synthesize RNA with electrophoretic mobilities ranging from greater than 28S to 4S. The variability we observe in the RNA patterns may be indicative of aggregation or ribonuclease degradation and may explain why investigators report significant differences in the RNA species synthesized by spermatozoa. Analysis of RNA under native and denatured conditions from separated populations of sperm heads and tails will help clarify the biological significance of transcription in these haploid cells.

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