Ribonucleic Acid Synthesis by Spermatozoa from the Rat and Hamster

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Spermatozoa from the cauda of the epididymis of the hamster and rat were incubated with [5-³H]uridine and glucose. By using a procedure avoiding bacterial and other cellular contamination, sonic extracts were prepared and digested with deoxyribonuclease and Pronase. Radioactive RNA of high molecular weight was isolated by two methods: (a) gel filtration on Sephadex G-75 columns and (b) polyacrylamide-gel electrophoresis in which it migrated in the region of 28S and 23S RNA markers. The macromolecules were alkali-labile and hydrolysed by ribonuclease. From ³H radioactivity and E_{260} of the isolated RNA the rate of incorporation of uridine into RNA of spermatozoa was calculated to be 0.1–0.5 nmol/h per mg of RNA.

It has long been known that RNA is present in spermatogonia and spermatocytes, but diminishes progressively in the developing gamete until, in the mature spermatozoon, it is no longer detectable by chemical and cytochemical methods. The absence of RNA in spermatozoa has therefore been widely accepted and past reports suggesting its presence have met with scepticism (see, Abraham & Bhargava, 1963; Mann, 1964).

By using the radioactive tracer technique, Abraham & Bhargava (1963) claimed to have demonstrated the presence of metabolically active RNA in ejaculated spermatozoa of the bull and the water buffalo. They incubated the spermatozoa with [³²P]orthophosphate, [¹⁴C]orotic acid and [¹⁴C]adenine, separated the total nucleotides and after hydrolysis of the reputed RNA calculated its amount from the loss of radioactivity. This indirect approach left doubts as to the identity of the product (which was not separated) and also its source because of the uncertainty of the extent of bacterial contamination, which can hardly be avoided when working with bovine ejaculates. The inconclusiveness of these findings and the technical difficulties may have discouraged subsequent work for an exceptionally long period of time.

In the present study, an attempt was made to isolate RNA from spermatozoa that had been incubated with [5-³H]uridine, which labels RNA specifically. The spermatozoa were taken from the cauda epididymidis of the hamster and the rat. These spermatozoa are almost fully mature and can be recovered under sterile conditions free from secretions of accessory glands. However, many spermatozoa carry cytoplasmic droplets and the suspensions also contain erythrocytes and cellular fragments derived from the epididymal tubules. After careful removal of the cellular contaminants and of the cytoplasmic droplets, the labelled spermatozoa were

ruptured by sonication, the extracts processed as described below and their endogenous RNA was isolated by Sephadex-gel filtration and by polyacrylamide-gel electrophoresis without the addition of carrier.

A preliminary report, which included the findings reported in the present paper, was presented to the 3rd Annual Meeting of the Society for the Study of Reproduction (Turner *et al.*, 1970).

Materials and Methods

Methods

Preparation of extracts of spermatozoa. Batches of 20 male hamsters or rats (retired breeders) were killed in groups of two or three by decapitation and the epididymides immediately removed and washed in 0.15 M-NaCl. The caudae were punctured with fine hypodermic needles and the contents of the tubules were extruded by gentle squeezing. The cells were immediately suspended in ice-cold phosphate saline, pH 7.4 (Peterson & Freund, 1968). After all the animals had been killed, the combined sperm suspension was centrifuged at 600g, resuspended in phosphate saline and the count adjusted to 10⁸/ml. The suspensions (20–40 ml) were incubated with [5-³H]uridine (1 mM; 20 mCi/mmol) and glucose (0.02 M) for 3 h at 37°C. In some experiments [³²P]-orthophosphate (0.1 mCi/ml) was used to label the RNA. After the incubation the flasks were chilled and the spermatozoa were sedimented by centrifugation and washed five times by resuspension in phosphate saline with addition of unlabelled uridine. Finally the spermatozoa were resuspended in a hypotonic solution prepared by mixing 30 ml of phosphate saline with 70 ml of water. This treatment lysed the cytoplasmic droplets on the tails of the spermatozoa and also the erythrocytes and other cells that

had been sedimented with the spermatozoa. After another washing and sedimentation the spermatozoa were resuspended in phosphate saline and polyvinyl sulphate (4 µg/ml) was added; the cells were sonicated with a Mullard probe at 1.6 kHz for three 15 s periods and the suspension was centrifuged at 50000g for 15 min. DNAase* (5 µg/ml) and Pronase (1 mg/ml) were added to the supernatant and after incubation for 1 h at 4°C the suspension was centrifuged again and the supernatant was freeze-dried.

Extracts of whole testis. Suspensions of testis cells were prepared by homogenizing decapsulated testes in phosphate saline, pH 7.4. The suspensions were incubated with [5-³H]uridine and glucose at either 37°C or at 4°C for 90 min. After incubation the cells were recovered by centrifugation, extracted and processed as outlined above, except that the treatment with hypo-osmotic saline was omitted. The macromolecular fraction was eluted from the Sephadex G-75 column as described above.

Isolation of RNA by gel filtration. The dry material was taken up in 1 ml of 0.1 M-ammonium acetate buffer, pH 5.2, and introduced into a Sephadex G-75 column (70 cm × 2.5 cm). The gel had been prepared by swelling in 0.1 M-ammonium acetate, pH 5.2, containing 0.1 % sodium dodecyl sulphate (Robins & Raacke, 1968). To minimize loss of RNA by degradation the procedures, from the killing of the animals to the application of the extract to the Sephadex column, were completed in 1 working day. The elution from the Sephadex column was then done at room temperature overnight. The column was run in the ascending direction. The fractions (1.1 ml) were monitored by measuring their extinction at 260 and 280 nm and by counting portions in Bray's (1960) solution in a liquid-scintillation counter (Packard Tri-Carb). The fractions constituting peaks were combined, concentrated and recounted. The total amount of RNA in the macromolecular peak was calculated from the absorption at 260 and 280 nm taking 20–23 E units at 260 nm as equivalent to 1 mg/ml.

Gel electrophoresis. Polyacrylamide gel, 2.6 % (w/v), was prepared by the method of Loening (1967) with the addition of 0.5 % agarose (Peacock & Dingman, 1968). The running buffer contained 0.04 M-Tris-HCl, pH 7.4; 0.02 M-sodium acetate; and 2 mM-EDTA. The gel in glass tubes was equilibrated in the buffer overnight and then pre-run at 5 mA/tube for 45 min at 4°C. The freeze-dried extract was dissolved in the running buffer and mixed with an equal volume of 40 % (w/v) sucrose containing 0.006 % Bromophenol Blue. Portions of 100 µl containing 75 µg of RNA (determined spectrophotometrically) were layered over 12–18 gels and run for 90 min at 5 mA/tube. Standards of RNA of known molecular weight

were included with each set. One or two of the gels from each set and the gels containing RNA standards were fixed in 1 M-acetic acid for 15 min and stained for 1 h in a solution of 0.2 % Methylene Blue in 0.4 M-potassium acetate, pH 5.5. They were destained in running water overnight. The untreated gels were cut into slices which were placed in scintillation vials and digested in 0.1 ml of H₂O₂ (30 %, v/v) at 55°C for 2 h. Bray's (1960) solution was added and the radioactivities of the samples were counted in the scintillation counter. Other gels were sectioned into zones corresponding to the stained areas of the marker tubes. The zones of a number of tubes were combined, eluted and their radioactivities counted.

Hydrolysis of RNA. The eluates were freeze-dried and taken up in a small volume of water. Portions were incubated with either 0.3 M-NaOH for 24 h at 37°C, or with ribonuclease (1 mg/ml, pretreated by heating at 80°C for 5 min) for 45 min. Unhydrolysed and hydrolysed samples were treated with cold 5 % (w/v) trichloroacetic acid and the precipitates collected on Millipore filters (0.45 µm). The filter discs were washed with trichloroacetic acid, dried, placed in Liquifluor (New England Nuclear Corp., Boston, Mass., U.S.A.) and counted for radioactivity in the scintillation counter. The loss of radioactivity owing to hydrolysis was taken as a measure of RNA that had been present. The trichloroacetic acid-soluble filtrates recovered after mild alkaline hydrolysis were concentrated and chromatographed on Whatman 3 MM filter paper in the solvent system: 95 % ethanol–1 M-ammonium acetate (60:40, v/v) for 30 h (Thach & Doty, 1965). The zone corresponding to UMP was cut out and the paper counted for radioactivity in the scintillation counter.

Reagents

The [5-³H]uridine (20 Ci/mmol) and carrier-free [³²P]phosphoric acid (5 mCi/ml) were purchased from New England Nuclear Corp.; polyvinyl sulphate from Eastman Kodak Co., Rochester, N.Y., U.S.A.; Pronase from Calbiochem, Los Angeles, Calif., U.S.A.; deoxyribonuclease (ribonuclease-free) from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; ribosomal RNA from *Escherichia coli* (5S, 16S and 23S) was purchased from Miles Laboratories, Kankakee, Ill., U.S.A. Mammalian RNA (18S and 28S) was prepared by Dr. W. L. Bourassa from rat liver by the method of Girard (1967).

Results and Discussion

Although in our earlier experiments the incubation of crude epididymal sperm suspensions with [5-³H]uridine had resulted in the isolation of an ³H-labelled macromolecule (Turner *et al.*, 1970), several pitfalls became apparent and the measures taken to avoid

* Abbreviation: DNAase, deoxyribonuclease.

Fig. 1. Uptake of [5-³H]uridine by epididymal spermatozoa

Hamster spermatozoa were incubated with [5-³H]-uridine. Portions of the spermatozoa suspensions were spotted on filter-paper discs; these were dried, washed in cold trichloroacetic acid and ether and counted for radioactivity in a liquid-scintillation counter. Spermatozoa were from the caput (○) and cauda (●) epididymidis.

these are discussed. Whereas the epididymal spermatozoa were free from bacteria, the sperm suspensions contained erythrocytes and epithelial cells. Many spermatozoa in the cauda epididymidis still carry cytoplasmic droplets, which have been reported to contain a variety of enzymes, among these ribonuclease (Dott & Dingle, 1968; Dingle & Dott, 1969; Garbers *et al.*, 1970). In the present study, exposure of the cell suspensions to hypo-osmotic saline was interposed as a step in the isolation of the spermatozoa. This treatment lysed the cytoplasmic droplets together with the erythrocytes and other cells recovered from the epididymal tubules, but left the spermatozoa intact. The removal of the cytoplasmic droplets was verified by examination of the spermatozoa suspensions by phase-contrast microscopy. Repeated sedimentation at slow speed before sonication of the spermatozoa freed them from the cytoplasmic fragments and removed any external RNA and ribonuclease that may have been present.

Another problem arose from the capacity of spermatozoa to bind uridine non-enzymically. When epididymal spermatozoa were incubated with [5-³H]-uridine, the nucleoside was taken up by the cells. By using the technique of spotting portions of the incubation mixture on filter-paper discs that were then washed in cold trichloroacetic acid, a progressive uptake of ³H was observed over a period of at least 2 h. As shown in Fig. 1, spermatozoa from the caput epididymidis took up uridine at a faster rate than spermatozoa from the cauda epididymidis.

Fig. 2. Isolation of RNA from spermatozoa

Spermatozoa from the cauda epididymidis were incubated with [5-³H]uridine and washed in hypo-osmotic saline to lyse cytoplasmic droplets and contaminant cells. The sonic extract was digested with DNAase and Pronase and chromatographed on a Sephadex G-75 column. A macromolecular peak labelled with ³H was eluted. ○, E₂₆₀, ●, ³H radioactivity of individual fractions (1.1 ml).

Labelling of the spermatozoa by [³H]uridine also occurred on incubation at 4°C. Zero-time controls, i.e. cold spermatozoa suspensions to which [4-³H]-uridine was added and which were immediately washed by repeated centrifugation, sonicated and spotted on filter-paper discs, were free from ³H radioactivity. When, however, these cold spermatozoa suspensions were not washed before being sonicated at zero time, high 'background' counts remained on the discs despite exhaustive washing with trichloroacetic acid. This suggests that the uridine was bound non-specifically after entering the cells and that in spermatozoa incubated at 37°C the gradual increase of the labelling with time could have been limited by the rate of diffusion of uridine across the cell membrane. Since in this series of experiments the spermatozoa had been washed with iso-osmotic saline, without lysis of cytoplasmic material, the greater binding capacity of spermatozoa from the caput epididymidis may have been due to the presence of a larger amount of cytoplasmic droplets than in spermatozoa from the cauda epididymidis.

Fig. 3. Polyacrylamide-gel electrophoresis of RNA

Rat epididymal spermatozoa were incubated with [32 P]orthophosphate and extracts prepared as outlined in Fig. 2. The carrier-free extracts were subjected to electrophoresis on polyacrylamide gels. The position of standards (28S and 23S RNA) is indicated by arrows.

In view of these observations, the procedure adopted for the isolation of RNA in large-scale experiments was designed to exclude binding effects. The spermatozoa that had been incubated with [$5\text{-}^3\text{H}$]uridine were washed repeatedly by centrifugation, first with iso-osmotic saline in the presence of carrier uridine, and then with hypo-osmotic saline. The cells were taken up in a small volume of iso-osmotic saline and sonicated. The soluble extract obtained by high-speed centrifugation was exposed to the combined action of Pronase and DNAase. The fragments of low molecular weight were then removed by either Sephadex-gel filtration or gel electrophoresis.

To test the effectiveness of this treatment, a testis homogenate was divided into two portions which were incubated with [$5\text{-}^3\text{H}$]uridine for 3 h under conditions identical with those described for spermatozoa suspensions, except that one portion was incubated at 37°C and the other at 4°C . The purpose of the incubation at low temperature was to allow sufficient time for the diffusion of uridine into the cells for binding to their proteins, but to keep to a minimum the enzymic incorporation of uridine into RNA. The subsequent treatment was as described above, except that the lysis by passage through hypo-osmotic saline was omitted, so that the binding power

of the cytoplasmic proteins was also preserved. After sonication, the extracts were incubated with DNAase and Pronase and chromatographed on Sephadex G-75 columns. The macromolecular peak isolated from the portion of the testes which had been incubated at 37°C was radioactive, whereas no radioactivity was found in the corresponding peak from the tissue that had been incubated at 4°C . The paper-disc experiments had shown that incubation for 90 min was sufficient to permit entry of uridine into spermatozoa, giving rise to high background counts. The experiment with the testis homogenate showed that after treatment of the extract with the proteolytic enzyme no protein-bound ^3H label accompanied the first peak emerging from the Sephadex column.

The ^3H label in the peak isolated from the spermatozoa by gel filtration can therefore be considered as the result of enzymic incorporation of uridine into a macromolecule. The molecule that was eluted from the Sephadex G-75 column at the void volume has a molecular weight of greater than 40000 (Robins & Raacke, 1968). Its specific labelling by [$5\text{-}^3\text{H}$]uridine and a ratio of 2:1 of the extinction at 260 and 280 nm support the conclusion that the macromolecule isolated was RNA (Fig. 2). Its elution from the Sephadex column was followed by a peak of ^3H -labelled material of low molecular weight which probably contained breakdown products of the macromolecule and smaller peptides binding uridine. Gel electrophoresis of ^3H - or ^{32}P -labelled extracts yielded one broad zone which migrated in the area of 28S and 23S RNA markers (Fig. 3). When the ^3H -labelled zone was eluted and subjected to hydrolysis by either ribonuclease or 0.3M-NaOH , 90% of its radioactivity was lost. Most of this radioactivity could be recovered from the trichloroacetic acid-soluble fraction of the mild alkaline hydrolysate; when this was chromatographed on paper, 70% of the lost radioactivity appeared in a zone corresponding to UMP. Since the zone was counted on the paper, this corresponds to complete recovery. In this system UMP separated clearly from CMP and other mononucleotides, whereas uridine migrated far ahead.

Since the technique employed was designed to extract endogenous RNA from spermatozoa suspensions that were free from bacterial, cellular and cytoplasmic contaminants, it is felt that the isolated RNA was an integral part of the spermatozoa and that its label was incorporated by their metabolic activity. The broad zone of endogenous RNA extending over the region of 28S and 23S RNA markers may be the result of incomplete suppression of nuclease activity during the processing of the material. This makes identification of the RNA as either ribosomal (28S) or mitochondrial (23S) uncertain.

During the development of the gamete the cytoplasm of the spermatocytes gradually shrinks with

loss of ribosomes, but a small number of these may persist in the spermatozoa. However, the spermatozoa retain a full complement of mitochondria and since this organelle is known to include ribosomes, it is possible that the RNA may be also of mitochondrial origin.

Because of the uncertainty of the extent of recovery by extraction and of subsequent losses during processing, no attempt was made to determine the RNA content of the spermatozoa. From the absorption at 260nm of the combined first peak recovered from the Sephadex column, its total radioactivity and the specific radioactivity of the [5-³H]uridine it was calculated that the spermatozoa incorporated 0.5 nmol of uridine/h into 1 mg of RNA at 37°C. When calculated from the u.v. absorption and radioactivity of the zone isolated by gel electrophoresis the value obtained was 0.1 nmol/h per mg of RNA.

Note added in proof (received 1 March 1973)

After this manuscript had been submitted for publication, a paper appeared in which the incorporation of [³²P]orthophosphate into RNA of bull spermatozoa was reported (Premkumar & Bhargava, 1972). Since ³²P label appeared in zones corresponding to 23S, 16S and 4S RNA isolated with RNA from *Escherichia coli* that had been added as carrier, the authors concluded that the RNA of spermatozoa is exclusively of mitochondrial origin.

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