Ribonucleic Acid Synthesis *in vitro* in Primary Spermatocytes Isolated from Rat Testis

By J. ANTON GROOTEGOED, ANNE H. GROLLÉ-HEY, FOCKO F. G. ROMMERTS
and HENK J. VAN DER MOLEN

Department of Biochemistry (Division of Chemical Endocrinology), Medical Faculty, Erasmus University
Rotterdam, Rotterdam, The Netherlands

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The incorporation of [^3H]uridine into RNA was studied quantitatively (by incorporation of [^3H]uridine into acid-precipitable material) and qualitatively (by phenol extraction and electrophoretic separation of RNA in polyacrylamide gels) in preparations enriched in primary spermatocytes, obtained from testes of rats 26 or 32 days old. The rate of incorporation of [^3H]uridine into RNA of isolated spermatocytes was constant during the first 8 h of incubation, after which it decreased, but the decreased rate of incorporation was not reflected in a marked change in electrophoretic profiles of labelled RNA. In isolated spermatocytes, [^3H]uridine was incorporated mainly into heterogeneous RNA with a low electrophoretic mobility. Most of this RNA was labile, as shown when further RNA synthesis was inhibited with actinomycin D. Spermatocytes *in vivo* also synthesized heterogeneous RNA with a low electrophoretic mobility. A low rate of incorporation of [^3H]uridine into rRNA of isolated spermatocytes was observed. The cleavage of 32S precursor rRNA to 28S rRNA was probably retarded in spermatocytes *in vitro* as well as *in vivo*. RNA synthesis by preparations enriched in early spermatids or Sertoli cells was qualitatively different from RNA synthesis by the spermatocyte preparations. It is concluded that isolated primary spermatocytes maintain a specific pattern of RNA synthesis, which resembles RNA synthesis in spermatocytes *in vivo*. Therefore isolated spermatocytes of the rat can be used for studying the possible regulation of RNA synthesis during the meiotic prophase.

In rat testis, spermatocytes arise from diploid stem cells via mitotic divisions. The spermatocytes go through the meiotic divisions and give rise to haploid spermatids which after transformation result in the formation of spermatozoa. Several steps of spermatogenesis, including the prophase of meiosis in primary spermatocytes and the meiotic divisions, require the presence of testosterone and/or follitropin (Clermont & Harvey, 1967). The molecular mechanisms of action of testosterone and/or follitropin on primary spermatocytes are not known. RNA synthesis in the spermatocytes may be involved in these actions because the action of hormones on cells or tissues in many systems is mediated via an effect on RNA metabolism (Woo & O'Malley, 1975; Means, 1971).

RNA synthesis during meiosis in male mammals has been studied previously in the rat (Söderström & Parvinen, 1976), mouse (Monesi, 1965; Kierszenbaum & Tres, 1974a), hamster (Utakoji, 1966) and ram (Loir, 1972), by using radioautographic techniques and the biochemical analysis of total testicular tissue or seminiferous tubules. Transcription during the meiotic prophase appeared to be almost completely restricted to the autosomes (Kierszenbaum & Tres, 1974a), and it has been suggested that this transcrip-

tion represented mainly the synthesis of heterogeneous nuclear RNA (Kierszenbaum & Tres, 1974b; Söderström & Parvinen, 1976). Radioautographic experiments have shown a low rate of RNA synthesis in nucleoli during meiotic prophase in the testis of mouse and rat (Kierszenbaum & Tres, 1974a; Stefanini *et al.*, 1974).

Biochemical analysis of RNA synthesis in total testicular tissue or seminiferous tubules, which contain many cell types, will give limited information about RNA synthesis in specific germinal cell types. We therefore decided to study RNA synthesis in isolated primary spermatocytes (pachytene stage, prophase of the first meiotic division) from rat testis, and the present results indicate that it is possible to analyse the metabolism of heterogeneous RNA and rRNA in such isolated germinal cells.

Materials and Methods

Animals

Male Wistar rats, substrain R-Amsterdam (26 days or, where indicated, 32 days old) were used in the

present study. In 26-day-old rats, spermatogenesis has reached the meiotic divisions and the first spermatids have formed. In testes from 32-day-old rats, spermatogenesis has progressed until stage 8, and a large number of early spermatids (steps 1–8) are present.

Chemicals

[5-³H]Uridine (specific radioactivities 5 Ci/mmol and 30 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Sodium tri-isopropyl-naphthalenesulphonate was supplied by Eastman Organic Chemicals, Rochester, NY, U.S.A. Sodium 4-aminosalicylate and colourless phenol (AnalaR) were purchased from BDH, Poole, Dorset, U.K. *m*-Cresol was distilled before use to obtain a colourless preparation. Formamide was deionized by shaking for several hours with a mixed-bed resin (Bio-Rad AG 501-X8; Bio-Rad Laboratories, Richmond, CA, U.S.A.; formamide/resin, 19:1, v/w). Acrylamide and *NN'*-methylenebisacrylamide of electrophoresis purity were purchased from Bio-Rad. Other chemicals used were of highest available purity. All glassware was heat-sterilized. Solutions were made in glass-distilled and autoclaved water. Media used for isolation of cells were sterilized by ultrafiltration (filter 0.2 µm pore size).

Preparation of cell suspensions

Rats were killed by cervical dislocation; the testes were collected at room temperature (22°C) and the tunica albuginea was removed from the testes. The testes (1.2–1.6 g wet wt.) were chopped into pieces about 0.5 mm³, and 20 ml of isolation medium [essentially Hanks balanced salt solution (Hanks & Wallace, 1949) but with 69.8 mM-NaCl and 85.8 mM-KCl, supplemented with penicillin (10⁵ i.u./litre), streptomycin (100 mg/litre) and Fungizone (1 mg/litre; Gibco, Grand Island, NY, U.S.A.)] was added to the tissue pieces in a 100 ml flask. Subsequently DNAase* (DN-25; Sigma Chemical Co., St. Louis, MO, U.S.A.) and trypsin (Code TRL; Worthington Biochemical Corporation, Freehold, NJ, U.S.A.), dissolved in isolation medium, were added to give final concentrations of 20 µg/ml and 0.1%. After shaking gently for 30 min, bovine serum albumin (fraction V, B grade; Calbiochem, San Diego, CA, U.S.A.) (0.1 g dissolved in 5 ml of isolation medium), soya-bean trypsin inhibitor (type I-S; Sigma) (20 mg) and DNAase (DN-25) (200 µg) were added to the cell suspension. Residual tissue fragments were removed by filtration through eight layers of aseptic gauze, and 24 ml of the cell suspension (2 × 10⁶ cells/ml) was

immediately used for separation of the different cell types.

Isolation of primary spermatocytes

The cells were separated by using the 'Staput' method of velocity sedimentation at unit gravity (Miller & Phillips, 1969; Meistrich *et al.*, 1973). Cell separation was performed at room temperature or, when indicated, at 4°C. The Perspex column (diam. 12 cm) was filled from the bottom successively with 60 ml of isolation medium, 24 ml of the cell suspension, and a non-linear albumin gradient in isolation medium [1% (w/v) (40 ml); 2% (240 ml); 3.2% (240 ml)]. Loading of the column was performed at a rate of 15 ml/min. Spermatocytes in the pachytene stage had sedimented 22–26 mm within 80 min (at room temperature) or 100 min (at 4°C) after the start of the loading procedure. During these periods, round early spermatids sedimented 10–13 mm. The sedimentation column was emptied at a rate of 15 ml/min and the spermatocyte fraction was collected. In some experiments, when 32-day-old rats were used, the early spermatid fraction was also collected. The fractions were centrifuged for 6 min at 200g and the cell pellet was suspended in incubation medium [essentially Eagle's Minimum Essential Medium (Flow Laboratories Ltd., Irvine, Ayrshire, Scotland, U.K.), but with 69.8 mM-NaCl and 56.9 mM-KCl, supplemented with 1 mM-sodium pyruvate, 6 mM-sodium DL-lactate, bovine serum albumin (4 g/litre), penicillin (10⁵ i.u./litre), streptomycin (100 mg/litre) and Fungizone (1 mg/litre)] to which pure DNAase (DN-CL; Sigma) (final concn. 2.0 µg/ml) was added.

After a second centrifugation for 4 min at 200g the cells were resuspended in incubation medium and 2 ml portions of the cell suspension (0.5 × 10⁵–2 × 10⁵ cells/ml) were incubated in a plastic Petri dish (diam. 3 cm) at 32°C in an atmosphere of air/CO₂ (19:1). The small amount of DNAase (0.05–0.1 µg/ml) that was still present after the washing procedure was necessary to prevent excessive cell clumping during the incubation.

The whole procedure from death of the rats until the incubation of the cells was completed within 3 h. Trypan Blue was excluded by 95% or more of the cells. The composition of the 'Staput' fractions was evaluated by microscopy of air-dried preparations fixed in Bouin's fluid and stained with Haematoxylin and Eosin.

Qualitative analysis of RNA synthesis

Cells (3 × 10⁵–4 × 10⁵) were preincubated for 2 h or longer, and RNA labelling was started by the addition of 10–50 µCi of [5-³H]uridine/ml (specific radioactivity 30 Ci/mmol). Incubation was stopped after 1–20 h of labelling by washing the cells twice at

* Abbreviation: DNAase, deoxyribonuclease.

4°C with 0.9% NaCl supplemented with unlabelled 0.5 mM-uridine, and the cells were frozen in solid CO₂. RNA was extracted by a modification of the method described by Parish & Kirby (1966), as follows. RNA from *Escherichia coli* (BDH) was added as carrier (25–50 µg). The cells were lysed at 0°C in 0.4 ml of solution A [6% (w/v) sodium 4-aminosalicylate, 1% (w/v) sodium tri-isopropyl-naphthalenesulphonate, 6% (v/v) butan-2-ol in water]. An equal volume of solution B (500 ml of phenol, 70 ml of *m*-cresol, 55 ml of water and 0.5 g of 8-hydroxyquinoline) was added and the mixture was shaken at room temperature for 10 min. After centrifugation (10000 rev./min, 10 min at 0°C) the water layer was removed and kept. The remaining interphase and phenol phase were mixed for 5 min with a second volume (0.2 ml) of solution A and after centrifugation (10000 rev./min, 10 min at 0°C) the water layer was added to the first water layer. NaCl (final concn. 3%, w/v) was added to the combined water layers and a second deproteinization with 0.2 ml of solution B was carried out. The nucleic acids in the water phase were precipitated by adding 2 vol. of ethanol containing 10% (w/v) *m*-cresol and leaving the mixture for 45 min at 0°C. The precipitate was washed twice with 0.2 ml of 0.2 M-sodium acetate in 75% (v/v) ethanol. DNA was removed by treatment of the sample with 50 µg of ribonuclease-free DNAase/ml (code DPFF; Worthington) in 7 mM-MgCl₂/100 mM-Tris/HCl (pH 7.5) for 30 min at 0°C (Spradling *et al.*, 1974). RNA was precipitated by adding 2 vol. of ethanol and leaving the mixture for 45 min at 0°C. The precipitated RNA was washed successively with 0.2 ml of 0.2 M-sodium acetate in 75% ethanol and with 0.2 ml of 96% ethanol. The RNA was dissolved in 50 µl of 95% (v/v) formamide, containing 2 mM-EDTA and 20 mM-Tris/HCl (pH 7.4). Before application to the gel the dissolved RNA was heated at 37°C for 10 min (Slack *et al.*, 1975) and cooled in ice.

Polyacrylamide gels (2.4%), cross-linked with *NN'*-methylenebisacrylamide, were prepared as described by Bishop *et al.* (1967). After washing and swelling of the gels during 72 h in buffer E (0.4 M-Tris, 0.02 M-sodium acetate, 0.001 M-sodium EDTA adjusted to pH 7.2 with acetic acid) (Bishop *et al.*, 1967) containing 0.2% sodium dodecyl sulphate, the gels were sucked into Plexiglass tubes (diam. 0.4 cm) and cut at a length of 9 cm. Buffer E containing 0.2% sodium dodecyl sulphate was used as electrophoresis buffer and the gels were pre-run for 30 min at 3 mA/gel and 70 V. The gels were then loaded with 20–40 µg of RNA dissolved in 40 µl of 95% formamide, and electrophoresis was performed for 130 min at 3 mA/gel. To localize 28S and 18S rRNA (and *E. coli* 23S and 16S rRNA) in the gels after electrophoresis, the gels were scanned in water at 260 nm with a Gilford spectrophotometer. *s* values of unknown peaks were calculated by the method of Lewicki & Sinskey (1970). The gels were subsequently frozen and cut into slices

of 1.4 mm. The slices were extracted in a glass liquid-scintillation-counting vial with 0.5 ml of Soluene-350 (Packard Instrument S.A. Benelux, Brussels, Belgium) for 2 h at 50°C, and the radioactivity in the extract was counted in toluene containing 4 g of PPO (2,5-diphenyloxazole)/litre and 0.04 g of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]/litre.

The amount of radioactivity in the gels was corrected for small differences in the amount of RNA applied to the gel, by using the *A*₂₆₀ of 28S rRNA as a reference.

Quantitative analysis of RNA synthesis

Cells (1.0×10^5 – 1.2×10^5) were preincubated for 0, 2, 4, 6, 10 or 20 h, after which they were incubated for 2 h with [5-³H]uridine (specific radioactivity 5 Ci/mmol) (10 µCi/ml). Incubation was stopped by the addition of ice-cold 0.9% NaCl containing 0.5 mM-uridine. The cells were washed twice with 15 ml of the 0.9% NaCl with 0.5 mM-uridine and subsequently the cells were frozen and thawed twice. To each sample 0.5 mg of RNA (type IV; Sigma) was added as a carrier and macromolecules were precipitated with 0.5 ml of 1.0 M-HClO₄ at 0°C. After centrifugation (2000 rev./min; 5 min) the supernatant was removed and kept, and the pellet was washed twice with 0.5 ml of 1.0 M-HClO₄. The supernatant and the washes were pooled for measurement of the amount of HClO₄-soluble radioactivity inside the cells. The pellet was washed with 0.5 ml of 96% ethanol, and RNA was hydrolysed in 0.5 ml of 1.0 M-NaOH at 37°C for 60 min. DNA and protein were precipitated by addition of 0.5 ml of 3.0 M-HClO₄ at 0°C. After centrifugation (2000 rev./min; 5 min) the supernatant was used for measurement of the amount of radioactivity incorporated into RNA (HClO₄-insoluble radioactivity). The pellet was washed with 96% ethanol, dissolved in 50 µl of 0.4 M-NH₃ at 0°C for 20 h and the amount of DNA was measured by using a fluorimetric assay (Robertson & Tait, 1971). Radioactivity present in HClO₄-insoluble and -soluble material (reflecting respectively [³H]uridine incorporated into RNA and [³H]uridine taken up by the cells but not incorporated into RNA) was counted in Instagel (Packard) and expressed as d.p.m./µg of DNA.

Results

Maintenance of isolated spermatocytes

Media for the isolation and incubation of spermatocytes from rat testis contained a high concentration of K⁺ (85.8 and 56.9 mM respectively), comparable with that in the tubular fluid (50–112 mM) that surrounds the spermatocytes *in vivo* (Tuck *et al.*, 1970). An

Table 1. Isolation of germinal cells from rat testicular tissue

Cell suspensions were prepared from total testicular tissue of the rat (see the Materials and Methods section) and the suspensions were fractionated by sedimentation at unit gravity (Meistrich *et al.*, 1973). The percentages of cell types present in the fractions collected from the sedimentation column are presented in this Table. Fractions A₁ and A₂, enriched in primary spermatocytes, sedimented over a distance of 22–26 mm during 80 min at room temperature or 110 min at 4°C respectively. Fraction B, containing mainly early spermatids, sedimented 10–13 mm during 110 min at 4°C. Fraction A₁ was obtained from 26-day-old rats, to minimize the contamination by (multinucleated) spermatids in this fraction; 32-day-old rats were used to obtain fractions A₂ and B, to prevent contamination by late spermatids in both fractions. Drops of the fractions were put on microscope slides, fixed in Bouin's fluid and stained with Haematoxylin and Eosin. The cells were identified by criteria developed for isolated cells from mouse testis (Meistrich *et al.*, 1973) as adapted for rat testicular cells (Platz *et al.*, 1975). Cells with more than one nucleus were counted as single cells. At least 600 cells were counted in each fraction.

	Fraction ...	Age of rats (days) ...	Cell types (%)		
			A ₁	A ₂	B
Cell type			26	32	32
Spermatogonia and preleptotene spermatocytes			3	2	2
Leptotene, zygotene and early-pachytene spermatocytes			3	2	1
Middle- and late-pachytene spermatocytes			80	77	1
Dividing cells and secondary spermatocytes			2	3	1
Early spermatids (steps 1–8)			5	10	89
Somatic cell types (including Leydig cells and Sertoli cells)			4	2	3
Unknown and broken cells			2	3	2
Cytoplasmic fragments			1	1	1

impression about the composition of the cell preparations from 26-day-old rats can be obtained from the results in Table 1 (Fraction A₁). The gross morphology of the isolated spermatocytes, evaluated by phase-contrast microscopy, remained intact for at least 20 h after isolation. The quantitative incorporation of [³H]uridine into RNA of spermatocytes was constant during the first 8 h of incubation, but incorporation decreased after 10–20 h of incubation (Fig. 1). The ratio of the amount of [³H]uridine incorporated into RNA to the amount of [³H]uridine taken up by the cells was relatively constant during the whole incubation period of 20 h (Fig. 1). The results in Fig. 1 indicate that the uptake of [³H]uridine by the cell

Fig. 1. Uptake of [³H]uridine by isolated spermatocytes and incorporation into RNA

Isolated spermatocytes were preincubated for different time periods. After preincubation the incubation was continued in the presence of [³H]uridine (10 μCi/ml, specific radioactivity 5 Ci/mmol) for 2 h. The amount of [³H]uridine incorporated into RNA (□) and the ratio of the amount of [³H]uridine incorporated into RNA to the total uptake of [³H]uridine (d.p.m. in HClO₄-precipitable material/d.p.m. in HClO₄-precipitable plus HClO₄-soluble material) (■) are presented. Means and range of individual mean values of two experiments are given. In each experiment the determinations were made in duplicate or in triplicate.

population decreased during the incubation period. The similarity of the two electrophoretic profiles (in Fig. 2) of RNA labelled during 8 h of incubation of the cells with [³H]uridine after either 4 or 12 h of preincubation illustrates the absence of marked qualitative changes in the pattern of RNA synthesis after prolonged incubation of the cells.

Time sequence of RNA labelling

Within the first hour after addition of [³H]uridine to isolated spermatocytes the label was incorporated into RNA with a heterogeneous electrophoretic mobility ('heterogeneous RNA'). Most of this labelled RNA moved more slowly than 28S rRNA, forming two peaks (calculated *s* value 50–55S and higher) (Fig. 3a). After 2 h of incubation in the presence of [³H]uridine, an additional labelled component (calculated *s* value 40–45S) was present (Fig. 3b), which probably represented 45S rRNA, the precursor of 28S and 18S rRNA (Penman, 1966). Labelled 18S rRNA was detected 4 h after addition of [³H]uridine. At the same time a radioactive component emerged with an electrophoretic mobility (calculated *s* value 32S) slightly less than that of 28S rRNA (Fig. 3c); this 32S RNA might represent the

Fig. 2. Effect of the preincubation period on qualitative RNA synthesis in isolated spermatocytes

Spermatocytes were incubated *in vitro* with [^3H]uridine ($25 \mu\text{Ci/ml}$) for 8 h after preincubation in the absence of labelled precursor for 4 h (a) or 12 h (b). RNA was extracted, and was separated by electrophoresis in polyacrylamide gels as described in the text.

immediate precursor of 28S rRNA (Penman, 1966). Within 20 h of incubation in the presence of [^3H]uridine the specific radioactivity of 28S rRNA was increased relative to that of 32S RNA (Fig. 3d). The results in Fig. 3 show an accumulation of labelled 32S RNA, 4 h and 20 h after addition of [^3H]uridine

to the isolated spermatocytes, but the amount of 32S RNA extracted from the spermatocytes (as determined from A_{260}) was small (Fig. 4).

Stability of heterogeneous RNA

After longer incubations of spermatocytes with [^3H]uridine the contribution of the slow-moving heterogeneous RNA to the profile of labelled RNA decreased relative to the amount of labelled 32S, 28S and 18S RNA, indicating that the heterogeneous RNA was not stable (Fig. 3). Moreover, no significant amount of the heterogeneous RNA was extracted from the spermatocytes, as determined from the A_{260} (Fig. 4). 'Chase' experiments were performed to investigate further the stability of the heterogeneous RNA synthesized *in vitro*. Because a 'chase' by isotope dilution was not sufficient to inhibit completely the incorporation of [^3H]uridine within a short time, actinomycin D was used for rapid inhibition of RNA synthesis (Perry & Kelley, 1970). Triplicate incubations of spermatocytes were labelled with [^3H]uridine for 1 h. Subsequently the cells from one incubation were frozen for RNA extraction (Fig. 5a) and actinomycin D (final concn. $10 \mu\text{g/ml}$) was added to the cells in the other two incubations, and these cells were frozen for RNA extraction after 1 h and 4 h (Figs. 5b and 5c). After 4 h in the presence of actinomycin D (Fig. 5c) the electrophoretic profile of labelled RNA showed a heterogeneous background and peaks of 32S and 18S rRNA. It is possible that part of the background activity represents non-ribosomal stable RNA. Most of the heterogeneous RNA synthesized during a short incubation period (Fig. 5a), however, disappeared in the presence of actinomycin D and was not converted into smaller and stable RNA molecules (Figs. 5b and 5c).

RNA synthesis in spermatocytes *in vivo*

In addition to the analysis of RNA synthesis in spermatocytes *in vitro*, we tried to label the RNA of the spermatocytes *in vivo*. [^3H]Uridine was injected into the testes of 32-day-old rats, and 4 h later a cell suspension was prepared at room temperature from these testes. Subsequently the spermatocytes were isolated at 4°C (Fraction A_2 in Table 1) and RNA was extracted.

The electrophoretic profile of the RNA labelled *in vivo* (Fig. 6a) was similar to the profile obtained after 4 h of labelling *in vitro* (Fig. 3c) with respect to the high rate of synthesis of heterogeneous RNA and the accumulation of labelled 32S RNA. Some turnover and processing of RNA may have occurred during the isolation of the cells after the labelling *in vivo*.

Fig. 3. Time sequence of RNA labelling in isolated spermatocytes

Spermatocytes were labelled *in vitro* with [^3H]uridine (a, b and c with $50 \mu\text{Ci/ml}$, d with $10 \mu\text{Ci/ml}$) and incubation was stopped after 1 h (a), 2 h (b), 4 h (c) or 20 h (d) of labelling. RNA was extracted and separated by electrophoresis in polyacrylamide gels. The labelled RNA peaks with a calculated *s* value of 32S and 40–45S are indicated as '32S' and '45S' respectively.

RNA synthesis in spermatids and Sertoli cells

To illustrate the specific features of RNA synthesis in spermatocytes, other testicular cell types were also labelled with [^3H]uridine, and the electrophoretic profiles of the labelled RNA were compared with those of the spermatocytes. A fraction enriched with early spermatids was collected from the sedimentation column (Fraction B in Table 1) after labelling testicular cells *in vivo*, by using 32-day-old rats. RNA was extracted from this spermatid fraction (Fig. 6b) as well as from Sertoli cells, which were

labelled with [^3H]uridine in a culture system as described by Fritz *et al.* (1976) (Fig. 7). The electrophoretic profile of labelled RNA from the preparation of early spermatids showed the presence of labelled heterogeneous RNA with an electrophoretic mobility similar to that of heterogeneous RNA synthesized in spermatocytes. In Sertoli cells the labelled heterogeneous RNA with a low electrophoretic mobility contributed little to the total of labelled RNA; labelled heterogeneous RNA from Sertoli cells moved more rapidly during electrophoresis (around 18S) than did the heterogeneous RNA extracted from the

Fig. 4. A_{260} of a polyacrylamide gel after electrophoresis of RNA extracted from spermatocytes

RNA was extracted from isolated spermatocytes and separated by electrophoresis in a polyacrylamide gel (2.4%, w/v). The gel was scanned at 260nm after electrophoresis. The calculated position of '32S' RNA in the gel is indicated by an arrow.

germinal cells. In the preparation of early spermatids as well as in the cultured Sertoli cells, 45S rRNA was processed to 28S and 18S rRNA, and no marked accumulation of labelled 32S RNA occurred in these cell types.

Discussion

In isolated spermatocytes, the rate of [3 H]uridine incorporation was constant during 8 h of incubation (Fig. 1). The observed decrease in the rate of incorporation after longer incubation periods was not accompanied by a change in the electrophoretic profile of labelled RNA (Fig. 2). Therefore the results suggest that, with respect to RNA metabolism, degeneration of isolated spermatocytes is reflected mainly in a decrease of the uptake of [3 H]uridine. We conclude that the analysis of RNA synthesis in isolated spermatocytes, under the conditions described, can be performed during at least 8 h after isolation of the cells (the period of 3 h required for isolation of the cells not included). The electrophoretic profiles of labelled RNA synthesized in spermatocytes *in vitro* (Fig. 3c) and *in vivo* (Fig. 6a) were similar, indicating that the synthesis of the different RNA species was not markedly altered after isolation. Moreover, RNA synthesis in spermatocytes was qualitatively different from RNA synthesis in preparations of early spermatids (Fig. 6b) and in cultured Sertoli cells (Fig. 7).

The present observations made on isolated spermatocytes show that the rate of synthesis of a heterogeneous class of RNA is much higher than the rate of synthesis of rRNA (Fig. 3), but the amount of slow-moving heterogeneous RNA extracted from spermatocytes was small (Fig. 4). It is concluded from the

Fig. 5. Stability of RNA in isolated spermatocytes during a 'chase' with actinomycin D

Isolated spermatocytes were labelled with [3 H]uridine (50 μ Ci/ml) for 1 h, after which actinomycin D (10 μ g/ml) was added to prevent further RNA synthesis. Incubation was stopped at 0 h (a), 1 h (b) or 4 h (c) after addition of actinomycin D. RNA was extracted and separated by electrophoresis in polyacrylamide gels.

results in Fig. 5 that most of the heterogeneous RNA in spermatocytes is labile. The rate of decay of this RNA in the presence of actinomycin D (average

Fig. 6. RNA synthesis in (a) spermatocytes and (b) early spermatids *in vivo*

[^3H]Uridine was injected into the testes of 32-day-old rats ($50 \mu\text{Ci/testis}$ in Hanks balanced salt solution). After 4 h of labelling *in vivo*, fractions of primary spermatocytes and early spermatids were isolated at 4°C (Fractions A₂ and B in Table 1), and RNA was extracted and separated by electrophoresis in polyacrylamide gels. The amount of 28S rRNA applied to the gels was five times the amount used for electrophoretic separation of RNA labelled *in vitro*.

Fig. 7. RNA synthesis in cultured Sertoli cells
Electrophoretic separation in a polyacrylamide gel of ^3H -labelled RNA extracted from cultured Sertoli cells (Fritz *et al.*, 1976). The Sertoli cells were incubated with [^3H]uridine ($25 \mu\text{Ci/ml}$) for 4 h.

cytes may resemble the heterogeneous nuclear RNA in somatic cells, although this has not been experimentally verified.

Evidence for a predominant synthesis of a heterogeneous class of RNA was also found, by biochemical methods, in total testis tissue of the hamster (Muramatsu *et al.*, 1968) and in seminiferous tubules of the rat (Söderström & Parvinen, 1976), and it was suggested that this synthetic activity originated from spermatocytes. Muramatsu *et al.* (1968) concluded that the heterogeneous 'rapidly labelled' RNA is not preserved as relatively stable mRNA or as rRNA, but is rapidly and completely degraded. In contrast, Söderström & Parvinen (1976) concluded that a considerable part of the RNA synthesized in the seminiferous tubules is heterogeneous nuclear RNA with a long lifetime (at least 36 h). They further suggested that the stable heterogeneous nuclear RNA, synthesized mainly in spermatocytes, includes precursors for very stable mRNA which might be translated during late spermiogenesis; in late spermatids the condensed genome is not transcribed, although proteins are synthesized (Monesi, 1965). The results of the present experiments, however, indicate that most of the heterogeneous RNA from spermatocytes has a short half-life and that only a

half-life 2–3 h) is roughly comparable with that described for nuclear RNA from HeLa cells (Scherrer *et al.*, 1970) and the heterogeneous RNA in spermato-

small portion of the heterogeneous RNA synthesized in spermatocytes may belong to a class of stable heterogeneous RNA (Fig. 5).

With respect to the transcription of ribosomal DNA, the present observations show that in spermatocytes the primary gene product, 45S rRNA, was probably cleaved into 32S and 18S rRNA (Figs. 3 and 6a), both presumably rRNA. The subsequent cleavage of 32S to 28S rRNA was retarded, as concluded from the accumulation of labelled 32S rRNA in the electrophoretic profile of RNA extracted from the whole cell. In somatic cells, 28S and 18S rRNA leave the nucleolus and pass into the cytoplasm (Penman, 1966) and the results obtained with spermatocytes could indicate, therefore, that the labelled 32S rRNA is located in the nucleolus and that labelled 18S rRNA is included in small ribosomal subunits in the cytoplasm. The 32S rRNA was probably not stable and might have been processed to 28S rRNA, as indicated by the time sequence of RNA labelling (Fig. 3) and the absence of a significant amount of 32S rRNA from the A_{260} profile (Fig. 4). In cell preparations containing mainly early spermatids (Fig. 6b) and Sertoli cells (Fig. 7), no accumulation of labelled 32S rRNA was observed.

Biochemical analysis of testicular tissue of the mouse labelled *in vivo* by Galdieri & Monesi (1974) led them to suggest a possible accumulation of rRNA precursors in the nucleolus during the meiotic prophase, although the evidence obtained was indirect and difficult to interpret. Parchman & Lin (1972) observed little synthesis of rRNA in nucleoli of lily microsporocytes during the later stage of meiotic prophase, and 'an RNA species involved in the ribosomal pathway' accumulated in the nucleoli. These data from the literature in combination with the present findings on the synthesis of rRNA in spermatocytes may indicate that the maturation of rRNA can be retarded during the pachytene stage in male germ cells.

In summary, the present biochemical results on RNA synthesis in spermatocytes indicate that the isolation and incubation of spermatocytes can be used to obtain information about RNA synthesis in these cells. On the basis of such information it should be possible to investigate whether hormones have an effect at the level of RNA synthesis in primary spermatocytes.

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