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Cellular content and biosynthesis of polyamines during rooster spermatogenesis

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The natural polyamines spermine and spermidine, and the diamine putrescine, were extracted from rooster testis cells separated by sedimentation at unit gravity, and from vas-deferens spermatozoa. The ratios spermine/DNA and spermidine/DNA were kept relatively constant throughout spermatogenesis, whereas the ratio putrescine/DNA rose in elongated spermatids. The cellular content of spermine, spermidine and putrescine decreased markedly in mature spermatozoa. Two rate-limiting enzymes in the biosynthetic pathway of polyamines, ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase, showed their highest activities at the end of spermiogenesis and were not detectable in vas-deferens spermatozoa. A marked reduction in cell volume during spermiogenesis without a parallel decrease in the cellular content of polyamines suggests the possibility that the marked changes in chromatin composition and structure occurring in rooster late spermatids could take place in an ambience of high polyamine concentration.

Polyamines are organic polyvalent cations present in both prokaryotic and eukaryotic cells (Cohen, 1978). Although spermine was originally isolated from testis and semen (Rosenheim, 1924) and the names spermine and spermidine evoke male genital function, few studies on polyamines during spermatogenesis have been published (MacIndoe & Turkington, 1973).

In rooster spermiogenesis, a massive removal of chromosomal proteins occurs during the transition 'nucleohistone–nucleoprotamine' (Mezquita & Teng, 1977*a*,*b*). The presence of unmasked DNA during spermiogenesis as a consequence of protein removal (Loir & Hochereau de Reviers, 1972; Barcellona *et al.*, 1974; Mezquita & Teng, 1977*a*,*b*; Loir & Lanneau, 1978) gives rise to the following questions. How can the negatively charged segments of the unmasked DNA be maintained in close proximity and how are the stiff DNA chains bent into a compact structure before the final nucleoprotamine complex is formed? And, on the other hand, how is the unmasked DNA protected against the action of nucleases and mutagens in order to preserve the genetic message delivered by the spermatozoa?

In several bacteriophages the compaction of DNA is facilitated by its interaction with polyamines (Gosule & Schellman, 1976). Spermine shows antimutagenic properties in prokaryotic and eukaryotic systems (Clarke & Shankel, 1975; Rajalakshmi *et al.*, 1978).

In order to investigate if polyamines might accomplish similar roles during rooster spermiogenesis, we needed to know first if polyamines and the rate-limiting enzymes in their biosynthesis were present in genetically inactive late spermatids. In the present paper we show that late spermatids do contain spermine, spermidine and putrescine, together with the enzymic activities ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase.

Experimental

Animals and chemicals

Hubbard White Mountain roosters (25–50 weeks old) were obtained from Las Planas farm, Manresa, Barcelona, Spain. The following chemicals were obtained from the sources indicated: DL-[1-^{14}C]-ornithine (sp. radioactivity 50 Ci/mol) and *S*-adenosyl-L-[1-^{14}C]methionine (sp. radioactivity 61 Ci/mol) were from The Radiochemical Centre (Amersham, Bucks., U.K.). Bovine serum albumin, soya-bean trypsin inhibitor and DNAase I were from Calbiochem (San Diego, CA, USA); minimum essential medium (Eagle) and trypsin were supplied by Grand Island Biological Co. (Grand Island, NY, U.S.A.). Spermine, spermidine, putrescine and dan-

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syl chloride were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). T.l.c. plates, silica-gel 60, Tris, EDTA, diphenylamine, glycerol, proline, dithiothreitol, L-ornithine and pyridoxal 5'-phosphate were from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Preparation of testicular cell suspensions

Testicular cell suspensions were prepared essentially as described by Meistrich (1977). Testes were decapsulated and minced finely with scissors. The minced tissue (20 g) was gently suspended in 10 vol. of minimum essential medium (Eagle) containing 0.1% (w/v) trypsin and 2 μg of DNAase I/ml. The suspension was incubated at 31°C for 30 min with gentle stirring in a water bath. After incubation the cell suspension was filtered through four layers of surgical gauze and centrifuged for 10 min at 1500 *g* in a JS-7.5 Beckman rotor. The sample was resuspended in 50 ml of Ca^{2+}/Mg^{2+}-free phosphate-buffered saline containing 0.02% (w/v) soya-bean trypsin inhibitor and 0.1% (w/v) bovine serum albumin.

Separation of testicular cells at unit gravity

The procedures for loading and running the Staput apparatus have been previously described (Mezquita & Teng, 1977*a*). Sedimentation chambers of 28 cm diameter were used. The cell suspension was diluted in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline to a final concentration of 16×10^6 cells/ml and 80 ml of the sample was loaded in the chamber. We have used a buffered step gradient of glycerol [3%/6%/12% (w/v)] generated as described by Miller & Phillips (1969). For comparative purposes in certain experiments Ficoll step gradients [0.5%/1%/3% (w/v)] were used.

Analytical methods

For polyamine assays rooster testis cells were extracted in 0.2 M-$HClO_4$. The extracts were dansylated (5-dimethylaminonaphthalene-1-sulphonylated) and the polyamine estimation was carried out by t.l.c. and scanning *in situ* of the fluorescent dansyl derivatives (Dion & Cohen, 1972).

The activities of ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase were assayed as described by Jänne & Williams-Ashman (1971*a*,*b*).

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard, and DNA was measured by the method of Burton (1956) with calf thymus DNA as the standard.

Results and discussion

Separation of testicular cells at unit gravity

Fractions obtained from the sedimentation chamber were identified by phase-contrast microscopy. The top of the sedimentation chamber (sedimentation velocity 0–0.5 mm/h) contained cytoplasmic fragments and residual bodies. Fractions with sedimentation velocity between 0.5 and 1 mm/h contained late spermatids and testicular spermatozoa. These fractions were contaminated by residual bodies (Plate 1*a*). Spermatozoa obtained from the vas deferens sedimented at the same region (Fig. 1). Elongated spermatids showed sedimentation velocities between 1 and 2 mm/h (Plates 1*b* and 1*c*). When the testicular cell suspension was centrifuged at 300 *g* for 5 min, a cell fraction enriched in elongated spermatids remained in the supernatant. This fraction sedimented in the region 1–2 mm/h (Fig. 1). Round spermatids and other spherical meiotic and premeiotic cells sedimented according to their differences in size (Plates 1*d*–1*f*).

The profile of the cell separation and the cellular polyamine content were similar when Ficoll was used instead of glycerol in the separation chamber.

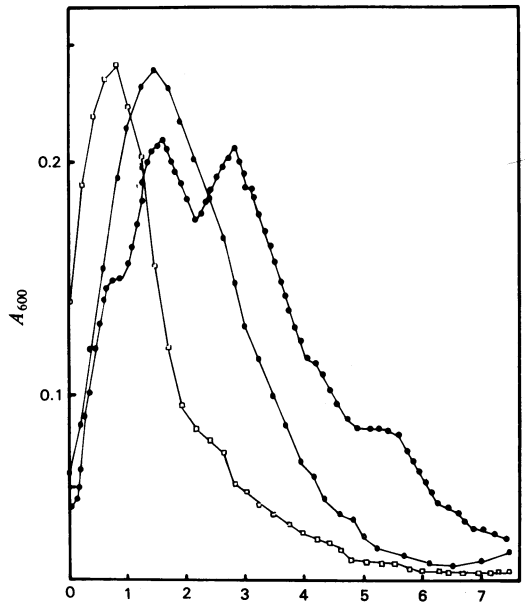
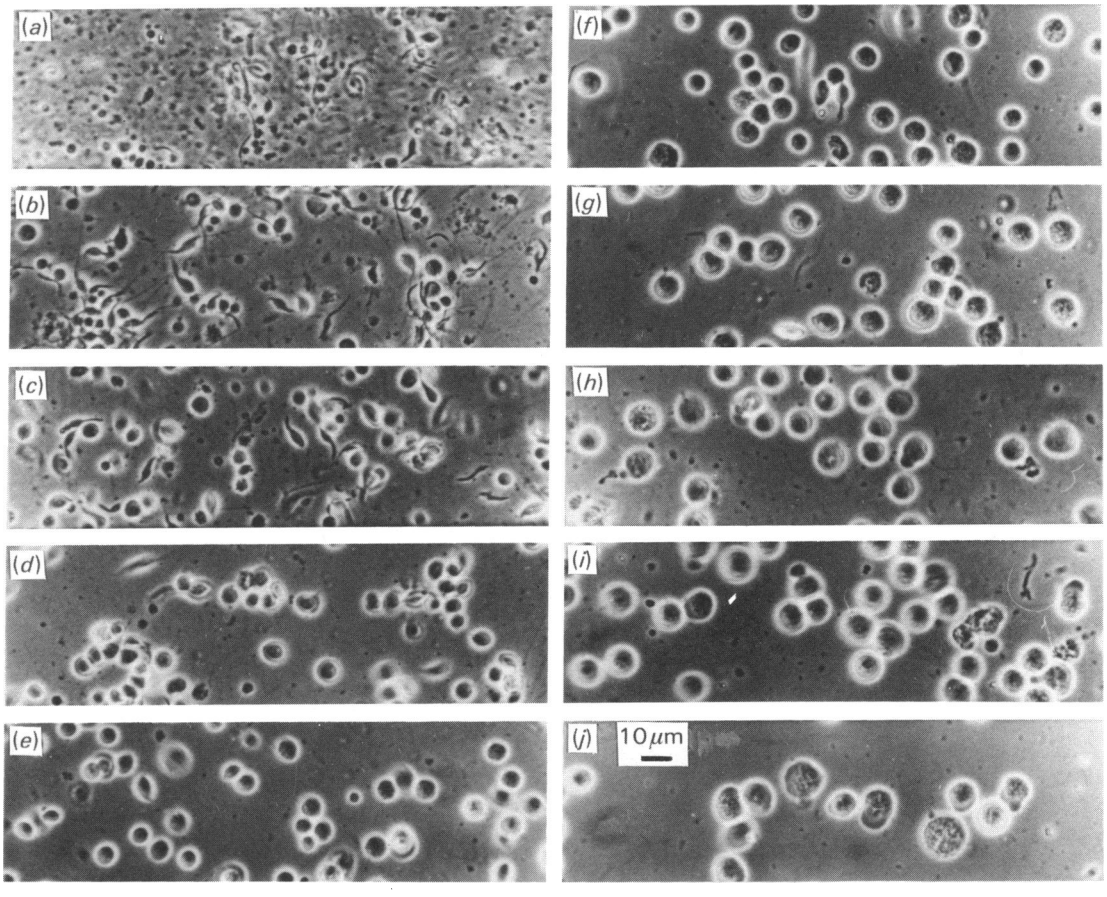


Fig. 1. *Distribution of rooster testis cells and spermatozoa of the vas deferens as a function of their sedimentation velocity at unit gravity*

Rooster testis cells were separated for 5 h at unit gravity through a buffered step gradient of glycerol (3%/6%/12%). The distribution of cells was monitored by absorption at 600 nm. □, Spermatozoa of the vas deferens; ○, fraction enriched in elongated spermatids; ●, suspension of cells prepared from sexually mature testes.



EXPLANATION OF PLATE 1

Phase-contrast photomicrographs of rooster testis cells separated by sedimentation at unit gravity

The cell suspension was loaded on the top of a buffered step gradient of glycerol (3%/6%/12%). Cell fractions were observed through a Zeiss ST-143 phase-contrast microscope. Fractions were enriched in (*a*) late spermatids and testicular spermatozoa, (*b* and *c*) elongated spermatids and (*d–j*) round spermatids, meiotic and premeiotic cells.

Since it was discovered that glycerol provided protection to avian spermatozoa during freezing, it has been extensively used as a cryoprotective agent (Polge, 1980). In addition to its low toxicity, a permeant solute such as glycerol does not possess osmotic activity and cells are not collapsed by the increasing concentrations of the gradient.

Polyamine content in rooster testis cells separated by sedimentation at unit gravity

The cellular content of the free polyamines spermine and spermidine was similar in testicular cells with different sedimentation velocities (Fig. 2), only decreasing markedly in spermatozoa obtained

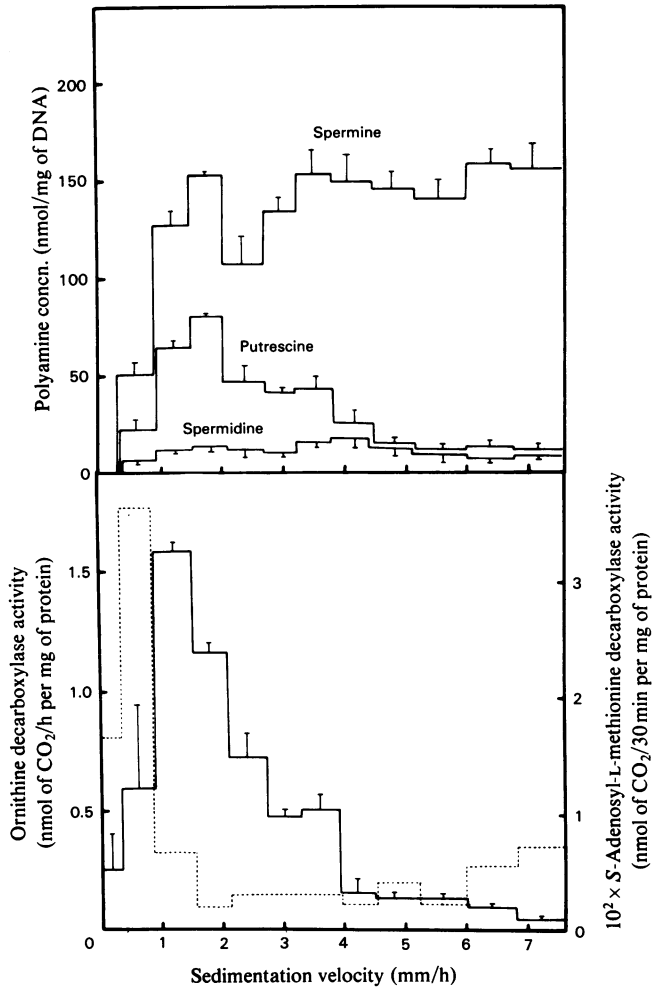


Fig. 2. *Cellular polyamine content, ornithine decarboxylase and S-adenosyl-L-methionine decarboxylase activities in rooster testis cells as a function of their sedimentation velocity at unit gravity*

Rooster testis cells were separated by sedimentation at unit gravity and the fractions, pooled as indicated in the Figure, were used for polyamine extraction or determination of the enzymic activities. Cell integrity assayed by Trypan Blue dye exclusion was 98%. Cells were harvested by centrifugation at 1000 *g* for 15 min. Polyamines were extracted with 0.2 M-$HClO_4$; the extracts were dansylated and the polyamine estimation was carried out by t.l.c. and scanning of the fluorescent dansyl derivatives. For the enzyme assays the crude homogenates of the different fractions were centrifuged at 48900 *g* for 20 min and the activities were measured in the clear supernatant by the rate of formation of $^{14}CO_2$. ——, Ornithine decarboxylase activity; – – –, *S*-adenosyl-L-methionine decarboxylase activity.

from the vas deferens (11 nmol/mg of DNA for spermine and 3 nmol/mg of DNA for spermidine). The amount of putrescine increased in fractions with low sedimentation velocity, reaching a maximum in elongated spermatids (Fig. 2). The cellular content of putrescine in vas-deferens spermatozoa was 1.8 nmol/mg of DNA.

The content of spermine in rooster testis cell nuclei separated by sedimentation at unit gravity (Mezquita & Teng, 1977*a*) varied in parallel with its cellular content (results not shown). However, the possibility of redistribution of free polyamines during nuclear isolation cannot be excluded.

The ratio of spermine/cell volume in meiotic and premeiotic rooster testis cells was in the same range as in somatic cells of different rat tissues (about 1 mM; Jänne *et al.*, 1964). This ratio increased about 10-fold in elongated spermatids as a consequence of the reduction in the cell volume. We do not know yet if the high ratio found in spermatids corresponds to an increase in free polyamine concentration, because at present there is no satisfactory technique by which the proportion of free and ionically bound polyamines can be measured. An increase of spermidine has been observed during spermatid maturation in rat testis (MacIndoe & Turkington, 1973).

A massive removal of chromosomal proteins with unmasking of DNA occurs in rooster spermatids undergoing differentiation (Mezquita & Teng, 1977*a*,*b*). Although the protamine galline is responsible for the final compaction of DNA in rooster spermatozoa, the transition from nucleohistone to nucleoprotamine could be facilitated in a similar way to that proposed for several bacteriophages (Gosule & Schellman, 1976). In this system during DNA compaction, the entropic force, the work required to bring the negatively charged segments of DNA into close proximity and the energy necessary to bend the stiff DNA chains would be strongly diminished by the presence of polyamines.

The presence of unmasked DNA in rooster spermatids could make the genome of these cells more susceptible to the attack of mutagens. Spermine is an antimutagenic agent in microbial systems (Clarke & Shankel, 1975) and it inhibits methylation of chromatin by the carcinogen *N*-methyl-*N*-nitrosourea (Rajalakshmi *et al.*, 1978). The presence of spermine in maturing spermatids could be important for the preservation of the genetic message delivered by spermatozoa.

Ornithine decarboxylase and S-adenosyl-L-methionine decarboxylase activities during rooster spermatogenesis

Rooster testis cell suspensions were subjected to sedimentation at unit gravity and the enzymic activities were determined as a function of the sedimentation velocity. Ornithine decarboxylase activity increased in parallel with putrescine content of testicular cells as the sedimentation velocity decreased, reaching a maximum in elongated spermatids (Fig. 2). The highest *S*-adenosyl-L-methionine decarboxylase activity was detected in a fraction of low sedimentation velocity, which contained late spermatids and testicular spermatozoa (Fig. 2). Neither enzymic activities were detectable in spermatozoa obtained from the vas deferens.

Similar increases in ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase activities have been noted during spermatid maturation in rat testis (MacIndoe & Turkington, 1973). These authors, however, were not able to detect ornithine decarboxylase activity in rat testis spermatids separated at unit gravity after mechanical dissociation of testicular tissue. This procedure removes the cytoplasm from many of the elongated spermatids and aggregates cells, reducing the quality of separation (Meistrich, 1977).

Ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase activities or even the whole pattern of enzymic activities responsible for polyamine biosynthesis cannot explain the actual polyamine pool, which also depends on other factors, such as the availability of precursors, the proportion of conjugated polyamines and the rate of elimination of these molecules. On the other hand, enzymic activities can be modified by their products or the concentration of other polyamines. Ornithine decarboxylase is subjected to feedback regulation by its immediate product putrescine (Jänne *et al.*, 1978). Adenosylmethionine decarboxylase is activated by putrescine and by a depletion of polyamines (Tabor & Tabor, 1976; Alhonen-Hongisto, 1980).

The presence of ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase in genetically inactive late spermatids offers an excellent system to study the post-transcriptional control of these enzymic activities.

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