Biochem. J. (1977) **164**, 99–111
Printed in Great Britain

Studies on Sex-Organ Development

CHANGES IN NUCLEAR AND CHROMATIN COMPOSITION AND GENOMIC ACTIVITY DURING SPERMATOGENESIS IN THE MATURING ROOSTER TESTIS

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(*Received* 2 *September* 1976)

We developed a technique to separate nuclei of rooster testis by centrifugation through a discontinuous sucrose density gradient and by sedimentation at unit gravity. Four different major fractions obtained from testicular nuclei and one from the vas deferens were characterized according to their velocity of sedimentation, morphology and DNA content. The ratios (w/w) of basic proteins, non-histone proteins and RNA to DNA decreased during spermiogenesis both in nuclei and chromatin. Changes in the electrophoretic patterns of histones and non-histone proteins were detected especially in the elongated spermatids. The lack of uptake of [^{3}H]uridine in elongating and elongated spermatids and in spermatozoa was demonstrated by radioautography and by the detection of labelled RNA extracted from different fractions of nuclei. Template activity for RNA synthesis and the binding of actinomycin D by testicular nuclei reached a peak in the elongated spermatid stage, when the histones are replaced by the protamine.

The most dramatic changes in chromatin structure and function observed in eukaryotes take place during spermatogenesis. Spermatogonia, spermatocytes and early spermatids are active in nuclear transcription, whereas spermatids undergoing differentiation (spermiogenesis) and spermatozoa are totally inactive (Henderson, 1964; Monesi, 1964; Bloch & Brack, 1964; Utakoji, 1966; Loir, 1972; Kierszenbaum & Tres, 1975). The typical structure of active chromatin in interphasic nuclei is lost at the beginning of spermiogenesis and a massive condensation of the chromatin occurs at the end of the process (McIntosh & Porter, 1967; Walker, 1971; Tingari, 1973; Subirana, 1975).

The genomic inactivation during spermiogenesis has been attributed to the masking of free sites on DNA by chromosomal basic proteins (Brachet & Hulin, 1969; Darżynkiewicz *et al.*, 1969*b*; Marushige & Dixon, 1969). However, the presence of unmasked DNA during spermiogenesis without concomitant synthesis of RNA *in vivo* (Loir & Hochereau de Reviers, 1972; Barcellona *et al.*, 1974) indicates that other control mechanisms are essential for genomic inactivation.

It is therefore necessary to reinvestigate the changes of genomic structure and their corresponding change in template activity *in vivo* and *in vitro*. Results from these observations should shed new light on the understanding of the mechanisms which control genomic expression during spermatogenesis.

Materials and Methods

Animals and chemicals

Sexually maturing white Leghorn roosters (25–50 weeks old) were obtained from Hendricks Grain Farms, Houston, TX, U.S.A., and used throughout this study. Testes from immature white Leghorn roosters (10 weeks old) were collected from a local slaughterhouse.

The following chemicals were obtained from the sources indicated: [^{3}H]UTP (specific radioactivity 17Ci/mmol), [^{3}H]uridine (specific radioactivity 40Ci/mmol), [^{3}H]actinomycin D (specific radioactivity 4.6Ci/mmol), actinomycin D, Tris, urea (ultra-pure), bovine serum albumin, ovalbumin and myoglobin were from Schwarz/Mann (Orangeburg, NY, U.S.A.); EDTA, diphenylamine, ammonium persulphate, and sucrose (analytical-reagent grade) were from Mallinckrodt (St. Louis, MO, U.S.A.); nucleoside triphosphates were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); acrylamide, *NN'*-methylenebisacrylamide and *NNN'N'*-tetramethylethylenediamine were from Eastman Kodak Co. (Rochester, NY, U.S.A.); SDS (sodium dodecyl sulphate) and Coomassie Blue were from Bio-Rad Laboratories (Richmond, CA, U.S.A.); collagenase was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.); aldolase and chymotrypsinogen were from Pharmacia (Uppsala, Sweden); Amido Black

10B was from Calbiochem (San Diego, CA, U.S.A.). All other chemicals were of analytical grade.

Preparation of testicular nuclei

(1) *Sucrose procedure.* The roosters were killed by cervical dislocation and the testes were removed immediately, weighed and placed in ice-cold solution A (containing 250mM-sucrose and 3mM-calcium acetate, pH5.8); all succeeding procedures were carried out at 0°C. Two to three testes were decapsulated and minced finely with scissors. The minced tissues were suspended in 5vol. of solution A and homogenized for 15 strokes with a Teflon/glass homogenizer (Glenco, Houston, TX, U.S.A.). Homogenates were filtered through one layer of nylon cloth (70μm pore size), and centrifuged at 1700g for 7min. The pellet was washed with 10vol. of solution A and again centrifuged at 1700g for 7min. The pellet was suspended in 10vol. of solution B (containing 2M-sucrose, 3.3mM-calcium acetate and 0.01% Triton X-100, pH5.8), homogenized for two strokes with a Glenco Teflon/glass homogenizer, and centrifuged at 52800g for 60min at 0°C in a Beckman model L5-65 ultracentrifuge in the SW27 rotor. The resulting nuclear pellet was dispersed and then resuspended in 10vol. of solution B by homogenization with two strokes in a Glenco Teflon/glass homogenizer.

(2) *Citric acid procedure.* Testes were removed and immediately placed in ice-cold solution C (containing 10mM-citric acid, pH2.7). Decapsulated testes, finely minced with scissors, were homogenized in 10vol. of solution C for ten strokes with a Glenco Teflon/glass homogenizer. Homogenates were filtered through one layer of nylon cloth (70μm pore size) and centrifuged at 1700g for 7min. The nuclear pellet was washed twice in the same citric acid solution by homogenization (five strokes) and centrifugation as before. Nuclei were washed in solution A, suspended by homogenization (two strokes) in heavy sucrose (solution B) and centrifuged at 52800g for 60min at 0°C.

Preparation of spermatozoan nuclei from ductus deferens

The ducti deferentes were minced and suspended in 10vol. of 0.15M-NaCl. The spermatozoa in the tissue were separated by filtering through four layers of cheesecloth and centrifuged at 1700g for 7min. The subsequent processes for isolation of nuclei from spermatozoa were exactly the same as those previously described for testicular nuclei.

In other experiments the ducti deferentes were excised and immediately placed in citric acid solution. The spermatozoa and other cells were separated by filtering through four layers of cheesecloth and centrifuging at 1700g for 7min. The following steps were the same as described for testicular nuclei.

Nuclear separation

Two steps were involved for the separation of nuclei.

Step 1. The discontinuous-sucrose-gradient technique used for separation of nuclei was as described by Utakoji *et al.* (1968) and Hotta & Stern (1971). Nuclei suspended in 14ml of solution B were layered

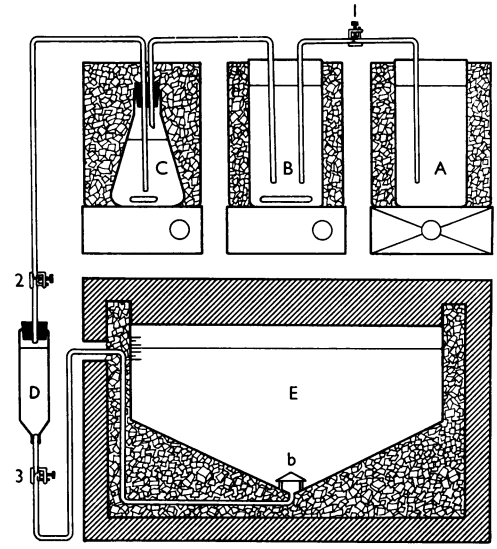


Fig. 1. *Apparatus for sedimentation of nuclei at unit gravity*
The nuclear-separation system consists of the gradient maker (beakers A and B and flask C), a syringe (D) for sample loading and the Lucite sedimentation chamber (E). Beakers A and B, flask C and chamber E were individually placed in the containers filled with ice. Beaker A was filled with 2 litres of 12% (w/v) sucrose solution, beaker B with the same volume of 6% sucrose solution and flask C with 200ml of 3% sucrose solution. The 12% sucrose solution used for separation was prepared in 3.3mM-calcium acetate and 0.01% Triton X-100, pH5.8; the 3 or 6% sucrose solution was prepared in 3.3mM-calcium acetate without Triton X-100. The nuclear suspension in 70ml (13×10^6nuclei/ml), obtained as indicated in the Materials and Methods section, was loaded into the chamber from D. The gradient was introduced into the chamber by gravity flow (110cm pressure head). Total sedimentation time was 300min. The unloading of the nuclear fractions was through the bottom of the chamber. The first volume of 2 litres was discarded and the remaining volume was collected manually into 60 fractions (25/ml fraction). The nuclear concentration in each fraction was detected by measuring the A_{600}.

on top of a discontinuous sucrose density gradient consisting of 12ml of 2.4M-sucrose and 12ml of 2.2M-sucrose, both in the 3.3mM-calcium acetate/0.01% Triton X-100, pH5.8, in Spinco SW27 rotor tubes. The tubes were centrifuged at 52800*g* for 10min at 0°C. The nuclei in the 2.0M-, 2.2M- and 2.4M-sucrose fractions were used immediately for step-2 separation.

Step 2. The separation of nuclei by sedimentation velocity at unit gravity was essentially a modification of the method of Miller & Phillips (1969). Before the separation, the nuclear preparation obtained from step 1 was washed with the solution containing 1.5% (w/v) sucrose and 3.3mM-calcium acetate, pH5.8, and centrifuged at 1000*g* for 7min. The sedimented nuclei were resuspended and washed again in the same solution once. Finally, the nuclei were suspended in the same solution by gently homogenizing for two strokes in a Glenco homogenizer and filtering through a layer of nylon cloth (70μm pore size). The nuclear concentration in the filtrate was adjusted to 13×10^6 nuclei/ml by counting with a haemocytometer or by measuring the turbidity of the nuclear suspension at 600nm with a Pye–Unicam SP.1800 spectrophotometer (approx. 0.8 A_{600} unit/ml is equivalent to 13×10^6 nuclei).

The procedures for the operation of the gradient chamber and obtaining the nuclear fraction were essentially those of Miller (1973) and Hymer *et al.* (1973). The apparatus for nuclear separation was constructed as indicated in Fig. 1 and kept at 0–4°C throughout the processes of operation.

Chromatin isolation

The nuclear pellet obtained from the previous procedure was suspended in 10vol. of 0.25M-sucrose (containing 3.3mM-calcium acetate, pH5.8, and 0.25% Triton X-100) for 15min. The nuclear suspension was centrifuged at 600*g* for 10min. The nuclei were washed twice with 0.14M-NaCl, or washed with 0.1M-Tris-HCl/0.14M-NaCl when isolated with citric acid.

The nuclei were suspended in 10vol. of 0.08M-NaCl/0.02M-EDTA and then collected by centrifugation at 600*g*. This NaCl/EDTA treatment was repeated once. The nuclear pellet was washed twice with 10vol. of 0.05M-Tris/HCl (pH7.5) and by homogenization with 21 strokes in a Dounce homogenizer with a loose pestle. The nuclear pellet was washed twice with 0.01M-Tris/HCl (pH7.5) and homogenized as before. After sedimentation the nuclei were suspended in 0.002M-Tris/HCl (pH7.5) by homogenization with 10 strokes in a Dounce homogenizer with a loose pestle, and the nuclear suspension was allowed to swell overnight at 0°C. After swelling, the chromatin in the nuclei was liberated into solution by homogenization for 200 strokes in a Dounce homogenizer equipped with a tight pestle. The chromatin in the solution was obtained by filtering through one layer of nylon cloth (30μm pore size).

Template-activity assay

Template activity of chromatin isolated from different fractions of nuclei was detected in an assay mixture of 0.25ml final volume, containing 62.5mM-Tris/HCl buffer, pH7.9, 1.25mM-$MnCl_2$, 2.5mM-β-mercaptoethanol, 0.15mM-GTP, 0.15mM-ATP, 0.15mM-CTP and 0.15mM-[^{3}H]UTP (specific radioactivity 74.85c.p.m./pmol). Then 5.9μg of *Escherichia coli* RNA polymerase, prepared by the method of Burgess (1969) as modified by Bautz & Dunn (1969), was added with 5μg of free DNA or with the equivalent amount of chromatin. Triplicate samples were incubated for 20min at 37°C. The tubes were then chilled in ice, and 50μl of bovine serum albumin (3mg/ml) was added to each tube, followed by the addition of 3ml of 5% (w/v) trichloroacetic acid containing 0.01M-sodium pyrophosphate. After 30min the precipitates were collected on Millipore filters (0.45μm pore size) and washed with 25ml of 5% trichloroacetic acid containing 0.01M-sodium pyrophosphate. The filters were dried and transferred to counting vials containing 4ml of scintillation fluid [6g of 2,5-diphenyloxazole, 0.15g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of toluene]. The radioactivity was determined at 60% efficiency in a Beckman model LS-250 scintillation spectrometer. For the assay of nuclear-template activity, nuclei (5μg of DNA) from different fractions were washed with 0.14M-NaCl and the activity was assayed by the procedures mentioned above.

Gel electrophoresis of chromatin proteins

Chromatin-associated proteins, comprising a mixture of histone and non-histone proteins, were resolved by electrophoresis in SDS/polyacrylamide gel according to their molecular weights as described by Laemmli (1970). Basic proteins were extracted and analysed by electrophoresis originally developed by Panyim & Chalkley (1969) and modified by Ruiz-Carrillo *et al.* (1974).

[^{3}H]Uridine incorporation

Approx. 5g of testicular tissues immediately removed from a rooster was incubated in 100ml of McCoy's medium (McCoy *et al.*, 1959) with 0.02Ci of [^{3}H]uridine/ml at 41°C in an air/CO_2 (19:1)-circulated incubator for 2h. The tissues were washed three times with the culture medium, and the nuclei were prepared and separated from the tissue by the procedure described above. The nuclear fractions were washed with 5% trichloroacetic acid, ethanol,

ethanol/ether and ether to remove free [^{3}H]uridine as described by Madgwick *et al.* (1972). The residue was suspended in 0.2M-$HClO_4$, and portions of the solution were used for determination of DNA, RNA and radioactivity. The incorporation of [^{3}H]uridine into different fractions of nuclei was expressed as c.p.m./mg of RNA or c.p.m./mg of DNA.

[^{3}H]Actinomycin D binding

Procedure 1. The soft testicular tissue was gently teased with a pair of forceps to liberate the cells, which then were suspended in Hanks solution (Hanks & Wallace, 1949). Cells were incubated in the solution at 37°C for 1h in the presence of 1μg of [^{3}H]actinomycin D/ml (final specific radioactivity 0.42mCi/mg). At the end of incubation the cells in the medium were diluted with 5vol. of Hanks solution. The cells were then centrifuged down at 700*g* and resuspended in 0.25M-sucrose/3.3mM-calcium acetate, pH5.8. Nuclei were prepared and fractionated by the procedure described above. Different fractions of nuclei in the sedimentation chamber were collected. A sample of each fraction was taken for DNA determination and the rest was precipitated on a fibre-glass filter (Reeve/Angel 934 AH). Each filter was washed with 100ml of 0.5% sucrose solution containing 3.3mM-calcium acetate, pH5.8. Filters were dried and assayed for radioactivity by the procedure previously mentioned.

Procedure 2. Nuclei at different stages of spermatogenesis isolated by the citric acid procedure and separated by sedimentation velocity at unit gravity were washed twice with 0.15M-NaCl/0.1M-Tris/HCl, pH7.5, and incubated for 1h at room temperature (24°C) in the same medium containing 2.2μg of [^{3}H]actinomycin D (final specific radioactivity 0.049mCi/mg). Different amounts of each fraction of nuclei (5–50μg of DNA) were used in the incubation mixtures. After the incubation, nuclei were collected in fibre-glass filters and washed with 100ml of 0.15M-NaCl/0.1M-Tris/HCl, pH7.5. Filters were dried and assayed for radioactivity.

Procedure 3. Roosters were anaesthetized with Nembutal (100mg in 2ml, injected intramuscularly). The left testis was injected with 100μCi of [^{3}H]-actinomycin D (specific radioactivity 3.6mCi/mg) in 1ml of Hanks solution. After 1h, animals were killed by cervical dislocation. Nuclei were isolated by the citric acid procedure and fractionated by sedimentation velocity at unit gravity. The different fractions of nuclei were collected in fibre-glass filters, washed with 100ml of 0.5% sucrose solution containing 3.3mM-calcium acetate, pH5.8, and assayed for radioactivity.

Radioautography

Fragments of testicular tissue (150–200mg) were incubated in 3ml of McCoy's medium containing 0.5% (v/v) of penicillin and streptomycin (5000i.u. of penicillin and 5000i.u. of streptomycin/ml of medium, supplied by Grand Island Biological Co., Grand Island, NY, U.S.A.) and with 0.01Ci of [^{3}H]uridine/ml in an incubator circulated with O_2/CO_2 (19:1) at 41°C for 2h. At the end of the incubation, the tissue was washed twice with Hanks solution, fixed with Bouin's fixative overnight, stored in 70% (v/v) ethanol, dehydrated and embedded in paraffin was. Kodak NTB_2 liquid emulsion was used for radioautography. After 30 days of exposure, radioautographs were developed and stained with Haematoxylin/Eosin.

General procedures

Protein was determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin (Sigma) as a standard. DNA was determined by the diphenylamine reaction (Giles & Myers, 1965), with calf thymus DNA (Schwarz/Mann) as a standard. RNA was determined by the procedure of Munro & Fleck (1966). Radioactivity was determined by adding 4ml of scintillation fluid in a mini counting vial (Rochester Scientific Co., Rochester, NY, U.S.A.). The radioactivity was determined at 60% efficiency in a Beckman model LS-250 liquid-scintillation spectrometer.

Results and Discussion

Isolation and separation of nuclei

The method of velocity sedimentation at unit gravity was successfully applied to separate the spermatogenic nuclei of the guinea-pig testis (Mezquita, 1974). We adopted this sedimentation method, with some modifications, for the preparation of nuclei from the maturing rooster testis during spermatogenesis. In our modified technique, the first step involved a crude nuclear separation in a discontinuous sucrose gradient. After centrifugation, the nuclei were distributed into three zones in the gradient, namely A, B and C (2.0M-, 2.2M- and 2.4M-sucrose fractions respectively). Sedimentation through a discontinuous gradient of sucrose requires a very short time (10min), and improves the resolution of nuclei during their subsequent separation in the unit-gravity chamber.

The second step of separation was accomplished in a unit-gravity sedimentation apparatus (Fig. 1). The design of this apparatus is based on the fact that a mixture of different-sized particles can be separated according to their sedimentation velocities in the earth's gravitational field. The decrease in nuclear volume during spermatogenesis in rooster testes ranged from 110 to 25μm^3 (from primary spermatocyte to round spermatid), yet no substantial change

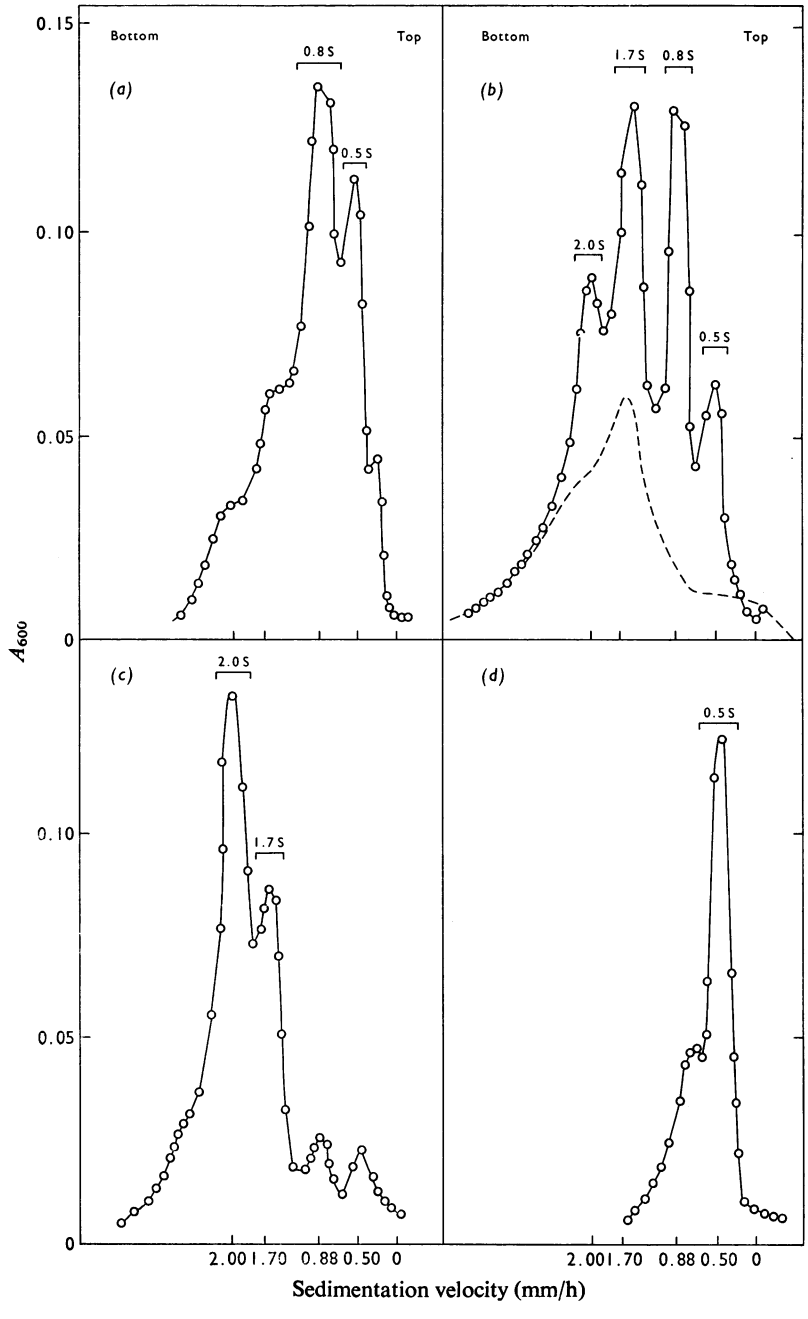


Fig. 2. *Distribution of nuclei isolated from testis or spermatozoa of the vas deferens as the function of their sedimentation velocity at unit gravity*

Nuclei isolated in a sucrose/calcium medium were separated in a discontinuous sucrose gradient. Three fractions of nuclei were obtained: fraction A from the top of the gradient (2M-+the interphase of 2M- and 2.2M-sucrose layers); fraction B from the middle portion of the gradient (2.2M-+the interphase of 2.2M- and 2.4M-sucrose layers); fraction C from the bottom of the gradient (pellet+2.4M-sucrose layer). (*a*) Nuclei (S 0.8 and 0.5mm/h) obtained from fraction A. (*b*) Nuclei (S 2.0, 1.7, 0.8 and 0.5mm/h) obtained from fraction B; ----, nuclei of immature testis (S>0.88mm/h). (*c*) Nuclei (S 2.0 and 1.7mm/h) obtained from fraction C. (*d*) Nuclei (S 0.5mm/h) isolated from spermatozoa of the vas deferens.

occurred in its spherical shape. The nucleus therefore becomes a prolate ellipsoid whose axial ratio increases as spermiogenesis proceeds. From the round-spermatid stage to the spermatozoa stage, the nuclear volume decreases from 25 to 2 μm^3 (McIntosh & Porter, 1967). The spermatogenic nuclei are suitable for gravitational separation.

The A_{600} showed that fraction A separated into two distinguishable nuclear fractions (Fig. 2*a*), fraction B separated into four fractions (Fig. 2*b*) and fraction C separated into two fractions (Fig. 2*c*). The total nuclear preparation of immature rooster testes recovered from the 2.2M-sucrose fraction was separated into one major single fraction (Fig. 2*c*). The spermatozoal nuclei isolated from the ductus deferens were sedimented at the region of 0.5S as a single major fraction (Fig. 2*d*).

The sedimentation velocity of the separated nuclei was determined by the following equation:

$$S\ (mm/h) = \frac{V-100}{\alpha t}$$

where V is the volume pooled since the last fraction (end of the gradient) to the top of the peak, $\alpha = 66$ (cm^3/mm; each mm in the cylindrical region of the chamber contains 66ml) and $t = 5$h (sedimentation time). The relation between log V and log S indicates that the separation of nuclei occurs mainly according to their differences in size.

Analysis of the morphological and chemical-composition changes of the nuclei

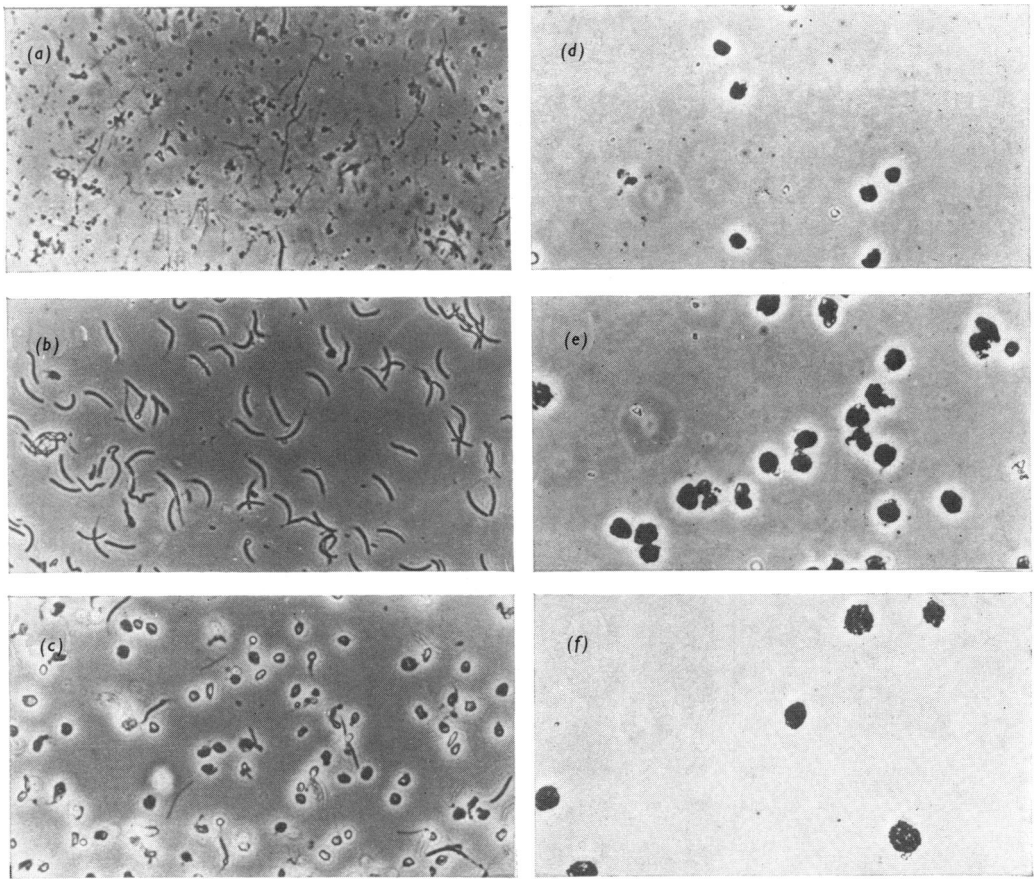
The five different sizes of nuclei obtained from the sedimentation chamber were identified by phase-contrast microscopy and acetic/orcein staining. The morphology of each nuclear fraction is presented in Plate 1.

The fragmentary particles and tail pieces were distributed in the 0.2S region (or the top of the sedimentation chamber) (Plate 1*a*). The fraction with S = 0.5mm/h contained slender elongated spermatid and testicular spermatozoal nuclei. The tails were not present in the fraction, yet 25–30% of the middle pieces remained attached to the head (Plate 1*b*). However, the preparation of spermatozoa by citric acid solution removes the tails and the middle pieces, which is in agreement with results reported by Daly *et al.* (1950). If the sedimentation time is shorter than 300min, this fraction will be contaminated by residual bodies (results not shown). Spermatozoal nuclei isolated from the vas deferens also sedimented in this fraction (Fig. 2*d*). The fraction with S = 0.8mm/h contained comparatively small round nuclei or nuclei with an irregular shape. This fraction was contaminated by less than 10% with the nuclei that sedimented at 0.5mm/h (Plate 1*b* and 1*c*). Plate 1(*e*) represents the fraction with a sedimentation

Table 1. *Chemical composition of nuclei and chromatin isolated from different stages of spermatogenesis*

Nuclei were prepared by (*a*) sucrose procedure or (*b*) citric acid procedure. Chromatin was isolated from nuclei prepared by both procedures. The preparation of nuclei, the isolation of chromatin and the determination of chemical composition were as described in the Materials and Methods section. Each value presented in (*a*) is the mean± S.D. of five determinations, and each value presented in (*b*) is the mean± S.D. of three determinations.

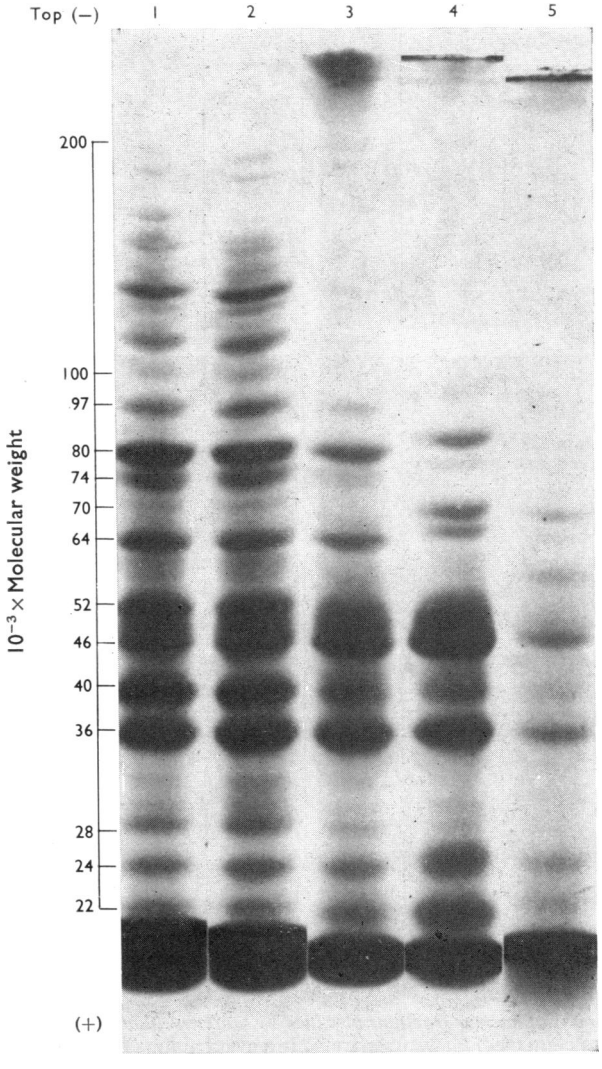
Stage of differentiation	S	DNA (pg/nucleus)	Basic proteins/DNA				Non-histone proteins/DNA				RNA/DNA			
			Nuclei		Chromatin		Nuclei		Chromatin		Nuclei		Chromatin	
			(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
1	2.00± 0.03	4.27	1.00± 0.06	1.09± 0.06	1.13± 0.10	1.06± 0.06	0.70± 0.02	0.91± 0.04	0.78± 0.06	0.80± 0.05	0.22± 0.03	0.21± 0.04	0.13± 0.04	0.15± 0.05
2	1.70± 0.03	2.57	1.04± 0.04	1.00± 0.05	1.17± 0.11	1.05± 1.07	0.88± 0.08	0.80± 0.07	0.76± 0.10	0.77± 0.06	0.27± 0.02	0.23± 0.06	0.11± 0.03	0.12± 0.04
3	0.88± 0.03	1.15	0.94± 0.03	0.90± 0.07	1.15± 0.07	0.90± 0.05	0.64± 0.08	0.59± 0.06	0.47± 0.11	0.48± 0.06	0.21± 0.02	0.17± 0.08	0.10± 0.03	0.10± 0.02
4	0.50± 0.02	1.20	0.54± 0.04	0.68± 0.03	0.58± 0.04	0.60± 0.05	0.32± 0.01	0.35± 0.04	0.42± 0.05	0.32± 0.06	0.17± 0.04	0.10± 0.05	0.06± 0.02	0.09± 0.02
5	0.50± 0.02	1.17	0.50± 0.03	0.61± 0.02	0.47± 0.05	0.59± 0.05	0.23± 0.01	0.22± 0.03	0.25± 0.07	0.23± 0.05	0.12± 0.03	0.09± 0.02	0.04± 0.01	0.05± 0.02



EXPLANATION OF PLATE 1

Photomicrographs of nuclear fractions obtained by sedimentation velocity at unit gravity

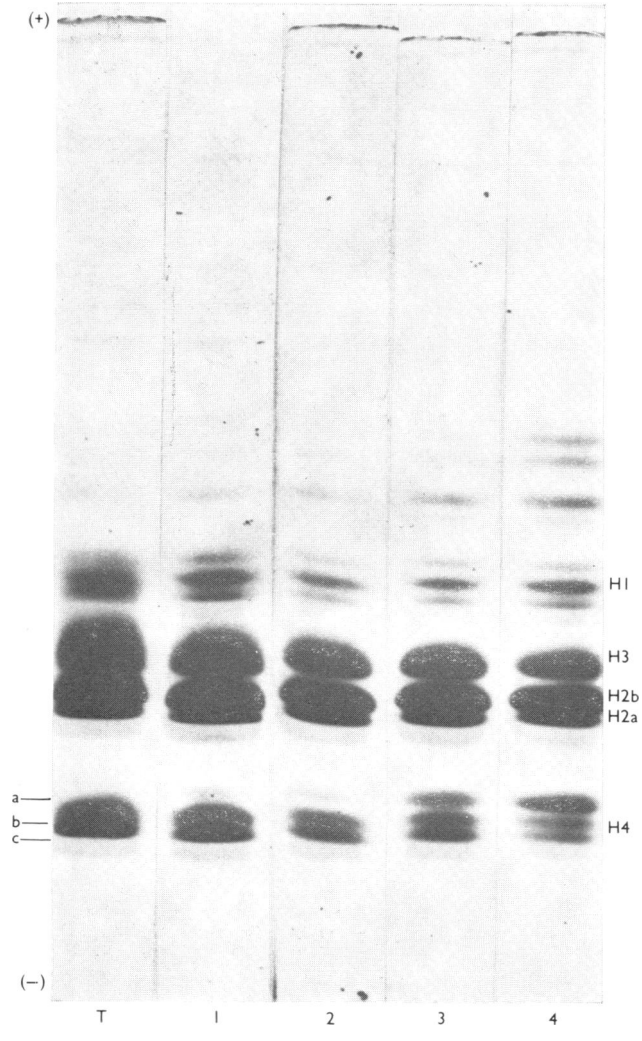
Nuclei isolated in a sucrose/calcium medium were separated in a discontinuous sucrose density gradient and then sedimented at unit gravity. The nuclear fractions were observed by a Zeiss S-14 phase contrast microscope. The samples in (*a*), (*b*) and (*c*) were magnified 375-fold, and the nuclei in (*d*), (*e*) and (*f*) were stained with acetic Orcein before observation and were magnified 562-fold. (*a*) Tails, residual bodies and debris that remained on the top of the gradient. (*b*) Nuclei (S = 0.5 mm/h) from the elongated spermatids and the testicular spermatozoa. (*c*) Nuclei (S = 0.8 mm/h) from the round and the elongating spermatids. (*d*) Nuclei of the round spermatids stained with acetic Orcein. (*e*) Nuclei (S = 1.7 mm/h) from the diploid meiotic and the gonial cells. (*f*) Nuclei (S = 2.0 mm/h) from the tetraploid primary spermatocytes.



EXPLANATION OF PLATE 2

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of chromosomal proteins from nuclei at different stages of spermatogenesis

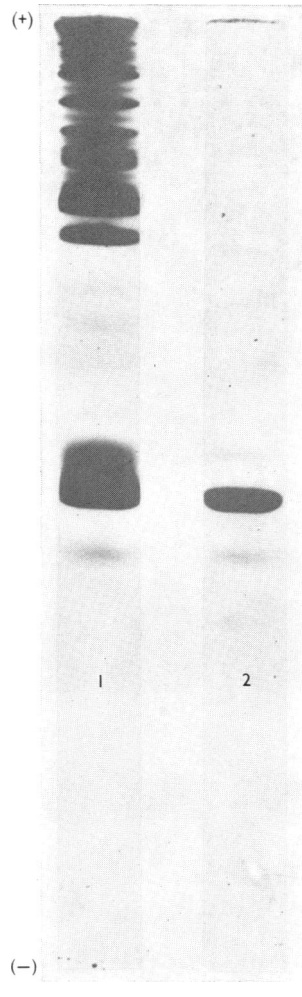
Nuclei were isolated in citric acid medium and separated by sedimentation velocity at unit gravity. Chromatin was prepared from each fraction of nuclei, and the chromatin proteins were solubilized with 2% SDS in 0.0625M-Tris/HCl, pH6.8, 0.7M-β-mercaptoethanol and 5mM-EDTA. (*a*) Gels (6mm × 85mm) were loaded with approx. 100μg of DNA as chromatin from the different stages of spermatogenesis and were simultaneously electrophoresed in 10% (w/v) polyacrylamide gels for 24h at 1mA per gel and stained with Amido Black. The molecular weights of the proteins were determined by the method of Weber & Osborn (1969) with the following proteins as standards: myoglobin (mol.wt. 17200), chymotrypsinogen (mol.wt. 23240), aldolase (mol.wt. 40000), ovalbumin (mol.wt. 45000), bovine serum albumin (mol.wt. 68000), collagenase (mol.wt. 109000) and myosin (mol.wt. 212000).



EXPLANATION OF PLATE 3

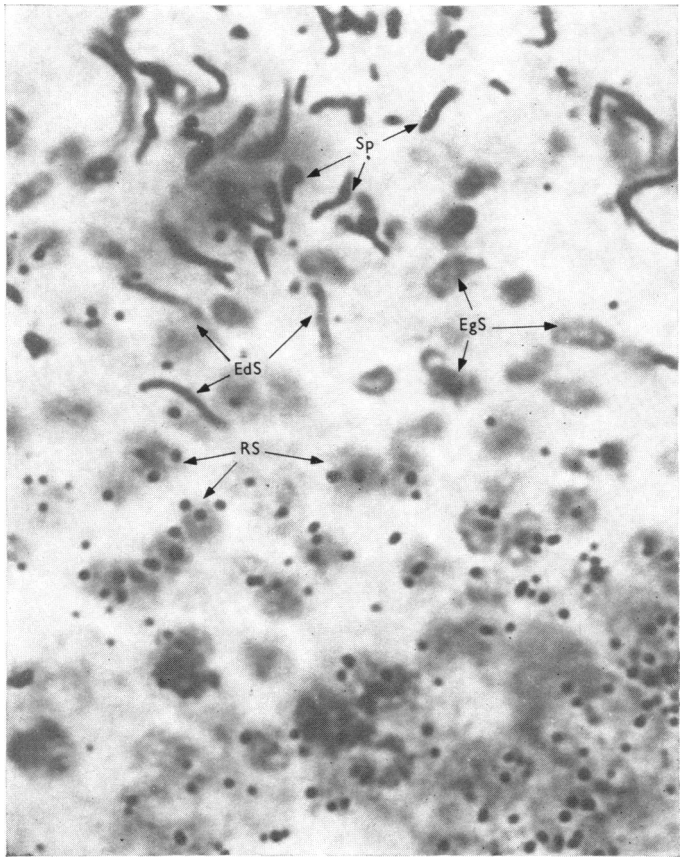
Polyacrylamide-gel-electrophoretic analysis of the acid-soluble chromosomal proteins from nuclei at different stages of spermatogenesis

Nuclei were isolated in sucrose/calcium medium containing 0.05 M-$NaHSO_3$ and separated in a discontinuous gradient of sucrose, then by sedimentation at unit gravity. Chromatin was isolated from each fraction of nuclei, and basic proteins were extracted with 0.2 M-H_2SO_4. Electrophoresis was performed in 15% (w/v) polyacrylamide gels containing 2.5 M-urea. Samples (20 μg) of protein were run for 6 h at 1 mA per gel. The detailed procedures are described in the Materials and Methods section.



EXPLANATION OF PLATE 4

Polyacrylamide-gel-electrophoretic analysis of acid-soluble chromosomal proteins from spermatids and spermatozoa
(*a*) Nuclei were isolated in citric acid medium and separated by sedimentation velocity at unit gravity. Chromatin was isolated from the nuclei, and basic proteins were extracted with 0.2M-H_2SO_4. Electrophoresis was performed in 15% (w/v) polyacrylamide gels containing 6.25M-urea. Samples (20–30μg) of protein were run for 3h at 1mA per gel. The procedures have been detailed in the Materials and Methods section. 1, Elongated spermatids and testicular spermatozoa; 2, spermatozoa from the vas deferens.



EXPLANATION OF PLATE 5

[^{3}H]Uridine incorporation into nuclei studied by radioautography

The detailed procedure for tissue culture, radioactivity incorporation and radioautography are described in the Materials and Methods section. The radioautography was observed under a model S-14 Zeiss phase-contrast microscope. RS, round spermatids; EgS, elongating spermatids; EdS, elongated spermatids; Sp, spermatozoa. The nuclei in the picture were amplified 1666-fold. The radioautography shows that the grains are located in the nucleus of premeiotic, meiotic and round spermatid cells.

velocity of 1.7mm/h; it contained smaller nuclei with the average diameter of small primary spermatocytes, secondary spermatocytes and spermatogonia, according to the description of Zlotnik (1947) and Miller (1938). Finally, the fraction with a sedimentation velocity of 2.0mm/h contained large spherical nuclei heavily stained with a nuclear network extending through the entire nuclear space. This fraction consisted of tetraploid primary spermatocyte nuclei (Plate 1*f*).

The various stages of differentiating nuclei, according to their sedimentation values, i.e. 2.0, 1.7, 0.8, 0.5 and 0.5S, were designated as stages 1, 2, 3, 4 and 5 respectively (Table 1). The nuclear DNA contents (in terms of pg/nucleus) of stages 1, 2, 3, 4 and 5 were 4.27, 2.57, 1.15, 1.20 and 1.17 respectively.

The amount of DNA per nucleus of rooster spermatozoa has been determined by Daly *et al.* (1950) and Vendrely *et al.* (1956). Their values of 1.26pg and 1.10pg are in good agreement with our results for spermatids and spermatozoa. The average diploid content for six somatic tissues of rooster was 2.45pg according to Mirsky & Ris (1950). The proportions of DNA/RNA/protamine for rooster spermatozoa are 10:1:5 (by wt.) (Nakano *et al.*, 1973).

The ratio of basic proteins to DNA remained at a constant range (1.0, 1.04 and 0.94) from stages 1 to 3 and then decreased to almost half of the original value (0.54, 0.50) in stages 4 and 5. Nuclear non-histone protein content remained high at stages 1 and 2 (ratios of non-histone proteins to DNA were 0.70 and 0.88), started to decrease at stage 3 (0.64) and finally decreased to 0.32 and 0.23 at stages 4 and 5. The amount of nuclear RNA compared with DNA was also observed to decrease as the cell reached the late stages of spermatogenesis. The chemical compositions of the nuclear preparations isolated with sucrose or citric acid methods are basically identical (Table 1).

The data presented above show a loss of nuclear proteins and RNA throughout the processes of spermiogenesis in the rooster testis. Similar losses have been reported to occur in developing spermatids in many species. In crustaceans, the developing spermatids lose a significant proportion of their nuclear proteins (Vaughn & Thomson, 1972). In the echinoderm *Holothuria tubulosa*, another example of a very primitive type of spermiogenesis, the histones remain in the sperm, but the ratio of basic proteins/DNA is also low (Subirana, 1972). Most of the non-histone proteins and RNA are lost after the early stage of spermiogenesis in the house cricket (Kaye & McMaster-Kaye, 1966). Non-histone proteins are removed during spermiogenesis of the frog (Zirkin, 1970). In rats, the non-histone proteins are discarded during spermiogenesis before the histones begin to leave the nucleus (Vaughn, 1966). Histochemical changes during avian spermatogenesis, showed that the granular basophilc substance, comprising RNA and protein, is present in spermatogonia, spermatocytes and early spermatids, and only disappears during the late stages of spermiogenesis when the nucleus begins to stain homogeneously for DNA and protein (Guraya, 1970).

Analysis of the chromatin composition change during spermatogenesis

Chromatin isolated from nuclei of different developmental stages showed changes in chemical composition. The pattern of change paralleled that observed in the nucleus. The chromatin basic protein/DNA ratio stayed constant at stages 1, 2 and 3 (1.13, 1.17 and 1.15 respectively) and decreased at stages 4 and 5 (0.58 and 0.47). The non-histone protein/DNA ratio remained at about the same value at stages 1 and 2 (0.78, 0.76), but started to decrease at stages 3 and 4 (0.47, 0.42) and fell to a minimum at stage 5 (0.25). In the later stage, the RNA/DNA ratio decreased by about 70% as compared with the early stage of spermatogenesis (Table 1). Similar findings were reported for sea-urchin *Arbacia punctulata* spermatozoa, in which chromatin has small amounts of non-basic proteins and RNA (Paoletti & Huang, 1969). During the maturation of trout testis, the content of RNA and non-histone proteins decreases, whereas the chromosomal basic proteins increase until the histones are replaced by protamine (Marushige & Dixon, 1969).

Analysis of the change in chromosomal non-histone proteins during spermatogenesis

The chromatin-associated proteins were analysed by SDS/polyacrylamide-gel electrophoresis. The electrophoretic separation of non-histone protein subcomponents from histones was distinguished by their mol.wt. range of 40000–200000, as presented in Plate 2. No obvious difference existed between the banding patterns of the non-histone proteins of stages 1 and 2. Approx. 28 of the total chromatin-associated protein subcomponents were present in these two stages; 14 characteristically high-molecular-weight non-histone protein subcomponents were present in the region of mol.wt. 80000–200000. The high heterogeneity of the non-histone proteins resolved by gel electrophoresis in the region above mol.wt. 75000–84000 has been observed in the meiotic nuclei of guinea-pig testis and rat testis (Mezquita, 1974; Kadohama & Turkington, 1974; Platz *et al.*, 1975).

In stage 3 (round and elongating spermatids) there were four proteins of high mol.wt. (100000–200000) which decreased in density. Also four

proteins of mol.wts. 72000, 52000, 40000 and 28000 decreased in density.

In stage 4 of spermatogenesis (elongated spermatids and testicular spermatozoa) approx. 12 protein components (in the mol.wt. range 80000–200000) plus three protein components (mol.wt. 80000, 74000 and 40000) were decreased drastically. There were four proteins with mol.wts. of 86000, 70000, 68000 and 46000 which increased in density. In stage 5 (spermatozoa from the vas deferens) only five proteins (in the mol.wt. range 36000–70000) remained visible; the remaining protein components disappeared (Plate 2).

A drastic decrease in the non-histone protein components has been reported in the elongated spermatids of guinea pig and rat (Mezquita, 1974; Platz *et al.*, 1975). Another similar observation was made in *Physarum polycephalum*, fibroblasts and HeLa cells. During the transition from proliferating and metabolically active cells to non-proliferating cells with inactive nuclei, a group of high-molecular-weight non-histone proteins disappear; also, a protein component of mol.wt. 52000 (similar in amino acid composition to tubulin) and a protein of heterogeneous nuclear RNA particles decrease and disappear, whereas actin, a protein of mol.wt. 46000, increases (LeStourgeon *et al.*, 1974). It is possible that the genetic quiescence of elongating and elongated spermatids is related to the loss of most of the nuclear non-histone proteins. The correlation between these proteins and gene activity has been reported in various developmental systems (Dingman & Sporn, 1964; Marushige & Ozaki, 1967; Teng, 1974; Ruiz-Carrillo *et al.*, 1974). Our observation of the remaining few non-histone proteins within the spermatozoon is consistent with previous observations of the spermatozoa of different species, e.g. sea-urchin, crab, newt and trout (Paoletti & Huang, 1969; Vaughn & Thomson, 1972; Picheral & Bassez, 1971; Marushige & Dixon, 1969). However, the function of this class of non-histone proteins is still unknown.

Analysis of the change in chromosomal basic proteins during spermatogenesis

Electrophoretic patterns of total histone from the 10-day chick embryo and of isolated chromatin of germ cells from various stages of differentiation are shown in Plate 3. The relative quantity and mobility of each of the five histone fractions are essentially similar and constant. However, there are detectable differences between histone fractions H1 and H4, especially during the late stages of spermatogenesis. H1 histone, isolated by the method of Johns (1964) from 10-day chick embryos, was run as a reference with H1 histone from various stages of differentiation (Fig. 3). H1-histone subcomponents with electro-

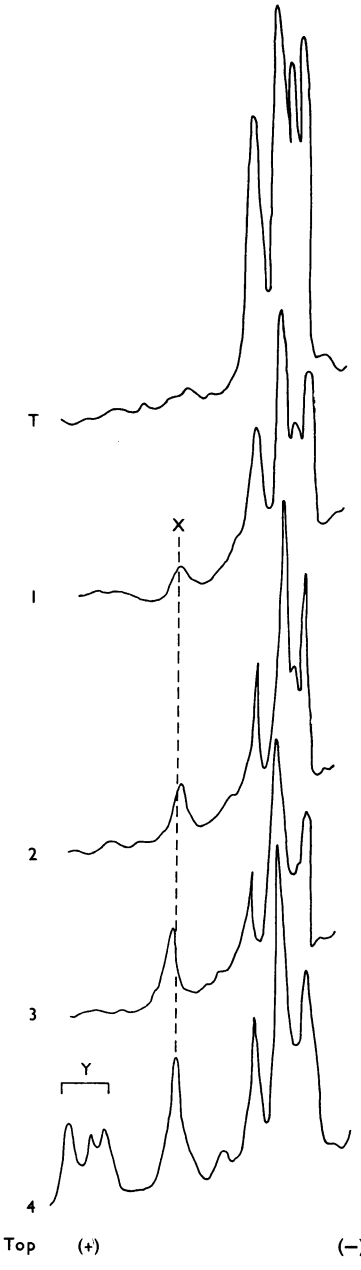


Fig. 3. *Polyacrylamide-gel-electrophoretic analysis of H1 histone*

Electrophoresis was performed in 15% (w/v) polyacrylamide gel containing 2.5 M-urea. Samples (20 μg) of protein were run for 10 h at 1 mA per gel. All other histones were run out of the gel. T, H1 histone extracted from 10-day chick embryos; 2, tetraploid primary spermatocytes; 3, diploid spermatocytes and gonial cells; 4, round and elongating spermatids; 5, elongated spermatids and testicular spermatozoa. X and Y bands are discussed in the text.

phoretic mobilities of 0.65, 0.67, 0.68 and 0.70 relative to histone H4 were present in all the chromatin. The observed heterogeneity of H1 histone is in general agreement with the observations of various organs of the hen by Panyim *et al.* (1971) and of chick embryos by Teng *et al.* (1974).

We have observed that there is one additional band, X, of lower mobility (0.57) than histone H1, and the increase in density of this band is greatest in the chromatin of stage-4 nuclei.

A similar observation has been reported in the rat testis (Branson *et al.*, 1975; Shires *et al.*, 1975). It was demonstrated that an extra lysine-rich histone band is closely related to H1 histone, and the concentration of this band, relative to the H1 histone, was greatest in the spermatid nuclei (Branson *et al.*, 1975; Kumaroo *et al.*, 1975). At present, we lack sufficient information to determine whether the additional X band in our system is equivalent to that observed in the others. We also lack information on the Y bands (with mobilities of 0.50 and 0.53) observed in the stage-4 spermatids. The characteristic nature and function of these bands have yet to be investigated.

It has been reported that histones H2a and H2b are present in amounts 33–50% lower in the testicular nuclei than in the somatic tissues of the house-cricket and rat (Kaye & McMaster-Kaye, 1974; Shires *et al.*, 1975). The amount of histone H3, on the other hand, is about 25% higher in the testicular than the somatic tissue (Kaye & McMaster-Kaye, 1974). In our system, the quantities of these three fractions of histones remained constant throughout differentiation as compared with their somatic counterparts.

The change in H4 histones during rooster spermatogenesis is apparent. Electrophoresis showed that one histone band (band a) of lower mobility than the doublet band of histone H4 (bands b and c) increased in concentration (Plate 3). In the developing sea-urchin *Arbacia lixula* embryo, the histone bands of lower mobility than histone H4 were identified as the acetylated products of the H4 histone (Wangh *et al.*, 1972). The ratio of one of the acetylated histones (trailing) to the leading member of the histone H4 group was greater in rat spermatid nuclei than in primary spermatocytes (Kumaroo *et al.*, 1975). It has been suggested that the acetylation of H4 histone functions physiologically as a part of the mechanism that removes the H4 histone during the replacement of histones by protamine (Marushige *et al.*, 1976).

The total basic protein extracted from the elongated spermatid and testicular spermatozoal nuclei (stage 4) has been analysed by polyacrylamide-gel electrophoresis; the analysis indicated the existence of both histones and protamine in the same fraction. The protamine appears to be a single major protein band in the gel. The basic protein isolated from spermatozoa from the vas deferens consisted only of protamine without histones (Plate 4). This observation confirmed previous reports that in rooster spermatozoa protamine is the only existing basic protein (Daly *et al.*, 1950; Fischer & Kreuzer, 1953).

The rooster protamine extracted from nuclei isolated with citric acid migrated in the polyacrylamide gel as a single band with the mobility of 0.64 relative to salmine. The single-banding pattern of protamine in gel electrophoresis was also observed in the sperm of various other species, e.g. human, rabbit, guinea pig, bull and rat (Bellvé *et al.*, 1975; Kumaroo *et al.*, 1975).

However, when protamine was extracted from nuclei prepared by the sucrose/calcium procedure, electrophoretic heterogeneity was evident (Fig. 4). This finding parallels the observation of rooster protamine by Nakano *et al.* (1973). Their experiment demonstrated the electrophoretic macroheterogeneity of protamine in the sperm. To gain more insight into the possible cause of the observed heterogeneity in the rooster, we have incubated the spermatozoal chromatin sample in 50mM-Tris/HCl, pH7.5, for 60min at 37°C and observed the degradation of the protamine (Fig. 4, trace 3). A similar phenomenon in bull spermatozoal chromatin was reported by Marushige & Marushige (1975). Further, when rooster spermatozoal chromatin was incubated with *N*-benzoyl-L-arginine ethyl ester, it could hydrolyse the latter (results not shown). All available information indicates that the degradation of protamine is probably due to the presence of proteolytic enzymes associated with the chromatin. The presence of proteolytic activity in rooster spermatozoa has been reported in several publications (Buruiana, 1956; Ho & Meizel, 1970; Yamagimachi & Teichman, 1972; McIndoe & Lake, 1974; Bernon & Buckland, 1975), and it is possible that the proteinase could contribute to this observed electrophoretic heterogeneity. Brown & Hartree (1976) reported that rooster spermatozoal acrosin was inactivated by pH2.7, and a precursor of the proteinase (proacrosin) was also extracted at the same pH. It is possible, then, that the citric acid procedure (at pH2.7) that we adopted for nuclear isolation provided an ideal condition for keeping the protamine intact.

Changes in [^{3}H]uridine uptake, template activity and actinomycin D binding during spermatogenesis

The incorporation of [^{3}H]uridine into RNA in the nuclei *in vitro* occurs in stages 1–3, but is almost undetectable in stages 4 and 5. The amounts of radioactive uridine incorporated into RNA (in terms of c.p.m. of [^{3}H]uridine incorporation/mg of DNA) are 3200, 2250, 3950, 550 and 350 for the respective five stages (Fig. 5*a*). The template activity of nuclei *in vitro* at different stages of spermatogenesis for RNA transcription in terms of pmol of [^{3}H]UTP incorporated into RNA/μg of nuclear DNA is 15.50,

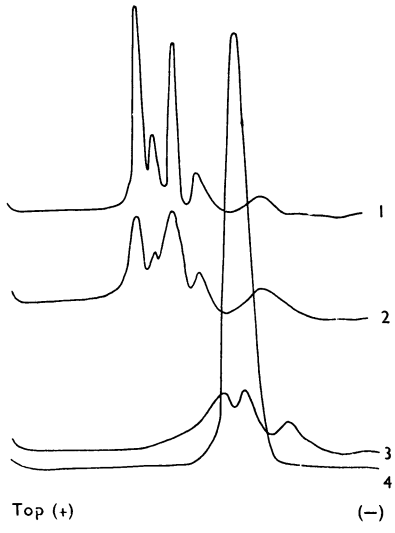


Fig. 4. *Electrophoretic pattern of basic protein extracted from chromatin prepared under various experimental conditions*

1, Nuclei of spermatozoa were isolated in a sucrose/calcium medium. Chromatin was prepared within 2h and the basic proteins were extracted with 0.2M-H_2SO_4; 2, nuclei of spermatozoa were isolated in a sucrose/calcium medium and sedimented for 300min at unit gravity; then the chromatin was prepared and basic proteins were extracted as before; 3, nuclei of spermatozoa were isolated in a sucrose/calcium medium, and chromatin was prepared within 2h and incubated in 50mM-Tris/HCl, pH7.5, for 60min before the extraction of the basic proteins; 4, salmine was electrophoresed in a similar condition as a marker.

15.06, 19.04, 24.27 and 3.01. The template activity of isolated chromatin followed the same pattern as the nuclei. The template activities of chromatin *in vitro* assayed in the presence of *E. coli* RNA polymerase as compared with the open template of chick DNA are 8.4, 8.0, 11.9, 19.0 and 0.4% for the respective spermatogenesis stages 1–5. Preincubation or lack of preincubation of the template (nuclei or chromatin) had no significant effect on the overall template activity *in vitro* (Fig. 5*b*).

The radioautography picture presented in Plate 5 confirmed our [^{3}H]uridine-incorporation study *in vitro* that the radioactive nucleotide was incorporated into the round spermatids, but no such incorporation was observed in the elongating and elongated spermatids or in the spermatozoa. Similar observations of RNA synthesis during spermatogenesis were documented in the hamster, orthopterans, mouse, ram and other species in which the [^{3}H]uridine incorporation was actively carried out in spermatogonia, meiotic cells and early spermatids and ceased in the differentiating spermatids (Henderson, 1964; Bloch & Brack, 1964; Utakoji, 1966; Loir, 1972; Kierszenbaum & Tres, 1975).

The inability of the elongating and the late-spermatid nuclei to synthesize RNA could be attributed to a lack of RNA polymerase in the nuclei. This possibility has gained support from the observation that in the mouse testis RNA polymerase activity was present in spermatogonia, meiotic cells and early spermatids, but was absent from the cells of later stages during spermatogenesis (Moore, 1971). In rooster spermiogenesis the inability of elongating and elongated spermatids to synthesize RNA coincides with the change in the texture of chromatin. It has been reported that the nuclei of early spermatids possess the characteristics of interphase nuclei. Later, just after elongation has begun and until the spermatids are fully elongated, the chromatin fibrils become uniform in appearance (McIntosh & Porter, 1967; Tingari, 1973). The uniform appearance of the chromatin can be related to the absence of substances involved in gene regulation and transcription (Subirana, 1975). In Plate 2 we have demonstrated the drastic decrease in nuclear non-histone proteins during these stages of spermiogenesis. It is possible that the absence of non-histone protein affected the chromatin's capacity for transcription. During spermiogenesis in the rooster testis we demonstrated that the inhibition of RNA synthesis in elongating spermatids preceded the appearance of arginine-rich protein (protamine) which takes place in the elongated spermatids. The non-correlation between the inhibition of RNA synthesis and the appearance of arginine-rich protein has been consistent in our system and has also been observed in other species (Monesi, 1964; Bloch & Brack, 1964; Loir & Hochereau de Reviers, 1972; Vaughn & Thomson, 1972; Pallotta & Tessier, 1976).

The actinomycin D-binding studies *in vivo* or *in vitro* indicated that the binding parallels the template activity for RNA synthesis. The highest actinomycin D binding corresponds to stage 4 (Figs. 5*c* and 6). Similar observations in the testis of the hamster, bull and ram have been reported (Barcellona *et al.*, 1974; Loir & Hochereau de Reviers, 1972). The stage-4 germ cells (elongated spermatids and testicular spermatozoa) therefore represent an interesting transitional state in the process of spermatogenesis. It is at this particular stage that protamine begins to replace histone. The observed high template activity *in vitro* and actinomycin D binding in this stage reflect unusual genomic structure. One of the contributing factors for the structural change is that histones undergo chemical modifications, such as

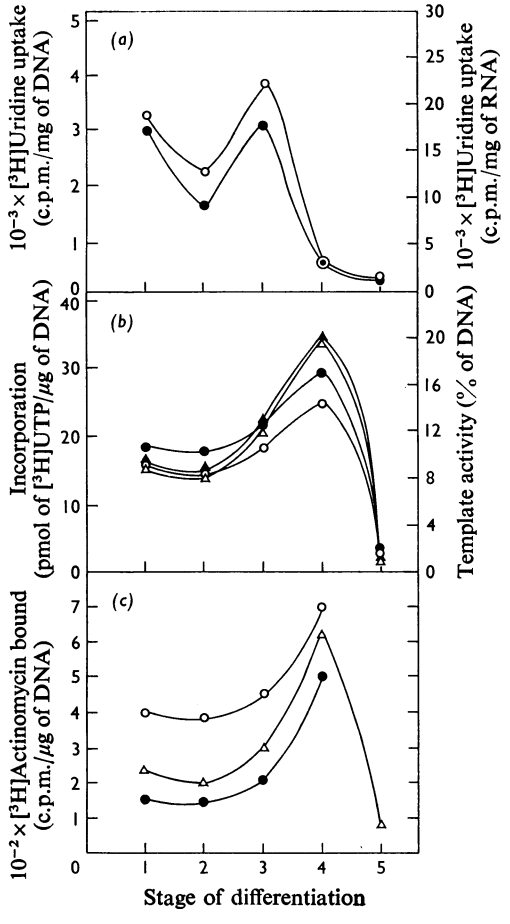


Fig. 5. [^{3}H]*Uridine uptake, template activity and* [^{3}H]*-actinomycin D binding of nuclei at different stages of spermatogenesis*

(*a*) Nuclear [^{3}H]uridine uptake. The procedures for the culture of testicular cell *in vitro*, the nuclei preparation and the removal of free [^{3}H]uridine were described in the Materials and Methods section. ○, [^{3}H]Uridine incorporation/mg of DNA; ●, [^{3}H]-uridine incorporation/mg of RNA. (*b*) Template activity of nuclei and chromatin assayed *in vitro*. Chromatin or nuclei (5μg of DNA) was preincubated at 37°C in the assay mixture without nucleotides and RNA polymerase for 20min. RNA synthesis was initiated by addition of the nucleotides and RNA polymerase. After incubation at 37°C for 20min the reaction was stopped. A group of identical samples was assayed without preincubation. ○, pmol of [^{3}H]UTP incorporated into nuclei/μg of DNA without preincubation; ●, [^{3}H]UTP incorporation into nuclei with preincubation; △, template activity (% of DNA) of chromatin with preincubation; ▲, template activity of chromatin without preincubation. (*c*) [^{3}H]Actinomycin D binding in nuclei. ○, Testicular cells were incubated with [^{3}H]actinomycin D (0.42mCi/mg) for 1h at 37°C. Nuclei were isolated in a sucrose/calcium medium, separated in a discontinuous sucrose gradient and sedimented at unit gravity. ●, Left testis was injected with 100μCi of [^{3}H]actinomycin D in 1ml of Hanks solution. After 1h the testis was removed and the nuclei were isolated in a citric acid medium and separated at unit gravity. △, Nuclei isolated in citric acid medium were separated by sedimentation at unit gravity. Different concentrations of nuclei (5–50μg of DNA) were incubated for 1h at room temperature in a medium containing 2.2μg of [^{3}H]actinomycin D (specific radioactivity 0.049mCi/mg) in 1ml of Hanks solution.

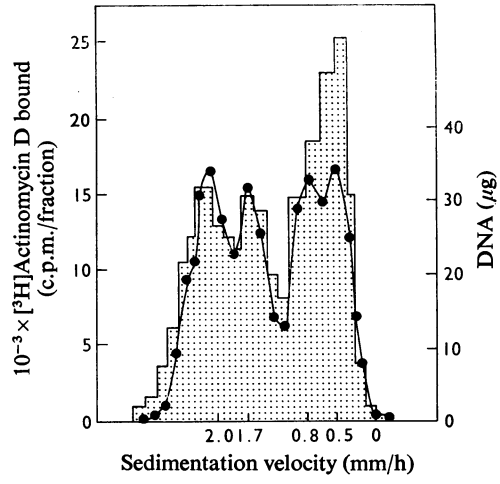


Fig. 6. *Histogram showing the variation in* [^{3}H]*actinomycin D binding as a function of the sedimentation velocity of testicular nuclei*

Testicular cells were incubated with 1μg of [^{3}H]-actinomycin D (0.42mCi/mg)/ml for 1h at 37°C. Nuclei were isolated in a sucrose/calcium medium and separated in a discontinuous sucrose gradient, followed by sedimentation at unit gravity. To obtain an approximately equal amount of DNA in each nuclear fraction, the chamber was loaded with 70ml of nuclear suspension (with 35ml of nuclear suspension obtained from fraction A+35ml of nuclear suspension obtained from fraction C of the discontinuous sucrose gradient). After sedimentation for 300min, 25ml fractions were collected. A sample of each fraction was taken for DNA determination, and the rest of the fraction was collected on fibreglass filters and washed before the determination of radioactivity. The detailed procedures for actinomycin D-binding assay are described in the Materials and Methods section. The histogram represents the [^{3}H]actinomycin D bound/fraction; ●, amount of DNA/fraction.

acetylation, as they disappear (Candido & Dixon, 1972; Grimes *et al.*, 1975). In the nucleus, the acetylation of histones increases the template activity (Allfrey *et al.*, 1964) and the actinomycin D binding (Darżynkiewicz *et al.*, 1969*a*) of the genome. In contrast, in the mature rooster spermatozoa, in which the basic proteins are completely replaced by protamines, the nucleoprotamine is unable to support RNA synthesis and has a low capacity for actinomycin D binding (Fig. 5*c*). Observations supporting these lines of evidence were made in studies of the trout and ejaculated mammalian sperm (Marushige & Dixon, 1969; Darżynkiewicz *et al.*, 1969*b*; Brachet & Hulin, 1969; Loir & Hochereau de Reviers, 1972). In the mature rooster spermatozoa, the nucleoprotamine is in a tightly packed state (1.2 pg of DNA in a volume of 2 μm^3 or 0.6 g of DNA/cm^3), which is equivalent to that observed in the trout (McIntosh & Porter, 1967; Tingari, 1973; Olins *et al.*, 1968). It has been proposed that the inability of nucleoprotamine to support RNA synthesis is probably due to its inaccessibility to RNA polymerase and water *in vitro* (Shih & Bonner, 1970; Subirana, 1975).

We thank Dr. Christina T. Teng for assistance with the nucleotide incorporation *in vitro* and radioautographic studies, and Mr. G. Andrews, Mr. R. Metivier and Mr. D. Kilgo for technical assistance. This study was supported by NIH grant HD-08218 and in part by the fellowships from the Program of Cultural Cooperation between the U.S.A. and Spain and Ministerio Español de Educacion y Ciencia to C. M.

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