Transformation of Sperm Histone during Formation and Maturation of Rat Spermatozoa*

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SUMMARY

Changes of chromosomal basic proteins of rats have been followed during transformation of spermatids into spermatozoa in the testis and during maturation of spermatozoa in the epididymis. Rat testis chromatin has been fractionated on the basis of differing sensitivity to shearing, yielding a soluble fraction and a condensed fraction. The sperm histone is found in the condensed fraction. Somatic-type histones are found in both fractions. The somatic-type histones in the condensed fraction contains much more lysine-rich histone I, than does the somatic-type histones in the soluble fraction. This may suggest that the lysine-rich histone I is the last histone to be displaced during the replacement of somatic-type histones by sperm histone.

After extensive shearing followed by sucrose centrifugation, the condensed portion of testis chromatin can be further fractionated into two morphologically distinctive fractions. One is a heavy fraction possessing an elongated shape typical of the head of late spermatids. The other is a light fraction which is presumably derived from spermatids at earlier stages of chromatin condensation and which is seen as a beaded structure in the light microscope.

Sperm histone of testis chromatin can be extractable completely by guanidinium chloride without a thiol, whereas 2-mercaptoethanol is required for extraction of sperm histone from caput and cauda epididymal spermatozoa. The light fraction of the condensed testis chromatin contains unmodified and monophospho-sperm histone. The sperm histone of the heavy fraction is mainly of monophospho and diphospho species, whereas unmodified and monophospho-sperm histones are found in caput and cauda epididymal spermatozoa. Labeling of cysteine sulfhydryl groups of sperm histone released by 2-mercaptoethanol treatment shows that essentially all of the cysteine residues of sperm histone in testis chromatin are present as sulfhydryl groups, while those of sperm histone isolated from mature (cauda epididymal) spermatozoa are present as disulfide forms and approximately 50% of the cysteine residues of sperm histone obtained from caput epididymal spermatozoa are in disulfide forms. These results suggest that phosphorylation of sperm histone is involved in the process of chromatin condensation during transformation of spermatids into spermatozoa in the testis and that the cross-linking of sperm histone through formation of disulfide bonds takes place during maturation of spermatozoa in the epididymis.

During transformation of spermatids into spermatozoa in terminal stages of spermatogenesis (spermiogenesis), chromosomal DNA becomes tightly packaged as a result of the replacement of somatic-type histones by sperm-specific basic proteins (1). Chromosomal basic proteins of spermatozoa (sperm histones) are known to vary widely among various organisms (2) ranging from a highly lysine-rich protein of the mollusk (3) to arginine-rich protamines of salmonid and related fish (4). When compared with the extensively studied system of rainbow trout (5-17), the formation of mature spermatozoa in mammals appears to involve rather different features. Sperm histones of several (perhaps all) eutherian mammals resemble protamines in being rich in arginine residues but, unlike protamines, are relatively rich in cysteine residues (18-21). It has been shown that isolated chromatin of mature bull spermatozoa consists principally of DNA and sperm histone. Moreover, essentially all of the cysteine sulfhydryl groups of mature sperm histone are present in the chromatin as disulfides (22). The packaging of DNA in mammals, therefore, involves cross-linking of sperm histone molecules through formation of disulfide bonds. In mammals, spermatozoa from the testis are known to be further processed during their passage through the epididymis. Much of the processing takes place in the proximal portion (caput epididymis), and mature spermatozoa are stored in the distal portion of epididymis (cauda epididymis). Morphological observations of spermatozoa in rat epididymis (23) have shown that the sperm heads become resistant to an anionic detergent during maturation of spermatozoa in the epididymis. As a step toward further understanding of the process of DNA packaging in mammals, we have investigated chromosomal basic proteins during formation and maturation of rat spermatozoa.

MATERIALS AND METHODS

Isolation of Testis Chromatin Testis chromatin was prepared in the presence of 5 mm iodoacetamide throughout all steps of the
process in order to prevent the oxidation of sperm histone. Light was avoided as much as possible during preparation. Forty frozen testes from sexually mature rats (Pel-Freez Biologicals) were thawed briefly. *T. amurensis* and blood vessels were removed. The testes were minced with scissors and homogenized by hand in 0.25 M sucrose containing 5 mM MgCl₂, 0.025 M KCl, and 0.01 M Tris-HCl (pH 8.0) with a Teflon homogenizer. The homogenate was passed through a layer of cheesecloth, and centrifuged at 1,500 × g for 10 min. The sediment was washed once with the homogenizing buffer by centrifugation (1,500 × g, 10 min). The sediment then was homogenized in saline-EDTA (0.075 M NaCl-0.024 mM EDTA, pH 8.0), using a motor-driven glass-Teflon homogenizer (TRI-R model SGSC, Setting 5) for 2 min and centrifuged at 1,500 × g for 10 min. The sediment was washed twice with saline-EDTA containing 0.5% Triton X-100 and once with 0.01 M Tris-HCl (pH 8.0) by resuspension and centrifugation (1,500 × g, 10 min). The sediment next was homogenized by hand in 0.01 M Tris-HCl (pH 8) in a Teflon homogenizer and centrifuged at 10,000 × g for 10 min. This step was repeated once. The gelatinous sediment of testis chromatin was resuspended in 60 ml of Tris-HCl (0.01 M, pH 8).

**Fractionation of Testis Chromatin** Testis chromatin was fractionated according to Marushige and Dixon (6). Each 18-ml portion of the chromatin suspension was sheared in a VirTis-45 homogenizer at 45 volts for 5 min. The sheared chromatin was diluted to 100 ml with the Tris buffer and centrifuged at 17,000 × g for 20 min. This supernatant is referred to as the soluble fraction of testis chromatin. The combined sediments were resuspended in 15 ml of the Tris buffer and sheared again under the same conditions as described above, diluted to 30 ml, and centrifuged at 17,000 × g for 20 min. The final sediment (the condensed fraction of testis chromatin) was resuspended in 0.01 M Tris-HCl (pH 8).

In some experiments, the condensed fraction of testis chromatin thus obtained again was sheared (VirTis-45 homogenizer) at 85 volts for 15 min. The suspension then was layered on an equal volume of 0.6 M sucrose containing 0.01 M Tris-HCl (pH 8) and centrifuged at 17,000 × g for 20 min. Two layers of sediments, the loosely packed upper layer, and the tightly packed bottom layer. Very little DNA was found in the supernatant. The upper layer was collected by gentle swirling with the Tris buffer, leaving the bottom layer behind. After thorough homogenization of each sediment in the Tris buffer, they were sedimented again through 0.6 M sucrose in the same manner. The upper layer of sediment was washed once with saline-EDTA and centrifuged at 17,000 × g for 20 min. The tightly packed sperm head fraction at the bottom was resuspended in the Tris buffer and again sedimented through 0.6 M sucrose in the same manner. In the case of cauda epididymal spermatozoa, however, separation of sperm heads from tails was found to be extremely difficult under these conditions, and the whole sediment, in which the sperm heads were heavily contaminated by tails, therefore was used for extraction of sperm histone.

The sperm head fraction was incubated in 5 mM guanidinium chloride containing 5 mM iodoacetamide and 0.05 M Tris-HCl (pH 8.0) at 37°C for 1 hour in the dark and centrifuged at 17,000 × g for 20 min. No sperm histone was extracted under these conditions. The sediment was washed once with 0.01 M Tris-HCl (pH 8), and sperm histone was isolated therefrom as described above. Sperm head fraction was first incubated in a mixture containing 0.2 M 2-mercaptoethanol, 1.2 M NaCl, 4 M urea (ultrapure grade, Schwarz-Mann), and 0.01 M Tris HCl (pH 8) at 37°C for 2 hours to dissociate sperm histone from DNA. The suspension was next treated with 0.2 M ICl (pH 8, 30 min). This was then centrifuged at 17,000 × g for 20 min. The extract thus obtained was desalted by chromatography on a Sephadex G-10 column (2 × 20 cm) using 0.01 M HCl as eluant. Proteins then were precipitated with 5% trichloroacetic acid. The precipitate was collected by centrifugation (17,000 × g, 20 min), washed successively with acetic acetate and acetone, and dried in a vacuum.

**Isolation of Rat Epididymal Spermatozoa** Heads of rat epididymal spermatozoa were isolated also in the presence of 5 mM urea, using a linearly increasing concentration of guanidinium chloride containing 0.05% sodium acetate (pH 5.0) at a flow rate of 90 ml per hour. Protein concentration was determined by absorbance at 230 nm. The resulting chromatographic fractions were appropriately combined, concentrated with a flash evaporator, desalted, and precipitated with 20% trichloroacetic acid as described above.

**Characterization of Chromosomal Basic Proteins**—Acid-soluble proteins were dissolved in 0.2 M guanidinium chloride containing 0.05 M sodium acetate (pH 5.0). Any insoluble material was removed by centrifugation. The proteins then were chromatographed on CM cellulose column (2 × 7 cm; Cellulose-CM, Bio-Rad), using a linearly increasing concentration of guanidinium chloride containing 0.05% sodium acetate (pH 5) at a flow rate of 60 ml per hour. Protein concentration was determined by absorbance at 230 nm. The resulting chromatographic fractions were appropriately combined, concentrated with a flash evaporator, desalted, and precipitated with 20% trichloroacetic acid as described above.

The protein fractions thus obtained were further characterized by electrophoresis in polyacrylamide gels.

**Disc electrophoresis in 15% polyacrylamide gels containing 6 M urea** was performed according to Bonner et al. (24). A sample of 0.01 to 0.04 mg (1 to 20μg of protein) in 0.2 M HCl containing 20% sucrose was applied to each gel and subjected to electrophoresis at 5 mA per tube for 70 min for 5-cm gels, and at 100 volts for 5 hours for 10-cm gels. The gels were stained with 0.1% Buffalo black in 40% ethanol containing 7% acetic acid for 4 to 5 hours and destained by an exhaustive washing in 40% ethanol-7% acetic acid.

**Comparison of Released Sulfhydryl Groups of Sperm Histone**—Sperm histone fraction obtained from testis chromatin by chromatography on a CM-cellulose column and then on a Sephadex G-75 column was treated with 0.2 M 2-mercaptoethanol in 8.5 M urea (pH 8) to reduce disulfide bonds, if any. Testis sperm histone was reisolated by Sephadex G-10 chromatography and precipitation with 5% trichloroacetic acid. Sperm histone then was subjected to electrophoresis at 5 mA per tube for 70 min for 5-cm gels, and at 100 volts for 5 hours for 10-cm gels. The gels were stained with 0.1% Buffalo black in 40% ethanol containing 7% acetic acid for 4 to 5 hours and destained by an exhaustive washing in 40% ethanol-7% acetic acid.

**Determination of Released Sulfhydryl Groups of Sperm Histone**—Sperm histone fraction obtained from testis chromatin by chromatography on a CM-cellulose column and then on a Sephadex G-75 column was treated with 0.2 M 2-mercaptoethanol in 8.5 M urea (pH 8) to reduce disulfide bonds, if any. Testis sperm histone then was reisolated by Sephadex G-10 chromatography and precipitation with 5% trichloroacetic acid. Sperm histone then was subjected to electrophoresis at 5 mA per tube for 70 min for 5-cm gels, and at 100 volts for 5 hours for 10-cm gels. The gels were stained with 0.1% Buffalo black in 40% ethanol containing 7% acetic acid for 4 to 5 hours and destained by an exhaustive washing in 40% ethanol-7% acetic acid.
histone was next purified by chromatography on a CM-cellulose column and a Sephadex G-75 column and assayed for protein and radioactivity. Moles of iodoacetamide reacted per mol of sperm histone were calculated.

**Differentiation Blocking of Sperm Histone with Iodoacetamide and Ethylenimine**—Testis sperm histone isolated in the presence of iodoacetamide in order to block its free sulfhydryl groups was incubated in a mixture containing 5 mM guanidinium chloride, 0.5 mM Tris-HCl (pH 8.5), 0.1 mM 2-mercaptoethanol, and 5 mM EDTA at 37° for 60 min. To the mixture ethylenimine then was added to a final concentration of 0.25 mM and incubation was continued for additional 90 min (37°). The incubation mixture next was dialyzed against 0.2 M HCl and sperm histone was precipitated from the acid extract with trichloroacetic acid. In order to isolate epididymal sperm histone differentially blocked with iodoacetamide and ethylenimine, the sperm head prepared in the presence of iodoacetamide and pretreated in 5 mM guanidinium chloride containing 5 mM iodoacetamide was incubated with 0.1 mM 2-mercaptoethanol and then treated with ethylenimine as described above. After dialysis of the incubation mixture against 0.2 M HCl, acid-insoluble materials were removed by centrifugation (17,000 x g, 20 min), and sperm histone was precipitated from the acid extract with trichloroacetic acid. The epididymal sperm histone fraction thus blocked was purified by CM-cellulose column chromatography and analyzed by gel electrophoresis.

**Chemical Analyses**—Protein was determined by the method by Lowry et al. (26), using bovine serum albumin as a standard. Phosphate was determined according to Ames (26). After hydrolysis of RNA in 0.3 M KOH (37°, 18 hours), DNA was hydrolyzed in 0.5 M perchloric acid at 100° for 10 min, and determined spectrophotometrically using an A, of 1 mg per ml of hydrolyzed DNA equal to 27.2.

**RESULTS AND DISCUSSION**

**Changes of Chromosomal Basic Proteins**—In mammals several germ cell generations are developing simultaneously. Hence at a given time testes contain spermatogonia, spermatocytes, and spermatids as well as various types of somatic cells (27). Chromatins isolated from whole testes are, therefore, a mixture of chromatins derived from these different types of cells. When the whole chromatin of rat testes is sheared (VirTis-45 homogenizer; 45 volts, 3 min), approximately 80% of the chromatin, as based on total DNA content, is solubilized (the soluble fraction of testis chromatin) and the remainder is obtained as an insoluble sediment (the condensed fraction of testis chromatin). As shown in Fig. 1A, the soluble fraction of rat testis chromatin has been found to be completely devoid of sperm histone and its basic protein fraction (Fig. 1A, a) exhibits an electrophoretic profile (Fig. 2A) similar to that of histones from somatic cells. It recently has been reported that the lysine-rich histone of rat testes consists of two major fractions, one of which is absent from histone fractions of rat liver (29). These two fractions of lysine-rich histones have not been resolved under the present electrophoretic conditions. Basic proteins extracted from the condensed fraction of rat testis chromatin with 5 M guanidinium chloride containing 5 mM iodoacetamide contain sperm histone (Fig. 1B, f and Fig. 2B) and somatic-type histones (Fig. 1B, b and Fig. 2B). Chromosomal basic proteins of this chromatin fraction are essentially totally extractable under these conditions. When the chromatin is sedimented by centrifugation at 36,000 rpm for 16 hours in a Spinco SW 50.1 rotor following treatment with 5 M guanidinium chloride-5 mM iodoacetamide, little acid-soluble proteins are additionally extracted from the sediment with either 5 M guanidinium chloride-0.2 M 2-mercaptoethanol or 1.2 M NaCl-1.4 M urea-0.2 M 2-mercaptoethanol. It has been found, however, that if rat testis chromatin is prepared in the absence of iodoacetamide, cross-linking of sperm histone apparently occurs as an artifact during preparation and only a small fraction of sperm histone is then extractable with 5 M guanidinium chloride in the absence of a thiold.

**Fig. 1.** Profiles of chromosomal basic proteins of rat testis and epididymal spermatozoa after chromatography on CM-cellulose columns (2 X 7 cm). Graphs show: basic protein fractions extracted from an 0.20 aliquot of the soluble fraction of rat testis chromatin obtained from 30 testes (A); the condensed fraction of testis chromatin obtained from 30 testes (B); the light (C) and the heavy (D) of condensed testis chromatin obtained from 40 testes; and the sperm heads obtained from 20 caput epididymides (E). Fractions indicated by arrows were combined and further analyzed by electrophoresis (Figs. 2 and 4).

**Fig. 2.** Electrophoretic profiles of chromosomal basic proteins obtained from rat testis and epididymal spermatozoa. Fractions a through k from CM-cellulose column chromatography in Fig. 1. I, II, III, and IV represent lysine-rich histone I, slightly lysine-rich histone II, and arginine-rich histones III and IV, respectively. Essentially all histone III is present as monomer. S, sperm histone; TP, TP protein (cf. Ref. 20). Electrophoreses were carried out on 5-cm gels at 5 ma per tube for 70 min.
sugest that lysine-rich histone I might be the last histone to be displaced during the histone change in rat spermatids. An identical observation has been made with trout testis chromatin during the replacement of somatic-type histones by protamine (9). Somatic-type histone fractions of the condensed chromatin contains a basic protein which is eluted at slightly higher concentrations of guanidinium chloride in a CM-cellulose column (Fig. 1B,e) than somatic-type histones and migrates as fast as sperm histone in polyacrylamide electrophoresis (compare Fig. 2,e and f). This component is not evident in somatic-type histone fractions isolated from the soluble fraction of rat testis chromatin (Fig. 2,g), as is likely to correspond to a spermatid-specific basic protein, TP, reported recently by Kistler et al. (20). A similar protein has also been reported in mice (29).

The condensed fraction of rat testis chromatin has been further fractionated into two morphologically distinctive chromatin fractions by extensive shearing (VirTis-45 homogenizer; 85 volts, 15 min) and sedimentation through 0.6 M sucrose. The slow sedimenting fraction (light fraction) which is presumable derived from earlier stages of chromatin condensation constitutes 10 to 15% of the total condensed testis chromatin on the basis of DNA contents and is seen as a beaded structure under a light microscope. The fast sedimenting fraction (heavy fraction) contains mainly highly condensed chromatin particles with the elongated shape characteristic of the head of late spermatids. As shown in Fig. 1, C and D, these two fractions of the condensed testis chromatin give markedly different profiles after chromatography on CM-cellulose columns. The light fraction contains slightly more somatic-type histones (Fig. 1C,g and Fig. 2,g) than sperm histone (Fig. 1C,h and Fig. 2,h), whereas chromosomal basic proteins of the heavy fraction consist mainly of sperm histone (Fig. 1D,j and Fig. 2,j). These results, consistent with the aforementioned morphological observations, indicate clearly that the light fraction of the condensed chromatin represents portions of spermatid chromatin undergoing active histone change, while the heavy fraction is a population of spermatid chromatin in which the replacement of somatic-type histones by sperm histone has essentially been completed. Somatic-type histone fractions of the heavy chromatin (Fig. 1D,i and Fig. 2,i) are found to be electrophoretically similar to those of the light fraction (Fig. 1C,g and Fig. 2,g). A small peak eluted after the main peak in CM-cellulose column chromatography (tubes 75 to 80 in Fig. 1D) appears to be the dimer of rat sperm histone. This component possesses a lower electrophoretic mobility (RF, 0.54) as compared with the mobility of the main component upon its incubation in 0.2 M HCl following a 2-hour incubation of spermatozoa from 17-cm cauda epididymides in 0.2 M 2-mercaptoethanol-1.2 M NaCl-4 M urea and treated with iodoacetamide. Acid-soluble proteins were dissolved in 0.6 M guanidinium chloride and applied to a CM-cellulose column (2 × 7 cm) equilibrated with 0.6 M guanidinium chloride. The column was washed with 1 column volume of 0.6 M guanidinium chloride and the proteins were eluted with a linear gradient of guanidinium chloride (A). Fractions indicated by arrows (l) were combined and further analyzed by chromatography on a Sephadex G-75 column (B) and by electrophoresis (Fig. 4). Profiles of sperm histone isolated in the same manner from bull spermatozoa (Inset, electrophoretic profiles of rat (R) and bull (B) sperm histone eluted on 5-cm polyacrylamide gels.

In contrast to sperm histone of the testis chromatin which can be extracted without a thiol, extraction of sperm histone of rat epididymal spermatozoa requires a thiol. Sperm histone has been totally extracted from the sperm head fraction obtained from caput or cauda epididymides with 1.2 M NaCl-4 M urea-0.2 M 2-mercaptoethanol followed by acid treatment. Acid-soluble proteins isolated in this manner from rat caput epididymides consist exclusively of sperm histone (Fig. 1E,h, and Fig. 2,h). Sperm histone similarly purified from cauda epididymal spermatozoa (Fig. 3A,j) gives an electrophoretic profile identical to that of caput epididymal sperm histone. As also shown in Fig. 3A, rat sperm histone is eluted at a slightly higher concentration of guanidinium chloride than bull sperm histone. This is consistent with their difference in amino acid composition. Bull sperm histone (18, 19) contains 51 mol % of arginine and 2 mol % of histidine, while rat sperm histone (20) contains 65 mol % of arginine and 4 mol % of lysine. The cysteine content of rat sperm histone (10 mol %, Ref. 20) is slightly less than that of bull sperm histone (13 mol % cysteine) which is known to contain 6 residues of cysteine (18, 19) and therefore the number of cysteine residues of rat sperm histone is likely to be 5. The minimum molecular weight of rat sperm histone calculated from its amino acid composition (20) is 6400. As shown in Fig. 3B, rat sperm histone obtained from cauda epididymal spermatozoa is eluted at essentially the same position in Sephadex G-75 chromatography as bull sperm histone whose molecular weight is known to be 6200 (18). The higher basicity of rat sperm histone as compared with bull sperm histone is reflected in its higher electrophoretic mobility (Fig. 3B, Inset).

**Phosphorylation of Sperm Histone**—It may be noted in Fig. 1 that sperm histone of the light fraction of the condensed testis chromatin (Fig. 1C,h) and that of caput epididymal spermatozoa (Fig. 1E,h) are eluted at guanidinium chloride concentrations slightly higher than that of sperm histone of the heavy fraction of the condensed testis chromatin (Fig. 1D,i). As may also be seen in Fig. 2, the electrophoretic band of the latter (Fig. 2,i) appears to be more diffused than the bands of the former (Fig. 2, h and k). These sperm histone fractions all have been, however, found to be eluted at an identical position when subjected to Sephadex G-75 column chromatography (cf. Fig. 3B). These
TABLE I

Phosphate contents of sperm histone during formation and maturation of rat spermatozoa

Sperm histone fractions of testis chromatin, and caput and cauda epididymal spermatozoa were purified by CM-cellulose column and Sephadex G-75 column chromatography, and determined for protein and phosphate. Moles of phosphate per mol of sperm histone were calculated using a value of 6400 as the molecular weight of rat sperm histone.

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<th>Sperm histone from:</th>
<th>Moles phosphate/mol sperm histone</th>
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<tr>
<td>Condensed fraction of testis chromatin</td>
<td>1.5</td>
</tr>
<tr>
<td>Light fraction</td>
<td>0.68</td>
</tr>
<tr>
<td>Heavy fraction</td>
<td>1.6</td>
</tr>
<tr>
<td>Caput epididymis</td>
<td>0.52</td>
</tr>
<tr>
<td>Cauda epididymis</td>
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observations together with an earlier finding that during spermogenesis of the salmonid fish newly synthesized protamine is extensively phosphorylated, while protamine obtained from mature spermatozoa lacks O-phosphoserine (5), have prompted us to investigate phosphorylation of rat sperm histone at various stages of maturation.

As shown in Table I, testis sperm histone contains more phosphate (1.5 mol/mol of protein) than do caput epididymal and cauda epididymal sperm histone, both of which contain 0.52 mol of phosphate per mol of protein. Data of Table I also show that sperm histone isolated from the light (minor) fraction of the condensed testis chromatin is phosphorylated to lesser extent (0.68 mol/mol of protein) as compared with sperm histone of the heavy (major) fraction (1.6 mol/mol of protein). Furthermore, electrophoretic analyses of these sperm histone fractions in 10-cm polyacrylamide gels have clearly revealed their heterogeneities due to their phosphate content, as summarized in Fig. 4.

Sperm histone of the whole fraction of the condensed chromatin and that of the heavy fraction of the condensed testis chromatin (Fig. 4, f and j, respectively) both give three bands, while sperm histone of the light fraction of the condensed testis chromatin as well as caput and cauda epididymal sperm histone (Fig. 4, h, k, and l, respectively) exhibit similar electrophoretic profiles and are resolved into two bands. The fastest migrating component of sperm histone obtained from the heavy fraction of the condensed testis chromatin corresponds to the slower migrating component of sperm histone obtained from the light fraction and to that of sperm histone obtained from the epididymal spermatozoa. When these sperm histone preparations are treated with *Escherichia coli* alkaline phosphatase, each gives a single band of an identical electrophoretic mobility (Fig. 4, f', k', and l') which, in turn, corresponds to the faster migrating band of sperm histone of the light fraction of the condensed testis chromatin and that of epididymal sperm histone, suggesting that the electrophoretic heterogeneities of sperm histone fractions at various stages of sperm maturation are due to their phosphorylation to various extents. Electrophoretic profiles (Fig. 4) together with the results of phosphate determination (Table I) now indicate that the heavy fraction of the condensed testis chromatin contains mainly mono- and diphospho-sperm histone with a small amount of triphospho-sperm histone, while epididymal sperm histone, as well as sperm histone of the light fraction of the condensed testis chromatin consist of monophospho and unmodified species in an approximately equal proportion. As discussed earlier in this report, the heavy fraction of the condensed testis chromatin possesses morphology typical for the late spermatids, and the light chromatin fraction represents portions of spermatid chromatin at earlier stages of condensation. Present results therefore suggest that phosphorylation of sperm histone occurs on the chromatin and is in some way associated with the process of condensation of chromosomal DNA during transformation of spermatids into spermatozoa. Sperm histone then is dephosphorylated, although not completely, later in the development. In spermogenesis of the salmonid fish, newly synthesized protamine is extensively phosphorylated (5, 7, 13, 16, 17), and phosphoprotamine is dephosphorylated during maturation of spermatids into spermatozoa (5, 17). Phosphorylated protamine has been shown to bind less tightly to DNA (7), and it has been suggested from kinetic studies of synthesis and phosphorylation of protamine in trout testes that controlled phosphorylation of protamine could play a role in its “correct” binding to DNA (17).

**Cross-Linking of Sperm Histone**—The fact that a thiol is required for extraction of epididymal sperm histone suggests that sperm histone molecules in the epididymal spermatozoa are intermolecularly cross-linked into a highly polymerized form through formation of disulfide linkages. On the other hand, such cross-linkings are evidently absent in the testis chromatin.
whose sperm histone is extractable without a thiol. In order to investigate disulfide formation of sperm histone in the processes of DNA packaging, the number of cysteine residues of sperm histone which are present as disulfides has been determined with testis chromatin and caput and cauda epididymal spermatozoa. Sperm histone fractions which had their free sulphydryl groups blocked with nonradioactive iodoacetamide were treated with 2-mercaptoethanol. Released sulphydryl groups then were blocked with iodo\[%\]acetamide, and the moles of iodoacetamide reacted per mol of sperm histone were calculated.

As can be seen in Table II, little reaction has been observed during incubation of testis sperm histone with radioactive iodoacetamide (0.024 mol per mol of protein), indicating that essentially all the cysteine residues of sperm histone in the testis chromatin are present as free sulphydryl groups. In contrast, sperm histone from cauda epididymal spermatozoa has reacted with 4.7 mol of iodoacetamide per mol of protein, indicating that average 4.7 cysteine sulphydryl groups per sperm histone molecule have been released by 2-mercaptoethanol during its extraction. The same value (4.7 mol of iodo\[\text{[14C]}\]acetamide per mol of protein) has been obtained for cauda epididymal sperm histone which have been extracted from the sperm head prepared in the absence of nonradioactive iodoacetamide, omitting its preincubation in 5 mM guanidine chloride containing 5 mM iodoacetamide. The number of cysteine residues per mol of rat sperm histone being five as discussed earlier in this report, essentially all the cysteine residues of sperm histone appear therefore to be present as disulfide forms in mature rat spermatozoa. An intermediate value (2.6 mol of iodoacetamide reacted per mol of protein) has been obtained for sperm histone obtained from caput epididymal spermatozoa, indicating that approximately half of cysteine residues of sperm histone are present as free sulphydryl groups in caput epididymal spermatozoa. These results have been further substantiated by electrophoretic analyses of sperm histone fractions whose free sulphydryl groups and sulphydryl groups released by 2-mercaptoethanol have been blocked differentially by iodoacetamide and ethylenimine. Sperm histone samples thus blocked were dephosphorylated by incubation with Escherichia coli alkaline phosphatase and subjected to gel electrophoreses. As can be seen in Fig. 4,6, in comparison with Fig. 4,6', an electrophoretic mobility of testis sperm histone is not affected by the ethylenimine treatment, while cauda epididymal sperm histone treated with ethylenimine (Fig. 4,6) migrates considerably faster than that blocked with iodoacetamide (Fig. 4,6'), presumably as a result of the introduction of five aminoethyls. Sperm histone of cauda epididymis (Fig. 4,6) migrates in between testis sperm histone (Fig. 4,6) and cauda epididymal sperm histone (Fig. 4,6), and its electrophoretic band appears to be more diffuse. This indicates that caput epididymal sperm histone blocked in the present manner consists of sperm histone with varying amounts of aminoethyls, and, in turn, suggests that sperm histone of the caput epididymal spermatozoa is heterogeneous with respect to its content of free cysteine sulphydryl groups.

It seems thus clear that cross-linking of sperm histone through formation of disulfide bonds is involved in maturation of mammalian spermatozoa and that the protein disulfide are formed during processing of spermatozoa in the caput and midportion of the epididymis. This is in good agreement with a conclusion obtained from morphological observations that sperm chromatin becomes stabilized by the formation of disulfide bonds during passage of rat spermatozoa through the epididymis (23). The cross-linking of sperm histone during sperm maturation appears to be so complete that in cauda epididymal spermatozoa essentially all the cysteine residues of sperm histone has been found as disulfide forms. We have already reported with highly purified chromatin of mature bull spermatozoa that such disulfide forms are almost exclusively those between sperm histone molecules (22). It is not clear at present as to why cysteine-rich rat sperm histone can exist in completely reduced form in the testis during the entire period of chromosomal transformation after its initial association with DNA. Although sperm histone of late spermatids (the heavy fraction of conditioned testis chromatin) contains, on the average, one additional phosphate group as compared with that of epididymal spermatozoa, such a difference does not seem likely to cause changes in structure of sperm histone in the chromatin in such a way that prevents the protein disulfide forms from forming. The nuclear environment of maturing spermatids may be responsible for maintaining a completely reduced state of sperm histone in the testis. Phosphorylation of sperm histone during condensation of spermatid chromatin could ensure proper disulfide formation through controlling the correct binding of sperm histone to DNA (cf. Ref. 17) or possibly through regulating proper folding of condensing chromatin, and thus could play a key role in proper packaging of DNA during formation and maturation of mammalian spermatozoa.

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TABLE II
Disulfide formation of sperm histone during formation and maturation of rat spermatozoa

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<th>Sperm histone from</th>
<th>Mole iodo[\text{[14C]}]acetamide reacted/mol sperm histone</th>
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<td>Testis ...............</td>
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<td>Cauda epididymis ...</td>
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