CHEMICAL DIFFERENTIATION OF NUCLEAR PROTEINS DURING SPERMATOGENESIS IN THE SALMON*, ‡

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Plates 19 to 21

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The characteristic morphogenetic changes which occur during sperm formation are at times accompanied by profound chemical alterations of the nuclear material. Thus it has been known since Miescher's pioneer studies during the 1870's (19) that nuclei in the immature salmon testis contain nucleohistones, while ripe salmon sperm contain nucleoprotamines. Basic proteins which are intermediate in composition between histones and protamines have been found in the sperm of some species (12, 18) while histones occur in others (11, 16); histones are generally also found in somatic nuclei of animals and higher plants (12). Both protamines and histones extracted from any type of material are not single compounds but represent mixtures of related substances (13, 23). All of those known to date form a spectrum of basic nuclear proteins among which the protamines represent the extreme in basicity and in simplicity of composition and structure.

More recent studies on salmon sperm composition have generally confirmed Miescher's results except for the finding that he overestimated the non-protamine protein content of the sperm nuclei. Pollister and Mirsky (21) reported that nucleoprotamines account for 91 per cent of the nuclear mass, while other workers (15) even claimed that these nuclei consist exclusively of nucleoprotamine. Such findings are in marked contrast to those typical for somatic nuclei (*e.g.* reference 6) and nuclei of sea urchin sperm (8) in which an appreciable fraction—at least about 25 per cent—of non-histone protein occurs. Compared to other nuclear types, the sperm nuclei of the salmon and related fishes thus may be characterized by two separate biochemical peculiarities: (*a*) the extreme qualitative modification of the basic proteins to protamines consisting predominantly of arginine residues, and (*b*) the extreme quantitative reduction of other nuclear protein fractions. When both of these changes

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‡ This paper is dedicated to Professor Franz Schrader on the occasion of his 65th birthday, March 11, 1956.

are considered together, they emphasize the probable role of sperm desoxyribonucleic acid (DNA) for transmission of paternal heredity to the egg (see Hotchkiss (17)).

In spite of the fact that the exact pattern of chemical differentiation of nucleoproteins during spermatogenesis is of interest to geneticists as well as to cell physiologists, little is known about the details of this process. Does the change in nucleoprotein composition occur gradually and progressively throughout the stages of meiosis, or is it a sudden change which takes place in a particular cell generation? This question could not be answered in Miescher's time when the process of meiosis was not understood; but even today such a problem cannot be solved unequivocally by routine biochemical procedures involving isolation and bulk analysis of nuclei from an organ as heterogeneous in cell composition as an immature testis: any differences in composition among the various nuclear types would be merged in an over-all average and lost. Cytochemical methods allowing the study of individual nuclei *in situ* are much better suited for such a case, provided that specific staining tests can be devised to permit visualization of the changes in which one is interested. The aforementioned biochemical peculiarities of salmon sperm actually make a direct cytochemical study feasible; its result is the subject of this paper.

Materials and Methods

Small pieces from immature testes of Chinook salmon (*Oncorhynchus tshawytscha*)[1] were fixed for 8 hours in 10 per cent neutral formalin; after washing them in running tap water overnight, the tissues were dehydrated in alcohols, embedded in paraffin, and sectioned at various thicknesses. All cytochemical procedures used were either applied in sequence to the same sections, or to sections which had been part of the same paraffin ribbon. Other materials with which the salmon testis was compared had been fixed and processed in the same manner.

In order to permit multiple sequential staining, a modified Feulgen procedure for DNA, as described by Bloch and Godman (9), was applied: this consists of substituting trichloracetic acid (TCA) for hydrochloric acid in all steps of the Feulgen technique (*i.e.* in the Schiff reagent, the hydrolysis bath, and the bleaches). Hydrolysis in 1 N TCA was performed at 60°C. for 12 minutes.

After photographing characteristic areas of the Feulgen-stained sections, the coverslip was removed, and the preparation was rehydrated and exposed for 12 minutes to 5 per cent TCA at 90°C. for extraction of nucleic acids and of the Feulgen dye. Basic proteins were subsequently stained by fast green at pH 8.1 according to the procedure of Alfert and Geschwind (3).

Other preparations were also stained in the same dye bath after enzymatic removal of DNA: for the latter purpose 0.5 mg./cc. Worthington desoxyribonuclease was made up in an aqueous gelatine-Mg^{++} solvent buffered at pH 6.8 and applied for 2 hours at 37°C. Formalin fixation greatly decreases the susceptibility of many tissue nuclei to desoxyribonu-

[1] Salmon testes were obtained through the courtesy of District Supervisor John Pelnar, from the United States Fish and Wildlife Service, Coleman Station, Anderson, California.

clease digestion. In accordance with a suggestion by Swift (22), this refractory state of the tissue against nuclease action was abolished by immersing the slides into distilled water at 90°C. for 5 minutes immediately prior to enzymatic digestion. Suitable controls (exposure to hot water followed by gelatin-Mg^{++} solvent without enzyme, and enzyme treatment without previous application of hot water) were also employed.

The effect of a protein group reagent (20) on the stainability of basic proteins was tested by the following procedure formerly used in connection with cytochemical staining experiments on nucleic acids (1) and proteins (14): slides previously exposed to hot water and desoxyribonuclease were dehydrated, blotted dry from absolute ethanol, and immersed for 2½ hours in acetic anhydride at room temperature. They were subsequently washed in distilled water and stained by the alkaline fast green reagent.

RESULTS AND DISCUSSION

The alkaline fast green reagent was developed for staining of basic proteins and results in a specific nuclear stain, presumably due to histones, in many tissues (3, 9). Before staining can occur it is necessary, however, to remove DNA from the nuclei and this can be achieved ordinarily by means of hot TCA extraction or by desoxyribonuclease digestion. In the original description of this staining method (3) it was noted that test spots of pure protamine were soluble in hot TCA. The use of desoxyribonuclease was therefore proposed for possible application of this method to protamine-containing sperm nuclei. However, the actual test on ripe salmon sperm has now revealed that neither of these procedures leads to staining of morphologically intact sperm nuclei: if TCA extraction is used the sperm heads dissolve completely and an unstained fibrous residue is left; after nuclease digestion some fast green stainable substance can still be demonstrated but the sperm heads appear badly damaged. These sperm heads also have the unusual property (noted previously also in sperm of the annelid *Sabellaria alveolata* (5)) of breaking down partly during Feulgen hydrolysis, so that confluent masses of Feulgen-positive material are produced. This behavior is illustrated in Figs. 1 *a*, 1 *b*, and 4 and can be explained on the basis of the composition of salmon sperm: protamine is extracted during acid hydrolysis for the Feulgen reaction and sperm head structure tends to break down (Fig. 1 *a-D*) owing to the lack or paucity of other protein components; alkaline fast green stainability is then also absent (Fig. 1 *b-D*) after even more vigorous acid hydrolysis; finally, even enzymatic removal of DNA under milder conditions leads to breakdown of structure and appreciable loss of the remaining water-soluble protamine (Fig. 4-*D*).

Somatic nuclei, mammalian sperm, rooster sperm,[2] and sea urchin sperm remain intact and are strongly stained by every one of the three procedures just discussed. The characteristic properties of the basic proteins in materials

[2] The staining behavior of rooster sperm is interesting. The basic protein of these sperm has been called a protamine on the basis of a certain solubility characteristic (12). In the present observations, however, it behaved like a histone. In composition it is intermediate between typical histones and protamines.

such as these can be determined by comparative microphotometric measurements (2, 4, 7, 9, 10), but they are not immediately evident on simple examination of the cells. In the salmon sperm, on the other hand, the occurrence of protamine can be detected directly by the absence of fast green staining as a consequence of the ease with which protamine is lost during the TCA–alkaline fast green procedure. A study of different stages of spermatogenesis may then indicate when the fast green stainable histone is replaced by protamine.

In Figs. 1 *a*, 2 *a*, and 3 *a* all stages of spermatogenesis are shown as they appear in Feulgen-stained sections. Primary spermatocytes (*A*), secondary spermatocytes (*B*), and spermatids (*C*) can be identified on the basis of nuclear size and staining intensity. Among the spermatids various stages of condensation may be distinguished: C_1 represents early spermatids with spherical nuclei and granular chromatin, C_2 a later stage in which nuclei are still spherical but smaller and of very homogeneous appearance, and C_3 a stage during which the nuclei become slightly elongated. Sperm cells are represented by the dark masses labelled *D*. In Fig. 2 *a* first meiotic division stages (*M I*) and in Fig. 3 *a* second meiotic division stages (*M II*) are shown. The same areas, restained with the alkaline fast green procedure after hot TCA extraction, were rephotographed and are represented by Figs. 1 *b*, 2 *b*, and 3 *b*. A cell by cell comparison of all stages can be made and demonstrates that the Feulgen and fast green pictures remain very similar through most of the spermatogenic stages. A striking difference becomes evident only at an advanced stage of spermatid condensation: the nuclei in stage C_3 have a vacuolated, ring-like appearance, most of the stainable substance is lost but their structure is still maintained. Finally the sperm heads in area *D* have become completely dispersed and no fast green stainable material can be demonstrated. This comparison indicates that the change from a histone-type nuclear protein to a protamine occurs rather abruptly at a late stage of spermiogenesis, probably somewhere in or between the stages designated C_2 and C_3. The fact that morphologically intact nuclei can still be recognized in C_3 may indicate that a non-basic protein component still occurs in appreciable quantity at that time, but is lost or reduced soon afterwards.

The results of alkaline fast green staining following desoxyribonuclease digestion (Fig. 4) and the effect of acetylation on such material (Fig. 5) lend strong support to the sequence of events just outlined. (These treatments cannot be used in sequence on one section and were therefore applied to different slides.) The enzyme-treated section is equivalent to the TCA-extracted one at all early stages; but "vacuolated" nuclei such as shown in Fig. 1 *b*, stage C_3, do not occur after enzyme digestion, and the sperm masses retain some stainability. Acetylation primarily affects ϵ-amino groups of lysine but not the guanidine group of arginine (20). One might therefore expect

that the fast green staining capacity of protamine, whose basicity is primarily due to arginine, would not be greatly changed by acetylation, while the stainability of much lysine-containing histone might be depressed. These effects can actually be observed when Figs. 4 and 5 are compared: acetylation (Fig. 5) substantially reduced the stainability of all stages during which histones are presumably present, up to an advanced stage of spermatid condensation; only this stage and the sperm have not been affected and continue to stain with similar intensity as without acetylation (Fig. 4).

The desoxyribonuclease control slides revealed an interesting point which concerns the mechanism of resistance to nuclease after formalin fixation. Without enzyme treatment no fast green staining occurred in any part of the section. When the hot water treatment was omitted prior to enzyme application, the ripe sperm masses alone, but none of the other nuclear types, became strongly stainable by the alkaline fast green reagent. This suggests that the effect of formalin fixation on susceptibility to desoxyribonuclease occurs indirectly, by way of a non-basic protein component lacking in sperm nuclei. It also gives a further indication of a substantial difference in composition between the nuclear stages designated C_3 and D.

SUMMARY

By means of qualitative staining experiments the characteristic protein changes which occur during maturation of salmon sperm can be followed. It can be observed that the replacement of histone by protamine takes place after completion of meiosis during an advanced stage of spermiogenesis.

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EXPLANATION OF PLATES

Photomicrography by Mr. Victor Duran. All photomicrographs were taken with a Zeiss 8.3 mm. apochromat and under yellow light from a Wratten No. 15 filter.

A, primary spermatocytes.

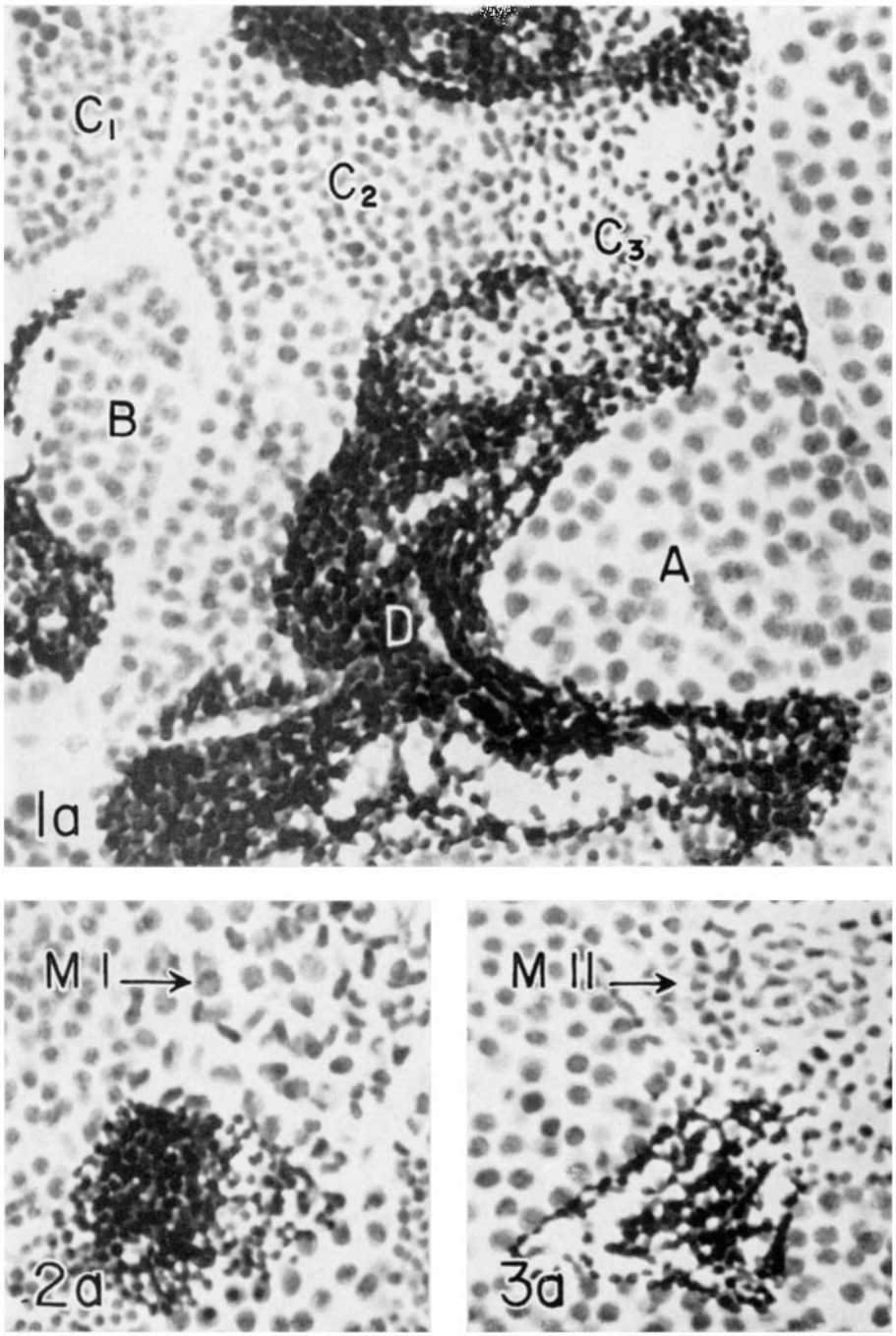
M I, first meiotic division figures.

B, secondary spermatocytes.

M II, second meiotic division figures.

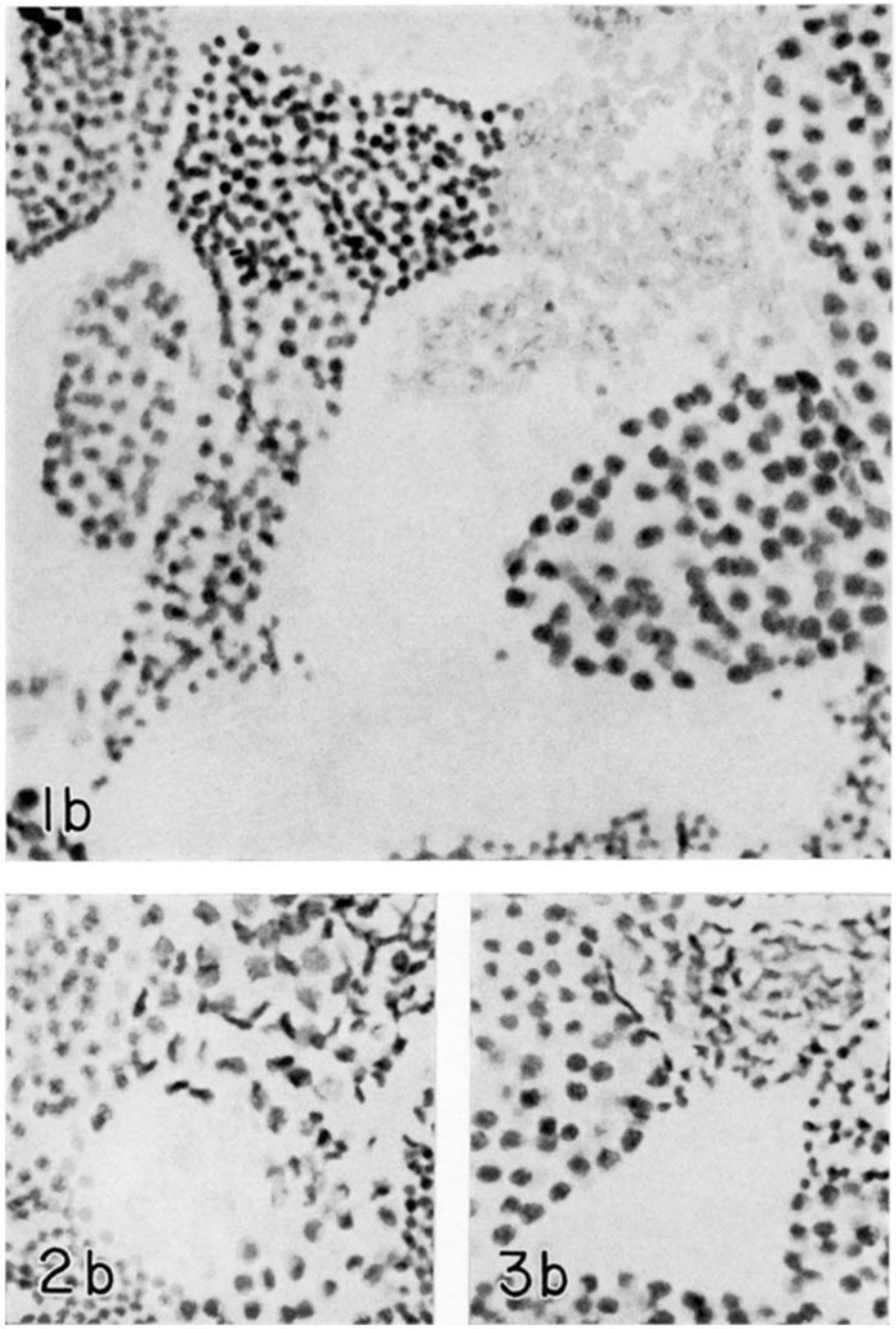
C_1, C_2, C_3, spermatids (progressive stages of condensation).

D, sperm.



FIGS. 1 *a* to 3 *a*. Salmon testis stained by the Feulgen procedure × 850

(Alfert: Nuclear proteins during spermatogenesis in salmon)



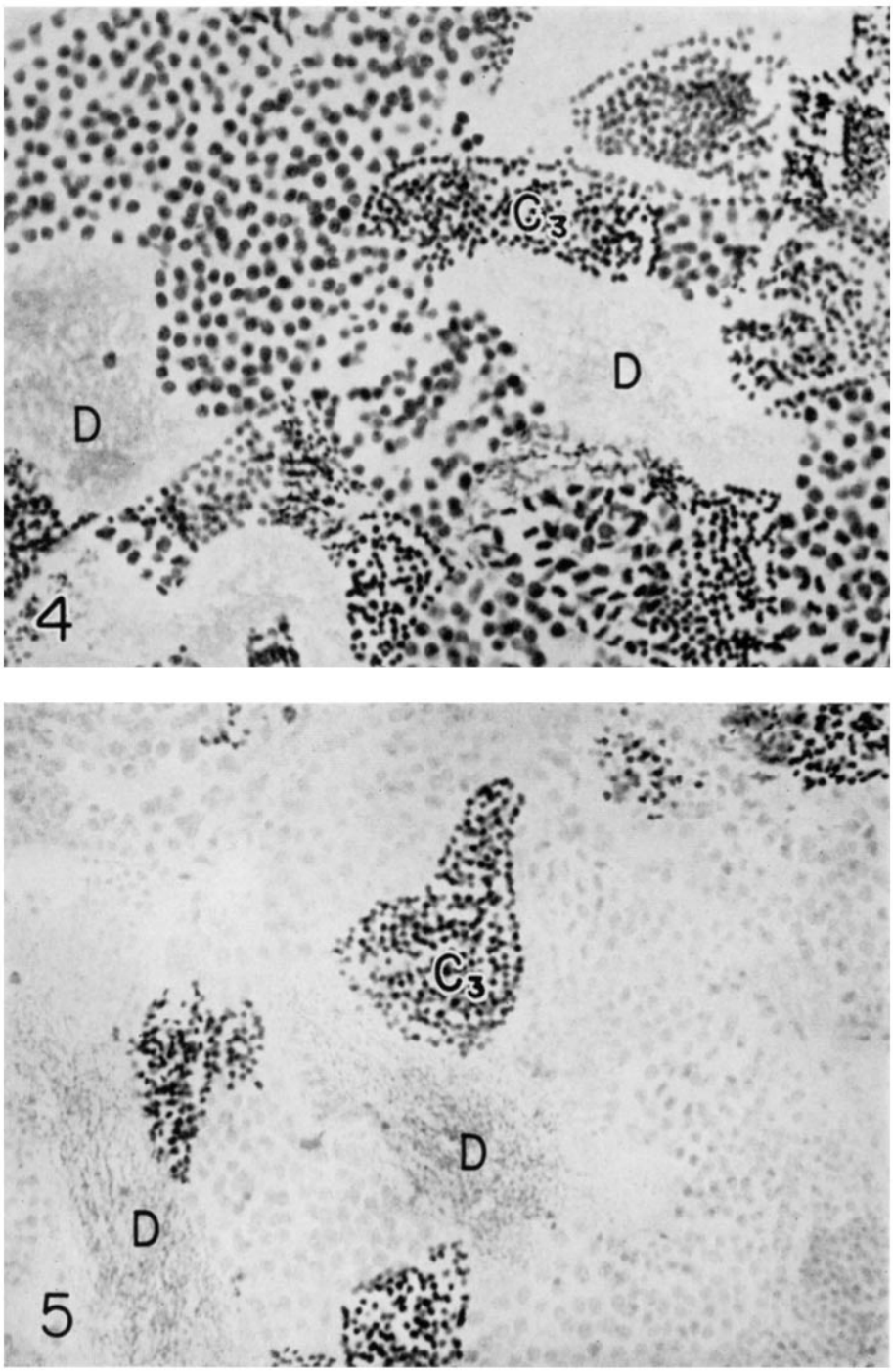
FIGS. 1 *b* to 3 *b*. The same areas as in Figs 1 *a* to 3 *a* restained by the alkaline fast green procedure after extraction with hot TCA. × 850.

(Alfert: Nuclear proteins during spermatogenesis in salmon)

PLATE 21

FIG. 4. Salmon testis stained by the alkaline fast green procedure after desoxyribonuclease digestion. × 530.

FIG. 5. Salmon testis digested with desoxyribonuclease, acetylated, and stained with alkaline fast green. × 530.



(Alfert: Nuclear proteins during spermatogenesis in salmon)