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STRUCTURE OF DEOXYRIBONUCLEOPROTEIN OF SPERM NUCLEI: NUCLEOPROTAMINE STRUCTURE

Introduction

The early investigations of birefrigence, ultraviolet dichroism (Caspersson, 1940; Pattri, 1932; Schmidt, 1937; Wilkins, 1951) and X-ray diffraction (Rinne, 1933) of sperm heads revealed the paracrystalline character of sperm head material. Shortly after the structure of DNA was established (Watson, Crick, 1953) the close similarity between the diffraction of DNA (B-form) and of the intact sperm heads of *Loligo* and *Sepia* was reported (Wilkins, Randall, 1953). Obviously, the molecular structure of DNA *in vivo* is little affected by protamine, and the X-ray diffraction is almost entirely determined by DNA, as the most ordered structure in sperm heads.

At present there are two main molecular models of DNP* structure: the first one is proposed by Wilkins et al. (Feughelman et al., 1955; Wilkins, 1956), the other, by Luzzati and Nicolaieff (1963). These models are based on different X-ray diffraction data. The main argument in favour of DNP Wilkins model is the intensity distribution on layer lines: the first layer line on the X-ray fibre diagram is considerably stronger than that of the DNA diagram. Spacefilling molecular model building and Fourier transforms of models indicate that the X-ray diffraction patterns are consistent with the structure in which the extended polypeptide chain of protamine is wrapped around the nucleic acid helix in a small groove, the basic end-groups of arginine residues are in contact with phosphate groups of both polynucleotide chains of DNA molecule.

However, this model of DNP structure is not consistent with the DNP swelling data of the same authors (Feughelman et al., 1955). They showed that the extent of DNP swelling is strongly limited. The upper value for the main equatorial reflection of DNP fibre diagrams is about 25 Å at 100% relative humidity (Feughelman et al., 1955; North, Rich, 1961). In contrast, the swelling of DNA is not limited at all, and there is a continuous transition from the dry DNA specimen to the solution. Obviously, there are links between DNA molecules in nucleoprotein limiting the extent of swelling (Ambrose, Butler, 1953). This point of view is especially advanced by small angle diffraction investigations of Luzzati and Nicolaieff (1963). The proposed structure consists of hexagonally arranged DNA molecules, crosslinked by protamine molecules into a uniform two-dimensional, practically infinite network. These results were obtained first

* DNP — deoxyribonucleoprotamine; DNH — deoxyribonucleohistone.

of all with the salt-extracted DNP, precipitated in 0.14M NaCl. Experiments with sperm nuclei confirmed these results and indicated that the molecular arrangement of DNP *in vivo* is very closely related to this structure (Luzzati, Nicolaieff, 1959; Nicolaieff, 1962).

However, the model of the infinite network is not supported by the following data. There are reports (Zubay, Wilkins, 1962) that it is possible to extract DNP by the "water" method from salmon sperm heads, if the sperm is previously treated with 0.024M ethylenediaminetetraacetate (EDTA) plus 0.075M NaCl, pH 8.

Possibility of DNP extraction from sperm nuclei after treatment with EDTA is obviously in contradiction with the DNP network model of Luzzati and Nicolaieff. Indeed, it is a well known fact that EDTA removes bivalent cations and cannot affect the linkage between DNA and protein. Thus the EDTA must not influence the solubility of this protamine-DNA complex.

Hence, some other approach to DNP structure is necessary, which takes into account the role of bivalent cations.

This investigation is concerned with the DNP structure in the sperm heads as revealed by electron microscopy and X-ray diffraction at small angles. The structure of native DNP is proposed as a compromise of two extremal viewpoints (DNP as 3-stranded helix (Feughelman et al., 1955) versus DNP as the infinite 2-dimensional network (Luzzati, Nicolaieff, 1963)).

Materials and Methods

Sperm material. The sperm of *Acipenser stellatus*, *A. güldenstädti* and *Huso huso* was obtained during the breeding season (end of April) from fresh milt in the Astrakhan fish cannery. In some experiments sperm was squeezed out from intact fishes. Sperm was kept at 0° C, transported by air to Moscow and investigated (or processed as described below) some days later.

Rainbow trouts (*Salmo irideus*) were obtained from the fish hatchery Skhodnya near Moscow in the first half of May. Sperm was extracted from excised testes of 3-year-old trout, kept at 0—4° C and used within a few days.

Sperm of *Salmo salar* was kindly supplied by N. S. Yandovskaya from the Nevski fish hatcheries near Leningrad. Sperm was squeezed out from intact fishes when spawning was vivacious in the first days of November. Sperm was kept at 0—4° C when transported to Moscow and used.

The dried nuclei of *Clupea harengus*, *Clupea pallasii* and *Oncorhynchus keta* sperm are a gift from Dr. T. Ando, University of Tokyo.

Preparation of sperm nuclei. *Acipenser stellatus*, *A. güldenstädti* and *Huso huso* sperm nuclei were prepared according to Felix (Felix, 1960; Felix et al., 1951). If a sperm from milt was used, the sperm was filtered through 1, 2 and 4 layers of the plankton gauze (40 holes/mm^2). The sperm was homogenized in the Dounce-type homogenizer (Dounce, 1955), and poured into a large volume of distilled water (about 1 g dry nuclei in 1 l *aq. dest.*). Mixing was carried out in cold for 30—60 minutes. The nuclear material was precipitated with 0.01% acetic acid and a loose sediment (about $^1/_5$ of total volume) was centrifuged. This sediment was homogenized while the entire cycle was repeated twice. The last sediment (pure nuclei) was used for X-ray diffraction analysis immediately or dried with alcohol and ether for later use (3—5 extractions).

Nuclear protein from any of three fish species mentioned belongs to triprotamine type. Amino acid composition determinations have given the following values (expressed as % of total N): arg 71—78, his 9—11, lys 5—7 (Лисицын, Александровская, 1940). More exact values for *Acipenser stellatus* sperm protamine (stelline) are given by

Dr. E. Kaverzneva (Raukas, 1966). Amino acid composition of these protamines is very similar to the composition of *Acipenser sturio* sperm protamine (Felix et al., 1952).

Similar procedures were used to prepare *Salmo irideus* and *Salmo salar* sperm nuclei. In some cases the sperm was treated with 0.14M NaCl instead of distilled water (see table).

Treatment of nuclei. To remove bivalent cations from sperm the following treatment was used, previously described by Zubay and Wilkins (1962) as a method for isolation of DNP. A fresh sperm of *Acipenser güldenstädti* (about 2 g dry weight) was washed once with large volume of 0.14M NaCl, precipitated in 0.01% acetic acid and 1 l of loose sediment centrifuged for 5 minutes at 2500 g. The sediment was dispersed in the Dounce-type homogenizer and mixed for 1 hour in 300 ml 0.024M EDTA plus 0.075M NaCl, pH 8 in cold. The homogenate was centrifuged for 15 minutes at 5000 g and the homogenization and sedimentation were repeated twice. After this the sediment was homogenized once more in a 300 ml EDTA solution and mixed overnight (12 hours). On the following day the suspension of nuclei was centrifuged and washed three times with distilled water (all the operations were performed similarly to the washing with 0.024M EDTA + 0.075M NaCl). The last, very loose sediment in distilled water had a volume of about 5—10 times that of the initial one and was used for electron microscopic and X-ray diffraction experiments. This treatment does not dissolve the nuclei completely: even in the light microscope very many intact or partially degraded nuclei were visible. It was not possible to prepare DNP from these nuclei in appreciable amounts, although ultraviolet spectra of the water extracts indicated the presence of DNP.

In the case of *Salmo irideus* and *Salmo salar* sperm, the swelling of nuclear sediment in distilled water was less pronounced than in case of *Acipenser* nuclei.

Preparation of DNP. DNP was prepared by the method of Pollister and Mirsky (1946). Purified sperm nuclei were extracted with 2M NaCl for 2 hours, centrifuged at 10,000 g for 2 hours, and precipitated by dilution with distilled water in 0.14M NaCl.

X-ray diffraction. For X-ray diffraction at small angles the samples of sperm or sperm nuclei were sealed in cuvettes covered with mica foils. Concentration of the specimens was determined after the exposition by drying to constant weight at 105—110° C. Kratky-type camera having line shaped primary beam with specimen-to-film distance 300 mm was used together with the semi-microfocus tube (Лемажихин, Лебедев, 1960), Cu K_α radiation (with Ni filter in most cases). Specimen cuvettes were cooled with tap water when exposed.

The spacings are given as measured on diffraction patterns with line shaped collimation unless otherwise stated. In some cases the line shaped collimation was transferred to the point collimation by Feodorov (Феодоров et al., 1965), making use of electronic computer.

Electron microscopy. A native sperm and a sperm treated with distilled water or EDTA as described above was used for electron microscopic investigations. The sperm or sperm nuclei suspensions in distilled water or physiological saline were centrifuged at 7000 g for 15 minutes, the sediments collected and fixed in OsO_4 buffered with phosphate according to Millonig (1961). Specimens were dehydrated in increasing concentrations of ethanol and embedded into mixture of butyl-methyl methacrylate. Ultrathin sections were prepared with LKB-4800 microtome, mounted on specimen grids and contrasted with lead salts according to Reynolds (1963). UIMB-100 electron microscope at 75 kV with 30 μ objective aperture was used, direct magnifications 10,000× and 22,000×.

In some cases the EDTA-treated nuclei were investigated by means of negative staining and shadowing. Micro-drops of suspensions were placed on specimen grids covered with nitrocellulose film and excess of suspension removed with filter paper. Specimens were stained on surface of 2% uranyl acetate solution pH 5 for 30 seconds. When the shadowing method was used the suspension was dropped upon carbon-coated grids, excess of suspension removed and specimens shadowed with Pt-Pd alloy. The shadowing was at an angle of 8 degrees from the plane of the supporting film

(approximate object height to shadow length ratio 1:7). JEM-6C electron microscope of Japan Electron Optics Laboratory Co, Ltd. was used, initial magnifications 25,000× and 50,000×.

Results

X-ray diffraction at small angles. X-ray diffraction of *Acipenser stellatus, A. güldenstädti* and *Huso huso* sperm nuclei at small angles reveals that there are some other reflections for sperm nuclei, besides those described earlier (Luzzati, Nicolaieff, 1959; Nicolaieff, 1962). Firstly, a pronounced reflection approximately 90 Å (corrected for collimation) and usually an unresolved or diffuse band between 45—60 Å are observed (Paykac, 1964) (fig. 1, *a*). This 90 Å reflection is relatively strong if the concentration is about 40—60% dry weight.

Besides these reflections there are two reflections described earlier: a very strong reflection with spacing of 25—27 Å and a considerably weaker one of 20 Å. These spacings correspond to the main equatorial reflection and the only strong reflection on the first layer line on the oriented fibre diagrams of DNP, respectively. In control experiments with 100 mm Kratky-type camera reflections corresponding to $1/\sqrt{3}$ and $1/\sqrt{4}$ of 27 Å were visible.

Attempt was made to find out whether reflections in the 100 Å region are common for other fish sperm nuclei of protamine type, widely used in experiments with DNP. For this purpose X-ray diffraction patterns from the following sperm nuclei were obtained (table) : *Clupea harengus, Clupea pallasii, Oncorhynchus keta, Salmo irideus* and *Salmo salar*. Besides 25—27 Å reflections from hexagonally packed DNA molecules all these specimens give reflection in the 100 Å region. In case of *Clupea pallasii* this reflection is a little more diffused as compared with *Acipenser* and has a mean spacing of 110 Å. Sperm heads of *Clupea harengus* in this region give a very diffused band of about 80 Å. On the contrary, the reflection from *Oncorhynchus keta* sperm heads is unusually sharp and has a spacing of 90 Å.

The reflections in the 100 Å region are also observed in experiments with the sperm heads of *Salmo salar*. However, these reflections are very diffused and usually only a band between 50 and 100 Å could be visualized. Such a band is observed also in diffraction patterns of sperm nuclei washed with 0.14M NaCl (table).

As only 20 Å and 25 Å reflections are described in X-ray diffraction patterns so far, a question arose, whether the small angle reflections might be caused by other components of sperm. In general, the small angle reflections from the sperm could be due to the following reasons:

1) presence of particles or substances other than spermatozoa;

2) other components of spermatozoa (possibly, sperm tails or residual proteins);

3) presence of nucleoprotein of DNH type having reflections in the small angle region;

4) these reflections are due to nucleoprotamine alone.

To test the first point, X-ray diffraction patterns of crude specimens (not purified with distilled water) were prepared. The sperm from *Salmo irideus* and *Salmo salar* was centrifuged (and sometimes slightly dried on a filter paper), closed into cuvettes without delay and examined for diffraction. In all cases one or two very intense small angle reflections

Results of small angle X-ray diffraction experiments

Species	Condition or treatment	% dry wt.	Spacings (Å) and intensity***				
Acip. stellatus	nuclei*	33	20.0 w.	24.8 s.	—	98 s.	—
Acip. güldenstädti	„	28	19.9 w.	24.8 s.	—	98 s.	—
Huso huso	„	46	20.0 w.	24.8 s.	—	98 s.	—
Clupea pallasii	nuclei**	29	—	26.8 s.	60 w.	108 s.	—
Clupea harengus	„	33	20.6 m.	25.9 s.	—	70— 90 w., d.	—
Oncorhynchus keta	„	42	20.8 w.	26.1 s.	—	88 m.	—
Acip. güldenstädti	washed 1 × 0.14M NaCl	32	20.0 w.	25.1 s.	50 w.	100 w.	—
	„ 1 × 0.14M NaCl + 1 × *aq. dest.*	32	20.0 w.	25.0 s.	50 w.	100 w., d.	—
	„ 4 × EDTA	24	21.0 w.	26.1 s.	—	—	—
	„ 4 × EDTA + 3 × *aq. dest.*	26	20.2 w.	24.8 s.	—	—	—
Acip. stellatus	DNP	44	20.0 w.	25.5 s.	—	45—100 w., d.	—
Salmo irideus	non-purified sperm	28	20.7 v. w.	26.4 s.	49 m.	68 v. s.	180 m.
	washed 1 × 0.14M NaCl 5 hours	37	20.6 v. w.	26.3 s.	—	50—100 m., d.	—
	„ 3 × 0.14M NaCl 40 „	33	—	27.0 s.	50 w.	70 m.	—
	„ 1 × *aq. dest.* 5 „	45	20.3 m.	25.5 s.	45 v. w.	90 v. w., d.	—
	„ 3 × *aq. dest.* 24 „	30	—	25.2 s.	—	45— 70 w., v. d.	—
	„ 3 × EDTA + 3 × *aq. dest.*	41	20.0 m.	24.7 s.	—	—	—
Salmo salar	non-purified sperm	18	20.5 w.	25.7 s.	45 w.	60 w.	180 m.
	washed 3 × *aq. dest.*	35	20.0 m.	24.8 s.	—	45— 80 m., d.	—
	„ 3 × *aq. dest.*	64	19.4 m.	23.9 s.	—	50—100 m., d.	—
	DNP	43	20.9 m.	26.3 s.	—	—	—

* Washed 3 times with *aqua destillata* in cold.
** Obtained from Dr. T. Ando.
*** v. s. — very strong; s. — strong; m. — medium; w. — weak; v. w. — very weak; d. — diffuse; v. d. — very diffuse.

were visible between 40 and 100 Å; spacings of these reflections strongly depend upon concentration of specimen. With a concentration of 40% dry weight, the spacings of these two reflections were 42 Å and 84 Å. Sometimes, with a small concentration (about 15%) a reflection was visible with a spacing of approximately 180 Å. However, when the sperm was washed with alcohol and ether or treated with distilled water only, these strong, concentration-dependent reflections disappear, leaving a reflection of medium intensity in the 100 Å region, spacing of which is considerably less sensitive to the variations in the concentration. The intensity of this reflection does not fall upon further extraction of sperm with solvents known to dissolve lipids (butanol, ethanol, ether). Extraction with butanol up to 24 hours was used to extract *Acipenser güldenstädti* nuclei, yet the 98 Å reflection still persisted.

To test the second point, tails of *Strongylocentrotus dröbachiensis* spermatozoa and residual protein of the *Acipenser güldenstädti* sperm nuclei have been isolated. The tails of spermatozoa were disrupted from the nuclei by ultrasound treatment and subjected to differential centrifugation; this process was followed by light microscopy. In the last sediment no more than 1 nucleus amidst 20 tails was counted. A joint fraction of residual and acidic protein was separated from a nuclear sediment in 2M NaCl after centrifugation, making use of DNase digestion and additional extractions and centrifugations in 2M NaCl. When these specimens were investigated for diffraction at small angles, no reflections or, at least, reflections having intensity comparable to that of the X-ray patterns of nuclei were visible in the micellar region.

Indeed, the cytolysis in distilled water is widely used to remove tails and cytoplasm of spermatozoa, so the specimens of nuclei have to be

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Fig. 1. Small angle X-ray diffraction patterns: *a* — nuclei of *Acipenser stellatus* in 0.14M NaCl, 46% dry weight; *b* — DNP from *A. stellatus* nuclei in 0.14M NaCl, 44% dry weight. (× 1.25.)

Fig 2. Ultrathin section of the water-treated sperm head of *Salmo salar*: only nucleus is visible, cytoplasm and tail have been removed. (× 53,300.)

Fig 3. Ultrathin section of the spermatozoa of *Salmo salar*. In light areas fibrils of about 20—100 Å in diameter are visible. (× 53,300.)

Fig. 4. Section through sperm head of *Acipenser güldenstädti*, treated with EDTA and distilled water. Sperm heads have been disintegrated and nucleoprotein fibrils go into solution. (× 55,500. Fig. 4a. × 148,000.)

Fig. 5. Section through sperm head of *Salmo salar*, treated with EDTA and distilled water. Sperm heads swell and large transparent areas appear, in which nucleoprotein threads are seen. (× 31,000.)

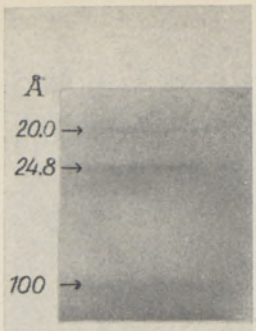
Fig. 6. Disintegrated nuclei of *Salmo salar*, stained with uranyl nitrate. (× 25,200.)

Fig. 7. Nucleus of *Salmo salar* with DNP fibrils after EDTA and distilled water treatment, negative staining. Mean diameter of the fibrils is about 70—80 Å. (× 100,000.)

Fig. 8. Another nucleus in EDTA and distilled water-treated *Salmo salar* sperm specimen. Negative staining. (× 60,000.)

Fig. 9. DNP fibrils at some distance from nuclei, stained with uranyl nitrate. Fibrils of various diameters ranging from DNA double helix up to 100 Å are visible. (× 225,000.)

Fig. 10. Gradual degradation pattern of DNP fibrils at the edge of EDTA and water-treated *Salmo salar* sperm nucleus. Thread with diameter of 25 Å obviously consists of one DNA molecule. Pt-Pd shadowing at the angle of 8 degrees from the plane of supporting film; arrow shows the direction of shadow. (× 75,000.)



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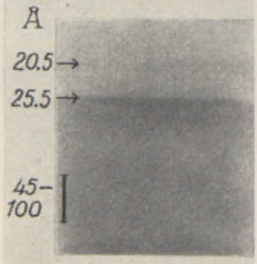

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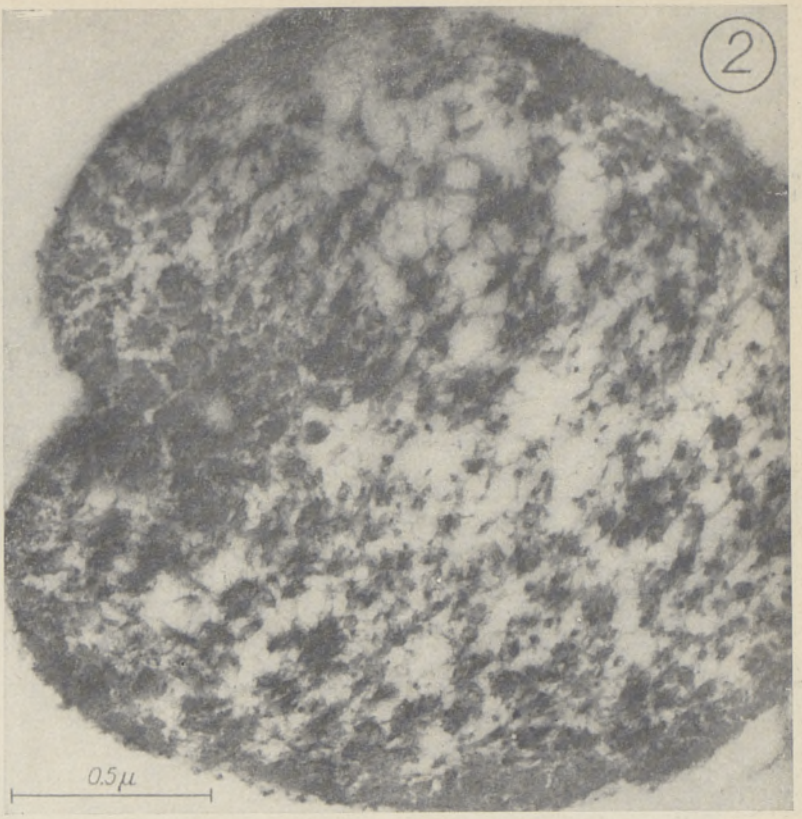

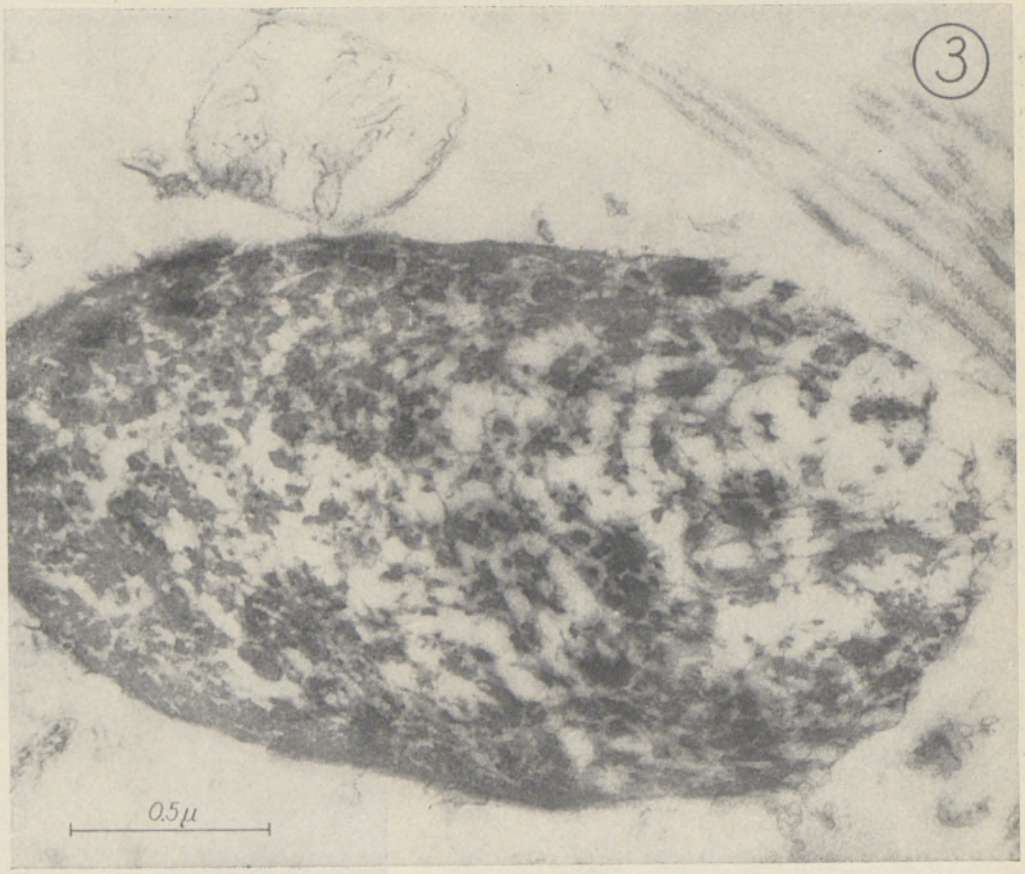

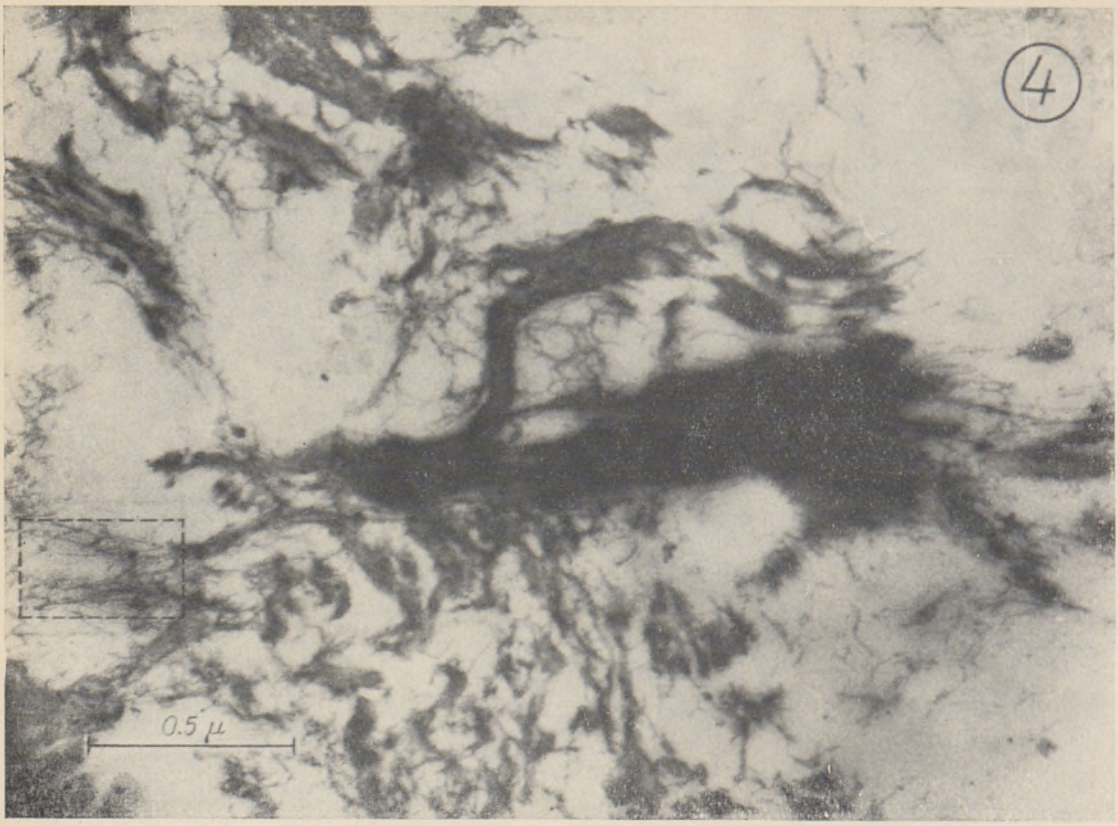
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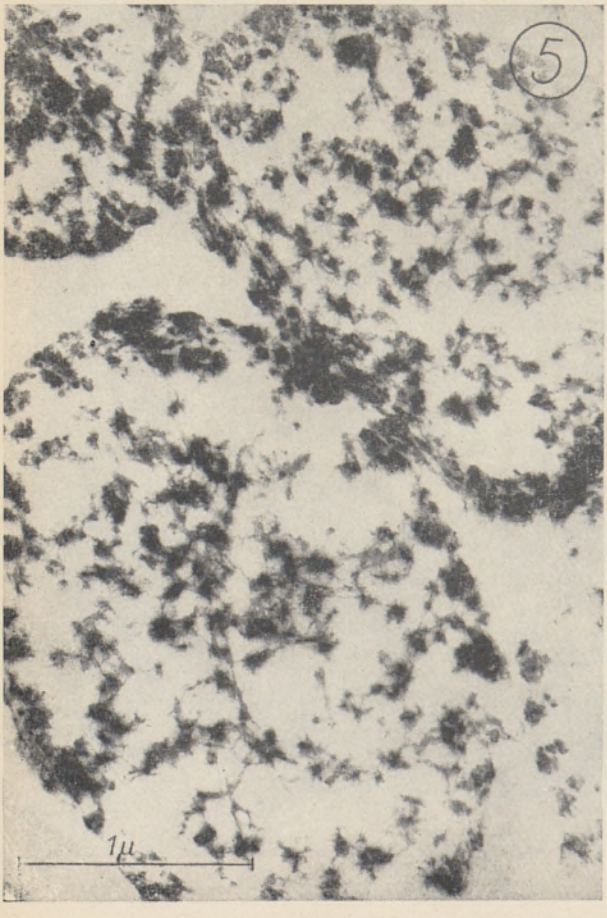
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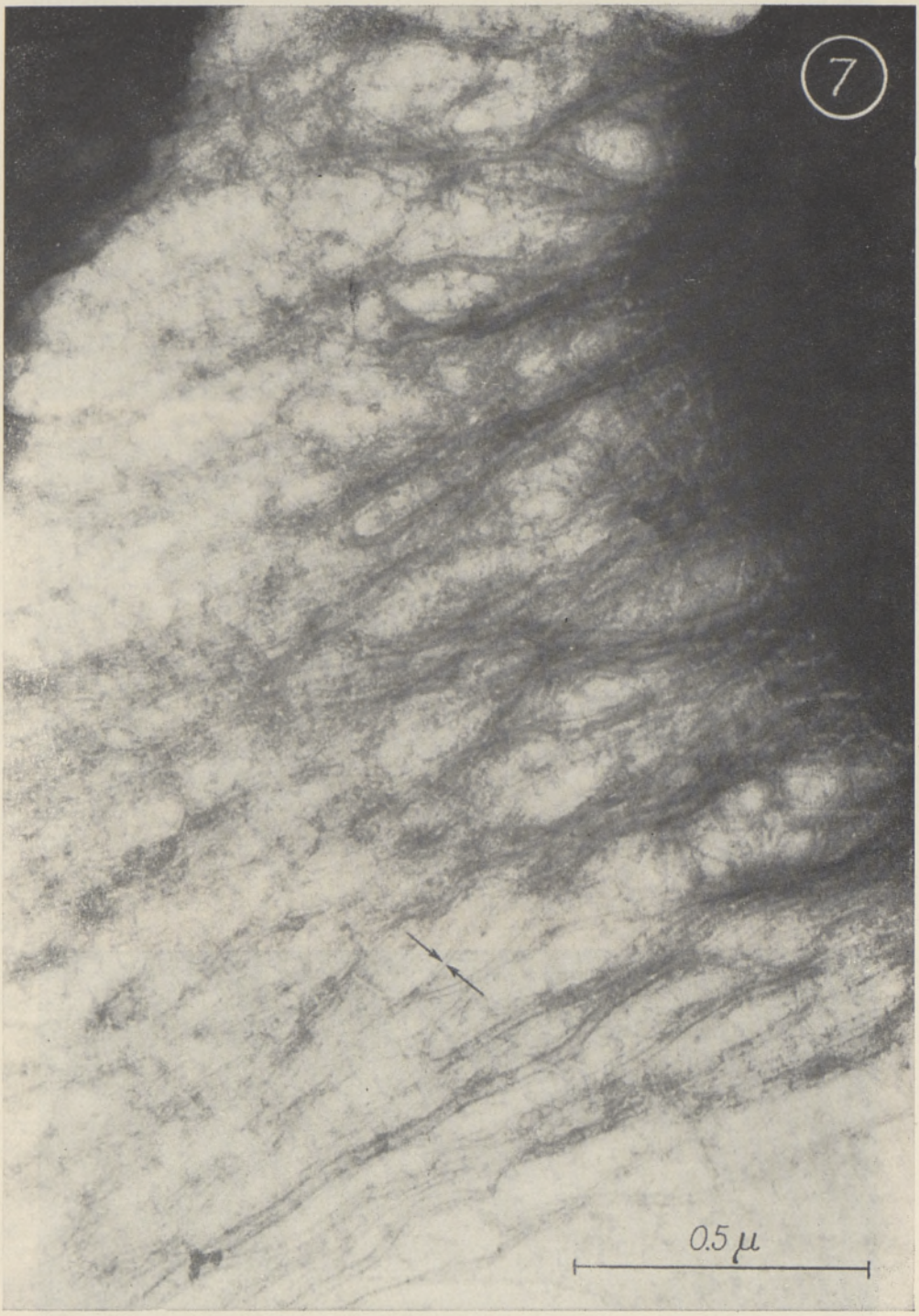
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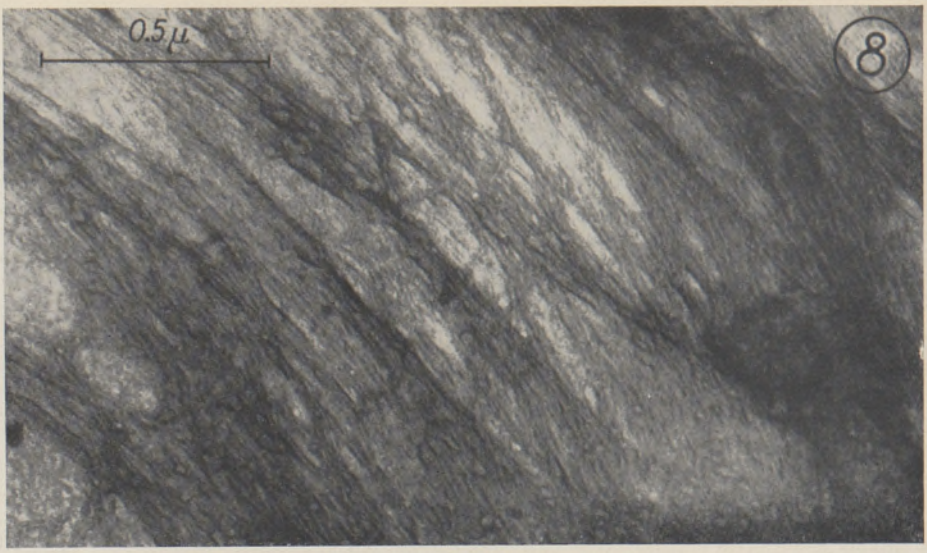
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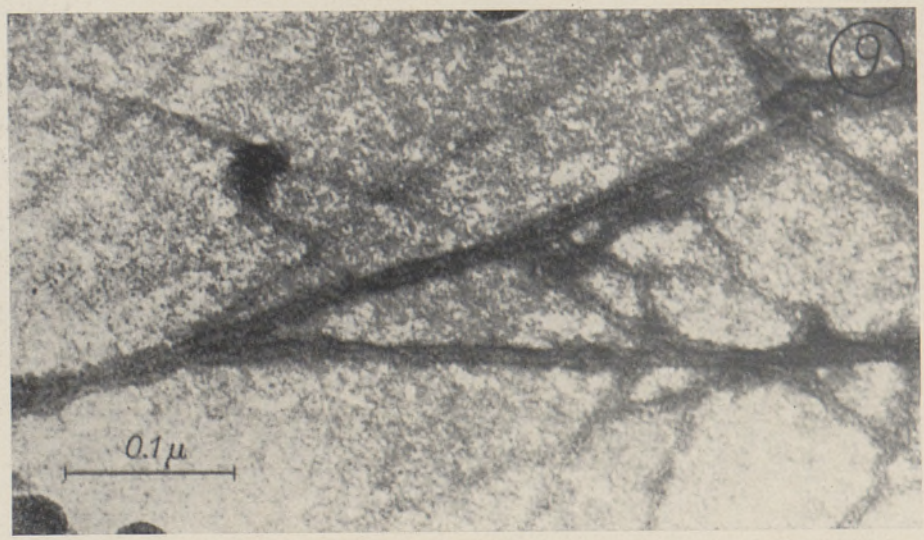
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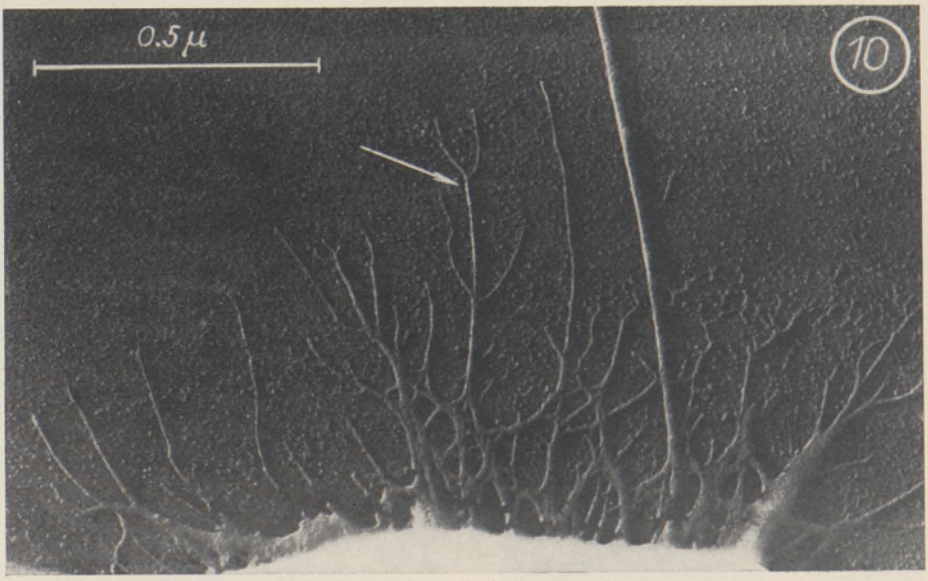
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essentially free from these components. This is also proved by means of microscopic examination during the course of this study.

Thus it must be concluded that reflections in the micellar region are not derived from the tails, nuclear envelopes or the cytoplasm of the spermatozoa.

To investigate the third possibility, the proteins were extracted from nuclei and examined, but no histone-like fractions were identified (Paykac, 1964).

In this manner it seems that the 100 Å reflection is not derived from other sperm components, and the fourth case remains to be proved.

If DNP is extracted from spermatozoa with 2M NaCl and precipitated in 0.14M NaCl, practically all reflections above 27 Å disappear (fig. 1, *b*). Only in a few cases traces of reflections in this region are visible (weak, diffuse band of 45—100 Å in *A. stellatus* DNP). However, the extraction of DNP with 2M NaCl leads to the dissociation of DNA-protamine complex and, consequently, to the disintegration of nucleoprotamine structure. It is reasonable to suppose that the micellar structures of DNP, if present in nuclei, must be badly damaged by this treatment.

Taking into account that attempts to attribute these reflections to other components of sperm have been unsuccessful, it might be supposed that 100 Å spacing corresponds to the structural periodicity of DNP in sperm nuclei.

However, several objections against this interpretation would be made. First of all, the intensity of micellar reflection varies markedly from one specimen to another. Secondly, after the treatment of sperm nuclei with 1% H_2SO_4 for 2 hours (in order to extract basic proteins) the intensity of micellar reflection increases and becomes more distinct, especially in the 50—75% concentration range. Thirdly, it is difficult to decide, whether the micellar reflection moves jointly with 25 Å reflection if the concentration is changed: when the 25 Å reflection shifts to 20 Å upon drying, the micellar reflection becomes diffused and indiscernible. On the contrary, the micellar reflection of acid-extracted nuclei would be followed up to 100% dry weight (during the drying the spacing of this reflection decreases from 100 Å to about 60 Å), but the 25 Å reflection vanishes after extraction.

Therefore, to draw any firm conclusions from these experiments would be premature; nevertheless, these experiments have been very useful, suggesting new approaches to this particular problem. A further investigation of the DNP structure was undertaken, making use of electron microscopy.

Electron microscopy. Electron micrographs of untreated sperm heads are usually dense, and very few (if any) details of nuclear structure might be seen. In longitudional and cross sections of *Acipenser* as well as *Salmo* sperm nuclei light areas are visible on a dense background if sperm is treated with distilled water in order to obtain pure nuclei (fig. 2). Occasionally, these regions are seen also in micrographs of untreated sperm (fig. 3) and thin fibrils of various diameters (20—100 Å) might be discerned. It is not known, if the sperm heads with less compact regions are caused by degradation of nuclear structures during the transportation, or these regions are presented sometimes in the native structure of DNP.

If sperm is treated with EDTA, most sperm heads swell and lose their integrity (fig. 4), and the DNP content of *Acipenser* sperm nuclei goes into the solution; this material seems to consist of fibrils with various diameters ranging from 20 Å to 100 Å. In the case of *Salmo* sperm less

drastic changes occur: the nuclei swell considerably, but usually do not break up (fig. 5). Only a part of *Salmo* sperm nuclei are disintegrated to an extent, comparable to that of the *Acipenser* nuclei (fig. 6).

When negative staining is used to study EDTA-treated nuclei, fibrils rather uniform in cross-section (about 70—80 Å) are visible (figs 7 and 8). These fibrils seem to be relatively unstable, and they split into thinner structures. Far from nuclei a variety of fibrils are visible (fig. 9). Electron micrographs of shadowed specimens reveal a very interesting gradual degradation pattern of these fibrils (fig. 10). Fibre of about 70 Å diameter separates into 4 fibrils, of 25 Å in each diameter. On the same electron micrograph a proximal part of a very long fibril, 70 Å in diameter is visible. There is obviously one DNA double helix per such a 25 Å strand; it follows that the number of DNA molecules per 80 Å strand is 4.

Discussion

It is well known that Mg^{++} and Ca^{++} are the main metallic constituents of cell nuclei (Mirsky, Osawa, 1961; Steffensen, 1961). As early as in the 19th century F. Miescher showed that somatic nuclei contain a considerable amount of Ca^{++}, varying in range from 0.3 to 0.7%: Ca^{++} content of sperm heads was found to be 0.23% (Miescher, 1897). Williamson and Gulick found the Ca^{++} content of thymus nuclei to be 0.7%, while the Mg^{++} content was considerably lower (0.08%) (Williamson, Gulick, 1944). Recently the Mg^{++} and Ca^{++} content and mode of interaction with nuclear constituents was investigated (Naora et al., 1961). It was shown that it is possible to release 75% of the total Mg^{++} free into the solution by digestion nuclei with DNase, while Ca^{++} remains firmly bound to nuclei. For every 24 atoms of phosphorus 1 ion of Mg^{++} is released. Experiments with histone indicated that Mg^{++} is not bound to it. On the contrary, Ca^{++} seems to be bound only with the protein part of DNH.

In chromosomes, most of Mg^{++} and Ca^{++} is located in the banded regions (Barigozzi, 1938). After treatment of *Drosophila* salivary gland chromosomes and grasshopper *Melanoplus femur rubrum* testes meiotic cells with EDTA and water (Mazia, 1954) chromosomes disperse and become soluble. Apparently, the bivalent cations link DNH molecules with each other in condensed chromosomes. Treatment with EDTA removes Ca^{++} and Mg^{++} and after exposing to low ionic strength repulsion forces between charged groups of macromolecules cause chromosome to disperse. If both conditions (removal of bivalent cations and low ionic strength) are fulfilled simultaneously, it is possible to disperse chromosomes in a single step in 0.001M EDTA.

The bivalent cations are also present in considerable amounts in sperm nuclei. Autoradiography experiments (Steffensen, 1959; 1961; Steffensen, LaChance, 1960) have shown that pollen of *Lilium longiflorum* as well as sperm nuclei of *Habrobracon* very strongly bind ^{45}Ca. When fertilized, *Habrobracon* sperm nucleus retains ^{45}Ca up to fusion of haploid male and female nuclei; the experiments with ^{90}Sr gave similar results. Thus bivalent cations, as distinct from their monovalent counterparts, are firmly bound to DNP and obviously have a definite structural role in sperm nuclei.

However, this structural role of Mg^{++} and Ca^{++} is never taken into account, when structure of DNP is considered. At the same time the importance of bivalent metal ions is especially clearly indicated in the case of nucleoprotamine. Indeed, there are reports that for DNP extraction by the "water" method, it is necessary to remove bivalent cations

with EDTA (Zubay, Wilkins, 1962). This "water" method of extraction is very common for use in DNH extraction from somatic tissues, but is earlier stated to be unappropriate to obtain DNP from salmon milt (Zubay, Doty, 1959). Analogous procedure has been previously used for extraction of DNH from sea urchin sperm (Bernstein, Mazia, 1953) (nucleoprotein of sea urchin sperm belongs to DNH type).

EDTA, known as a chelating agent, strongly binds bivalent cations. Hence, we may suppose that after multiple treatment of sperm heads with 0.024M ETDA plus 0.075M NaCl, considerable amounts of Mg^{++} and Ca^{++} are removed. Electron micrographs of sperm heads, which are treated with EDTA and dispersed in distilled water, indicate that they swell and lose their integrity (figs 4, 5, 6, 7 and 8). Sometimes the fibrils from EDTA-treated nuclei are rather uniform in diameter (about 70—80 Å). The presence of nucleoprotein fibrils of such a diameter is in good agreement with X-ray diffraction data: all the sperm heads investigated had a reflection in the 100 Å region. It is natural to suppose that bivalent cations serve as linkages between these nucleoprotein fibrils. Possibly, the elementary chromosome fibrils do not lose their individuality during the maturation of sperm, but are rather laterally aggregated by means of bivalent cations. After treatment with EDTA followed by washing in distilled water, the sperm heads broke up into elementary fibrils.

These fibrils are obviously too thick to be single DNA molecules coated with protamine. The number of DNA molecules per such a bundle may be determined by the electron micrographs of partially degraded DNP. As it is seen in fig. 10, a fibre of about 70 Å in diameter splits into 4 fibrils of 25 Å diameters each. As this 25 Å fibril obviously consists of one DNA molecule, the 70 Å fibril has to contain 4 DNA double helices. When DNA is extracted with mild methods from these sperm heads, fibrils consisting of 4 DNA double helices in a cable-like fashion are obtained (Raukas et al., 1966).

Let us consider now the internal structure of these elementary fibrils. From the plots of DNP swelling and experiments of DNP salt extraction it follows that molecules of DNA in DNP structure are cross-linked by protamine bridges. Similarity of DNP and sperm nuclei diffraction patterns requires also that DNP in intact sperm heads has a structure close to that of the extracted material. It is reasonable to assume that in the elementary fibrils the DNA double helices are held together by protamine molecules in a fashion similar to that of the extracted DNP. Thus the distances between DNA molecules within these fibrils are apparently determined by side-chain length of arginine residues in protamine (Paукас, 1965a), as revealed by X-ray diffraction experiments at small angles, using DNA complexes with poly-l-ornithine, poly-l-lysine and poly-l-arginine.

However, it is difficult to decide whether protamine bridges between DNA molecules in these fibrils are the only links. As seen in some electron micrographs (fig. 9 and 10), elementary fibrils frequently split into more thin fibrils. It must be concluded that either the bivalent cations play some role in the structure of elementary fibril or the protein linkages between DNA molecules are relatively weak and fibrils break up when homogenized. Thus the role of bivalent cations is not determined unequivocally in the present investigation.

The protamine network hypothesis is indirectly supported by the recent amino acid sequence analysis of protamines (Ando et al., 1962; Felix, 1960). This analysis reveals that the sequence ... $X_2Arg_4X_2Arg_4$ ... proposed earlier (Felix et al., 1956) for protamine is not correct and that

the sequence of amino acids in protamines is far more complicated. In particular, there are single non-basic amino acid residues between tetra- and diarginines, and, on the contrary, the single arginine residue between non-basic amino acid dipeptides (Ando et al., 1962). At the same time the 3-stranded DNP model (Feughelman et al., 1955) requires the non-basic amino acid residues to occur in pairs in order to compensate all phosphate groups of DNA. Therefore, for such a sequence in this DNP model some phosphate groups remain noncompensated by given protamine molecule and may be compensated by some arginine residue of another protamine molecule, wrapped around the neighbour DNA molecule. On the other hand, the single non-basic amino acid residue between arginine residues may serve as bridge between two neighbouring DNA molecules. This possibility is suggested by the X-ray data. The mean distance between DNA molecules in DNA-protamine complex is about 2 Å longer than that in DNA-poly-l-arginine complex (Paукас, 1965a).

It is very interesting to compare these results with the data about amino acid composition and heterogeneity of the very common protamine, the clupeine (from *Clupea pallasii*). When the crude clupeine hydrochloride is fractionated in the alumina column with 0.48M aqueous K_2HPO_4 two fractions were obtained (Ando, Sawada, 1962). One of these fractions (designated as Z) has been proved to be homogeneous upon N-terminal analysis (only alanine is found as N-terminal), whereas the other fraction Y is heterogeneous, N-terminal of which consists of proline and alanine residues (Ando, Sawada, 1961). Afterwards the amino acid sequence of component Z was reported (Ando et al., 1962). Recomputation of mole per cent arginine residues into per cent of total N gives 89.5% for this fraction, which is essentially the same as unfractionated clupeine (89.0%) (Ando et al., 1959). Therefore the arginine content of both fractions (Z and Y) must be considered practically equal. As the relative content of Z and Y fractions is roughly 1 : 3 (Ando, Sawada, 1962) and fraction Z consists of 21 arginine residues, structural unit of DNP could not be less than 84 arginine residues, *i. e.* 4 pitches of a 2-stranded DNA helix, or possibly the distance of one DNA pitch (34 Å) along the fibre axis, consisting of four helices. In any case the nucleoprotamine complex has not to be regarded as a single DNA molecule coated with protein. The nucleoprotamine complex, consisting of one DNA molecule, in the small groove of which protamine molecules are located, may arise only in a solution of low ionic strength and low concentration of DNA. This model of DNP obviously does not bear so much upon the DNP structure *in vivo* as generally accepted.

The following tentative molecular model is proposed on these grounds. In the course of spermiogenesis elementary chromosome fibrils have been linked together by bivalent cations to yield a compact structure of DNP in mature sperm head. These fibrils in mature sperm heads, about 70—80 Å in diameter, consist of probably 4 DNA double helices. The protamine polypeptide chain is wrapped around each DNA molecule in these fibrils, but in some places there are protamine bridges between DNA molecules. There are two possible ways to extract DNP from the sperm. The first, a milder method, with the use of EDTA and distilled water (Zubay, Wilkins, 1962), yields perhaps intact elementary chromosome fibrils, and may be regarded as the most native DNP. The other, more common method is to dissolve nuclei at high salt concentrations (Pollister, Mirsky, 1946); this involves complete or nearly complete dissociation of DNA and protamine. After precipitation of DNP in 0.14M NaCl the sediment is formed, described more closely by Luzzati and Nicolaieff as a two-dimens-

ional network of DNA molecules, with maximum distance between DNA molecules about 31 Å. X-ray diffraction patterns of this DNP have no reflections in the 100 Å region. Thus, the structure of salt-extracted DNP must not be considered identical with DNP structure in sperm heads.

Summary

Sperm heads of *Salmo salar*, *S. irideus*, *Acipenser stellatus*, *A. güldenstädti* and *Huso huso* (fishes) were investigated by means of electron microscopy and X-ray diffraction at small angles.

Upon X-ray diffraction at small angles all the sperm head specimens mentioned above give reflections in the 60—100 Å region. These spacings seem to be connected with native DNP structure and not with other constituents of sperm nuclei.

In order to remove bivalent cations the sperm heads were treated with 0.024M EDTA plus 0.075M NaCl, pH 8, and washed with distilled water. Electron micrography shows that after this treatment sperm heads swell and lose their integrity. The nucleoprotein fibrils released from these nuclei seem to vary in diameter from 20 Å to 100 Å, but sometimes the fibrils of about 70—80 Å in diameter are a predominant feature of electron micrographs.

It is concluded that 100 Å elementary chromosome fibrils do not lose their individuality during the sperm maturation and histone → protamine transition, but are rather laterally aggregated by means of bivalent cations.

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NUKLEOPROTEIIDI STRUKTUUR SPERMATOSOIDIDE TUUMADES: NUKLEOPROTAMIINI STRUKTUUR

Resümee

Röntgenidifraktsioonimeetodil väikestel nurkadel ja elektronmikroskoopiliselt uuriti kalade *Acipenser stellatus, A. güldenstädti, Huso huso, Salmo irideus* ja *Salmo salar* spermatosoidide tuumades nukleoprotamiini struktuuri.

Elektronmikroskoopilisel vaatlusel selgus, et spermatosoidide mõjustamisel etüleendiaminotetraatsetaadi ja destilleeritud veega nende tuumad lagunevad ja nukleoprotamiin läheb osaliselt lahusesse. Mõnedel juhtudel sedastati tuumade lagunemist suhteliselt konstantse diameetriga fibrillideks (70—80 Å).

Spermatosoidide tuumade uurimisel röntgenidifraktsioonimeetodil leiti reflekse piirkonnas 80—110 Å. Need refleksid on seostatavad nukleoprotamiini struktuuriga spermatosoidide tuumades.

Järeldatakse, et spermatogeneesil ja histooni asendumisel protamiinga ei kaota elementaarsed kromosoomsed fibrillid oma individuaalsust, vaid ainult seostuvad omavahel kahevalentsete katioonide kaudu.

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ЭРГО РАУКАС, В. МИТЮШИН, А. КАФТАНОВА

СТРУКТУРА НУКЛЕОПРОТЕИДА В ЯДРАХ СПЕРМАТОЗОИДОВ: СТРУКТУРА НУКЛЕОПРОТАМИНА

Резюме

При помощи рентгеновской дифракции под малыми углами и электронной микроскопии исследовали структуру нуклеопротамина в головках сперматозоидов осетровых (*Acipenser stellatus, A. güldenstädti, Huso huso*), форели (*Salmo irideus*) и лосося (*Salmo salar*).

Электронная микроскопия сперматозоидов, обработанных версеном и дистиллированной водой, показывает, что головки сперматозоидов разрушаются и нуклеопротамин частично входит в раствор. В некоторых случаях отмечается распад ядер спермиев на фибриллы сравнительно одинакового диаметра (70—80 Å).

При рентгеновском исследовании ядер сперматозоидов обнаружены рефлексы с расстояниями 80—110 Å, которые могут быть отнесены к структуре нуклеопротамина в ядрах сперматозоидов.

Сделан вывод о том, что в процессе сперматогенеза и замены гистона протамином элементарные хромосомные фибриллы не теряют индивидуальности, а связываются между собой посредством двухвалентных катионов.

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