THE NUCLEOPROTAMINE OF TROUT SPERM

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PLATES 1 and 2

(Received for publication, June 20, 1946)

Constituents of the cell nucleus were extracted and isolated for the first time by Miescher (1). Sperm was the material used in most of Miescher's classical investigations on nuclear constituents; and in the long series of subsequent investigations on sperm, by Kossel (2) and his numerous associates, by Steudel (3), and finally by Rasmussen and Linderstrøm-Lang (4), the methods of extraction were essentially the same as those used by Miescher. These methods involved the use of such strong acid and alkali that the nuclear materials were drastically changed in the course of extraction. We have extracted nuclear constituents from sperm by a far milder procedure. In this paper we describe our method of extraction, some of the properties of the extracted material, and at the same time the cytological changes wrought by the extractant. At one stage in the extraction the highly condensed sperm nucleus changes so that it shows a striking resemblance to a resting nucleus or early prophase, thread-like chromosomes now being clearly visible.

Sperm was selected by Miescher as a material for study of the cell nucleus because the sperm cell contains relatively little cytoplasm, and the sperm of fish was used because this is available in such large quantities. In practically all of his work Miescher used salmon sperm; other workers have frequently used herring sperm. Two of the constituents of the sperm nucleus are a basic protein and nucleic acid. The basic proteins in salmon and herring sperm are protamines, proteins having a nitrogen content of 31.6 per cent, and 89.2 per cent of this is in the form of one basic amino acid, arginine. Protamine was extracted by treating sperm with 0.2 N HCl. The residue remaining after acid extraction still contained all the nucleic acid and this was later extracted with sodium hydroxide. Within the nucleus, protamine and nucleic acid are combined. They are separated by the hydrochloric acid used to extract protamine. It is possible that protamine is altered by treatment with 0.2 N HCl, but this is not known because protamine has never been prepared by a mild procedure. It is also possible that nucleic acid is modified by the treatment first with hydrochloric acid and then with sodium hydroxide.

We have found that both nucleic acid and protamine can be extracted by a neutral 1 M solution of NaCl.

Material.—For our work neither fresh salmon nor herring sperm were available. The trout (*Salmo fario*, the brown trout) is a member of the same genus as the Atlantic salmon (*Salmo salar*), and trout sperm was available.¹ A disadvantage of this material is that one is obliged to work with relatively small quantities. For one preparation Rasmussen and Linderstrøm-Lang used 20 kilos of herring testes; no more than 20 gm. of trout sperm were available to us at one time. A considerable advantage of working with trout sperm is that the material is obtained by stripping live fish, so that all of the sperm is ripe and therefore relatively uniform in composition; other investigators have worked with excised testes which, even in the breeding season, may contain some immature sperm. While stripping the trout, care is taken that no feces become mixed with the sperm. Within 2 hours after being collected the sperm were in the laboratory and while being transported they were packed in ice. All the experiments to be described, unless otherwise noted, were done in a cold room kept between 0 and 1°C. Most of our work has been on trout sperm, but, as will be mentioned later, we have also used the sperm of other animals—the shad, key-hole limpet, fresh-water clam, sea urchin, frog, fowl, and bull.

Extraction.—Before extraction the trout sperm are washed to free them of the small quantities of intercellular material present. The wash fluid contains in 1000 ml.; 7.8 gm. NaCl, 0.664 gm. KCl, and 0.687 gm. K_2SO_4 (5). After stirring, the sperm suspension is centrifuged at 5000 R.P.M. for 15 minutes. The supernatant is water-clear.

The washed sperm are extracted with 1 M NaCl, final concentration after mixing. On adding salt solution, the sperm mass immediately becomes sticky and gelatinous, so that it appears at first as if the cells were merely swelling. It is necessary to add a large volume of solution and to stir vigorously in a Waring mixer before it becomes apparent that the cells are breaking up as their contents pass into solution. For a mass of sperm with a dry weight of 900 mg. the volume of the extraction mixture should be about 500 ml. Even so, the mixture is quite viscous. After vigorous stirring the mixture is centrifuged at 12,000 R.P.M. for 60 minutes. A perfectly clear, viscous supernatant and a scanty residue are obtained. The material extracted from the sperm is precipitated by pouring the supernatant into 6 volumes of water. The precipitate is in the form of long fibrous strands, so fibrous that they can easily be wound around a rod and, if the rod has a crook at its lower end, the fibers can be transferred to another vessel while still clinging to the rod. If the fibers are not twisted around a rod they soon settle and after the supernatant is decanted much fluid can be pressed from the fibrous mass. The fibrous precipitate redissolves in 1 M NaCl. Vigorous stirring shortens the time needed to dissolve the precipitate. The solution is as viscous as it was when the material was initially extracted from the sperm. Any suspended particles are removed by centrifugation and the dissolved material is then reprecipitated by pouring the solution into 6 volumes of water. The precipitate has the same fibrous character as when first formed. The fibrous material

¹ Trout sperm was obtained from the New York State Fish Hatchery at Cold Spring Harbor, Long Island, and from the New Jersey State Hatchery at Hackettstown. We are much indebted to the superintendents of these hatcheries, Mr. Stanley Walters and Mr. C. O. Hayford.

is soluble in 1 M NaCl and insoluble in 0.14 M NaCl and, because of these properties can easily be reprecipitated and redissolved any number of times. Dissolved in 1 M NaCl it keeps well without any preservative at 0°.

A fibrous nucleoprotein has also been extracted in 1 M NaCl from the sperm of another fish, the shad. From the sperm of the sea urchin, key-hole limpet, and fresh-water clam no nucleoprotein was extractable with 1 M NaCl. With 2 M NaCl, however, there was no difficulty extracting fibrous nucleoproteins from these sperm. From frog and mouse testes nucleoproteins were extracted with 1 M NaCl. No nucleoproteins could be extracted from bull sperm (obtained by centrifuging bull semen) with either 1 M or 2 M NaCl. It would be interesting to know the nature of the change that occurs in the maturation of mammalian sperm that prevents extraction of nucleoproteins with concentrated salt solutions.

Composition.—For analytical purposes the whole trout sperm and the fibrous precipitate prepared from it are washed first with 65 per cent alcohol to remove salt, then with hot 95 per cent alcohol, and finally with ether. The dehydrated preparations are dried at 106°. Phosphorus (6) and nitrogen (7) analyses give the following results:

Material	Phosphorus content	Nitrogen
	<i>per cent</i>	<i>per cent</i>
Trout whole sperm.....	5.02	18.2
Trout whole sperm.....	5.04	18.33
Nucleoprotein.....	5.93	18.1
Nucleoprotein.....	6.14	18.4

This is the elementary composition that would be expected of a nucleoprotamine. If it is assumed (and it will be shown below that this assumption is justified) that all of the phosphorus is in the form of nucleic acid, it can be calculated that the substance joined to the nucleic acid has a nitrogen content of somewhat more than 25 per cent, a value high for any protein other than a protamine. And there can be little doubt that the fibrous material extracted from trout sperm consists largely if not entirely of nucleoprotamine, for the quantity obtained from trout sperm is of about the same order as the quantity of protamine and nucleic acid extracted from salmon sperm by Miescher; the fibrous material forms 81.6 per cent of the whole trout sperm and the amounts of protamine and nucleic acid in salmon sperm as reckoned by Miescher account for 81 per cent of the weight of the sperm head. This comparison does not exclude the possibility that there is some other protein in addition to protamine in the fibrous material. But no other protein can in fact be detected in this material. One of the characteristics of a protein such as salmine (the prota-

mine of salmon sperm) is its lack of tyrosine, tryptophane, and certain other amino acids. The Millon reaction, an exceedingly sensitive test for tyrosine and furthermore a test with which nucleic acid does not interfere, is altogether negative when applied either to purified salmine or to the fibrous material derived from trout sperm.

There are other indications, to be described more fully below, that the fibrous nucleoprotamine contains no other protein than protamine. If an aqueous solution of protein is shaken with a chloroform-octyl alcohol mixture a layer of protein soon appears at the interface, and as the shaking is continued, more and more of the dissolved protein accumulates at the interface (8). When a solution of nucleohistone is shaken in this way nearly all of the histone gradually accumulates at the interface, nucleic acid remaining dissolved in the aqueous phase. On shaking a nucleoprotamine with a chloroform-octyl alcohol mixture, it is found that very little material appears at the interface even after prolonged shaking. This is to be expected because it is known that a protamine does not spread at the surface of water. The experiment also shows that the fibrous nucleoprotamine contains no considerable quantity of protein that does spread at the surface of water.

The presence of nucleic acid in the nucleoprotamine is demonstrated at once by the ultraviolet absorption spectrum; and from the magnitude of the extinction coefficient at 2600 A. u. the quantity of nucleic acid present can be estimated. In Text-fig. 1 are shown absorption spectra in the ultraviolet of a sample of thymus nucleic acid (prepared by a procedure which will be described in another paper) and of trout nucleoprotamine. It is apparent that the nucleoprotamine has the characteristic nucleic acid absorption spectrum and that when concentrations of material are expressed in terms of phosphorus contents, that the absorption coefficients at the position of maximum absorption (2600 A. u.) are the same for nucleic acid and nucleoprotamine. This means that all of the phosphorus in the fibrous nucleoprotamine is in the form of nucleic acid. As seen in Text-fig. 1, the absorption curves of nucleic acid and nucleoprotamine differ only at wave-lengths below 2440 A. u. Below this wave-length the effect of protamine absorption can be seen.

All of the nucleic acid in the nucleoprotamine is of the desoxyribose type. This can be demonstrated by use of the Dische diphenylamine reagent (9). The intensity of blue color given by the nucleoprotamine is precisely the same as that given by a quantity of desoxyribose nucleic acid (prepared from the thymus) with an equivalent phosphorus content.

Separation of Nucleoprotein into Its Component Parts.—To separate nucleic acid from protamine it was found, to our great surprise, that dialysis suffices. Dialysis against water is ineffective because the nucleoprotamine complex precipitates and remains combined within the cellophane membrane, as the sodium chloride in which it is dissolved is removed. But if the solution of

nucleoprotamine in 1 M NaCl is dialyzed not against water, but against 1 M NaCl, protamine slowly passes through the cellophane membrane leaving nucleic acid behind. Even when dialysis takes place in a rocking dialyzer in which the fluid inside the cellophane membrane is constantly stirred and the outer fluid is frequently changed the removal of protamine proceeds very slowly. The process can be followed by nitrogen and phosphorus analyses on the solution within the membrane. At the outset the nitrogen:phosphorus

TEXT-FIG. 1. Absorption spectra in the ultraviolet of thymus nucleic acid, trout nucleoprotamine, and trout nucleoprotamine dialyzed free of protamine. In each solution the concentration of phosphorus is 0.005 mg. per ml.

ratio is 3.45 and as protamine passes through the membrane the ratio drops. After 2 days the nitrogen:phosphorus ratio is 2.40, after 4 days it is 2.08, and after 15 days 1.72.

At the same time that the nitrogen content of the solution within the membrane drops the phosphorus content remains unchanged. In one experiment, for example the solution put into the cellophane tube had 0.092 mg. P per ml. and after 10 days' dialysis it had 0.093 mg. P per ml. The unchanged phosphorus content shows that no nucleic acid passes through the cellophane membrane. Two other observations also show that all the nucleic acid is

retained by the membrane: the desoxyribose content of the solution, as estimated by the diphenylamine reaction remains unchanged; and the ultraviolet extinction coefficient at 2600 A. u. remains constant.

After dialysis the nucleic acid is precipitated by pouring the solution into 5 volumes of alcohol. A fibrous precipitate is obtained. The fibers are washed quickly in 65 per cent alcohol to remove adhering sodium chloride, and then after washing with alcohol and ether, are dried in a vacuum. Phosphorus content 8.97 per cent; nitrogen 14.47 per cent. The nitrogen:phosphorus ratio is 1.72. This composition is close to the theoretical for the sodium salt of desoxyribose nucleic acid: 9.28 per cent P; 15.58 per cent N; and a nitrogen:phosphorus ratio of 1.68.

Removal of protamine from nucleoprotamine causes no significant change in the originally high viscosity of a nucleoprotamine solution. Measurements in an Ostwald viscosimeter show that the relative viscosity in 1 M NaCl of a nucleoprotamine solution containing 0.052 mg. P per ml. is 2.85; after a prolonged dialysis in which all the protamine is lost the phosphorus content is 0.051 mg. P per ml. and the relative viscosity is 2.80. The high viscosity of nucleoprotamine solutions is obviously due entirely to the nucleic acid component of the complex and it is clear that the nucleic acid must be highly polymerized.

Removal of protamine from a nucleoprotamine complex by dialysis in presence of 1 M NaCl shows that the components of the complex readily dissociate in 1 M NaCl. The fact that protamine has no influence on the viscosity of nucleic acid in 1 M NaCl strongly suggests that dissociation is in fact complete.

Solubility of the nucleoprotamine complex is, on the other hand, much influenced by presence of protamine. The nucleoprotamine, though soluble in 1 M NaCl is insoluble in distilled water and in physiological saline (0.14 M NaCl). After removal of protamine the nucleic acid is soluble under all of these conditions. These observations on solubility show clearly that in low concentrations of salt protamine and nucleic acid are firmly combined. Increasing the salt concentration dissolves the complex because dissociation occurs. Within the sperm cell the electrolyte concentration is low and consequently protamine and nucleic acid combine to form an insoluble complex; extraction with M NaCl is effective because in the presence of so much salt the complex dissociates. In a recent paper on "The enzymatic degradation of thymus nucleohistone," Cohen (10) repeats on somewhat different material many of our experiments (which we have briefly mentioned in earlier publications (11)) on the nucleoprotamine complex and comes to essentially the same conclusion concerning the effects of M NaCl.

The dissociating effect of sodium chloride on the nucleoprotamine complex was clearly foreshadowed by Miescher. He found that when 10 per cent sodium chloride is added to the complex formed by adding protamine to

nucleic acid, the insoluble complex swells. (When this experiment is done with a nucleic acid preparation that has not been degraded, as Miescher's was, the complex dissolves.) And he discovered that when sodium chloride is added to sperm defatted by extraction with alcohol and ether that they too swell. In the clear saline fluid in which the gel-like clumps are suspended considerable quantities of dissolved protamine were detected. This fluid contained no nucleic acid, Miescher said, or only traces of it. As more salt solution was added, more protamine went into solution, but there was never more than a fraction of the total present. When the gel with its surrounding fluid was poured into a large volume of water and shaken for a moment the clumps contracted and combined with all the protamine in the surrounding fluid, so that not even a trace of dissolved protamine could be found. Our experiments show that if the sperm had not been treated previously with alcohol and ether practically all of the protamine, and the nucleic acid as well, would have been dissolved by the concentrated sodium chloride and that the dissociated components would recombine and precipitate on addition of water.

The nucleic acid isolated from salmon sperm by Miescher and his successors has the same elementary composition as has the nucleic acid that we have prepared from trout sperm. The difference between the two preparations can, however, be perceived at once in their appearance even when dry; the older preparations are powders whereas our preparation consists of fibers. The fibers dissolve readily in water to form a viscous solution which exhibits birefringence of flow; the powder dissolves only on addition of a little alkali to form a solution hardly more viscous than water and not birefringent. These observations show clearly that the strong acid and alkali used in the older preparations depolymerize the nucleic acid. And the alteration produced goes beyond depolymerization; the desoxyribose nucleic acid has been changed to some extent. The intensity of blue color given in the diphenylamine reaction by the depolymerized nucleic acid is considerably less than that given by an equivalent quantity of polymerized nucleic acid.²

The highly polymerized nucleic acid prepared from sperm resembles the nucleic acid prepared from the thymus gland by the Bang-Hammarsten method (12). In this procedure the material is extracted with water and so a drastic depolymerization is avoided. Extraction with water is altogether ineffective when applied to trout sperm. It is of interest to note that Miescher himself came very close to discovering that nucleoprotamine can be extracted from salmon sperm by a 1 M solution of sodium chloride. He noticed (page 63) in his first experiments on salmon sperm in 1874 that when sperm are treated with concentrated salt solutions they are immediately transformed into "transparent, slimy lumps of jelly" and that at the same time the sperm heads swell

² These experiments will be described in another paper.

and disappear. He did not pursue the matter, fearful apparently of the slimy mass he had produced.

The protamine that diffuses through the cellophane membrane has not yet been collected and studied. Rasmussen and Linderstrøm-Lang (4) have shown that the equivalent weight of the protamine clupein determined by electro-metric titration lies between 4000 and 4100. This determination was on clupein prepared by acid extraction. Our observations show that a protamine preparation that has not been exposed to such drastic treatment has a very low molecular weight, low enough to allow the protamine to pass through a cellophane membrane. It is therefore probable that the equivalent weight determined by Rasmussen and Linderstrøm-Lang is the molecular weight of the intact protamine.

Composition of the Sperm Nucleus.—There is such a large quantity of nucleoprotamine in the sperm head that the question arises of how much other material there is in this part of the sperm. Miescher was the first to consider this problem and it has fascinated all later workers who have approached it. The composition of the sperm head is of great interest because this portion of the sperm is very nearly a bare nucleus, the surrounding cytoplasm being so scanty. The nucleus, in turn, consists almost entirely of chromatin so that a study of the composition of the sperm head is virtually a study of the composition of chromatin.

We have attempted to determine the relative volumes of major morphological constituents of the salmonid spermatozoon for comparison with the amount of the nucleoprotamine fraction. These measurements have been made on sperm of the rainbow trout, *Salmo irideus*. The photographs upon which the measurements were based were made with sealed drops of unwashed and unfixed spermatozoa, which were stained with crystal violet (0.05 per cent in normal saline). Comparison of these photographs with those of unstained spermatozoa mounted in seminal fluid indicate that the crystal violet staining causes no measureable change of volume.

Study of the sperm head by critical focusing and by observation of heads as they are rotated by flow makes it clear that the head is a symmetrical bell-shaped body. In cross-section the head is approximately an ellipse. There are three profiles which are significant to a determination of its volume: first, the view directly on end, from which it is learned that the head is a symmetrical ellipse at all focal levels, and from which also one can accurately determine the principal axes of the ellipse where these are maximal; second and third are the widest and narrowest, full length profiles. At any point along the principal axis of the head the latter two profiles will give the major and minor axes of the elliptical cross-section of that level. Profile one is a check on the correctness of the identification of the two longitudinal profiles, for the points of greatest widths of the latter coincide (as they should) with the lengths of the

two axes (32.0 mm. and 26.0 mm. respectively) when the focus is at the level of greatest cross-sectional area. Another check on the determination of the longitudinal profiles comes from measurements of sperm heads that are oriented at random. These should approach, but not exceed, the measurements of the full length profiles; that is, the vertical (longitudinal) axis should approach 37.0 mm. and the horizontal axis, at greatest width, of any single sperm head on the photographs should lie between 32.0 mm. and 26.0 mm. Caliper measurements of photographs of 34 sperms agree with this expectation. The mean maximum width of the 34.0 specimens was 29.6 mm. which is not far from 29.0, the calculated mean between 32.0 and 26.0 mm.

From the three profiles described above the volume of the nucleus was determined as follows:³ Photomicrographs of the three profiles were enlarged to 11,400 diameters. By strong transmitted light these enlarged profiles were projected, without change of magnification, onto a millimeter graph paper, on which outlines of one-half profiles were traced. Caliper measurements of the profiles on the photographs were compared with the tracing to check the accuracy of the latter. These measurements were made to the *middle* of the blurred marginal diffraction band. We have never actually obtained longitudinal profiles in which both sides were in perfect focus at all points along the margin, but checking by comparison with the cross-section profile and by comparison with various oblique views indicates that the side in good focus in several of the best profiles has not been appreciably distorted by the slight cant of the head shown by the softer focus of the opposite side of the profile; and that, therefore, the half of the profile on the better focus side of the central axis is fairly close to a perfect one-half profile. A whole sperm head diagram was constructed from two of these half-profiles. Since the calculations involve only one-half the total width at any one level, a good half profile is all that is needed. The actual volume calculations are made from the longitudinal profiles as follows: the total length of the enlarged head was 37.0 mm. (corresponding to an actual length of 3.25μ). This is considered to be a series of 37 elliptical cylinders of 1 mm. height and with axes greatest (from the widest profile) and least (from the narrowest profile). The volume of the total head is obtained by adding together the volumes of the 37 cylinders. The total volume of the head we have thus determined as $15,743 \text{ mm.}^3$ For nuclear volume this figure is too high; for there is a central cone of cytoplasm projecting into the base of the nucleus the volume of which is determined from the profiles to be 291 mm.^3 The real nuclear volume then is $15,452 \text{ mm.}^3$ From this figure determined from the photographs the actual nuclear volume is calculated to be $10.430\mu^3$.

Next to the nucleus the principal piece of the tail is the largest part of the

³ We are indebted to Dr. K. J. Arnold for suggesting this method of determining the nuclear volume.

spermatozoon. On the accuracy of the determination of its volume, then, rests the chief value of any determination of volume of the nucleus relative to the whole cell. The tail is considerably less than 1.0 micron in diameter, and although it appears to be of equal width from any profile, it can only be said to be cylindrical within the limits imposed by the resolution of the microscope, which in the case of so small an object leaves considerable room for variation. The determination of the length of the sperm principal piece offers no special difficulty. Photographs, at magnification $\times 11,400$, of six complete principal pieces ranged from 339 μ m. to 364 μ m., with the mean at 347.7 μ m. (corresponding to an actual length of 30.5 μ m.).

Measuring to the middle of the marginal diffraction images, the diameter of the tail is 5.0 μ m. Calculating the tail volume as that of a cylinder of height 347.7 μ m. and radius 2.5 μ m., we reach a figure of 6824 μ m.³. In the

TABLE I

Part of sperm	Volume at $\times 11,400$	Actual volume	Total sperm volume
	<i>mm.³</i>	<i>μ^3</i>	<i>per cent</i>
Nucleus.....	15,452	10.430	66.92
Tail.....	6,824	4.606	29.55
Nebenkern.....	524	0.354	2.27
Cone in head.....	291	0.196	1.26
Total sperm volume.....	23,091	15.586	100.00

suspended spermatozoa the nebenkern is an approximately spherical body, the diameter of which, on the photographs, is about 10 μ m. Calculated as a sphere its volume is 524 μ m.³. The measurements of the volumes of parts of the salmonid spermatozoon are summarized in Table I. Significant in connection with our analytical studies of the nucleoprotein content of these spermatozoa are the relative volumes of sperm "head" (or nucleus plus cone) and "tail" (or principal piece plus middle piece). The former is 68.2 per cent of the total sperm, the latter 31.8 per cent. From our analyses it appears that the fat-free content of the sperm heads makes up 89.6 per cent of the total weight of the sperm, see page 113. The remaining 10.4 per cent of the total weight of the spermatozoon must include the total lipid of all parts plus the non-lipoidal content of the "tail." It appears, then, that 31.8 per cent of the volume of the spermatozoon can constitute at most 10.4 per cent of the dry weight. This low specific gravity of the non-nuclear parts of the sperm is not unexpected, in view of the reported high water and lipid content of mitochondria (13) (of which the nebenkern is constituted), and of the high lipid content which Schmiedeberg (1) found in isolated tails of the Atlantic salmon sperm.

In his final work on the composition of the sperm nucleus, Miescher's procedure was first to remove the tail and midpiece from the head by washing the sperm with water. The suspension of sperm nuclei was extracted with alcohol and ether and so converted into a white powder. From a weighed quantity of the powder protamine was extracted with hydrochloric acid and finally isolated and weighed. The nucleic acid content of the residue left after acid treatment was computed by assuming that all of the phosphorus present in the residue was in the form of nucleic acid. In Miescher's experiments the sum of the isolated protamine plus the computed nucleic acid accounted for 81 per cent of the sperm nuclear material.

With 1 M NaCl nucleoprotamine can be extracted from water-washed sperm cells, and there is no difficulty in gathering the extracted material. In this way it would be possible to compute what part of the sperm nucleus consists of nucleoprotamine, if one could be confident that a water-washed sperm cell is actually a sperm nucleus.

There are two reasons for caution in considering a water-washed sperm cell to be equivalent to a sperm nucleus: first, it is possible that some of the material removed by washing may be derived from the sperm nucleus; and secondly, careful microscopic examination of water-washed sperm shows that by no means all of the sperm tails have been washed away.

A more reliable method of finding what part of the sperm nucleus consists of nucleoprotamine would be to extract intact sperm cells with 1 M NaCl and refer the quantity of extracted nucleoprotamine not to the whole sperm mass but to the mass of nuclear material only. For this purpose it is necessary to know: (1) that the nucleoprotamine extracted is derived from the nucleus only; (2) that practically all of the nucleoprotamine is extracted by 1 M NaCl, and (3) what fraction of the sperm cell is occupied by the nucleus. These three problems will now be considered.

It has long been realized that one cannot account for more than a small part of the nucleic acid and protamine which may be extracted from suspensions of salmonid sperms unless one assumes that there has been material extracted from the nucleus. From our cytological study of the effect of strong saline upon the spermatozoa it is clear that probably the whole nucleoprotamine fraction comes from the nucleus. Even after long treatment with 1 M NaCl the sperm tails are unaltered; the heads (nuclei), however, swell and apparently become completely dissolved immediately upon exposure to the sodium chloride.

The following simple experiment demonstrates the effect of salt solution on the spermatozoa. A small drop of sperm suspension (Figs. 1-3) and another of 2 M NaCl are placed a few millimeters apart on a cover-slip, which has a small amount of vaseline around its edge, except at one point. A slide is brought in contact with the vaseline and is then inverted carrying the cover-slip. Pressure on the edges of the cover-slip seals the preparation except at one point; and further pressure flattens the drops until

they come in contact with one another. The seal is then completed by adding vaseline at the point which has served as an air escape valve during the flattening of the drops. Since this technique has involved no vigorous stirring of the drops of salt solution and sperm suspension, there is a sodium chloride gradient across the latter. The sperms farthest from the concentrated salt are quite unaltered. By contrast, the spermatozoa adjacent to the salt drop are represented only by a mass of sperm tails (Fig. 6).

In areas between these two extremes one finds a whole sequence of stages in swelling and solution of the highly condensed sperm nuclei. As the salt content rises the nuclei first become swollen, with little change of shape (Fig. 4). With further increase in size (to approximately four times the original volume) the nucleus becomes a sphere, which internally shows a most striking resemblance to a resting nucleus (Fig. 8)—or rather more to an early prophase, for it has none of the heterochromatic masses which are so characteristic of most resting nuclei. At any one focal plane the nucleus appears at first sight granular, but focussing quickly shows that the granules are actually the endwise views of threads. This observation is confirmed by the next events, for upon further swelling (*e.g.* to about 12 times the size of the sperm head) the nuclear membrane breaks down; and in place of the nucleus an entangled mass of threads, the chromosomes, then lies at the anterior end of the sperm tail. Release of the chromosomes occurs with dramatic speed; and they actually spring out of the nucleus and writhe about as if they were so many steel springs which had been compressed within the nuclear membrane. The threads appear somewhat more slender than those first seen within the swelling nucleus; and when isolated it can be seen that the released threads have the form of loose helices. There is good reason, therefore, to regard them as the chromonemata. When the salt content of the medium continues to rise a stage is soon reached where the chromosomes are no longer visible (Fig. 5).

These interesting structural changes in the sperm nucleus proceed quite rapidly when the cells are in a region of rising salt concentration. However, if sperms are placed for some time in various salt strengths below 1.0 M, the sequence of nuclear changes becomes arrested at one particular stage for each concentration. In 0.8 M NaCl the nuclei are finally slightly swollen, but never become spherical. The spherical nucleus, with chromosomes visible within it, is reached in 0.9 M NaCl. The final swelling and disappearance take place only when the salt concentration is very nearly 1.0 M.

Cytological examination shows that the residue which sediments after 1 M NaCl extraction consists largely of sperm tails. The mitochondrial body, or nebenkern, likewise is not dissolved by the sodium chloride. Nucleus, tail, and mitochondrial body make up practically the total volume of the salmonid sperm. The cytological observations indicate that, of these, only the nucleus contributes to the 1 M NaCl extract, and that this contribution is all, or nearly all, of the nuclear substance.

Practically complete extraction of nucleoprotamine can be accomplished with 1 M NaCl. The residue remaining after extraction gives only a faint Feulgen reaction, indicating that all but traces of desoxyribose nucleic acid have been extracted. The low phosphorus content of the residue is further

evidence that practically all the desoxyribose nucleic acid has been extracted. The residue represents less than 20 per cent of the sperm mass and its phosphorus content is only 0.35 per cent, to be compared with the 5.03 per cent P content of the whole sperm. And the residue contains little, if any, protamine, for the residue has only 13.1 per cent N, as against the 18.2 per cent of the whole sperm.

Being able to extract practically all of the nucleoprotamine from a known mass of sperm, our next problem is to determine what fraction of that mass consists of nuclei. This can be accomplished by washing a suspension of sperm with dilute citric acid, for the treatment removes tails and midpieces leaving a suspension of clean nuclei (as shown in Fig. 7). We used citric acid to isolate the sperm nucleus because we had previously found this technique effective in isolating the nuclei of liver, kidney, thymus, and other cells. The use of citric acid to remove cytoplasm and leave the nuclei of tissue cells was introduced by Crossmon (15) and Stoneburg (16), but the equivalent of citric acid had been used many years earlier by Miescher. He found that dilute acetic acid removes the tail and midpiece of the salmon sperm, and there can be little doubt that in using citric acid we are simply replacing acetic acid with another weak acid. After treating sperm with citric acid (and the same holds for acetic acid) the nucleoprotamine is changed so that it is no longer readily extracted by 1 M NaCl. Because of this, our procedure is to take a suspension of trout sperm, determine at once the mass of dried sperm material per cubic centimeter, and then divide the suspension into two parts: from one of these nucleoprotamine is extracted with 1 M NaCl, isolated, dried, and weighed; the other part of the suspension is treated with citric acid and the isolated nuclei are dried and weighed. In these experiments preparations are carefully washed with alcohol and ether before being dried, so that all the results obtained refer to lipid-free material. The quantity of nucleoprotamine isolated is 81.5 per cent of the mass of the sperm. The nucleus is found to be 89 per cent of the mass of the sperm cell, and nucleoprotamine accounts, therefore, for 91 per cent of the mass of the nucleus.

The trout sperm used in this experiment were centrifuged and washed once with 500 cc. of the saline mentioned above and then brought to a volume of 200 cc. Of this two 10 cc. samples were taken for dry weight determinations. The weights were 103.0 mg. and 103.4 mg., so that 1 cc. of the sperm suspension contained 10.34 mg. of sperm substance. For determination of the nuclear mass 80 cc. of the suspension (containing 825.6 mg. sperm) were taken. The sperm were washed three times with 0.2 per cent citric acid, 500 cc. being used for each washing. The sperm heads were washed with 65 per cent alcohol, hot 95 per cent alcohol, and ether. Dry weight was 740 mg. or 89.6 per cent of the sperm mass. Nucleoprotamine was extracted from two samples of the whole sperm suspension. In one case 30 cc. of the suspension (containing 309.6 mg. sperm) were taken. To this were added 30 cc. of 2 M NaCl and

190 cc. of 1 M NaCl. To another sample of the sperm suspension consisting of 70 cc. (containing 722.4 mg. sperm) were added 70 cc. of 2 M NaCl and 160 cc. 1 M NaCl. These mixtures were stirred in the Waring blender for a few minutes, stirred overnight with an ordinary mechanical stirrer, and then before being centrifuged they were again placed in the Waring blender. Centrifugation was for 60 minutes at 12,000 R.P.M. The supernatants were almost water-clear. Of the first sample 225 cc. of the supernatant were taken and of the second 250 cc. were taken. These fluids were added to 6 volumes of water. The fibrous precipitates were carefully collected, washed with 65 per cent alcohol, hot 95 per cent alcohol, ether, dried, and weighed: first sample, 227.8 mg. or 81.7 per cent of the sperm used; second sample, 491.8 mg. or 81.5 per cent of sperm used.

There are two sources of error in estimating the nucleoprotamine content of the trout sperm nucleus. In extracting and isolating nucleoprotamine a small quantity is lost. Another error may be introduced when tail and midpiece are separated from the sperm by citric acid, for some material other than nucleoprotamine may be removed from the nucleus at the same time. This would make the nucleus appear to be smaller than it actually is, and so render the estimate of the nuclear content of nucleoprotamine higher than it should be. The two errors would, accordingly, tend to balance each other.

The estimate of 91 per cent for the nucleoprotamine content of the trout sperm nucleus is considerably higher than Miescher's estimate of 81 per cent, made by an entirely different, and less direct, method. In this method protamine is extracted with dilute HCl, and the extraction is far from complete. This difficulty was recognized by Schmideberg, who edited Miescher's papers, and he attempted to compute how much protamine failed to be extracted, but the assumptions introduced in making this correction are unsatisfactory. High as the nucleoprotamine content of the trout sperm nucleus is, the remaining nuclear constituents are not negligible quantitatively. We have already isolated one other nuclear constituent. It will be described in another paper.

SUMMARY

The nucleoprotamine of trout sperm can be extracted completely with 1 M sodium chloride. On reducing the salt concentration to 0.14 M, physiological saline, the nucleoprotamine precipitates in long, fibrous strands. When the nucleoprotamine, dissolved in M NaCl, is dialyzed all the protamine diffuses through the membrane leaving behind highly polymerized, protein-free desoxyribose nucleic acid. The nucleoprotamine constitutes 91 per cent of the lipid-free mass of the sperm nucleus. While nucleoprotamine is being extracted by M NaCl a stage is reached at which the sperm chromosomes are clearly visible.

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EXPLANATION OF PLATES

PLATE 1

All figures are photomicrographs of unfixed salmonid spermatozoa, stained with crystal violet. Figs. 1 to 7 are *Salmo fario*. Fig. 8 is from *Salmo irideus*. All figures are magnified $\times 1960$. The illumination was the 5460 Å line from an H4 Mercury arc lamp isolated by Wratten Filter No. 62.

Figs. 1 to 3. Various views of normal spermatozoa, showing the three conspicuous morphological features. The nucleus is in the shape of a bell, ellipsoidal in cross-section: the narrowest profile is on the right in Fig. 3, while two of the three complete sperms of Fig. 2 are oriented with the nucleus in nearly its widest profile. The mitochondrial body, nebenkern, is a small approximately spherical structure at the broad base, or posterior end, of the nucleus. The tail is a filament approximately 11 times the length of the head.

FIG. 4. Spermatozoon from an area where the salt concentration was just high enough (approximately 0.8 M) to cause the first step in swelling.

FIG. 5. The same sperm as Fig. 4, 40 minutes later, when the salt concentration had risen because of diffusion from the 2 M droplet (to 1 M or higher), and the nucleus had dissolved.

FIG. 6. From a region adjacent to the 2 M NaCl droplet. The sperm nuclei have disappeared, leaving tails only. (The mitochondrial bodies also remain, but they are not shown here since they have become detached and have floated upward to the lower surface of the cover-slip.)

FIG. 7. Isolated nuclei, after citric acid treatment has dissolved the sperm tails.

(Pollister and Mirsky: Nucleoprotamine of trout sperm)

PLATE 2

FIG. 8. Sperm nuclei in a zone where the NaCl concentration is approaching 1 M. The nuclei have all become spherical. In the less swollen nuclei the chromosomes may be seen.

(Pollister and Mirsky: Nucleoprotamine of trout sperm)