STUDIES ON SPERM HISTONES IN AMPHIBIA AND CHONDRICHTHYES

by

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ABSTRACT

The basic protein composition of sperm, as well as the change in basic proteins during spermiogenesis, has been studied in a number of organisms, using both cytochemical and biochemical techniques.

The sperm of the seven anurans studied are divided on cytochemical criteria into three of the five classes proposed by Bloch (1969). <u>Rana palustris</u> and <u>R. pretiosa</u> are of the <u>Rana type. Xenopus laevis</u>, <u>Hyla versicolor</u>, and <u>H. regilla</u> are of the <u>Mytilus</u> type while <u>Bufo</u> <u>americanus</u> and <u>B. boreas</u> appear to be of the Salmon type.

Electrophoresis of testicular histones from representatives of these three types reveals significant differences. Testis specific components are absent in <u>R. pipiens</u>. In <u>X. laevis</u>, three testis specific bands, migrating between salmon protamine and the somatic histones, are present. A testis specific band migrating close to salmon protamine is found in B. americanus.

The basic protein changes during spermiogenesis in the eastern red spotted newt, <u>Diemictylus viridescens</u>, resemble the transitions described in the snail, <u>Helix aspera</u>, (Bloch and Hew 1960a), the squid, <u>Loligo opalescens</u> (Bloch 1962) and <u>Pleurodeles waltii</u> (Picheral 1970). The early stages of spermiogenesis contain somatic type histones which in later spermatids are replaced by the Mouse/grasshopper type of protein. In turn, these proteins are replaced by the Salmon type of protein in the spermatozoa.

Electrophoresis of testicular histones of the newt supports the cytochemical events outlined. Two testis specific bands are found.

Spermiogenesis in three cartilaginous fish (dogfish, skate and ratfish) is characterized by unusual changes in basic proteins. Early spermatids contain somatic type histones. However, late spermatids contain the Salmon type of sperm histone while spermatozoa contain the Mouse/grasshopper type.

Electrophoresis of testicular histones indicates that protamines are present in elasmobranch testes. However, a Mouse/grasshopper type of protein is not revealed.

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I GENERAL INTRODUCTION

While the history of the basic proteins found in the sperm nucleus is a long one, the function of these proteins, despite many hypotheses, remains unknown. Bloch (1969) approached the problem of function by cataloging the variety of basic proteins found in sperm and searching for possible correlations with protein type. This approach, although promising, is frustrated by the lack of information on the basic protein composition of most sperm, by the difficulty in interpretating older work in the light of recent biochemical techniques (Vendrely and Vendrely 1966), and by the problem of comparing conclusions from biochemical work with those drawn from a cytochemical approach. Therefore, the basic protein composition of sperm, as well as the changes in basic proteins during spermiogenesis, has been studied in a number of organisms, using both cytochemical and biochemical techniques.

The history of the basic proteins in the cell nucleus began in 1874 when Miescher isolated from salmon sperm a nitrogenous base which he termed protamine (from Felix 1960). From the immature testes, he isolated a protein but he was unable to find protamines. Later Kossel demonstrated that protamines were made up of amino acids and therefore were proteins. Kossel also showed that the basic protein from the unripe testes belonged to the class of basic proteins to which Kossel had given the name histone (from Luck 1964). However, the distinction between the histones and the protamines was not always apparent.

Presently, the most common definition of histones is one given by Murray (1964); "histones are basic proteins that at some time are associated with DNA." This definition includes protamines and deliberately makes no distinction between the two. Since in many respects sperm nuclei are unique, the term protamine might be used for the basic proteins associated with the DNA of sperm (Johns 1971). Even this definition causes difficulties since protamines are generally thought of only in connection with the basic proteins found in fish

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sperm. The most explicit term for the basic proteins associated with sperm DNA is sperm histone (Bloch 1969), and, therefore, the term will be used in this study. Protamines will refer to the very basic proteins found in salmon sperm and the proteins that behave similarly to salmon sperm histones.

The early work on sperm histones was largely confined to teleost fish. Kossel and his co-workers (1928) studied protamines from many species of fish and the work was continued by Felix and his students (1960). From these studies fish sperm histones were shown to contain very few kinds of amino acids, to contain about two basic amino acids to every non-basic amino acid, and to have arginine constitute about two-thirds of all amino acids. Based on these facts, the sperm histones were classed by Kossel as either monoprotamines, diprotamines, or triprotamines. Monoprotamines contained only one basic amino acid:arginine. The diprotamines contained arginine and either lysine or histidine,while triprotamines contained all three basic amino acids.

Recent work on the sperm histones of fish has been confined largely to a few laboratories. Ando and Swada (1961) separated the protamine from Pacific herrings into two main fractions, Y and Z, and then further resolved Y into Yl and Y2. The complete amino acid sequence of these three fractions was determined (Ando and Suzuki 1967) and with recent work by Ando and Watanabe (1969) the sequences of seven protamines are now known. A fundamental pentapeptide module was present in all protamine sequences elucidated. This subunit may be designated as XR_h, where X is alanine, glycine, isoleucine, proline, serine, threenine or valine and R represents arginine. Ling, Trevithick, and Dixon (1969) demonstrated that the protamine of Salmo gairdnerii was synthesized in the cytoplasm of early spermatids and rapidly transferred to the nucleus. By use of various inhibitors, these workers were able to show that protamine was synthesized on ribosomes like other proteins and that the protamine mRNA was very stable. Ingles and Dixon (1967) reported extensive phosphorylation of protamine from S. gairdnerii. All the serine residues

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were phosphorylates in vivo. The reason for this phosphorylation is unknown.

Work on the basic proteins found in the sperm of other organisms proceeded much slower than the work with fish sperm. Perhaps the echinoderms received the most attention. The work of Kossel (1928), Hamer (1955), and Vendrely and Vendrely (1966) indicated that the basic proteins in the sperm of echinoderms were very similar to those found in somatic cell nuclei. Daly, Mirsky, and Ris (1951) studied the basic proteins from rooster sperm and found them rich in arginine. Since histidine was present but lysine absent, these proteins would be classed as diprotamines.

Information on the basic protein composition in the sperm of other organisms likely proceeded slowly for two reasons. Sperm histones are most easily studied if sperm may be obtained free of other cell types. "The best starting material for the preparation of protamines is freshly drawn milt from fish that are ready to spawn, since it contains only one type of cells, the spermatozoa" (Felix 1960). With echinoderms, sperm also could be collected free from other cell types. The second requirement was the availability of large quantities of sperm in order to do biochemical analysis. Once again fish satisfied this requirement.

The development of cytochemical techniques to demonstrate basic proteins and to distinguish between histones and protamines was a major advance in attempts to study the protein types in a wide variety of organisms. Alfert and Geschwind (1953) used an anionic dye, fast green, at a basic pH (8.1) to selectively stain proteins having high isoelectric points. Histones and protamines have much higher isoelectric points than most proteins in the cell. This method does appear to be specific for histones and protamines since the selective staining of the nucleus is usually achieved and the removal of DNA is required for staining. Since DNA must be removed for staining to occur, the actual dye binding sites are likely the basic groups of histones and protamines which are normally bound electrostatically with the phosphate groups of DNA. The removal of DNA with hot 5% TCA resulted in a technique to distinguish protamines from histones. Alfert (1956) found that while the early stages of spermiogenesis in Chinook salmon stained with fast green after the extraction of DNA with hot trichloroacetic acid, the mature sperm did not. Since model experiments on filter paper had indicated that hot TCA removed protamines and retained histones (Alfert and Geschwind 1953), the lack of staining suggested the presence of protamines. A subsequent biochemical study (Callanan, Carroll, and Mitchell 1957) confirmed that protamines were indeed present in the sperm of this species. Thus protamines can be extracted from formalin fixed sections while histones cannot.

The use of cytochemistry to determine the basic protein composition of sperm was further refined by Bloch and Hew (1960a). They were able to stain protamines by removing DNA with picric acid. Picric acid is a strong precipitant of protamines (Felix 1960) and retains protamines in the sections. Deamination or acetylation of lysine residues was also carried out in order to "permit the specific staining of histones which have a very high ratio of arginine to lysine" (Bloch 1966). Under these conditions protamines did stain while the basic proteins found in the somatic cells did not. The proteins that do stain have been termed the very arginine-rich histones to distinguish them from the arginine-rich histones of somatic cells (Pipkin 1969).

Through the use of these techniques a number of very interesting observations were made on a wide range of organisms. Bloch and Hew (1960a) found that the mature sperm of the snail, <u>Helix aspersa</u>, contained protamines. However, in the transition from the normal histone complement to the protamine, spermatids went through a stage where they were not extractable with hot 5% TCA but yet contained proteins very rich in arginine. The proteins for this stage have been termed the "stable protamines" Bloch (1969). Subsequent studies showed that this transition was common to squid (Bloch 1962)

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and <u>Pleurodeles waltii</u> (Picheral 1970). In other organisms the transition proceeded only as far as the "stable protamine." This was the case in <u>Drosophila melanogaster</u> (Das, Gay, and Kaufmann 1964), the grasshopper (Bloch and Brack 1964) the coccid (Berlowitz 1965), the cricket (Kaye and McMaster-Kaye 1966), the mouse (Monesi 1964), and the rat (Vaughn 1966). In other organisms the sperm did not stain at all after deamination or acetylation. This was the case with <u>Rana</u> <u>pipiens</u> sperm which were therefore not rich in arginine. Perhaps the most exciting finding was that the sperm of many crabs had no cytochemically demonstrable basic proteins (Bloch 1966, Chevaillier 1967, Langreth 1969, Vaughn 1968). This conclusion was also supported by some biochemical evidence (Vaughn, Chaitoff, Deleen, and Garland 1969, Vaughn and Hinsch 1970).

Bloch (1969) attempted to bring order to this array of information by putting sperm into classes "according to the nature and the disposition of their histones." Since the characterization of these proteins was largely incomplete, the classes were designated by species that served as typical examples of the protein type. In order of decreasing basicity, these are the Salmon type, the Mouse/ grasshopper type, the <u>Mytilus</u> type, the <u>Rana</u> type and the Crab type.

Salmon and organisms of this type have sperm which contain the monoprotamines of Kossel. Arginine constitutes the sole basic amino acid and makes up to two-thirds of the amino acid residues. Cytochemically, these proteins are characterized by being extracted with hot 5% trichloroacetic acid, yet are retained by picric acid hydrolysis, and give a bright red reaction with the Sakaguchi reagent for arginine.

The Mouse/grasshopper type of sperm contain proteins that "are very rich in arginine but are much more complex than the monoprotamines." Although not explicit in Bloch's article, this type is characterized strictly on cytochemical critera. These sperm contain the "stable protamines." These proteins are not extracted by 5% trichloroacetic acid and they are not affected by the blocking of lysine amino groups, indicating that they are rich in arginine.

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This is confirmed by the bright red reaction these nuclei give after the Sakaguchi test for arginine.

Sperm of the <u>Mytilus</u> type contain proteins that were called diprotamines or triprotamines by Kossel. These histones may be called intermediate "because they fall between the true histones and the monoprotamines" (Bloch 1969). Cytochemically, Bloch did not define this type. However, he gave many examples of this type, citing unpublished cytochemical data as a reference. Bloch (1966) reported the sperm of the Pacific coast mussel, <u>Mytilus</u> <u>edulis</u>, did stain with fast green after trichloroacetic acid hydrolysis and continued to stain despite deamination of lysine residues. However, this staining was not as bright as that obtained with sperm of the Mouse/grasshopper type. Therefore, this will be used as the cytochemical definition of this type.

The <u>Rana</u> type of sperm contain histones that are similar to those of somatic cells. They do not stain after the amino groups have been blocked by deamination or acetylation. This is how somatic histones behave. The amino acid composition of these sperm is similar to that obtained with somatic nuclei (Vendrely 1957).

Sperm of the Crab type contain no cytochemically detectable basic proteins. Perhaps proteins of low basicity similar to the "cleavage histones" in the developing snail embryo (Bloch and Hew 1960b) are present.

The most commonly held view on the function of the basic proteins in the sperm nucleus is that they are somehow involved in the complete repression of the sperm genome. The exact origin of this view is unknown. However, this view most likely stems from the hypothesis of Stedman and Stedman (1950) that the function of the basic proteins in the cell nucleus "is to act as gene repressors." Although considerable evidence has accumulated (Georgiev 1969) that histones do inhibit DNA-dependent RNA synthesis and thus gene action, the almost total lack of histone tissue specificity argues against histones being specific gene regulators. The discovery that the arginine-rich histone (f2al) is remarkably similar in calf and pea (De Lange <u>et al. 1969</u>) also argues against specificity in the repression of the genome by histones. However, the basic proteins found in the sperm nucleus are of considerable interest since they are one of the few examples of histone tissue specificity, as well as displaying considerable species specificity. Thus the basic proteins found in sperm nuclei are unique and might prove useful in elucidating the function of histones in general.

Another popular hypothesis is that protamines are necessary for the proper "packaging" of the sperm DNA. Thus the pattern of nuclear condensation, the shape of the nucleus, and the amount of DNA present in the nucleus are of interest in order to test this hypothesis.

Sperm histones may have a "protective" role (Bloch 1969). Nebulous as this term is, possible correlations might exist between sperm histone type and external or internal fertilization. A correlation might exist between short or long-lived sperm and the histone type.

Olins, Olins, and Von Hippel (1968) have suggested that the function of the protamines is to erase the developmental history of the cell in order to restore totipotency.

Another possibility is that the type of sperm histone present is related to the beginning of RNA synthesis in the embryo or to the cleavage pattern (Bloch 1969).

On the other hand, the sperm histones might have no function and the "variability of the protein reflects an evolutionary indifference to a relatively unimportant protein in an inert nucleus" (Bloch 1969).

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II. BASIC PROTEIN COMPOSITION OF ANURAN SPERM

A. INTRODUCTION

While a <u>Rana</u> class has been designated, work on anuran sperm histones has been minimal. Referring to unpublished cytochemical data, Bloch (1969) classified the sperm histones of <u>Bufo vulgaris</u> and <u>Xenopus laevis</u> as the <u>Mytilus</u> type. Only the sperm histones of <u>Rana pipiens</u> have been studied biochemically (Vendrely 1957; Bloch 1962) and cytochemically (Bloch 1962; Zirkin 1970). The histones of these sperm were found to be similar to those of somatic cells.

Although little information is available on anuran sperm histones, Bloch (1969) used examples from anurans to test many hypotheses on sperm histone function. In order to properly test these hypotheses in the future, the variety of anuran sperm histones has been further examined.

B. MATERIAL AND METHODS

1. CYTOCHEMISTRY

a. Frogs

Sexually mature <u>Rana palustris</u> and <u>Hyla versicolor</u> were obtained from the Connecticut Valley Biological Supply Co. (Southampton, Massachusetts) and sexually mature <u>Bufo americanus</u> from the Steinhilber Co. (Oshkosh, Wisconsin) in May 1971. <u>Xenopus laevis</u> were purchased from the South African Snake Farm (Cape Province, South Africa) in October, 1970, and were maintained with a laboratory stock. <u>Rana</u> <u>pretiosa</u>, <u>Hyla regilla</u>, and <u>Bufo boreas</u> were collected in the lower Fraser Valley, British Columbia, during their breeding seasons in 1971.

The animals were etherized and the testes quickly removed and fixed in 10% neutral buffered formalin (Pearse 1968, page 601) or in absolute ethanol-glacial acetic acid (3:1). Formalin fixation was for 4-6 h at room temperature followed by a 14-17 h wash in running tap water. Tissues were then dehydrated in a graded series of ethanol, cleared in benzene, and embedded in Paraplast or Paraplast Plus (Fisher Scientific Co.). Fixation in absolute ethanol-glacial acetic acid was for 2 h at room temperature followed by 2 rinses (1 h each) in absolute ethanol. Tissues were then cleared in benzene and embedded in Paraplast. Sections (6-10u)were cut on a Spencer "820" microtome.

With the exception of <u>X. laevis</u>, results were recorded for the sperm found in the testes of sexually mature frogs in, or just before, their breeding season (see Wright and Wright 1949, for breeding seasons and a table of breeding sizes). For <u>X. laevis</u>, testes were fixed 24 h after the frogs had received injections of chorionic gonadotrophin. This is a standard method for obtaining fertile <u>X. laevis</u> males (Gurdon 1967). Thus, mature sperm were present in all frogs.

b. Feulgen reaction

Dexoyribonucleic acid (DNA) was demonstrated by the Feulgen reaction. Sections were hydrolyzed for ll minutes in l N HCl at 60° C. After two rinses (5 minutes each) in distilled H₂O, sections were stained in Schiff's reagent for 30 minutes. This was followed by many short rinses in distilled H₂O, a five minute rinse in acidbisulfite wash, and then two rinses (5 minutes) in distilled H₂O again. Sections were dehydrated, cleared, and mounted. DNA was also demonstrated by the Feulgen procedure after Bloch and Godman (1955) and by basic fuchsin in acid alcohol (Horobin and Kevill-Davies 1971). The three techniques gave comparable results.

c. Alkaline fast green reaction

The presence of histones was demonstrated by the Alfert and Geschwind (1953) procedure. Deoxyribonucleic acid was removed from sections with hot $(85-89^{\circ}C)$ 5% trichloroacetic acid (TCA), before staining for 30 minutes with 0.1% (w/v) alkaline fast green (AFG) at pH 8.1-8.3. The correct pH was obtained by titrating with 0.1N NaOH. The AFG solution was used immediately after preparation. Hydrolysis with 5% TCA was carried out with fresh reagent on each occasion. Repeated use of the same TCA solution resulted in the incomplete re-

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moval of DNA which in turn lead to reduced staining. Since the sperm of R. pretiosa, H. regilla, and H. versicolor appeared to be destroyed at 94-100°C and their morphology altered between 90-94°C. DNA was removed at lower temperatures (85-89°C) which left these sperm nuclei intact. The removal of DNA was checked by the Feulgen procedure for DNA. After hydrolysis the sections were immersed in three changes of 70% ethanol (10 minutes each) to remove TCA. Staining was followed by a five minute rinse in distilled H₂O, dehydration, and mounting. Deoxyribonucleic acid was also removed with lN trichloroacetic acid for 3 h at 60°C (Bloch 1966) and the same staining procedure followed. The two methods gave identical results. However. with this method, the difference between protamine and nonprotamine containing cells is less distinct. Treatment of sections with hot 5% TCA causes the complete removal of protamine. Being a stronger precipitant than 5% TCA, 1N TCA, while removing most protamine, causes the retention of a small amount of protamine. The 1N TCA - treatment also alters nuclear morphology, particularly the sperm muclei of R. pretiosa, H. versicolor and H. regilla.

Since protamines are washed out by trichloroacetic acid treatment, DNA was also removed by treatment with saturated picric acid for 6 h at 60°C (Bloch and Hew 1960). Sections were then stained with alkaline fast green. The picric acid solution was made just before use. As with the TCA method, the hydrolysis reagent gave poor results if reused. Cytoplasmic staining was observed unless measures were taken to remove picric acid from the sections before staining. Two methods were tried. As recommended by Pipkin (1968), picric acid was removed by rinsing sections briefly (5 seconds) in acetone containing 1% concentrated hydrochloric acid (v/v). However, this procedure resulted in reduced staining which is perhaps due to the extraction of basic proteins. Also, many sperm nuclei appeared distorted as a result of this step. However, picric acid could be removed from sections without these complications by long rinses in distilled H₂O (3-24 h). The AFG reaction was performed only with material fixed in formalin.

d. Eosin Y

Eosin Y was used after picric acid hydrolysis (Bloch and Hew 1960a) to corroborate the AFG staining. The slides, following the rinsing after the picric acid hydrolysis, were stained with 0.1% (w/v) eosin Y buffered at pH 8.1-8.3 with 0.01M tris-HCl buffer. The sections were stained for 3 h, rinsed in distilled H_20 for 5 minutes, dehydrated, and mounted. In some cases, in order to improve specificity, the distilled H_20 was brought to pH 8.3. Only formalin fixed material was used for this procedure.

e. Deamination

To determine whether very arginine-rich histones were present, deamination of lysine residues (Bloch and Hew 1960a) was carried out after TCA hydrolysis and prior to staining with AFG. Two changes of nitrous acid, prepared just before use by combining equal volumes of (10% w/v) trichloroacetic acid and 10% (w/v) sodium nitrite, were used for 15 minutes each. The stock solutions of trichloroacetic acid and sodium nitrite were never kept for more than one week. Old solutions gave erratic results. The procedure of Pipkin (1968) was also tried. This consisted of three successive 15 minute changes at 4° C in freshly prepared nitrous acid solution. However, deamination was judged incomplete in the control <u>R. pipiens</u> testis. The deamination procedure after picric acid hydrolysis gave inconsistent results. This was due to the incomplete removal of picric acid, which appeared to inhibit the deamination reaction.

f. Acetylation

Also, to determine whether very arginine-rich histones were present, acetylation of lysine residues (Bloch and Hew 1960a) was performed after picric acid hydrolysis and before staining with AFG or eosin Y. After hydrolysis, the slides were rinsed in three changes of distilled H_20 (5 minutes each), dehydrated in ethanol, and transferred to a solution containing 1% (v/v) glacial acetic acid in pure acetic anhydride. Time and temperatures were varied in order to find acetylation conditions which gave the most consistent results. Slides were left in the acetylation reagent for 1, 2, 3, 4, 8, 12, and 24 h at 60°C and at room temperature. Acetylation at room temperature for 24 h was found to be best. However, acetylation for 1 h at 60°C gave adequate results if picric acid was completely removed from the sections prior to acetylation. Since acetylation at room temperature for 24 h was used first and did give consistent results, this procedure was adopted for the rest of the study.

g. Alkaline fast green without hydrolysis

To test for free basic proteins, alkaline fast green staining was used without prior hydrolysis for the removal of DNA. Sections were brought to water, stained for 30 minutes in AFG, rinsed in distilled H₂O for 5 minutes, dehydrated, and mounted. Initially, this technique was carried out with fast green in citrate-phosphate buffer at pH8.1 as recommended by Chayen et al. (1969) for general alkaline fast green staining. This method gave unusual results. Therefore. 0.01M tris-HCl buffer was tried; yet the same results were achieved. To determine whether these observations were due to salt concentration, sections were stained in AFG made up in increasingly higher concentrations of NaCl. A range from no NaCl to 2.4M NaCl was tried. From these experiments, the general practice of using AFG adjusted to pH 8.1-8.3 with O.1N NaOH was adopted. For this test, material fixed in 10% NBF and ethanol-glacial acetic acid (3:1) was used.

h. Feulgen - alkaline fast green procedure

The method of Vaughn (1966) was followed to demonstrate non-DNA-associated basic proteins. Slides were brought to water and then hydrolyzed for 25 minutes in lN trichloroacetic acid at 60° C. Staining in trichloroacetic acid-Schiff's reagent (made up according to Pipkin 1968) for 45 minutes at room temperature followed. Next, three 5 minute rinses in sulfite bleach (prepared with trichloroacetic acid) and then three 10 minute rinses in 70% ethanol were carried out. Sections were then stained with alkaline fast green in the normal manner. After staining they were differentiated for 5 minutes in two rinses of absolute methanol, cleared in xylene, and mounted. This procedure was carried out on formalin fixed material only.

1. Sakaguchi reaction

Arginine was demonstrated by the Sakaguchi reaction as modified by Deitch (1961). Stock solutions of 4% barium hydroxide (w/v) and 1.5% 2.4-dichloro-naphthol (w/v) in tertiary butanol were made up in advance and used over a period of a month. Just before use, 1% sodium hypochlorite (v/v) was prepared. Instead of "clorox," "white magic bleach" (Safeway Ltd.) was used as a source of 5% NaOCl. Slides were brought to water and placed in an empty Coplin jar. Immediately the staining reagent was prepared (5 parts barium hydroxide, 1 part hypo-chlorite, and then 1 part dichloronaphthol) and poured on the slides. Staining took place at room temperature for 10 minutes. Slides were then transferred through 3 changes of tertiary butanol containing 5% (v/v) of tri-N-butylamine. The first change was for 5 secs and the next two for 30 secs each. Slides were cleared in two changes (30 secs each) of xylene containing 5% (v/v) tri-N-butylamine and mounted in Permount containing 10% (v/v) tri-Nbutylamine. The Sakaguchi reaction was performed on material fixed in formalin as well as material fixed in absolute ethanol-glacial acetic acid (3:1).

j. Dinitrofluorobenzene procedure

Protein-bound lysine was demonstrated by the dinitrofluorobenzene (DNFB) procedure as outlined by Pipkin (1968). Material fixed in 10% neutral buffered formalin was hydrolyzed for 15 minutes in 5% TCA at 86-89 C to remove formaldehyde. In some cases, formaldehyde was removed by immersing sections in boiling water for five minutes. This step insured the retention of all basic proteins. After three 10 minute changes of 70% ethanol, the sections were stained in DNFB solution (0.60ml DNFB in 26 ml of ethanol, 4.0ml 1M sodium bicarbonate, and 20.ml of distilled water) for 30 minutes at room temperature. The reagent was used immediately on preparation. The sections were rinsed in several changes of 70% ethanol, dehydrated, cleared in xylene, and mounted.

k. Controls

As a control for histone staining, sections were subjected to

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trichloroacetic or picric acid hydrolysis and then treated with 0.1N HCl for 15 hr to remove histones. Staining was very light or absent. In all staining procedures, sperm from a number of different species were stained at the same time in order to facilitate comparisons. A number of organisms in which the basic protein composition of the sperm was known were used as controls for most reactions. The following organisms were used: Salmo gairdnerii, whose sperm cells contain protamines (Ingles et al. 1966); R. pipiens, which contains histones in the sperm similar to those of somatic cells (Block 1962; Zirkin 1970); and Carassius auratus, whose sperm are similar to those of R. pipiens (Zirkin 1971). Since the staining pattern observed with B. boreas and B. americanus appeared unusual, alkaline fast green staining with both trichloroacetic acid and picric acid hydrolysis was repeated with material fixed in absolute ethanol-glacial acetic acid. Also, AFG staining was tried for different lengths of time, for different periods of hydrolysis, and at different temperatures.

All photographs were taken on a Zeiss photomicroscope.

2. BIOCHEMISTRY

a. Frogs

R. pipiens were obtained from E. G. Steinhilber & Co. (Oshkosh, Wisconsin) in December 1971 while B. americanus were obtained from the same company in May 1972. With R. pipiens, the animals were ether-The testes and livers were quickly removed and stored at -20°C ized. until the time of use. In December, the most common stage of spermatogenesis in the R. pipiens testis is mature sperm (Rugh 1939). The toads, B. americanus, were pithed; the testes and livers were quickly removed and used almost immediately. Since B. americanus breeds in May and June (Wright and Wright 1949), the testis at this time of the year should contain many mature sperm. A light microscope inspection of the testis confirms that sperm are the predominant cell type present. Sexually mature X. laevis males were obtained originally from the South African Snake Farm and were maintained with a laboratory stock for at least two years. During this time, they periodically received

injections of chorionic gonadotrophin which is a standard method for obtaining fertile <u>X. laevis</u> males (Gurdon 1967). The <u>X. laevis</u> males were pithed. The testes and livers were quickly removed and used almost immediately.

b. Preparation of chromatin

With slight modifications, chromatin was isolated by the procedure of Marushige and Bonner (1966). All steps were performed at 4°C or on ice. "Testes and livers were homogenized in a Sorvall Omni Mixer with saline-EDTA (0.075 M NaCl, 0.024 M EDTA, pH 8.0) at a speed setting of 5 for 2 minutes, filtered through 4 layers of washed cheesecloth, and centrifuged at 1,500 g for 15 minutes. In some cases, several drops of 2- octanol was added to prevent frothing. The nuclear pellet was washed twice by resuspending in saline-EDTA (on Vortex-Genie for 1 minute) and centrifugation. Since the nuclear pellets from livers were heavily contaminated with pigment, additional washings were employed for this tissue. The nuclear pellet was homogenized with a Dounce hand homogenizer in 0.01 M Tris buffer (pH 8.0). Chromatin was sedimented at 10,000g for 15 minutes and washed once with the same buffer. In a few cases, chromatin was further purified by centrifugation through sucrose. Chromatin was homogenized in 0.01 M Tris buffer (pH 8.0) with a Dounce homogenizer. Six ml of this chromatin was layered over 25ml of 1.7 M sucrose. The upper one-third of the tube was mixed gently and centrifuged at 22,000 rpm for 2 h in the SW-27 rotor of a Spindo L2-65 preparative ultracentrifuge. Purification through sucrose was omitted in most cases since Marushige et al. (1969) found that with trout testes in the late stages of development the purity of the chromatin was not significantly altered by this step.

c. Extraction of histones

Chromatin was homogenized in 0.2 M H_2SO_4 with a Dounce homogenizer and stirred in the same solution for 1 h at 4°C. This was followed by centrifugation at 10,000 g for 20 minutes. To the supernatant, 3-4 volumes of cold 100% ethanol was added and the mixture was placed in the freezer (-20°C) where precipitation usually occurred

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within 24 h. The precipitate was recovered by centrifugation, washed once with ethanol, and dried in vacuo. In most cases, the chromatin pellet was extracted a second time by the above procedure.

d. Polyacrylamide disc gel electrophoresis

Histones were fractionated on 15% polyacrylamide disc gels by the method of Bonner et al. (1968). Protein samples were dissolved in 8 M urea at 1 mg/ml. This concentration was approximated in cases where only a small amount of protein was available. Dithiothreitol (DTT) was added (0.05 ml of 1 M DTT/1) to the samples which were then incubated at 37°C for 30 minutes. Each gel was loaded with 0.02-0.06 ml of sample and run at 4-5 milliamperes. While gels from 5.0 - 7.4 cm in length were tried, the long gels (7.4 cm) were most suitable since these gels could be run for 80-90 minutes, which allowed for the separation of the histones, and still retain the fast moving protamine bands. Gels were stained in 1% Buffalo Black NBR in 7% acetic acid for at least two hours and destained in 7% acetic acid for approximately 40 h. A number of known histones were run as markers in order to give possible identities to the bands obtained. Calf thymus histone was obtained from Worthington Biochemical Co. and salmon protamine (grade 1) from Signa Chemical Co. Trout protamine and histone T were obtained from Dr. G. Dixon while histone 1V from pea was obtained from Dr. D. Fambrough. The X. laevis erythrocyte histone used in this study was prepared by Dr. H. Kasinsky. Trout and salmon protamine migrated together.

e. Alkaline fast green staining of gels

Gels were also stained with 0.1% fast green (w/v) buffered at pH 8.0 with 0.07 M Tris-HCl. This was performed on the Bonner et al. (1968) gel system (described above) essentially as outlined by Berlowitz et al. (1970) for the Johns (1967) gel system. After electrophoresis, the gels were removed from the tubes and placed in 1 N acetic acid for 2 h. Gels were then washed in distilled H_20 for 15 minutes and in 0.07 M Tris-HCl at pH 8.0 for 45 minutes. Some of the gels were then stained for 16 h. The remainder of the gels were deaminated with nitrous acid. Deamination consisted of immersing the gels in a freshly prepared solution of equal volumes of 10% sodium nitrite (w/v) and 10% trichloroacetic acid (w/v). This step was carried out for 45 minutes and then repeated for another 45 minutes with a fresh solution. The deaminated gels, which were exceedingly fragile, were washed in water for 5 minutes, placed in 0.07 M Tris-HCl (pH 8.0) for two 15 minute washes, and stained 16 h in alkaline fast green. Diffusion destaining was carried out in 0.07 M Tris-HCl (pH 8.0).

T. Photographing and scanning gels

Gels were photographed with a Polaroid camera. A Gilford Spectrophotometer 2400 was used to scan gels at 660 nm with a scan rate of 1 cm/min and a chart speed of 0.5 min/inch.

C. RESULTS

1. CYTOCHEMISTRY

The sperm nuclei of R. pretiosa are rod-shaped (Fig. 1) as are those of R. palustris. Feulgen staining for DNA is intense and uniform. Alkaline fast green staining for histones with 5% trichloroacetic acid hydrolysis parallels the DNA staining and is similarly uniform and intense. These nuclei also stain with alkaline fast green after 1N trichloroacetic acid hydrolysis at 60°C. However, the nuclear morphology is seriously altered (Fig. 2). With trichloroacetic acid hydrolysis the protamines in the control Salmo gairdnerii sperm are washed out. This indicates that protamines are not present in the sperm of R. pretiosa and R. palustris. After deamination the Rana sperm nuclei do not stain or stain very slightly (Fig. 3), suggesting that arginine-rich histones are absent. The nuclei stain with alkaline fast green and eosin Y after picric acid hydrolysis but not if acetylation is carried out before staining. This reinforces the conclusion that protamines and arginine-rich histones are not present in these sperm and that these sperm contain histones of the somatic type. The nuclei show intense dinitrofluorobenzene staining for lysine but stain only lightly after the Sakaguchi reaction for arginine. Protamines and arginine-rich histones of the

mouse and grasshopper type give a bright red reaction with the Sakaguchi test (Bloch 1969). With no hydrolysis, alkaline fast green staining is absent (Table 1), showing that non-nucleic acid associated basic proteins are missing. Alkaline fast green after Feulgen staining results in a uniform purple stain; thus non-DNA-associated basic proteins are also absent from the Rana sperm.

The sperm nuclei of H. versicolor (Fig. 4) as well as H. Regilla are rod-shaped but slightly bent. Feulgen staining is intense and uniform. Alkaline fast green staining after trichloroacetic acid hydrolysis is intense (Fig. 5) and follows the Feulgen staining pattern. This indicates that protamines are not present. The staining is reduced only slightly after deamination (Fig. 6), which suggests that arginine-rich histones are present. The nuclei stain with alkaline fast green or eosin Y after picric acid hydrolysis in both species and continue to stain even with prior acetylation (Table 1); this, also, suggests that arginine-rich histones are present. In addition, these nuclei stain with dinitrofluorobenzene but respond moderately to the Sakaguchi reaction. The weak Sakaguchi reaction demonstrates that the sperm histones are not of the salmon, or mouse and grasshopper type. Non-nucleic acid-associated basic proteins and non-DNA-associated basic proteins are absent.

The sperm nuclei of <u>X. laevis</u> are wavy (Fig. 7). Feulgen staining is intense and uniform; the alkaline fast green staining corroborates the Feulgen staining (Fig. 8). Alkaline fast green staining occurs with TCA hydrolysis (Table 1 and Fig. 8). This demonstrates that protamines are not present, since they are washed out by such treatment. Alkaline fast green staining, although reduced, is still evident even with prior deamination (Fig. 9), which indicates the presence of arginine-rich histones. The nuclei stain with alkaline fast green and eosin Y after picric acid hydrolysis and even with prior acetylation, which is another indication that arginine-rich histones are present. The nuclei stain with dinitrofluorobenzene and give a moderate reaction with the Sakaguchi test. Thus, like the <u>Hyla</u> sperm, the <u>X. laevis sperm</u> contain histones that are more basic

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than the <u>Rana</u> type; but they are not as basic as those of the Salmon, or Mouse/grasshopper types which give a strong Sakaguchi reaction. Non-nucleic acid-associated basic proteins and non-DNAassociated basic proteins are absent from Xenopus sperm.

As in Rana, the sperm nuclei of B. boreas and B. americanus are also rod-shaped and Feulgen staining is intense and uniform (Fig. 10). The nuclei do not stain with alkaline fast green after TCA hydrolysis (Fig. 11 and Table 1). They do stain with alkaline fast green or with eosin Y after picric acid hydrolysis in each instance (Fig. 12), which strongly suggests that protamines are present. However, the nuclei do not stain under any of the conditions of acetylation (Fig. 13) which indicates that protamines and argininerich histones are absent. This paradox persists with both fixatives and even when alkaline fast green staining is tried for different lengths of time, for different periods of hydrolysis, and at different temperatures. The nuclei stain with dinitrofluorobenzene but only moderately after the Sakaguchi reaction. However, when the Sakaguchi reaction is performed on formalin fixed material these nuclei stain strongly. Picheral (1970) found that the sperm of Pleurodeles waltlii showed a similar staining pattern, except that the sperm gave an intense red reaction with the Sakaguchi test. He therefore concluded that protamines similar to those in salmon were present in this urodele. Perhaps, Bufo sperm also contain histones of the salmon type. The nuclei of Bufo sperm are unusual in another respect. The staining observed after picric acid hydrolysis is not uniform; instead, the nuclei show clumps of intense staining (Fig. 14). Bloch and Hew (1960a) observed a coagulation effect with the sperm of the snail Helix aspersa after picric acid treatment. Perhaps a similar phenomenon is being observed with the Bufo sperm. Non-nucleic acid-associated basic proteins are absent in Bufo sperm as are non-DNA-associated basic proteins.

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TABLE 1

Cytochemistry of anuran sperm nuclei

Results using

Staining and pre- treatment	Reactive material	Rana palu- stris	Rana pret- iosa	<u>Bufo</u> ameri- canus	<u>Bufo</u> boreas	<u>Hyla</u> <u>reg-</u> <u>illa</u>	<u>Hyla</u> <u>versi-</u> color	<u>Xenopus</u> <u>laevis</u>
Feulgen	DNA	+	÷	+	+	+	+	+
AFG-TCA 86°C ¹	Basic pro- teins other than prot- amines	+	+	-	-	+	+	+
AFG-TCA 86°C, deamin- ation	Basic pro- teins rich in argin- ine	-	-		_	+	+	+
AFG-picric acid	Basic pro- teins in- cluding protamines	÷	+	•	÷	+	+	+
AFG-picric acid, ac- etylation	Basic pro- teins rich in arginine	-	-		-	+	+	+
Eosin-Y picric acid	Basic pro- teins except "cleavage histones" ²	t +	+	+	+	+	+	+
Eosin-Y picric acid, ac- etvlation	Basic pro- teins rich in arginine		*3	_	-	+	+	+
AFG	Non-nucleic acid-assoc- iated basic proteins	_	-	_	63	-	-	_

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TABLE 1 (continued)

Results using

Staining and pre- treatment	Reactive material	Rana palu- stris	Rana pret- iosa	Bufo ameri- canus	<u>Bufo</u> boreas	<u>Hyla</u> <u>reg-</u> illa	Hyla versi- color	<u>Xenopus</u> laevis
AFG after Feulgen	Non-DNA- associated basic pro- teins	***	CPS			-	-	
Sakaguchi	Protein- bound arginine	+	÷	+	+	+	+	+
Dinitro- fluoro- benzene	Protein- bound lysine	+	+	+	+	+	4	+

NOTE: AFG = alkaline fast green; TCA = trichloroacetic acid. Identical results were obtained with AFG-1 N TCA, 60°C. Bloch and Hew (1960b). Figures 1 - 3. Sections of <u>Rana pretiosa</u> testis. Scale in Fig. 1 denotes 10µ and applies to Figs. 2 and 3 as well.

Figure 1. Feulgen staining showing clusters of rod-shaped sperm nuclei.

Figure 2. Staining with alkaline fast green, 1N trichloroacetic acid hydrolysis at 60°C. Sperm nuclear morphology has been seriously altered.

Figure 3. Staining with alkaline fast green, 1N trichloroacetic acid hydrolysis, after deamination. No staining can be seen.



Figures 4 - 6. Sections of <u>Hyla versicolor</u> testis. Scale in Fig. 4 denotes 10,0 and applies to Figs. 5 and 6 as well.

Figure 4. Feulgen staining demonstrating clusters of curved sperm nuclei.

Figure 5. Staining with alkaline fast green, lN trichloroacetic acid hydrolysis at $60^{\circ}C$.

Figure 6. Staining with alkaline fast green, lN trichloroacetic acid hydrolysis at 60° C, after deamination. Sperm nuclei continue to stain although staining is reduced.



Figures 7 - 9. Sections of Xenopus laevis testis. Scale in Fig. 7 denotes 10μ and applies to Figs. 8 and 9 also.

Figure 7. Feulgen staining showing clusters of wavy sperm nuclei.

Figure 8. Staining with alkaline fast green, lN trichloroacetic acid hydrolysis at $60^{\circ}C$.

Figure 9. Staining with alkaline fast green, 1N trichloroacetic acid hydrolysis at 60°C, after deamination.
Figures 10 - 13. Sections of <u>Bufo</u> <u>americanus</u> testis. Scale in Fig. 10 denotes 10 µ and applies to Figs. 11, 12 and 13 as well.

Figure 10. Feulgen staining. Clusters of rod-shaped sperm nuclei are evident.

Figure 11. Staining with alkaline fast green, 5% trichloroacetic acid hydrolysis at 90°C. Sperm nuclei do not stain.

Figure 12. Staining with alkaline fast green, picric acid hydrolysis. Sperm nuclei stain unevenly.

Figure 13. Staining with alkaline fast green, picric acid hydrolysis, after deamination. Staining is absent.



Figure 14. A sperm nucleus of <u>B. boreas</u> stained with alkaline fast green, picric acid hydrolysis. Staining is uneven. Scale denotes 10 µ.



2. BIOCHEMISTRY

An electrophoretic comparison of the basic proteins from the testes of R. pipiens, X. laevis, and B. americanus indicates significant differences (Figs. 15, 16, 17 and 18). The region on polyacrylamide gels bound by the fastest and slowest moving bands obtained with histones from R. pipiens testes is defined as the somatic histone region (H) since previous biochemical studies had indicated that the amino acid compositions of frog sperm and somatic tissues were similar (Vendrely 1957) and the electrophoretic mobility (in an undescribed system) of histones from frog testes and somatic tissue were also similar (Bloch 1962). The H region should contain the five main histone fractions - I, IIb, IIb, III, and IV (Johns 1971). Fambrough and Bonner (1966) report that electrophoresis (as performed in this section) will separate whole calf thymus histone into three The fastest moving band contains histone IV while histone I bands. makes up the slowest moving band. The third band is composed of histones IIb,, IIb, and III. Bands migrating slower than the H region are likely to be non-histone contaminating proteins or aggregates of histones. In this study the fastest moving band in the histone region co-electrophoresed with histone IV from pea. Only histone T (Wigle and Dixon 1971), protamine (Marushige and Dixon 1969), and protamine-like proteins (Lam and Bruce 1971) are reported to migrate faster than histone IV. With X. laevis testes three bands (X_1, X_2, X_3) were found to migrate faster than histone IV while with B. americanus one band (B) has a greater mobility than IV. (Figs. 16 and 17).

A comparison on polyacrylamide gels of the histone components of testis and liver from <u>R. pipiens</u> supports the contention that the basic proteins of testes are similar to those of somatic tissues (Fig. 19). Four bands are present in both tissues, although the amounts vary. The fastest moving band is very faint in liver. The contamination of liver chromatin with pigment might account for some of this variation since certain histones might be selectively bound

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by pigment. Electrophoresis of these frog histones reveals one more band than does the electrophoresis of commercial calf thymus histone or trout testis histone. Perhaps, histone V, which is present in <u>R</u>. <u>catesbiana</u> liver (Nelson and Yunis 1969), accounts for the additional band. Four bands are less evident in <u>R</u>. <u>pipiens</u> testis. Here three bands are clearly visible with possibly a fourth band being present as a shoulder on the main band (Fig. 15). Four components also appear to be present when the gels are stained with alkaline fast green (Figs. 25 and 26). However, the two slowest moving bands do not stain when the gels are deaminated prior to staining (Fig. 26). This indicates that these bands are not rich in arginine.

An electrophoretic comparison of the histones from testis, liver, and erythrocyte of X. laevis reveals that the three fast moving bands (X1, X2, X3) are specific to the testis (Figs. 16 and 20). Three main bands are present in the testis and liver histone region (H) while four bands are present in the erythrocyte histone region (Fig. 20). The erythrocytes of frogs have been reported to contain histone V (Nelson and Yunis 1969) and this could account for the extra band in the erythrocyte H region. The fastest moving band in the histone region co-electrophoreses with histone IV from pea. (Fig. 20). When the gels are stained with alkaline fast green, the same pattern is revealed (Figs. 25 and 27), except X1 appears in amounts too small to be detected. Three bands in the histone region and bands X2 and X3 continue to stain even if the gels are deaminated prior to staining with alkaline fast green (Fig. 27). Therefore, these bands are rich in arginine. None of the testis specific bands co-electrophorese with protamine from trout or salmon but trout histone T migrates in the region of the X2 doublet (Fig. 22). However, trout histone T does not stain with alkaline fast green after deamination as does X2 which indicates that X2 is more rich in arginine than the lysine-rich histone T (Fig. 28).

A comparison of the histone components of testis and liver from <u>B. americanus</u> discloses that the fast moving band, B, is specific to the testis (Figs. 17 and 21). In the histone region (H) at least two

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major bands are present in both tissues with possibly two minor bands also present in the testis histone region. The fastest moving band in the histone region (H) co-electrophoreses with histone IV from pea (Fig. 21). A similar electrophoretic pattern is revealed when the gels are stained with alkaline fast green (Fig. 25). Deamination of the gels prior to staining with alkaline fast green does not prevent the two fastest moving bands in the histone region and band B from staining. These bands are therefore rich in arginine. Band B migrates in the protamine region (Fig. 23), although coelectrophoresis with salmon protamine is not quite achieved. Electrophoresis of X. laevis testis histones together with the testis histones of <u>B. americanus</u> indicates that band B migrates faster than the three testis specific histones from <u>X. laevis</u> (Fig. 24). Figures 15 - 17. Densitometer tracings of histones from anuran testes run on 15% polyacrylamide gels for 85 minutes. Arrow indicates band that co-electrophoresed with histone IV from pea. H indicates somatic histone region.

Figure 15. Rana pipiens testis.

Figure 16. Xenopus laevis testis.

Figure 17. Bufo americanus testis.



Figures 18 - 28. Polyacrylamide gel electrophoresis patterns of histones from anurans. Gels were run for 85 minutes and stained with buffalo black unless indicated otherwise. Gel patterns in each figure were obtained during the same run. H indicates somatic histone region. The direction of electrophoresis is from the anode (top of gel) towards the cathode (bottom of gel).

Figure 18. A. Rana pipiens testis.

- B. Bufo americanus testis.
- C. Xenopus laevis testis.
- D. Same as C, except a greater quantity of protein was placed on the gel.
- Figure 19. A. R. pipiens testis.
 - B. R. pipiens liver.
 - C. R. pipiens liver run with histone IV from pea.
- Figure 20. A. X. laevis testis.
 - B. X. laevis liver.
 - C. X. laevis erythrocyte.
 - D. X. laevis erythrocyte run with histone IV from pea.
- Figure 21. A. B. americanus testis.
 - B. B. americanus liver.
 - C. B. americanus liver run with histone IV from pea.





Figure 22. Histones from Xenopus laevis testis.

- A. Testicular histones alone.
- B. Testicular histones plus salmon protamine (P).
- C. Testicular histones plus trout histone T.

Figure 23. Histones from Bufo americanus testis.

- A. Testicular histones alone.
- B. Testicular histones plus salmon protamine (P).
- Figure 24. Testicular histones from <u>X</u>. <u>laevis</u> and <u>B</u>. <u>americanus</u> run together.



Figure 25. Histones from anuran testes stained with either buffalo black or alkaline fast green.

A, C, and E. Stained with buffalo black.

B, D, and F. Stained with alkaline fast green.

A and B. Rana pipiens

C and D. Bufo americanus

E and F. Xenopus laevis

Figure 26. Testicular histones from <u>R. pipiens</u> stained with alkaline fast green.

A. Without deamination of gels.

B. With deamination of gels.

Figure 27. Testicular histones from X. <u>laevis</u> stained with alkaline fast green.

A. Without deamination of gels.

B. With deamination of gels.

Figure 28. Trout histone T and salmon protamine (P) stained with alkaline fast green.

A. Without deamination of gels.

B. With deamination of gels.





3. SALT CONCENTRATION AND THE ALKALINE FAST GREEN REACTION

Alkaline fast green, made up in citrate-phosphate buffer, gives unusual results when used without prior hydrolysis. The sperm of trout, <u>Salmo gairdnerii</u>, stain while the sperm of <u>R. pipiens</u> do not. This is unusual since the protamines of trout sperm are not free basic proteins but instead are electrostatically bound to the phosphate groups of DNA (Fredericq 1971). If the experiment is repeated using 0.01M tris-HCl buffer in place of the citrate-phosphate buffer, the same results are achieved. However, when the experiment is repeated using AFG brought to the correct pH by titrating with 0.1N NaOH, trout sperm, as well as the sperm of <u>R. pipiens</u>, do not stain. This suggests that salt concentration is the cause of these unusual results. Since the possibility of developing a new technique to distinguish between protamine and non-protamine containing cells existed, the phenomenon was studied further.

Salt concentration is indeed necessary for staining without prior hydrolysis. As can be seen in Table II, staining only occurs at high salt concentrations. Staining is dependent on the mixture of salt and dye (see Table III). Staining is absent if sections are treated with a salt solution prior to staining with AFG made up in distilled water.

Staining appears to be confined to very arginine-rich sperm nuclei (Table IV). Deamination of trout sperm does not prevent staining and thus protamines are responsible for staining in this case. Since at high salt concentrations nuclei become distorted, the technique is used on a variety of sperm (Table IV) at an intermediate salt concentration. As has been shown in the previous sections, the sperm that give a positive reaction are rich in arginine. Despite this initial success, the technique was abandoned since a low level of staining (cytoplasmic as well as nuclear) always existed. However, this study does point out a possible source of error in testing for free basic proteins.

TABLE II

Salt Concentration and the AFG Reaction *

Stain_

Results with sperm nuclei from

AFG made	<u>Salmo gairdnerii</u>	<u>Xenopus</u> <u>laevis</u>	<u>Rana pipiens</u>
up in			
distilled H ₂ O	-	-	t 2
0.0012 M NaCl	-	-	-
0.0024 M NaCl	-	-	-
0.0047 M NaCl		-	-
0.0094 M NaCl	_ =	· •	***
0.0188 M NaCl	slight		—
0.0375 M NaCl	· +		-
0.0750 M NaCl	+	slight	-
0.1500 M NaCl	+	+	-
0.3000 M NaCl	**	+	-
0.6000 M NaCl	++	+	634
1.2000 M NaCl	++	+	-
2.4000 M NaCl	++	+	æ

* These results are obtained without hydrolysis

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TABLE III

Pretreatment and the AFG Reaction *

AFG made up in	Pretreatment	Trout sperm		
distilled H ₂ 0	none			
distilled H ₂ O	30 minutes in 0.300 M NaCl	· _		
0.30 M NaCl	none	+		
0.30 M NaCl	deamination	+		

* These results were obtained without hydrolysis.

TABLE IV

Species Results with Sperm Nuclei Sperm Rich in Arginine Xenopus laevis + Bufo boreas +

Staining with AFG made up in 0.30 M NaCl *

Rana pipiens-R. pretiosa-R. palustris-

* These results were obtained without hydrolysis.

B. americanus

D. DISCUSSION

On the basis of cytochemical criteria, the sperm of the seven anurans studied may be classified into three groups. The sperm histones of X. laevis are of the Mytilus type, which is in agreement with the data of Bloch (1969). These sperm nuclei stain with alkaline fast green under all conditions of hydrolysis, but they stain slightly less intensely after acetylation or deamination. This distinguishes them from the Mouse and grasshopper type of sperm histone which shows no reduction in staining intensity after deamination or acetylation. The sperm of H. regilla and H. versicolor demonstrate a staining pattern similar to that of X. laevis and therefore also belong to the Mytilus type. The sperm of R. palustris and R. pretiosa stain like the sperm of R. pipiens (Bloch 1962; Zirkin 1970) and thus are of the Rana type. These sperm do not stain or stain only faintly with alkaline fast green or eosin Y after deamination. The basic proteins in the sperm of B. americanus and B. boreas are unusual from a cytochemical point of view. These sperm satisfy three criteria for the Salmon type. They do not stain after TCA hydrolysis, do stain after picric acid hydrolysis, and in formalin fixed material give a strong Sakaguchi reaction. However, these sperm do not stain after picric acid hydrolysis if the sections are acetylated, which suggests that protamines are absent. Therefore, on the basis of this cytochemical data these sperm are classed as the Salmon type with reservation. Bloch (1969), on the other hand, classified the sperm histones of B. vulgaris as the Mytilus type on the basis of unpublished cytochemical data.

An electrophoretic comparison of the histones from <u>R. pipiens</u> testis and liver reveals no qualitative differences. This is in agreement with the studies of Vendrely (1957) and Bloch (1962). Vendrely found that the amino acid compositions of histones extracted from frog sperm and from somatic tissues were similar while Bloch found no electrophoretic differences between histones from testes and histones from somatic tissues. The testes of <u>R. pretiosa</u> and <u>R.</u> palustris would be expected to give electrophoretic patterns very similar to those obtained with R. pipiens since they stain similarly.

Unlike R. pipiens, electrophoresis of histones from X. laevis reveals three fast moving bands specific to the testis. These bands are not likely to be degradation products since similar bands were not found in other tissues of Xenopus nor in the other anurans examined by the same procedure. Since the sperm nuclei were the only nuclei in the Xenopus testis that did not show staining properties similar to Rana type sperm nuclei or somatic cell nuclei, these bands are likely specific to sperm. This is also supported by the observation that bands X2 and X3 do stain after deamination, indicating, that like the sperm nuclei, they are rich in arginine. On the gel system employed in this study bands X1, X2, and X3 demonstrate mobilities intermediate between protamines and somatic histones. Thus the Mytilus type of sperm histone does appear to be an "intermediate" type.

These observations with <u>X. laevis</u> are very similar to those obtained by Bloch (1966) with <u>Mytilus edulis</u>. He reported that electrophoresis of testicular histones showed three bands (β , γ , δ) whose mobilities were faster than those of somatic histones (although somatic tissues were not studied in this organism). How these three bands are related to the bands (X1, X2, and X3) obtained in this study is unknown since the electrophoretic system was not described by Bloch. However, Bloch did determine the amino acid composition of the two fastest moving bands. They contained all three of the basic amino acids: arginine, lysine, and histidine. Although the amount of histidine was small, these proteins appear to be similar to the triprotamines of Kossel. Since techniques are being developed to do amino acid analysis of bands obtained on polyacrylamide gels (Houston 1971), the amino acid composition of bands X1, X2, and X3 can also be determined.

How these bands are related to the process of spermiogenesis and how they are related to each other is unknown. As a result of labelling patterns with C^{14} -amino acids, Bloch (1966) believes that the three proteins in <u>M. edulis</u> are synthesized at the same time in the early spermatid; however, as development proceeds they are successively incorporated into the sperm chromatin. Bloch's data could also be interpreted to indicate a precursor-product relationship. Thus band β could be converted to δ and this in turn to γ . This raises the question as to whether bands X1, X2, and X3 are specific to various stages of <u>Xenopus</u> spermiogenesis or whether all three are found in mature sperm? Are the normal somatic histones completely removed during spermiogenesis or are small amounts present along with the sperm specific basic proteins? These questions remain to be answered.

One fast moving band, B, is specific to the testis of B. amer-This band migrates similarly to salmon protamine and, like icanus. salmon protamine, continues to stain despite deamination of the gels. The only cells in the testis that behave cytochemically like protamine containing cells are sperm. Therefore, this band is most likely specific to sperm nuclei. Only amino acid analysis of this band will determine how closely related band B is to salmon protamine. However, the present data support the contention that sperm histones of B. americanus are of the Salmon type. Why these nuclei do not stain after acetylation is still unexplained. The argument might be advanced that band B could be a meiotic protein, perhaps similar to the protein obtained by Sheridan and Stern (1967) from the meiotic cells in the anthers of lily and tulip. However, the latter protein is low in arginine. Also, the absence of band B in R. pipiens and X. laevis testis would be difficult to explain.

Basic proteins free from DNA have been observed in the sperm of a number of animals. Vaughn <u>et al.</u> (1969) found such proteins in the capsule of the sperm cells of <u>Emerita analoga</u>. Das <u>et al</u>. (1967) demonstrated basic proteins in the acrosomes of the sperm of <u>Urechis caupo</u>. In <u>Pleurodel waltlii</u>, Picheral (1970) saw free basic proteins in the neck and tail of the sperm. In the present study, free basic proteins were not observed in the sperm cells of the seven anurans examined. Vaughn (1966) observed basic proteins associated with RNA in the "sphere chromatophile" of the rat sperm.

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Similar proteins in the sperm of frogs were not found. Since the appearance of non-DNA-associated basic proteins has been correlated with the removal of somatic histones, such proteins would not be expected in an organism with the somatic type of sperm histone. However, such proteins might be expected in sperm with the <u>Mytilus</u> or Salmon type of sperm histone.

An explanation for the effect of salt concentration on the AFG reaction is difficult to envision. Specific staining of sperm rich in arginine is achieved without the removal of DNA at high salt concentrations. Since increasing salt concentrations are known to selectively remove histones (Fambrough and Bonner 1968), one possibility is that the dye binding sites of histones very rich in arginine are selectively exposed by high salt concentrations. In vitro dissociation of nucleohistone and nucleoprotamine by salt indicates that lysine-rich histones are removed from DNA at low salt concentrations; the arginine-rich histones at much higher concentrations; and the protamines (very rich in arginine) at intermediate concentrations (Marushige and Dixon 1971). Thus, the selective exposure of histones very rich in arginine appears an unlikely mechanism, since at low salt concentrations somatic cells would be expected to stain due to the exposure of lysine-rich histones. At high salt concentrations staining would be expected due to the exposure of arginine-rich histones. In fact, somatic cells usually do not stain under any conditions.

A direct comparison between sectioned material and chromatin may be misleading since in sectioned material the fixative must be considered. The experiments in this study were performed on material fixed in formaldehyde, which is thought to function as a fixative by forming methylene bridges between amino groups of neighboring proteins (Bowes and Carter 1965). However, besides protein-protein binding, formaldehyde links histone molecules to DNA. Brutlag <u>et al</u>. (1969) found that the histones of formaldehyde-treated nucleohistone were not dissociable from DNA by salt. The protein-DNA interaction was no longer mainly ionic as in native nucleohistone. Instead,

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methylene bridges were formed between the amino groups of histones and the amino groups of DNA. This might explain why somatic cells are not stained despite high salt concentrations. Protamines, however, contain few, if any lysine residues (Ling <u>et al.</u> 1971) and thus few free amino groups. Therefore, in formaldehyde treated material, protamines would be less capable of forming methylene bridges with DNA and might still be dissociated from DNA with salt. On this basis, the selective staining of very arginine-rich sperm histones with AFG at high salt concentrations might be explained.

In formaldehyde-treated sections, the dye binding sites of protamines and proteins very rich in arginine are exposed by high salt concentration due to the lack of protamine-DNA cross linking while the dye binding sites of somatic histones are not exposed due to extensive histone-DNA cross linking. Possibly, the protamines are not completely removed by the salt concentrations used due to some protamine-protein linkage. The requirement for the salt and the dye to be together for staining to occur can also be explained by this mechanism. When salt dissociation occurs in the presence of the dye, the stain competes successively with negative ions for positive sites on the protamine and staining occurs. When this dissociation occurs prior to staining, all the staining sites are blocked by negative ions and staining does not occur.

The mechanism is testable. If cross linking due to formaldehyde is the basis of the differential exposure of protamine, then the effect should be observed only in material fixed in formaldehyde. If protamine is not capable of forming methylene bridges with DNA in the presence of formaldehyde, then the protamine of formaldehyde-treated nucleoprotamine should be dissociable from DNA by salt. This could lead to a very selective method for isolating protamine.

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III. CHANGES IN BASIC PROTEINS DURING SPERMIOGENESIS IN THE EASTERN RED SPOTTED NEWT, DIEMICTYLUS VIRISDESCENS

A. INTRODUCTION

The histone transitions observed during spermiogenesis in the urodele, <u>Pleurodeles waltii</u>, (Picheral 1970) are very similar to the changes found in the snail (Bloch and Hew 1960a) and the squid (Bloch 1966). In these organisms the mature sperm are of the Salmon type. However, Bloch (1969) reported that the urodele, <u>Diemictylus viridescens</u>, showed a transition only as far as the "stable protamine" or the Mouse/grasshopper type of sperm histone. To check the diversity of sperm histone types in the urodeles, the basic protein changes during spermiogenesis in the eastern red spotted newt have been reexamined.

B. METHODS AND MATERIALS

1. CYTOCHEMISTRY

Sexually mature <u>Diemictylus (Triturus) viridescens</u>, the eastern red spotted newt, were obtained from E. G. Steinhilber & Co. Animals, maintained in the laboratory on beef heart and <u>X. laevis</u> tadpoles, were sacrificed in February, May, and October. The testes were removed and fixed in 10% neutral buffered formalin or absolute ethanolglacial acetic acid (3:1) as outlined in section II. All staining procedures employed on this material have been described previously.

2. BIOCHEMISTRY

Newts were obtained from the same company in the first week of January 1972. On arrival, the animals were etherized and the testes, livers, hearts, and spleens were removed and stored at -20° C until use. With one exception all biochemical procedures were carried out as described in section II. In order to collect enough material for the extraction of histones, somatic tissues were combined and then

extracted.

C. RESULTS

1. CYTOCHEMISTRY

The newt testis is organized into seminiferous tubules which, in turn, are organized into clusters of cells or cysts. All cysts within a seminiferous tubule are approximately at the same stage of spermatogenesis. In September and October all stages of spermiogenesis may be found in the newt testis while in December mature sperm predominate (Baker 1966). As a result, most observations were made on material fixed in October.

Spermiogenesis has been divided into five stages on the basis of nuclear shape and basic protein composition. These stages are shown in Figs. 29-32 and the cytochemical results for each stage are summarized in Table V.

Stage 1 nuclei, the earliest spermatids observed, are round (Fig. 29) and demonstrate uneven staining for DNA. AFG staining for histones parallels the Feulgen-staining pattern. Treatment of these nuclei with deamination or acetylation reagents abolishes staining for basic proteins, indicating the absence of very arginine-rich histones. These nuclei stain lightly after the Sakaguchi reaction for arginine. These results indicate that the histones which occur in this stage are similar to those of somatic cell nuclei.

Spermatids beginning nuclear elongation have been designated as stage 2. These nuclei are oval to cigar-shaped (Fig. 29). For stage 2 nuclei, the Feulgen and AFG-staining patterns are uneven and parallel each other. These nuclei do not stain with AFG if the sections are deaminated or acetylated and like stage 1 nuclei, they give a light response to the Sakaguchi reaction. Therefore, histones of the somatic type are present in these nuclei.

Stage 3 nuclei are narrow and cylindrical and have almost completed nuclear elongation. The arrangement of these nuclei in cysts is more precise than in stages 1 and 2 (Fig. 30). Within a cyst, the nuclei lie approximately parallel with the apices pointing towards a Sertoli cell (Fig. 30). Feulgen-staining as well as AFG-staining of stage 3 nuclei is intense and uniform. As with stages 1 and 2, AFG-staining of this stage occurs after trichloroacetic acid hydrolysis (Fig.33), indicating that protamines are absent from these nuclei. While AFG staining in the apical region of stage 3 nuclei is abolished by deamination or acetylation, the basal region continues to stain (Fig. 34). A gradient is actually observed. In some cysts only the most basal regions of the nuclei stain; in others, the posterior halves of the nuclei stain; and in some, the nuclei are completely stained except for the apices. These staining patterns suggest that the very arginine-rich histones gradually replace the somatic histones from the base to the apex of the nucleus. Within a cyst, all nuclei show the same staining pattern, suggesting that this transition is synchronous. However, the cysts within a seminiferous tubule are not quite synchronous with one another (Fig. 34) and different steps in the transition to the very arginine-rich form are present. This transition is more difficult to follow with the Sakaguchi reaction, yet the basal portion of stage 3 nuclei appear to stain more intensely than the apical regions.

Stage 4 nuclei appear as very thin cylinders, which in some cysts are slightly curved. These nuclei occur in more tightly packed cysts (Fig. 31) than stage 5 nuclei. Feulgen staining is very intense and uniform in these nuclei while the AFG staining is similarly intense and uniform. Since staining with AFG occurs after trichloroacetic acid hydrolysis, stage 4 nuclei do not contain protamines. AFG or eosin Y staining of these nuclei remains intense and uniform despite deamination or acetylation of these cells. These nuclei also stain very strongly after the Sakaguchi reaction. Therefore, they contain very arginine-rich histones of the Mouse/grasshopper type ("stable protamine").

Stage 5 nuclei appear as thin, curved rods and occur in very tightly packed cysts (Fig. 32). These nuclei represent the most advanced stage observed and were found in October and February testes. Thus, they are probably mature sperm. These nuclei demonstrate a very intense, uniform staining for DNA. However, these nuclei do not stain with AFG after trichloroacetic acid hydrolysis, which suggests that protamines are present. They do stain with AFG and eosin Y after picric acid hydrolysis and continue to stain strongly even when acetylation is carried out prior to staining. Stage 5 nuclei stain intensely after the Sakaguchi reaction. These results indicate that protamines are present in mature sperm. Unlike the previous change, the transition from the Mouse/grasshopper type of sperm histone in stage 4 nuclei to the protamines in mature sperm is very sudden and no transition steps are observed.

With formalin fixed material, AFG staining without prior hydrolysis is absent in all stages of spermiogenesis. This indicates that free basic proteins are not present. However, with the urodele, Pleurodeles waltii. Picheral (1970) observed free basic proteins in the neck and tail regions of spermatozoa. The appearance of free basic proteins in these regions was correlated with the transition to the very arginine-rich histones and then to the protamines. Therefore, in the present study the staining procedure was repeated on material fixed in absolute ethanol-glacial acetic acid (3:1). Under these conditions the tails of mature sperm do stain with AFG without hydrolysis (Fig. 35). This suggested that formalin was hiding free basic proteins by binding with the amino groups of lysine, the primary dye binding sites of lysine-rich histones. However, when formalin fixed material was treated with boiling water to remove formaldehyde, AFG staining was still absent in all stages of spermiogenesis. The tails of mature sperm do stain after the Sakaguchi reaction for argin-Thus free basic proteins that are only retained by Clarke's fixine. ative (absolute ethanol-glacial acetic acid) appear to be present in the tails of mature spermatozoa.

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TABLE V

Cytochemistry of newt spermiogenesis

Staining and pretreatment	Reactive material	1	2	Stages 3 a b	4	5
AFG-TCA 86°C	Basic proteins other than protamines	+	+	+ +	+	-
AFG-TCA 86 ⁰ C, deamination	Basic proteins rich in arginine	æ	4	\$-	+	434
AFG-picric acid	Basic proteins including protamines	+	÷	+ +	 +	+
AFG-picric acid, acetylation	Basic proteins rich in arginine	-	-		+	÷
Eosin-Y picric acid	Basic proteins except "cleavage histones"	+	+	+ +	÷	+
Eosin-Y picric acid, acetylation	Basic proteins rich in arginine	-	-	- +	+	+
AFG	Non-nucleic acid- associated basic proteins ²	-		44 ga	-	-
Sakaguchi	Protein-bound arginine	+	+	+ +	++	++
NOTE: AFG = alka	line fast green; TCA = t	richloro	acet	ic acid.		

²Bloch and Hew (1960b). These results are for formalin fixed material.

a = apical region.b = basal region.

Figures 29-32. Stages of spermiogenesis in the eastern red spotted newt. Feulgen stained. Scale denots 50 μ .

Figure 29. Cysts of round stage 1 nuclei to the left and cysts of -cigar-shaped-stage 2 nuclei to the right.

Figure 30. Cysts of stage 3 nuclei. Arrow indicates Sertoli cell nucleus.

Figure 31. Cysts of stage 4 nuclei.

Figure 32. Cysts of stage 5 nuclei.

Figures 33-34. Consecutive sections of cysts of stage 3 nuclei stained with alkaline fast green, 5% trichloroacetic acid hydrolysis. Scale denotes 50 μ .

Figure 33. Without deamination.

Figure 34. With deamination.



Figure 35. Sperm of <u>Diemictylus viridescens</u> stained with alkaline fast green without prior hydrolysis of DNA. The material was fixed in absolute ethanol: glacial acetic acid (3:1). The tail regions stain while the nuclei do not. The scale denots 50 μ .



2. BIOCHEMISTRY

The histone components of newt testis are compared electrophoretically with those from a combined preparation of newt liver. heart, and spleen (Figs. 36, 37 and 38). The histone region (H) is defined by the slowest and fastest moving bands obtained with the somatic tissues and is similar to the pattern obtained with R. pipiens testes. Four bands are present in both the testis histone region and the histone region from a combination of tissues (Fig. 38). However, two main histone bands are visible after electrophoresis for only 70 minutes (Fig. 37). In the H region the fastest moving band, which co-electrophoreses with histone IV from pea, runs as a doublet in the testis preparation. A number of bands migrating slower than the histone region are present in the testis and may represent degradation products. Two very fast moving bands, Nl, which migrates as a doublet, and N2, which migrates slightly faster than Nl, are specific to the testis. Occasionally, a faint band appears in front of N2 and only this band co-electrophoreses with protamine from salmon or trout (Fig. 37). Electrophoresis of newt and X. laevis testis histones together reveals that N1 and N2 migrate faster than the testis specific bands from X. laevis (Fig. 39). If B. americanus histones are run with a newt testis preparation. band B migrates with N2 (Fig. 39), strongly suggesting that they are similar.

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Figures 36 and 37. Densitometer tracings of histones from the eastern red spotted newt run on 15% polyacrylamide gels for 70 minutes and stained with buffalo black. H indicates somatic histone region.

Figure 36. Histones from a combination of somatic tissues -- heart, liver and spleen.

Top: run alone.

Bottom: run with histone IV from pea.

Figure 37. Testicular histones. Arrow in the histone region indicates band that co-electrophoresed with histone IV from pea. Second arrow designates the faint band which is occasionally present and which co-electrophoresed with protamine from salmon or trout.

Top: run alone. Bottom: run with salmon protamine.


Figure 38. Polyacrylamide gel electrophoresis patterns of histones from the eastern red spotted newt. Gels were run for 85 minutes and stained with buffalo black. H designates somatic histone region.

- A. testicular histones.
- B. testicular histones plus salmon protamine (P).
- C. liver histones.
- D. liver histones plus histone IV from pea.

Figure 39. Testicular histones from the newt run with testicular histones from Xenopus laevis (A) and from <u>Bufo</u> americanus (B).





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D. DISCUSSION

The basic protein changes during spermiogenesis in the eastern red spotted newt resemble the transitions described in the snail, <u>Helix aspera</u>, (Bloch and Hew 1960a), the squid, <u>Loligo opalescens</u>, (Bloch 1962), and <u>Pleurodeles waltii</u> (Picheral 1970). The early stages of spermiogenesis contain somatic type histones which in later spermatids are replaced by basic proteins very rich in arginine but not extractable with hot trichloroacetic acid. In turn, these proteins are replaced by protamines, proteins that are soluble in trichloroacetic acid and very rich in arginine.

In the eastern red spotted newt the transition from the somatic type of histone to the "stable protamine" is progressive and very similar to the conversion observed in <u>P. waltii</u> (Picheral 1970). Besides monitoring this transition by staining techniques, Picheral observed the sequential incorporation of H^3 -arginine into nuclei undergoing this histone change. Therefore, the gradual change in nuclear staining does not represent an unmasking of the very arginine-rich histones but a progressive accumulation of newly synthesized proteins. As with the nuclear basic proteins found in the sperm of the grasshopper (Bloch and Brack 1964) and trout (Ling <u>et al.</u> 1969), Picheral (1970) found that in <u>P. waltii</u> both the Mouse/grasshopper type of protein ("stable protamine") and protamines were synthesized in the cytoplasm of spermatids.

The change in <u>D. viridescens</u> from the Mouse/grasshopper type of protein to protamines is sudden and no transitional stages are observed. Picheral (1970) found a similar situation in <u>P. waltii</u>. In this species even the H^3 -arginine incorporation showed no transition.

The Salmon type of sperm histone might be characteristic of Urodeles. Bloch (1969) indicated that the sperm of <u>Amphiuma</u> were of the Salmon type while Picheral (1970) reached the same conclusion for the sperm of <u>P. waltii</u>. While Bloch (1969) (citing unpublished cytochemical data) classified the sperm of <u>D. viridescens</u> as the Mouse/grasshopper type, the present study indicates that these sperm are actually of the Salmon type. Bloch might have sampled testes in which the most advanced stage of spermiogenesis was late spermatid with the Mouse/grasshopper type of protein. This could account for his erroneous conclusion.

Picheral (1971) was also able to correlate these basic protein changes during spermiogenesis with ultrastructural observations. In the early spermatid chromatin fibers were organized in a loose network while as the gradual transition to the "stable protamine" began dense granules appeared at the base of the nucleus and moved up towards the central and apical part of the nucleus. At the same time a dense matrix was seen to spread between the granules from the tip to the basal part of the nucleus. No change in nuclear fine structure was observed on the transition to protamines.

Perhaps the most interesting observation made by Picheral (1970) was the presence of free basic proteins in the neck and tail regions of sperm. As the transition from the somatic type of histone to the "stable protamine" occured, free basic proteins appeared in the neck region of the spermatid. Subsequently, when the "stable protamine" was replaced by protamines, free basic proteins appeared in the tail region. These results strongly imply that the free basic proteins represent sloughed off nuclear histones. In the present study, free basic proteins were not observed at any stage in formalin fixed mater-Picheral employed a similar fixative; however, his fixation time ial. was considerably longer than the one used in this study. On the other hand, with Clarke's fixative, free basic proteins were present in the tail region of stage 5 spermatozoa in D. viridescens. Unfortunately, not all stages of spermiogenesis were fixed in Clarke's so whether free basic proteins are present in other stages of spermiogenesis remains unknown. Why these proteins are evident only after Clarke's fixative is not clear. However, Vaughn (as reported by Bloch 1966) found that some basic proteins displaced from the nucleus during rat spermiogenesis were retained by Carnoy's fixative (very similar to Clarke's) and not by formalin.

The electrophoretic comparison of testicular histones from \underline{D} . viridescens with somatic histones indicates that two testis specific

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bands, N1 and N2, are present. Since N2 migrates closest to salmon protamine, this band is likely confined to mature sperm which cytochemically have been shown to behave like salmon sperm. In addition. N2 migrates with band B from Bufo americanus testes, which also contain cells that stain similar to the mature sperm of D. viridescens. The NI doublet, then, is likely specific to stage 3 and 4 nuclei and represents the "stable protamine." The interpretation of these results is similar to the one given by Bloch (1962) to results obtained with the squid, which show a staining pattern similar to the eastern red spotted newt. Starch gel electrophoresis showed four major groups of histones from squid testes. The two slowest moving bands corresponded to histones from somatic tissues while the two fastest moving bands were confined to late spermatid and sperm nuclei (these stages were separated by centrifugation in a sucrose gradient). The fastest moving band was very similar in amino acid composition and molecular weight to typical protamines and was also found in a preparation consisting entirely of mature sperm. Therefore, Bloch indicated that this protein was present in cells that were cytochemically extractable with hot trichloroacetic acid and very rich in arginine. The slightly slower moving band was thought to occur in the late spermatids and to represent the "stable protamine." This protein contained all the amino acids of typical histones but had an arginine to lysine ratio of about six. This very arginine-rich histone did not contain cysteine. Picheral (1970), on the other hand, reported that in P. waltii the nuclei containing the "stable protamine" were rich in cysteine while the mature sperm were not. Whether the late spermatids of the eastern red spotted newt contain cysteine is unknown.

Two arguments may be advanced against the above interpretation of the testicular histone bands in <u>D. viridescens</u>. First, one would expect band N2 to occur in greater amounts since at the time the testes were extracted mature sperm should have been the predominant cell type (Baker 1966). However, spermiogenesis might have been delayed or interrupted if the animals were improperly maintained by the commercial dealer. Secondly, since the sperm of D. viridescens

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and salmon are cytochemically similar, one would expect the protamines of these cells to migrate together. Yet, band N2 migrates slightly slower than salmon protamine. Perhaps, an "amphibian protamine" is present that differs just slightly from salmon protamine in charge and molecular weight causing the slight difference in mobility.

IV. CHANGES IN BASIC PROTEINS DURING SPERMIOGENESIS IN THREE CARTILAGINOUS FISH

A. INTRODUCTION

In order to biochemically characterize the <u>Mytilus</u> type of sperm histone large quantities of starting material are required. An ideal organism for such a study must have large testes, be easily obtained, and of course, have the <u>Mytilus</u> type of sperm histone. Citing Kossel, Bloch (1969) classed the shark, <u>Centrophorus granulosa</u>, as containing the <u>Mytilus</u> type of sperm histone. Bloch also classed the ray, <u>Dasyatis sabina</u>, as the <u>Mytilus</u> type on the basis of unpublished cytochemical data. The elasmobranchs then appear to be suitable organisms for studying the <u>Mytilus</u> sperm histone type. Therefore, a study of the basic protein changes during spermiogenesis in the dogfish (<u>Squalus sucklevi</u>) and in the long nose skate (<u>Raja rhina</u>) was undertaken. Later, when the <u>Mytilus</u> type of sperm histone was not found in these elasmobranchs, the study included another cartilaginous fish, the ratfish (Hydrolagus colliei).

B. METHODS AND MATERIALS

1. CYTOCHEMISTRY

<u>Squalus suckleyi</u> the dogfish, <u>Raja rhina</u>, the long nose skate, and <u>Hydrolagus collici</u>, the ratfish, were collected off Comox, British Columbia, in May and November. Collections were made by means of a trawl operated from the Canadian Fisheries Research vessel, the "Investigator No. 1." After a drag of approximately 15 minutes, the net was emptied onto the deck where mature-looking specimens of each species were chosen for dissection. The testes were excised and thin sections were cut at right angles to the long axis of the testes for fixation in either 10% neutral buffered formalin or in absolute ethanol-glacial acetic acid (3:1). Since material could not be promptly returned to the laboratory, fixation occurred for 2⁴ to 32 h. However, with the ratfish, an occasion arose where material was fixed for 6, 8, 10, 12, 16, and 32 h. Generally, the tissues were washed in running tap water for 24 to 32 h in order to compensate for the increased time spent in formalin. Otherwise, the tissues were dehydrated and embedded as described in the anuran section. All staining procedures performed on this material have been described previously.

2. BIOCHEMISTRY

For biochemical work, the same organisms, collected in the same manner, were used. The testes and livers were removed as quickly as possible, under conditions that were sometimes difficult, and stored on dry ice in Thermos bottles for 24-32 h, although some material was stored in this manner for only a 6-10 h period. In the laboratory this material was stored at -20° C and in some cases at -70° C until use. Since just a small portion of a testis was used for cytochemical studies, biochemical and cytochemical work was often performed on material from the same testis. Also, for dogfish, testes were obtained in August and September as well as in November and May.

Initially, attempts were made to extract basic proteins from the dogfish testis by the procedure used by Ingles <u>et al</u>. (1966) to show the presence of protamine in trout testes. The sample of testis was homogenized with a Dounce homogenizer in the presence of 0.2 N HCl and centrifuged at 12,000g for 30 minutes. The supernatant was dialyzed against distilled water for 24 h and then lyophilized. The proteins obtained were examined by polyacrylamide disc electrophoresis as previously outlined. This procedure was tried three times and then abandoned.

Testes were also extracted in the manner used to demonstrate protamine in the mouse testis (Lam and Bruce 1970). A tissue slice (2 grams), obtained by cutting at right angles to the long axis of the testis, was homogenized gently with a Dounce homogenizer in distilled water and left to lyse for 30 minutes. The homogenate was centrifuged at 10,000g for 10 minutes and the pellet obtained was homogenized again in distilled water. This homogenate was centrifuged

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at 12,000g for 20 minutes. The pellet was homogenized in 35 ml of 0.25 N HCl and left to stand at room temperature for 2 h. The suspension was centrifuged at 12,000g for 20 minutes and the supernatant lyophilized. The proteins in the lypholized extract were examined by polyacrylamide disc electrophoresis. This same procedure was followed with livers as well as with testes.

Testes were also extracted with acid after the cells had been first disrupted with β -mercaptoethanol and urea (Lam and Bruce 1970). Pellets obtained from the first 12,000g centrifugation as described above were homogenized with a Dounce homogenizer at room temperature in 5% β -mercaptoethanol (v/v) and δ M urea in 0.1M Tris at pH 8.5. The suspension was incubated at 37°C for 2 h in a Dubnoff metabolic shaking incubator and then brought to a final concentration of 0.5 N HCl by the addition of concentrated HCl. The homogenate was left for 1 h at room temperature and then centrifuged at 15,000g for 20 minutes. The supernatant was brought to pH 5.0 by the addition of NaOH and applied to a CM30-cellulose column (1x10 cm, H⁺ form). The column was first washed with 200 ml of distilled H₂O and then the basic proteins were eluted with 0.1N HCl. The HCl fraction was lyophilized and examined by gel electrophoresis.

Basic proteins were also extracted from a crude nuclear prepar-Essentially, the first four steps used to obtain chromatin ation. (described previously) were followed in order to get nuclei. The tissue was homogenized on the Sorvall in saline-EDTA (pH 8.0), filtered through 4 layers of washed cheesecloth, centrifuged at 1,500g for 15 minutes, resuspended in saline-EDTA, and centrifuged again at 1,500g for 15 minutes. The nuclear pellet was checked under a light microscope. To each gram of nuclei, 5 ml of 0.2 M H₂SO₄ was added (as recommended for trout testis nuclei, Ling et al. 1971). After being homogenized and left at room temperature for 20 minutes, the mixture was centrifuged at 12,000g for 20 minutes. Cold 100% ethanol (3-4 volumes) was added to the supernatant which was then stored at $-20^{\circ}C$ for approximately 24 h. The precipitate which formed in this time was recovered by centrifugation (12,000g for 20 minutes), washed once

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with ethanol, and dried in vacuo.

Chromatin was prepared and extracted with acid as described in the anuran section.

C. RESULTS

1. CYTOCHEMISTRY

a. Elasmobranchii

The testes of elasmobranch fishes are composed of spherical follicles or ampullae (Stanley 1966), also termed tubules by some authors (Simpson and Wardle 1967). Within a spherical follicle all the germ elements are at the same stage of differentiation and are organized into a number of spermatocysts or cysts. The follicles arise from fixed germinal sites on the lateral or dorso-lateral aspect of the testis and move steadily away as they develop, followed closely by successively younger stages (Stanley 1966). Follicles that have reached the ventral and ventro-medial area of the testis contain the late stages of spermiogenesis and mature spermatozoa. When spermiogenesis is completed, the follicles open to the collecting ductule system and the spermatozoa are released. Thus in a section cut at right angles to the long axis of the testis all stages of spermatogenesis are present (Fig. 40).

Generally, the elasmobranch testis contains all stages of spermatogenesis throughout the year (Simpson and Wardle 1967). While an annual cycle in the dogfish testis has been observed (Simpson and Wardle 1967), only the abundance of various stages changed. Mature sperm were most frequent in testes caught between July and December and were least common in testes caught in February and May. No information is available on the presence or absence of a seasonal cycle in the skate testis.

In the present study spermiogenesis in the dogfish and skate has been divided into eight stages on the basis of nuclear morphology and basic protein composition. The stages are illustrated in figures 41-52 and the cytochemical results for these stages are summarized in Tables VI and VII.

The process of nuclear elongation has been divided into 4 stages (Figs. 41-44). Stage 1 nuclei for both species are round and loosely organized in the follicles. Stage 2 nuclei are shaped like tear drops while stage 3 nuclei are rod like. Stage 4 nuclei are thin and cylindrical and are organized into definite cysts, particularly in the case of skates. The stage 4 nuclei of the skate appear longer and straighter than those of dogfish.

Spiralization has been divided into two stages. Stage 5 nuclei are spiralized at the base and rod-like at the apexes. This process is more noticeable with skates (Fig. 49) than with dogfish (Fig. 45). Stage 6 nuclei are completely spiralized (Figs. 46 and 50).

Stage 7 and 8 nuclei are morphologically indistinuishable from each other (Figs. 47 and 51); yet differ cytochemically. They are distinguished from stage 6 nuclei by being organized into very densely packed cysts (Figs. 47, 48, 51 and 52).

Stages 1, 2, and 3 in both skate and dogfish are cytochemically similar (Tables VI and VII). These nuclei stain with AFG after all types of TCA hydrolysis, indicating the absence of protamines. However, these nuclei do not stain with AFG if they have been deaminated or acetylated indicating the absence of very arginine-rich histones. These nuclei stain weakly after the Sakaguchi reaction for arginine. Therefore, these nuclei contain histones similar to those of somatic cells.

Stage 4 nuclei stain with AFG after 1N TCA hydrolysis and after 5% TCA hydrolysis at 89°C Table VII). However, staining is light or absent after 5% TCA hydrolysis at 97°C (Table VII). This suggests the presence of a protamine. These nuclei stain with AFG-picric acid despite acetylation indicating the presence of very argininerich histones and stain moderately after the Sakaguchi reaction for arginine.

Stage 5 and 6 nuclei show identical staining properties (Tables VI and VII). They both stain weakly with AFG and 1N TCA hydrolysis

at 60°C. With 5% TCA hydrolysis at 89°C and at 97°C they do not stain at all. They stain with AFG after picric acid hydrolysis and continue to do so even after acetylation. This indicates the presence of very arginine-rich histones. This conclusion is reinfroced by the intense staining after the Sakaguchi reaction. Since staining is absent after TCA hydrolysis at 97°C, these results suggest that protamines are present in these nuclei.

Stage 7 nuclei stain well with AFG after 1N TCA hydrolysis at 60° C and stain moderately well after 5% TCA hydrolysis at 89° C (Table VI and VII). However they do not stain with AFG when the hydrolysis temperature is raised to 97° C. These nuclei are very arginine-rich since they stain with AFG - picric acid despite acety-lation. The intense red staining observed after the Sakaguchi reaction supports the contention that the very arginine-rich histones are present. These nuclei, like stages 5 and 6, appear to contain protamines.

Stage 8 nuclei stain well with AFG under all conditions of TCA hydrolysis (Figs. 48 and 52). Thus protamines are absent. These nuclei continue to stain intensely despite deamination or acetylation of sections prior to staining. This indicates the presence of very arginine-rich histones and the conclusion is reinforced by the intense red reaction these nuclei give after the Sakaguchi reagent for arginine. These results suggest that the mature sperm of dogfish and skate contain the mouse and grasshopper type of histone.

Free basic proteins were absent in all stages of both dogfish and skate spermiogenesis. This was true both for formalin and absolute ethanol-glacial acetic acid fixed material.

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Figure 40. From Stanley (1966). The zonation of the testis of <u>Scylorhinus caniculus</u> as seen in transverse section. At the left the lateral area of the testis contains the germinal zone (GZ) from which seminiferous follicles are continuously formed. Zones 1-5 indicate areas in which follicles of progressively later development are found. 1. spermatogonia; 2. primary spermatocytes; 3. secondary spermatocytes; 4. spermatids; 5. zone of sperm release and follicular degeneration. E efferent ductule. Figures 41-47. Follicles containing stages of dogfish spermiogenesis. Feulgen staining. Scale denotes $50 \,\mu$.

Figure 41. Follicles containing round stage 1 nuclei.

Figure 42. Tear drop-shaped stage 2 nuclei. L designates lumen of a follicle.

Figure 43. Follicles of rod like stage 3 nuclei.

Figure 44. Follicles of stage 4 nuclei. The organization of nuclei into cysts is noticeable.



Figure 45. Stage 5 nuclei.

Figure 46. Stage 6 nuclei.

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Figure 47. Stage 7 and 8 nuclei. These nuclei occur in densely packed cysts.

Figure 48. Alkaline fast green staining, 5% trichloroacetic acid hydrolysis at 97° C. Nuclei that stain are stage 8 while those that do not (see arrow) are stage 7. Scale denotes 50 μ .



Figures 49-51. Follicles containing late stages of skate spermiogenesis. Feulgen staining. Scale denotes 50 µ .

Figure 49. Follicle containing stage 5 nuclei which have begun spiralization. L indicates lumen of the follicle.

Figure 50. Stage 6 nuclei. Spiralization has been completed.

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Figure 51. Stage 7 and 8 nuclei. These nuclei occur in densely packed cysts.

Figure 52. Alkaline fast green staining, 5% trichloroacetic acid hydrolysis at 97° C. Nuclei that stain are stage 8 while those that do not (see arrow) are stage 7. Scale denotes 50 μ .



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TABLE VI

Staining and Reactive Stages 4 6 8 pretreatment material 1 2 3 5 7 AFG-TCA Basic proteins 97°C other than protamines AFG-TCA 97°C, Basic proteins rich in ardeamination ginine AFG-picric Basic proteins acid including protamines . AFG-picric acid, Basic proteins acetylation rich in arginine AFG Non-nucleic acidassociated basic proteins Sakaguchi Protein-bound arginine

Cytochemistry of dogfish and skate spermiogenesis

NOTE: AFG = alkaline fast green; TCA = trichloroacetic acid.

TABLE VII

Effect of hydrolysis conditions on AFG staining in

Hydrolysis conditions	1	2	3	st 4	ages 5	6	7	8
Picric acid for 6 h at 60°C	+	+	+	+	+	+	+	+
lN TCA at 60 ⁰ C for 3 h	+	+	÷	+	. <u>+</u>	±.	+	+
5% TCA at 89 ⁰ C for 15 mins	+	+	+	+	-	-	+	. +
5% TCA at 97 ⁰ C for 15 mins	+	+	÷	· • ••	-		-	

dogfish and skate spermiogenesis

NOTE: TCA = trichloroacetic acid.

b. Holocephali

Like the elasmobranch testis, the ratfish testis is composed of spherical ampullae and within each ampullae germ elements are at the same stage of development (Stanley 1963). All stages of spermatogenesis are seen in sections cut at right angles to the long axis of the testis. A seasonal cycle has not been reported in the ratfish testis.

In this study spermiogenesis in the ratfish has been divided into seven stages on the basis of nuclear morphology and basic protein composition. The stages are illustrated in figures 53-59 and the cytochemical results are summarized in Tables VIII and IX. In addition, cytochemical results are recorded for sperm in the epididymis.

Stages 1-4 of spermiogenesis represent steps in the process of nuclear elongation. Stage 1 nuclei are round and loosely organized in the follicles (Fig. 53) while stage 2 nuclei are tear drop shaped and have begun to migrate to the periphery of the follicles (Fig. 54). The rod shaped nuclei aligned at the periphery of follicles have been designated as stage 3 (Fig. 55) while the thin, cylindrical nuclei organized into cysts are stage 4 (Fig. 56). Each cyst of stage 4 nuclei points toward a Sertoli nucleus, which is not always apparent due to the diffuse chromatin staining, while the tails of these spermatozoa protrude into the central lumen of the follicle.

After nuclear elongation, spermatids go through a process of spiralization. Spiralization begins at the base of the nucleus and proceeds to the anterior tip and nuclei demonstrating this transition have been termed stage 5 (Fig. 57). Stage 6 nuclei have completed spiralization (Fig. 58) while stage 7 nuclei are organized into much more tightly packed cysts than stage 6 (Fig. 59).

The follicles as a whole appear to change in size as spermiogenesis proceeds. Follicles containing early stages of spermiogenesis are considerably larger than those containing later stages. Stanley (1966) observed a similar phenomenon in <u>Scyliorhinus</u> and <u>Torpedo</u>.

Stages 1, 2, and 3 give similar cytochemical results (Tables VIII

and IX. These nuclei stain with AFG after TCA hydrolysis, indicating the absence of protamines; do not stain with AFG if sections are previously deaminated or acetylated, indicating the absence of very arginine-rich histones; and stain lightly after the Sakaguchi reaction for arginine. These nuclei exhibited the typical somatic cell staining pattern and therefore contain the somatic type of histones.

Stage 4 nuclei give unusual staining results (Tables VIII and IX). Staining is dependent on the temperature at which TCA hydrolysis is carried out. Stage 4 nuclei stain with AFG after 1N TCA hydrolysis at 60°C or at 89°C; and stain faintly or not at all after TCA hydrolysis at 97°C. These nuclei stain with AFG after picric acid hydrolysis and continue to stain even if acetylated. This suggests they contain very arginine-rich histones. However, these nuclei give only a moderate Sakaguchi reaction.

Stages 5 and 6 nuclei also give unusual staining results (Tables VIII and IX). These nuclei stain weakly after 1N TCA hydrolysis at 60°C and do not stain at all after 5% TCA hydrolysis at 89°C or 97°C. These nuclei stain with AFG after picric acid hydrolysis and continue to do so despite acetylation, indicating the presence of very arginine-rich histones. These nuclei also stain strongly after the Sakaguchi reaction for arginine. This staining pattern suggests the presence of protamines.

Stage 7 nuclei and nuclei found in the epididymis give identical cytochemical results. These nuclei stain under all conditions of TCA hydrolysis and continue to do so even if the sections are deaminated prior to staining. Staining with AFG after picric acid hydrolysis continues despite acetylation. An intense red reaction is given by these nuclei after treatment with the Sakaguchi reagent. These results indicate the presence of a very arginine-rich histone similar to the mouse and grasshopper type.

Free basic proteins were not observed in any of the stages of spermiogenesis.

The effect of fixation on AFG staining and deamination was

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studied on nuclei found in the epididymis. Fixation times between 6-32 h did not affect the results.

Figures 53-59. Follicles containing stages of ratfish spermiogenesis. Feulgen staining. Scale denotes 10μ .

Figure 53. Round stage 1 nuclei. L indicates lumen of the follicle.

Figure 54. Tear drop-shaped stage 2 nuclei.

Figure 55. Rod-shaped stage 3 nuclei.

Figure 56. Stage 4 nuclei organized into cysts.



Figure 57. Stage 5 nuclei. These nuclei are undergoing nuclear spiralization.

Figure 58. Stage 6 nuclei. Spiralization has been completed.

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Figure 59. Stage 7 nuclei.

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TABLE VIII

Staining and	Reactive		•		Stages		r	* *
pretreatment	material	T.	۲	<u> </u>		2	0	-7-
AFG-TCA 97°C	Basic proteins other than protamines	` +	. +	. +	+	e #	-	+
AFG-TCA 97°C, deamination	Basic proteins rich in arginine	-		-	-	-		÷
AFG-picric acid	Basic proteins including protamines	+	+	+	+	•	+ .	+
AFG-picric acid, acetylation	Basic proteins rich in arginine	-	-	• 	+	+	÷	. +
AFG	Non-nucleic acid- associated basic proteins	_	-	-	-	-	-	-
Sakaguchi	Protein-bound arginine	÷	+	÷	+	++	++	++

Cytochemistry of ratfish spermiogenesis

NOTE: AFG = alkaline fast green; TCA = trichloroacetic acid.

* Sperm found in the epididymis gave identical results to stage 7.

TABLE IX

Effect of hydrolysis conditions on AFG staining in ratfish spermiogenesis

Hydrolysis	Stages								
conditions	۲ 	2	<u> </u>		2 	6			
Picric acid for 6 h at 60°C	+	+	+	+	+	+	+		
lNTCA at 60 ⁰ C for 3 h	+	+	+	+	+ -	+	+		
5% TCA at 89 [°] C for 15 mins	+	+	+	+	-	-	+		
5% TCA at 97 ⁰ C for 15 mins	+	+	÷	-	-	-	+		

NOTE: TCA = trichloroacetic acid.

2. BIOCHEMISTRY

The histone components of skate testes and livers are compared in figures 60-62. Two major bands are evident after the electrophoresis of liver histones on polyacrylamide gels and the region bound by these bands will be defined as the histone region. The histone region of testes also contains two bands. The fastest moving band in the histone region from both tissues co-electrophoreses with histone IV from pea. A number of bands migrating slower than the histone region are likely non-histone contaminating proteins. Band S which migrates faster than histone IV is specific to the testis and co-electrophoreses with salmon protamine (Fig. 62). Therefore, band S has two characteristics of protamine - specificity to the testis and identical electrophoretic mobility with protamine.

The electrophoresis of histones from dogfish liver and dogfish testis is illustrated in figures 63-65. Liver histones migrate as two bands and the area bound by these bands will be defined as the dogfish histone region. Two bands are also present in the testis histone region and the fastest moving band in this region from both testes and livers migrates with histone IV from pea. The bands migrating slower than the histone region are contaminating proteins. A very fast moving band (D) is specific to the testis and migrates with salmon protamine (Fig. 65). This protein appears identical with band S of skate.

The use of other extraction methods reinforces the conclusion that only 1 protamine band is present in skate and dogfish testes. Figures 66 and 67 illustrate the electrophoretic patterns obtained after the acid extraction of nuclei. In both skate and dogfish one fast moving band that co-electrophoreses with salmon protamine is present. In the case of the skate testis, basic proteins were extracted by the two methods used by Lam and Bruce (1971) to extract mouse protamine. Both methods yielded only one major band in the protamine region (Fig. 68). Extraction of livers by these methods did not yield a protamine-like protein.

Band D is present in testes caught in May, August, and November.

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Although no attempt was made to quantitate the results, band D appears to be most easily extracted from testes caught in May.

Since the stages of spermatogenesis in the elasmobranch testis are arranged in layers from the lateral to the ventral areas (Stanley 1966), attempts were made to isolate cell types by cutting the testis into long strips. However, this method proved unsuccessful since the correct orientation of frozen testes was very difficult. The application of the sedimentation velocity technique used by Lam and Bruce (1970) to separate mouse spermatogenic cells might prove useful. Figures 60-62. Densitometer tracings of skate histones run on 15% polyacrylamide gels for 80 minutes and stained with buffalo black. The histones were extracted from chromatin. H indicates somatic histone region. Arrow indicates that the band co-electrophoreses with histone IV from pea.

Figure 60. Liver histones.

Figure 61. Testicular histones.

Figure 62. Testicular histones run with salmon protamine.



Figures 63-65. Densitometer tracings of dogfish histones run on 15% polyacrylamide gels for 80 minutes and stained with buffalo black. The histones were extracted from chromatin. H indicates somatic histone region. Arrow indicates that the band co-electrophoreses with histone IV from pea.

Figure 63. Liver histones

Figure 64. Testicular histones.

Figure 65. Testicular histones run with salmon protamine.


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Figures 66 and 67. Densitometer tracings of testicular histones from skate and dogfish run on 15% polyacrylamide gels for 80 minutes and stained with buffalo black. The histones were extracted from crude nuclear preparations. H indicates somatic histone region. Arrow indicates that the band co-electrophoreses with histone IV from pea.

Figure 66. Skate testicular histones. Top: electrophoresed alone. Bottom: electrophoresed with salmon protamine.

Figure 67. Dogfish testicular histones.

Top: electrophoresed alone.

Bottom: electrophoresed with salmon protamine.



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Figure 68. Polyacrylamide gel electrophoresis patterns of histones from the skate. Gels run as in Figures 60-67.

- A. Liver histones extracted from a crude nuclear preparation.
- B. Testicular histones extracted as in A.
- C. Testicular histones extracted by the method of Lam and Bruce (1971).
- D. Testicular histones extracted from chromatin.



D. DISCUSSION

The basic protein changes during spermiogenesis in the three cartilaginous fish studied are remarkably similar and very unusual. In all three specie the transition to a very arginine-rich histone begins at the ends of nuclear elongation and the beginning of nuclear spiralization. However, instead of the appearance of "stable protamines" or the Mouse/grasshopper type of sperm histone followed by a transition to protamines, as is the case in the snail (Bloch and Hew 1960a), <u>Pleurodele waltii</u> (Picheral 1970), the squid (Bloch 1962) and the eastern red spotted newt (part III of this thesis), protamines appear first followed by stable protamines.

Three lines of evidence suggest that this is the correct sequence of events and not just an error in staging. If sections cut at right angles to the long axis of the testis are scanned from the germinal zone to the opposite edge of the testis, a layer of protamine containing cells appears first, followed by the appearance of stable protamine containing sperm at the very outer edge of the testis. The direction of this scan is from the most immature stage of spermatogenesis to the most mature (Stanley 1966). In the case of the dogfish, sperm of the Mouse/grasshopper type are found in collecting ductules while in both dogfish and skate sperm of the Mouse/grasshopper type are observed in the lumen of follicles, indicating that they are mature and are being released (Stanley 1966). Finally, the sperm in the epididymal region of the ratfish are found to be the Mouse/grasshopper type while the testis displays earlier stages of spermiogenesis where protamines are present. Thus in cartilaginous fish the basic protein changes during spermiogenesis appear to be the reverse of those reported in other organisms (histone protamine stable protamine).

The principal argument against this unusual transition is the lack of supporting biochemical evidence.Using the same gel system as in the present study, Lam and Bruce (1971) reported that the basic protein isolated from mouse sperm migrated slightly slower than salmon

protamine. Since sperm were found in dogfish and skate testes that stained exactly like mouse sperm, a similar protein would be expected to be found in these testes. No such band was obtained, although a hint of such a band was obtained with one skate preparation. This protein might have been missed for at least two reasons. Perhaps, the cells containing the Mouse/grasshopper type of protein were absent or present in only small numbers in the tissues extracted. However, in some cases cytochemistry was performed on a small portion of the testis to be extracted and the presence of the stable protamine was confirmed. This protein might have been missed for another reason. In some cases, basic proteins from mammalian sperm, the Mouse/grasshopper type, can only be isolated under extremely acid conditions and even the methods of Lam and Bruce (1971) will fail (Ti Wing Wu, personal communications). A similar situation might exist for the mature sperm of cartilaginous fish. The observation that protamines were most easily extracted from testes obtained in May would be consistent with this explanation. The testes at this time would be expected to contain a predominance of cells in the middle of spermiogenesis, which would be easy to extract, while testes in November would contain an abundance of mature sperm (Simpson and Wardle 1967) which would contain the difficult to extract Mouse/grasshopper type of protein.

An alternate possibility is that the appearance of stable protamines is an artifact. Perhaps, as the sperm nuclei become very condensed they resist trichloroacetic extraction for reasons other than the presence of the Mouse/grasshopper type of protein. This would explain why despite numerous extraction methods only a protamine band was obtained with skate and dogfish testes. However, why these nuclei would be resistant to extraction is difficult to explain.

These possibilities may be tested by the extraction of basic proteins from mature sperm only. Sufficient mature sperm might be collected from the epididymal region of the ductus deferens. If a protein is obtained from these sperm similar in amino acid composition and electrophoretic mobility to the testis specific bands, the

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unusual sequence of events is likely an artifact. However, if from these sperm a protein is obtained which is different from the testis specific protein, the sequence of events outlined above is likely to be the correct one.

A third possibility is that the appearance of protamines is an artifact. Some stages of spermiogenesis might be sensitive to trichloroacetic acid hydrolysis for reasons other than the presence of protamines. The reorganization of nuclear material in the process of spiralization might be the cause of this. However, if the appearance of protamine is an artifact, the co-electrophoresis of testis specific bands with salmon protamine must be regarded as merely fortuitous. This is an unlikely possibility.

Therefore, on the basis of the cytochemical results, the sperm of the dogfish, long nose skate, and the ratfish will be tentatively classed as the Mouse/grasshopper type. Future studies should clarify the discrepancies between this conclusion and the biochemical results.

The conclusion of this study is not in agreement with the little available information on the basic protein composition of sperm from other cartilaginous fish. On the basis of Kossel's (1928) work, Bloch (1969) classified the shark, <u>Centrophorus granulosus</u>, as the <u>Mytilus</u> type. However, Vendrely and Vendrely (1966) have pointed out that much of Kossel's work should be repeated since the isolation techniques utilized in his day were harsh and artifacts in amino acid composition were possible. On the basis of unpublished cytochemical data, Bloch (1969) classified the ray, <u>Dasyatis sabina</u>, as the <u>Mytilus</u> type. Comment on unpublished results is difficult. However, in this study no variation in sperm histone type was observed in a wide range of cartilaginous fish. Therefore, differences between this study and Bloch's results are not likely to be explained on the basis that different organisms have been studied.

The observation that protamines are present in elasmobranchs might lead to some fascinating comparative studies. Can the protamines of elasmobranchs be fractionated, Protamines from teleost fish have been fractionated by a number of workers (Ando and Sawada 1961, Ando and Suzuki 1966, Ling <u>et al</u>. 1971). The amino acid sequence of protamines from elasmobranch fish would make an exciting study since a fundamental pentapeptide has been found in all the protamines so far elucidated. This sub-unit occurs some three or four times per molecule, while other pieces are fragments of the unit resulting from partial duplication of the cistron (Black and Dixon 1967). Protamines have been sequenced from the rainbow trout (<u>Salmo gaird</u>nerii), the Pacific herring (<u>Clupea pallasii</u>), and Chum salmon (Oncorhynchus keta) (Phillips 1971). These organisms are bony fish.

The basic protein changes of this study may be related to recent ultrastructure observations on dogfish spermiogenesis (Stanley 1971a, 1971b). Stanley observed that the chromatin of early spermatids was finely granular and evenly dispersed. While nucleoprotein fibers were initially randomly oriented, fibers at a later stage were aligned anteroposteriorly. When the nucleus reached a length of around 19 μ the intra nuclear fibers were joined together laterally to form a maze of sheetlike configurations. These fibrous sheets followed a helical course along the length of the nucleus. Later these sheets were compacted into a solid column of chromatin. At this point the nucleus was almost completely elongated and the nuclear volume had decreased from about $180 \mu^3$ to approximately $10 \mu^3$.

The transition to the very arginine-rich histones begins at about this stage which indicates that these proteins are not involved in early nuclear condensation. Next, Stanley observed the spiralization of chromatin, beginning at the posterior end of the nucleus and proceeding to the anterior tip. Although the appearance of protamines is correlated with the process of nuclear spiralization, a cause and effect relationship is unlikely, since the spiralization is foreshadowed much earlier by the helical alignment of fibrillar nuclear sheets. The chromatin material of mature sperm was highly compacted, but thin sections still gave evidence of longitudinal fibrils. The center of the sperm nucleus appeared more electron dense than the periphery and Stanley suggested that the peripheral material may be residual nuclear protein. Such protein might be the protamines that this study suggests are replaced in the late stages of spermiogenesis

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by the Mouse/grasshopper type of protein.

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TABLE X

The Variety of Sperm Histones

Organism *

Sperm Histone Type

CLASS CHONDRICHTHYES

Subclass Elasmobranchii

Squalus suckleyi (or acanthias)

Raja rhina

Subclass Holocephali

Hydrolagus colliei

Mouse/grasshopper Mouse/grasshopper

Mouse/grasshopper

CLASS AMPHIBIA

Subclass Lepospondyl: <u>Diemictylus viridescens</u> Subclass Apsidospondyli <u>Xenopus laevis</u> <u>Rana pretiosa</u> <u>Rana palustris</u> <u>Bufo boreas</u> <u>Bufo americanus</u> <u>Hyla regilla</u> Hyla versicolor

Salmon

<u>Mytilus</u> <u>Rana</u> <u>Rana</u> Salmon Salmon <u>Mytilus</u> Mytilus

* Classification is from Romer (1967).

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V. GENERAL DISCUSSION

The variety of sperm histone types which have been demonstrated in the present study are summarized in Table X. These results are discussed below with respect to hypotheses on the function of sperm histones.

While the results in Table X yield no information on the postulated repressor role of sperm histones, the data can be used in the future to critically test this hypothesis. If the function of the histone transition is to repress the sperm genome, one would expect the sperm genome to be repressed only in organisms which showed a trans-Indeed, RNA synthesis is not observed in sperm of the Mytilus ition。 type (Das et al. 1965), the Mouse/grasshopper type (Bloch and Brach 1964) and the Salmon type (Marushige and Dixon 1969). RNA synthesis is also absent in sperm of the crab type which are free of basic proteins (Vaughn and Thomson 1972). In all these cases a transition from the somatic histone type occurs. On the other hand, in the Rana type no transition is observed. Whether the sperm genome is completely repressed in this type remains to be seen. However, if the sperm histone transition serves to repress the sperm genome, one would predict that the sperm genome is not repressed in the case of the Rana type.

The sperm histone types might reflect different packaging requirements for sperm DNA. This hypothesis may be discussed in terms of nuclear shape, nuclear condensation pattern, and the amount of DNA per nucleus.

No correlation appears to exist between sperm histone type and nuclear shape. Goldfish (Zirkin 1971b) and frogs of the genus <u>Rana</u> have sperm histones similar to somatic cells. Yet, the sperm nuclei of goldfish are round while those of the frogs are rod-shaped. The sperm of trout contain protamines, as do the sperm of <u>Bufo americanus</u> and <u>B. boreas</u>. Despite this similarity in protein type, trout sperm nuclei are round while Bufo sperm nuclei are rod-shaped.

A correlation between nuclear condensation pattern and sperm histone type might exist. As noted in the sections on the eastern red

spotted newt and the cartilaginous fish, basic protein transitions are accompanied by marked changes in nuclear fine structure. A similar observation was made by Bloch and Hew (1960a) for the snail. Zirkin (1971a, 1971b) has studied the nuclear fine structure in sperm that contain the Rana type of histone. Unlike the above organisms, highly oriented fibers and sheets were not evident. Instead randomly oriented fibers with diameters slightly smaller than those from somatic cell nuclei were present. In crab sperm the loss of basic nuclear proteins was correlated with changes in chromatin (Langreth 1969). The chromatin changed from homogenous fine granules and fibrils of fairly low electron opacity to clumps of fibers in an electron-translucent nucleoplasm. Thus changes in nuclear fine structure do appear to be correlated with histone type. This correlation suggests the DNA is packed into the sperm head in a manner dependent on the sperm histone type.

Walker (1971) believes three basic patterns of nuclear condensation are evident in spermiogenesis. The first pattern is the "fibrous" type. Fibers or filaments occur in the early spermatid and become thicker and eventually fuse to give the mature sperm head. In the second pattern, the "lamellar" type, the fibers present in the early spermatid fuse into sheets or lamellae as spermiogenesis proceeds. The third pattern is the "granular" type. In this type the spermatid undergoes all alteration in shape prior to the start of any condensation of the nucleoprotein. Walker found little correlation between these condensation patterns and sperm histone type.

A relationship between sperm histone type and the amount of DNA per nucleus is not evident. Olins <u>et al.</u> (1968) have calculated that the density of packaging in trout sperm is $0.7g \text{ DNA/cm}^3$. Since in the sperm head the ratio of protamine arginine/DNA phosphate was about one, they suggested that protamines may facilitate the packing of DNA by reducing phosphate-phosphate electrostatic repulsions. Thus the formation of aggregated structures would be favored. However, using the nuclear volume given by Zirkin (1970) and calculating the DNA content from the results of Bachman (1970), one may compute the density

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of packaging in <u>R. pipiens</u> sperm. In these sperm with the somatic histone type the density of packaging is $0.66g \text{ DNA/cm}^3$ and very similar to that in trout sperm which contain protamine.

Bloch (1969) postulated that the variety of sperm histone types might reflect different protective roles. Thus organisms which have external fertilization might require a specific histone type. Alternatively, organisms with internal fertilization might require a unique histone. Perhaps, sperm which are stored in the female for long periods before fertilization need a special sperm histone.

No correlation exists between sperm histone type and external or internal fertilization. The anurans studied all have external fertilization; yet, they display a wide range of histone types. The eastern red spotted newt and the three cartilaginous fish studied have internal fertilization. The Salmon type of sperm histone is found in the newt while the cartilaginous fish have the Mouse/grasshopper type. The honey bee also has internal fertilization and yet, contains the Rana type of sperm histone (Bloch 1969).

Long-lived sperm do not appear to require a special sperm histone. Sperm in the spermatheca of the queen honey bee have been reported to remain viable for upwards of a year (Taber and Blum 1960). Snakes have been reported to continue bearing offspring more than four years after mating (Wright and Wright 1957). Sperm storage has been reported in the female dogfish (Metten 1939) and might be of general occurrence in elasmobranchs (Grover 1970). Yet bees have the <u>Rana</u> type of histone, snakes the Salmon type (Bloch 1969), and the dogfish the Mouse/grasshopper type.

Bloch (1969), among others (Olins <u>et al.</u> 1968), has suggested that the sperm histone transition may erase the developmental history of the cell, thereby restoring totipotency to a highly specialized cell. This view requires that "spermatogonial cells and spermatocytes be considered differentiated in the same sense as somatic cells " (Bloch 1969). In a series of nuclear-transplant experiments Di Berardino and Hoffner (1970) demonstrated that the nuclei of spermatogonial cells from <u>Rana pipiens</u> underwent developmental restrictions during their process of cell differentiation. They behaved like somatic nuclei. However, in <u>R. pipiens</u> no sperm histone transition occurs. Therefore, the histone transition does not appear to be necessary to erase the developmental restrictions.

Little information is available as to what happens to sperm histones in early development. Work on rat sperm suggests that they are lost very early in pronuclear formation (Kopecny 1970). However, a correlation might exist between characteristic events of early development and sperm histone type.

Perhaps the sperm histone type is related to the cleavage pattern of the egg. However, among the amphibians, <u>Diemictylus</u>, <u>Xenopus</u>, and <u>Rana</u>, cleavage is holoblastic. Yet the sperm of these organisms are of the <u>Salmon</u>, <u>Mytilus</u>, and <u>Rana</u> types, respectively. The cartilaginous fish have meroblastic eggs as do most teleosts. The cartilaginous fish have the Mouse/grasshopper type of sperm histone while the teleosts demonstrate the same range in sperm histones as the amphibians do. Thus no relation appears to exist between sperm histone type and cleavage pattern.

The beginnings of RNA synthesis during early embryonic development appears to be quite variable and might be correlated with sperm histone type (Bloch 1969). However, due to the lack of data on RNA synthesis in the organisms of the present study a correlation is not possible.

Bloch (1969) noted that sperm histone types did not show an evolutionary trend. Within most of the broad taxa studied all types were represented. Indeed, in the present study a wide range of sperm histones was observed in the amphibians. However, no variation was observed in the cartilaginous fish. An explanation for these results cannot be advanced. Bloch also observed little variation in sperm histones within tightly defined taxomic groups. Similarly, in the present study variation in sperm histone type was not found among frogs of the same genus.

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Since no correlation presently exists between histone type and any one aspect of function, one is forced to conclude with Bloch (1969) that the variability of sperm histones "reflects an evolutionary indifference to a relatively unimportant protein in an inert nucleus." The only general requirement of these proteins appears to be basicity.

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