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**BASIC CHROMATIN PROTEIN CHANGES DURING SPERMATOGENESIS IN
XENOPUS LAEVIS**

by

MICHAEL S. RISLEY

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This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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CHAPTER 1

INTRODUCTION

Multicellular eukaryotes are usually composed of cells of diverse phenotype. Since the common ancestor of these diverse cell types is the fertilized egg, questions arise concerning the nature of mechanisms which permit diversification during development. The elucidation of these mechanisms should also provide insight into the manner in which phenotypes may be altered following appropriate stimulation (carcinogenic, mitogenic, hormonal, etc.). Several theoretical hypotheses concerning mechanisms of differentiation exist. First, the quantitative alteration of the informational content (DNA) of various cells may occur differentially. Exemplary mechanisms of quantitative alterations of DNA are gene amplification (for review, see 1) and chromosome diminution or elimination (for review, see 2). Second, extensive translational and post-transcriptional control mechanisms may provide means for editing informational output in a cell-type specific manner (for review, see 3). Third, transcriptional controls may specifically select certain bits of the total information to be available for a particular cell type. This third level of control forms the basis of the theory of differential gene activity (for review, see 4). Although examples of the first two possibilities are available, it may be that those mechanisms are ultimately a result of differential gene activity.

While elegant studies in prokaryotic systems have provided models for understanding the mechanisms of differential gene activity (for examples, see 5-7) direct parallels

cannot be made with eukaryotic systems. First, only 5-30% of the DNA of a eukaryotic cell is utilized for transcription (see 4,8), while a much larger percentage of bacterial DNA is transcribed in growing populations (9,10). Second, eukaryotic cells contain multiple forms of DNA-dependent RNA polymerases (for review, see 11) while prokaryotic cells contain a single, albeit mutable enzyme (for review, see 12). Third, eukaryotic DNA is approximately one-third the mass of a highly complex component known as chromatin, with the other two-thirds of its mass being protein and RNA. Prokaryotic DNA has a much smaller mass of protein associated with it and does not exhibit the organizational complexity of chromatin.

Many investigators have adopted the working hypothesis that many of the proteins associated with DNA in chromatin may be involved in regulating gene activity. This has led to the biochemical dissection of chromatin in attempts to characterize the chromatin proteins in terms of their structure and function. This avenue of investigation has produced the finding that chromatin isolated from most eukaryotic nuclei contains two major classes of proteins: the histones (for reviews, see 13,14), and the nonhistone chromosomal proteins (for reviews, see 14-16). Of these two classes of proteins, evidence favors the involvement of certain nonhistone proteins in the specific mechanisms of gene regulation (see 15,16). Histones appear to be structural proteins involved principally in "packaging" DNA within the

cell nucleus. While histones do not appear to be specific regulators of gene activity, it is important to consider the influence that histones may have on gene activity through their structural role.

The relatedness of chromatin structure to chromatin function has been recognized for some time now. Classically, interphase chromatin exists in two different structural states, namely euchromatin and heterochromatin (17). Heterochromatin is typically transcriptionally inactive and highly compact, while euchromatin contains actively transcribed DNA sequences and is more extended and diffuse (18, 19). The activities of certain genes have been noted as being dependent upon their proximity to heterochromatin and may be influenced by the structure of heterochromatin. Such structural effects have been termed position effects (20, 21). An additional demonstration of the relationship between chromatin structure and function is provided by the observations of X chromosome inactivation (for review, see 22). Here, one of the X chromosomes in the cells of mammalian females becomes pycnotic and transcriptionally inert while the other remains diffuse and active. The example of polytene chromosome "puffing" is also appropriate to demonstrate correlates of chromatin structure and function. Early events of gene activation in polytene chromosomes are commonly preceded by structural alterations in which particular chromosome bands undergo a decondensation to form transcriptionally active puffs (for review, see 23,24). A simi-

lar decondensation phenomenon is typical of the transcriptionally active loops of lampbrush chromosomes (for reviews, see 25,26).

While it is recognized that gene activity need not always be a direct function of chromatin structure, adequate examples exist to demonstrate that such a relationship often does exist. To understand this relationship it is important to understand the molecules (i.e., histones) responsible for maintenance of chromatin structure and the mechanisms through which chromatin structure may be altered.

Histones--General Properties

The histones are a group of small, basic ($pI > 9$) proteins associated with the DNA of most eukaryotes. Dinoflagellates and possibly some fungi may lack typical histones (27). Generally, five main histones are found in this group: lysine-rich histone H1, slightly lysine-rich histones H2B and H2A, and the arginine-rich histones H3 and H4. All of the five histones have a ratio of basic to acidic amino acids ≥ 2 and lack tryptophan. The mass ratio of histones to DNA ranges from 0.8 to 1.35 (28). The molar ratios of individual histones relative to one another is found to be slightly variable from tissue to tissue and organism to organism (29,30) with the histones appearing in order of increasing amounts as $H1 < H2A \approx H3 \approx H4 < H2B$. Technical problems have made it difficult to discern a clear relationship between the molar ratios of individual histones from chro-

matins of various tissues. However, the molar ratios of H3, H4, H2B, and H2A approximate 1; while that of H1, relative to the others, is nearly 0.5. The molecular weights of the various histones are: H4, 11,282 daltons; H3, 15,324 daltons; H2B, 13,774 daltons; H2A, 14,004 daltons; and H1 21,500 daltons (taken from [31] for calf thymus histones).

The amino acid sequences of the five histones isolated from several species have been examined (for review, see 31-33). Interestingly, the comparison of sequences for individual histones isolated from diverse species indicates a high degree of sequence conservation, with the exception of histone H1. The calculated mutation rate (.06 per 100 amino acid residues per 100 million years) for histone H4 is the lowest observed to date (32). The relative degree of sequence conservation for the individual histones is H3, H4 > H2B, H2A > H1. Such evolutionary conservatism suggests that most of the histones perform a vital role common to most eukaryotes.

In addition to the conservative nature of histone primary structure, sequence analyses have demonstrated the presence of an asymmetric distribution of amino acid residues in histones (32,33). The terminal portions of all histones contain the greatest density of basic amino acids. Histone H1 has its basic residues clustered in the C-terminal portion of the molecules. The clustering of basic amino acids in the other histones occurs principally in the N-terminal region. Hydrophobic and acidic residues tend to

dominate the central regions of histones.

The dramatic chemical differentiation within histone molecules suggest that the physical and perhaps biological properties of the N-terminal, C-terminal, and central regions of histone molecules may be different. Histone conformation has been studied in solvents favoring formation of ordered structures (for reviews, see 13,31,34,35). Briefly, the formation of structures such as α -helices, or intra- and intermolecular structures occurs in the regions of histones low in basic amino acids and enriched in hydrophobic residues. Regions rich in basic amino acids are relatively unaffected by solvent induced transitions and may occur in a random coil conformation. The regions of histones rich in basic residues most likely interact with DNA. Regions which form ordered structures may be involved in protein-protein interactions. Ordered structures, such as α -helices, may form in basic regions following charge neutralization which occurs upon interaction with DNA, or following acetylation of specific lysine residues. However, evidence for this is meager, due to the overlapping contributions of structure from DNA and histone which cannot be discerned by circular dichroism (CD) or optical rotatory dispersion (ORD), techniques commonly used to examine molecular conformation.

Histone Synthesis

Histones are synthesized in the cytoplasm of cells (36,37) and then rapidly transported into the cell nucleus.

The synthesis of histones takes place on small polyribosomes containing a messenger RNA with a sedimentation coefficient of 9S (37-39). Electrophoresis on polyacrylamide gels has been used to fractionate this population of mRNAs (from sea urchins) into 3 heterogeneous groups (37,39,40). In an in vitro protein synthesis system, fraction A coded for histone H1, fraction B coded for histones H2B, H2A, and H3, and fraction C coded for histone H4 (40). Histone mRNA appears to lack a poly (A) segment at their 3'-OH termini (41). Recently, the mRNA for histone H4 has been isolated from two different sea urchin species, purified, and compared (42,43). Extensive code degeneracy was noted between the H4 mRNAs from the two species. However, the H4 mRNA of a single species was found to be highly conserved. These observations suggest that a cellular correction mechanism exists to depress heterogeneity within the H4 mRNA of a single species.

The genes coding for histones have been shown to be moderately repetitive (hundred to thousand fold) and clustered in the genomes of sea urchins (44-46), Drosophila (47, 48) and Xenopus (49). Curiously, human (50) and mouse (51) genomes contain only 10-20 repeats of the histone genes. The difference in histone sequence repetition between lower eukaryotes and mammals may be related to the large difference in the mitotic rate during early development (51).

Histone synthesis appears to be temporally and functionally coupled to DNA synthesis. Histone mRNA appears in the cytoplasm of cells during the S phase of the cell cycle

(36,37,52). Examination of histone turnover has demonstrated a very low rate of turnover of these chromatin proteins (53). The close relationship between DNA synthesis and histone synthesis has been further demonstrated through the use of inhibitors of DNA synthesis (36,37). Inhibition of DNA synthesis with hydroxyurea or cytosine arabinoside results in a rapid inhibition of histone synthesis and rapid loss of histone mRNAs from the cytoplasm. Transcriptional and translational control mechanisms have been proposed to account for the coupling of histone and DNA synthesis (54). However, the demonstration of histone synthesis in large, diplotene stage oocytes of Xenopus laevis, which do not synthesize chromosomal DNA, indicates that histone synthesis may be uncoupled at times from DNA synthesis (55).

Enzymatic Modifications

Histones are subject to enzymatic modifications such as phosphorylation, acetylation, and methylation (for review, see 14,34,56). These modifications usually occur at specific sites within the histone molecule. Phosphorylation of α -N-acetylserine residues has been noted in histones H2A and H4 (57,58). Histone H1 may be phosphorylated at several residues in the C-terminal region (59) as well as at serine 37 (60,61). Acetylation may occur at the N-terminal serine of histones H1, H2A, and H4 (62). This acetylation is irreversible and occurs during the synthesis of these histones via the involvement of N-acetylseryl-tRNA (63). Acetylation of

internal lysine residues also occurs. Histone H4 may be reversibly acetylated at lysines 5, 8, 12, and 16 (64,65). Histone H3 from calf thymus may be acetylated at lysines 14 and 23 (66), while that of trout testes may also be acetylated at lysines 9 and 18 (67). Histone H2B is little acetylated in many systems, but trout testis histone H2B may be acetylated at lysines 5, 10, 13, and 18 (67). Similarly, trout testis histone H2A can be acetylated at lysine residue 5 (68), while little internal acetylation of this histone occurs in other systems (69). Histone H1 is usually not acetylated. Methylation of histones may occur on lysine residues at positions 9 and 27 in histone H3 (70,71) and the lysine at position 20 in histone H4 (64). Minor sites of methylation may also occur at lysine 4 and lysine 36 of histone H3 (72). Methylation of histone H2B may occur as methyl arginine (73). Specific residues subject to methylation may exist in either the unmethylated, mono-, di-, or trimethylated forms.

Phosphorylation adds a negative charge to histone molecules, while acetylation results in the removal of a positive charge. Methylation tends to increase positive charge somewhat. Since histones interact with DNA through ionic bonds, enzymatic modifications may alter histone-DNA binding and play a significant role in regulating the biological function of histones.

Numerous correlations have been found between histone phosphorylation, acetylation, and the metabolic state of

eukaryotic cells (see 14,74). Phosphorylation of histones has been correlated with the growth rate of cells. Greater amounts of phosphorylated histones appear in rapidly growing cells than in nondividing cells (75,76). In some instances phosphorylation has been correlated with increased RNA synthesis, such as that which occurs following hormonal stimulation of rat liver (77), during rat liver regeneration (78-80), or following mitogen stimulation of lymphocytes (81,82).

Histone H1 is usually the most highly phosphorylated histone. Phosphorylation of this histone appears to occur throughout the cell cycle, except early G1, in both hepatoma tissue culture (HTC) cells (83) and chinese hamster ovary (CHO) cells (84). Massive phosphorylation of this histone during the G2 and M phases of the cell cycle in Physarum polycephalum (85), HTC cells (86), and CHO cells (87) has prompted hypotheses for the involvement of histone H1 phosphorylation in the process of chromatin condensation. However, a recent study (88) has shown that histone H1 may be dephosphorylated without an accompanying decondensation of metaphase chromosomes.

The other histones also display cell cycle dependent phosphorylation patterns. Histone H4 phosphorylation appears to be coupled to the synthesis of this protein, during S phase (74,89,90). Phosphorylation of histone H2A occurs throughout the cell cycle in CHO cells (91). Studies of the phosphorylation of histone H3 in CHO cells (87) have shown that this histone is phosphorylated only during the M phase

of the cell cycle and, therefore, may be involved in chromosome condensation. That phosphorylation can modify histone-DNA interactions is suggested by the demonstration that phosphorylated histone H1 has an altered capacity to induce characteristic conformational changes in DNA (92).

Histone acetylation has been temporally correlated with active RNA synthesis and spatially correlated with diffuse chromatin. A much higher rate of acetylation of histones H3 and H4 occurs in the diffuse, transcriptionally active chromatin of calf thymus lymphocytes, relative to the condensed, inactive chromatin (93). The transcriptionally active macronuclei of Tetrahymena pyriformis contain acetylated forms of histones H3 and H4, while the inactive micronuclei do not (94). Histones H3 and H4 are also unacetylated in transcriptionally inert, sea urchin sperm (95) and avian erythrocyte nuclei (90). However, precursor-cells for both the sperm and the erythrocytes are active in RNA synthesis and contain acetylated forms of histones H3 and H4. Increased levels of acetylation accompany, and often precede, increased levels of RNA synthesis following hormonal stimulation (96,97), during rat liver regeneration (98), and following mitogen stimulation of lymphocytes (99). A recent report (100) has indicated that reduced levels of acetylated histones H3 and H4 are associated with nontransforming host range mutants of polyoma virus, relative to transforming, wild type polyoma and SV40 viruses.

Newly synthesized or "old" histone molecules may be

acetylated (74). Acetylation of "old" histones during protamine replacement in trout spermatids has been suggested as a mechanism for the modification of histone-DNA interactions and subsequent removal of histones (101). Acetylation and/or phosphorylation of newly synthesized histones may be involved in the "correct" binding of histones to DNA (74,102). Circular dichroism studies of the binding of histone H4 (acetylated to different degrees) to DNA have provided evidence that acetylation can alter the influence of histone binding on the conformation of DNA (103).

It is evident that enzymatic modifications of histones provide a means to achieve a greater degree of complexity than can be found in the primary sequences of histones alone. Such microheterogeneity in the histones could produce a wide spectrum of histone-DNA and/or histone-histone interactions. This may provide a mechanism for the fine control of chromatin structure and perhaps some level of control of gene activity.

Variations in Histone Composition

While constancy in histone composition is more the rule than the exception, several examples of diversity in histone composition are available. Such diversity adds an additional level of complexity to these structural molecules. As mentioned earlier, dinoflagellate chromatin does not contain typical histones (27). Representatives of the ascomycetes, phycomycetes, and fungi imperfecti have also

been found to lack typical histone patterns (104,105). Yeast chromatin has been reported to lack histone H1 (106). The micronucleus of Tetrahymena pyriformis lacks histone H1 and H3 while the macronucleus contains all five of the basic types of histones (107).

In addition to examples of the absence of certain histones on chromatin, examples of the presence of variant or unique histones in chromatin are available. Nucleated erythrocytes of birds contain a lysine, arginine, serine-rich histone (H5) which replaces much of histone H1 during the maturation of avian erythrocytes (108,109). Histone H1 is composed of several components which often appear species-specific, organ-specific (29,110), and cell-specific (111). Heterogeneity has been found in histone H2A of rat chloro-leukemia cells (112) and Friend-leukemia cells (113). Histone H3 is heterogeneous in pea embryos (114) and calf thymus (115). Polymorphism has also been reported for chicken erythrocyte histone H5 (116). Alterations in the relative amounts of the different forms of Friend-leukemia cell histone H2A occurs during dimethylsulfoxide treatment (113). Modifications in the amounts of different histones also occur during early development (117-119). Extremely large variations in the histone composition of cells are commonly found in the cells of the male germ line (see section on the basic chromatin proteins of spermatogenic cells).

Systems which display variations in the composition of histones provide an opportunity to examine chromatin struc-

ture in the absence of certain histones or during modulation of the relative amounts of particular histones. The information derived from such studies may prove valuable to the elucidation of the functions of particular histones.

Histone Function

The evidence leading to the conclusion that histones function primarily as structural molecules, and perhaps as general repressors of gene activity, is reviewed in this section. The ability of histones to render DNA inaccessible to exogenous RNA polymerase is well documented. As early as 1962, it was demonstrated that histones added to DNA in vitro decrease the capacity of the DNA to act as a template for RNA synthesis (120). This finding has been confirmed numerous times (121-123). Moreover, stepwise removal of histones from DNA results in an increased template efficiency (123-126). Histones do not exhibit a capacity for recognition of specific DNA sequences other than preferences for A-T rich or GC rich DNA (127-128).

These combined results led to the hypothesis that histones act as generalized repressors of transcription and therefore may only be associated with inactive regions of DNA (see 28). However, evidence favors a random distribution of histones on DNA. Moreover, recent studies utilizing electron microscopy (129) and micrococcal nuclease digestion (130) have demonstrated the existence of histone containing nucleosomes on actively transcribed genes.

A study of the number of RNA polymerase initiation sites in chromatin and the elongation rates has indicated that only 10% of the initiation sites available in DNA are available in chromatin (131). Once initiation occurs in chromatin, elongation proceeds, although at a rate 33% (131) to 50% (132) lower than in DNA. The reduced RNA elongation rates seem to be due to interference of RNA polymerase translocation by chromatin proteins (132). These results suggest that repression of transcription on specific genes by histones is principally due to the blockage of specific promoter sites for DNA-dependent RNA polymerase, rather than blockage of the remainder of the genes. Particular configurations of promoter sequences appear to be required for proper initiation by E. coli and mammalian RNA polymerase (133-135). Histone binding in promoter regions may alter the conformation of promoter DNA in such a way that RNA initiation would be prevented. Modification of histone-promoter binding, either specifically (by nonhistones and/or enzymatic modification) or non-specifically (by polyanions), may then permit RNA chain initiation.

Whether alterations of histone-histone or histone-DNA associations are required for RNA chain elongation is unclear at present. The decrease in RNA chain elongation in chromatin relative to free DNA suggests that such alterations may be required. Recent studies of actively transcribed ribosomal RNA gene chromatin by electron microscopy have been unable to demonstrate the existence of histone containing

nucleosomes in the active regions of these genes (136). However, micrococcal nuclease digestion of ribosomal RNA genes produces DNA fragments grossly typical of those produced by digestion of the bulk of chromatin (137-139). The apparent paradox may be resolved by assuming that nucleosome structures exist in an extended or relaxed state on the active regions of the ribosomal RNA genes. In such a state, histones may remain associated with the DNA, providing protection against micrococcal nuclease digestion. However, they would not appear as typical nucleosomes in the electron microscope. A model for such a relaxed nucleosome structure has been proposed (140). Relaxation may also occur during RNA chain elongation on other genes. The visualization of unrelaxed nucleosomes on other genes (129) may occur because the low density of RNA polymerase on these genes relative to ribosomal RNA genes (see 141), does not require a high degree of nucleosome relaxation. Recent studies (142) have demonstrated that actively transcribed genes are more sensitive to DNase I digestion than inactive genes. This observation provides evidence for altered chromatin structure and histone-DNA interactions in actively transcribed genes.

The influence of histones on RNA synthesis is most likely related to their affect on the structure of the DNA template. Therefore, the structural role of histones will be reviewed. Hydrodynamic studies have shown that DNA is more compact when associated with histone than when free in solution (143,144). Electron microscopic and X-ray analyses

of chromatin have been interpreted as suggestive of a DNA supercoiling (146,147). Circular dichroism studies indicate that DNA in chromatin contains a significant amount (50-65%) of C-character, while DNA in solution contains a high degree of B-character (148,149). Removal of most histones by salt (150,151) or detergent (152) causes chromatin DNA to revert to B-form. Removal of histone H1 has little influence on the CD spectra of chromatin (153).

In addition to compacting DNA and altering nucleotide stacking in DNA, histones increase the stability of DNA against thermal denaturation (154-156). All histones seem to have similar capacities for stabilizing DNA in chromatin (124). The degree of stabilization of DNA is most likely due to factors other than ionic shielding alone (124) since half-molecules of histone H2B stabilize DNA to lesser extents than a complete histone H2B (157). This may be due to interstrand crosslinking of DNA. Stabilization of DNA base-pairing may be involved in regulating RNA synthesis.

Binding of histones to DNA appears to take place primarily in the major groove of DNA. Olin (158) has shown that phage T2 DNA does not become glucosylated well if combined with histone H1. Glucosylation occurs in the major groove of T2 DNA. Actinomycin D, which binds the minor groove of DNA (159), binds equally well to free DNA and DNA-histone complexes (158,160). Similar results have been obtained with a dicationic chromophoric "reporter molecule" (161). Models for the binding of histone H4 in the major

groove have been proposed (155,162).

Recent observations have resulted in a major breakthrough in the study of chromatin structure and the role of histones (for review, see 33). Hewish and Burgoyne (163) reported that an endonuclease, endogenous to rat liver nuclei, cleaves DNA at regular intervals into a series of discrete DNA fragments all being a multiple of a basic fragment size. Staphylococcal nuclease digests of nuclei or chromatin produce similar DNA fragments which are multiples of a 140-200 base pair fragment (164-166). Extensive nuclease digestion yields several DNA fragments varying in size from 45-145 base pairs (165-168). This limit digest pattern results from degradation of approximately 50% of DNA to an acid-soluble form. The acid insoluble monomeric DNA fragments produced by mild nuclease digestion can be isolated as chromatin fragments (164,165,167,169,170). Further treatment of monomer size (140-200 bp) chromatin fragments with staphylococcal nuclease produces DNA fragments 45-145 base pairs in length, similar to those produced from whole chromatin and nuclei (165,167,171,172). Evidently, staphylococcal nuclease preferentially attacks open or accessible DNA regions at regular intervals (every 200 base pairs) and then produces discrete cleavages within the 200 base pair DNA regions which are (histone) protected. These observations suggest that chromatin is organized into a repeating, fundamental substructure.

Repeating units of chromatin structure have recently

been demonstrated by electron microscopy. Olins and Olins (173) first demonstrated the existence of spherical chromatin bodies (ν -bodies) arranged in linear array on DNA. These bodies were reported as being $60-83 \text{ \AA}$ in diameter and separated in chromatin by connecting strands of $15 \text{ \AA}$ DNA. Since this initial report, several electron microscopic studies have confirmed the existence of such repeating chromatin structures in the chromatin isolated from various sources (129,136,174-179). The size of these ν bodies (nucleosomes) was what may be expected if they represented the nuclease resistant structures of chromatin. Several studies have confirmed the expected relationship between nucleosomes and the nuclease-resistant subunits of chromatin (175,177,178,180). Controversy about the exact size of nucleosomes, and whether internucleosome DNA strands exist in chromatin, remains (173,175,178,181). Potential artifacts in electron microscopic preparations have made it difficult to resolve reported discrepancies.

Kornberg and Thomas (182) and Kornberg (183) proposed a model of chromatin structure relating chromatin repeat units to histones. Kornberg and Thomas (182) employed dimethylsuberimidate as an agent to cross-link histones in solution and in chromatin. Their data, along with previous observations, suggested that each 200 base pair nuclease resistant complex may be composed of 2 each of histones H3, H4, H2B, and H2A. Histone H1 was proposed as not being involved in forming the repeat structure. Since histones

H3 and H4, and histones H2B and H2A could be crosslinked, it appeared that these histones could be involved in intimate associations with each other in chromatin. These histone-histone interactions could then act to stabilize nucleosome structure. Examinations of the histones isolated from nucleosomes, prepared from whole chromatin or H1 depleted chromatin, have demonstrated that histones H3, H2B, H2A, and H4 are present in a molar ratio of 1, and that histone H1 is absent, and hence not required for nucleosome structure (171, 172, 184, 185). The presence of histones in nucleosomes has also been demonstrated by immunoelectron microscopy (181).

A problem remaining unresolved is whether nucleosomes are relatively homogeneous or heterogeneous in histone composition. Kornberg and Thomas (182) have provided evidence that all four histones (H3, H2B, H2A, H4) are required in histone DNA complexes to produce x-ray patterns characteristic of native chromatin. Similarly, evidence has been presented to demonstrate that all four histones (H2A, H2B, H3, and H4) are required in histone-DNA complexes to yield nuclease digest patterns similar to those from chromatin (186, 187). These studies emphasized a central role for histones H3 and H4. However, nuclease digests of Tetrahymena micronuclei, which lack histones H1 and H3, contain DNA fragments similar to those obtained from chromatin with a complete histone complement (188). In addition, nucleosome fragments (produced by nuclease and trypsin treatment) lacking histones H2B and H2A have been found (189).

Several studies have demonstrated that the circular dichroic spectra of nucleosomes are anomalous compared to free DNA spectra (169,190-191). This may indicate the presence of large amounts of C-form DNA in nucleosomes. Nucleosomes also display increased T_{ms} , relative to free DNA, when subjected to thermal denaturation (190-192). Additionally, nucleosomes display similar neutron-diffraction patterns to those of chromatin (193-194). The similarities of the physical characteristics of whole chromatin and isolated nucleosomes suggest that nucleosomes are indeed the fundamental units of chromatin structure.

A recent electron microscopic analysis of chromatin produced by nuclease digestion has led to the suggestion that a filament or linear array of nucleosomes may form helices of approximately $300 \text{ \AA}$ dia. (195). The formation of such higher order folding was found to be dependent upon the presence of histone H1 and $MgCl_2$. Such a "solenoidal" structure would have a central hollow core and a pitch of $110 \text{ \AA}$. This interpretation is consistent with data obtained from neutron-diffraction (193,194), X-ray scattering (196,197), and electron microscopy (see 198,199). Such a model provides an understanding of the manner by which high levels of DNA folding and packing may occur. That nucleosomes do persist in such highly compacted states of chromatin has been demonstrated by nuclease digest studies on metaphase chromosomes (200).

Histone-histone interactions most likely play a major

role in the maintenance of nucleosome structure. That such interactions can occur between histones has been demonstrated by D'Anna and Isenberg in a series of papers (201-204). These authors have shown that histones readily interact to form aggregates in solution. The strengths of these interactions are dependent upon the individual histones involved. Histone pairs H3 and H4, H2B and H2A, and H2B and H4 interact strongly in solution. Sperling and Bustin (205) have also shown that histones may self-assemble in solution and that the histone complexes form bent rods ($22 \overset{\circ}{\text{\AA}}$ dia.) or fibers ($44 \overset{\circ}{\text{\AA}}$ dia.) depending upon ionic strength. These observations led to a proposal for such rods forming a central core which DNA could wrap around (183,193,205).

Evidence that histones form such intimate associations in chromatin has been provided by cross-linking studies. Dimers of histones H2B and H2A, as well as histones H2B and H4, have been demonstrated following treatment of chromatin with formaldehyde (206). Dimethylsuberimide treatment of chromatin produces tetramers of histones H3 and H4, and oligomers of histones H2B and H2A (183,207). Tetranitromethane treatment of chromatin crosslinks histones H2B and H4 (208). A carbodiimide has been used to produce dimers of histones H3 and H4 (209), and UV light induces dimer formation between histones H2B and H2A (210). These results demonstrate that histones (H3,H4,H2B,H2A) are intimately associated with each other in a specific manner in chromatin. Histones H1 and H5 never form crosslinks with any of the

other histones. However, they may interact with each other since evidence has been provided for the occurrence of oligomers of histones H1 and H5 in chromatin (209). Apparently, histones H1 and H5 associate with the nuclease sensitive, extranucleosomal DNA.

Treatment of chromatin with increasing urea concentrations results in a disruption of nucleosome structure (191,211). Urea treatment probably weakens the associations of histones in chromatin by disrupting hydrophobic and/or hydrogen bonds (212). Hydrophobic and hydrogen bonds may stabilize histone-histone associations. Recent studies of the effects of trypsin treatment on nucleosome structure demonstrate that cleavage of histones H3, H4, H2B, and H2A occurs approximately 20-30 residues from the basic N-terminal residues (166,189). Such a cleavage produces a trypsin resistant core consisting of the hydrophobic C-terminal regions of the four histones. Trypsin treatment also destroys the native nucleosome structure (166,189). These observations indicate that hydrophobic interactions between histones play a major role in maintaining nucleosome structure. It is probable that histones H2B, H2A, H3, and H4 associate with each other through hydrophobic interactions of the C-terminal and central regions of the proteins. These associations may lead to the formation of the histone core of nucleosomes. Basic N-terminal regions may then remain free to interact with DNA electrostatically.

Questions remaining unanswered as yet pertain princi-

pally to the control of nucleosome and higher order chromatin structure. The details of intranucleosome structure appear to be close to resolution. However, little is known of alterations in nucleosome structure which may occur during transcription or DNA replication. Several other questions also remain unanswered. How do enzymatic modifications of histones affect inter- and intranucleosome structure? What effects do histone variants have on nucleosome structure? How do nonhistones interact with nucleosome DNA? How do nonhistones influence histone-DNA interactions in nucleosomes?

Basic Chromatin Proteins of Spermatogenic Cells

Unlike the relative constancy of histones in somatic cells (exceptions previously noted), the basic DNA-associated proteins of cells in the male germ line are exceptionally variable (for reviews, see 213-215). These proteins range in character from histones, to histone-like proteins, to protamines, depending upon the organism and spermatogenic cell type examined. Protamines are small, basic proteins containing a large percentage of lysine and/or arginine.

Five categories have been proposed to classify the protein types found in spermatzoa (213). These are: (1) Salmo type, (2) mammalian type, (3) Mytilus type, (4) Rana type, and (5) crab type. Salmo type sperm contain arginine-rich protamines only. Mammalian type sperm contain arginine, cysteine-rich protamines. The sperm of the Mytilus category

contain proteins intermediate in character (basic amino acid content) to histones and protamines. Rana type sperm only contain histones, while crab type sperm lack a significant amount of basic proteins altogether. The ability to identify five categories, based on sperm protein content alone, is demonstrable of the variability which exists in the basic chromatin proteins of the male germ line.

Several other levels of variability also exist in the basic chromatin proteins of the male germ line. Within the categories listed above the proteins are not homogeneous. For example, several types of fish (215-218) and bird (219-220) monoproamines have been characterized. Moreover, mammalian, cysteine-rich protamines are heterogeneous on interspecific (221,222) and intraspecific (221) levels.

Significant diversity of histones and histone-like proteins also occurs in spermatocytes and spermatids. Marushige and Dixon (223) described the presence of three unusual basic proteins (R,S,T,) in trout testis chromatin. Wigle and Dixon (224) have since characterized the T protein as a lysine-rich histone, specific to trout spermatids. Variants of histones H1, H2B, and H2A have been described in sea urchin sperm (225,226). Two histone-like proteins, TH1 and TH2, have been extracted from the chromatin of house cricket (Acheta domesticus) spermatids (227). A unique H1 histone has been extracted from sperm and spermatids of Rana pipiens (228). A unique H1 histone (111,221), an unusual H2B histone (221), and other histone-like proteins (222,229-231) have been de-

scribed in primary spermatocytes of mammals. An arginine, lysine-rich protein (TP) is specific to spermatid stage cells in mammals (232).

While the variation of basic chromatin proteins in the male germ line has made it difficult to obtain insights into the function and evolution of these variants, it also provokes several questions. Do the variant histones and histone-like proteins serve similar roles to the histones of somatic cells or roles specific to spermatogenic cells? Does the presence of variant histones and histone-like proteins in chromatin alter typical histone-DNA or histone-histone interactions? The process of spermiogenesis, in certain organisms, provides an opportunity to examine chromatin structure during the displacement of histones from DNA. The mechanisms involved in histone displacement may be similar to mechanisms operative in the alteration of histone-DNA and histone-histone interactions in somatic cells.

The only spermatogenesis system to be analyzed in detail has been that of the rainbow trout (Salmo gairdnerii or S. irideus). Marushige and Dixon (223,233) demonstrated that trout sperm contain monoproamines and lack histones. The proamines are synthesized by mid-and late stage spermatids (89,234,235) on small polysomes (235). Proamine mRNA appears to be 165-170 nucleotides in length and may exist in both the polyadenylated and non-polyadenylated forms (236). Proamines undergo extensive phosphorylation following their synthesis and ultimately become triphosphorylated (89,237, 238). Proamine phosphates (and a methionyl residue) are

then slowly removed during spermatid maturation (223,238). It has been postulated that protamine phosphorylation may be required to achieve correct protamine-DNA binding (238). Dephosphorylation may then result in a controlled process of chromatin condensation. The protamine of mammalian sperm also appears to undergo a similar phosphorylation-dephosphorylation cycle (239).

During protamine binding to DNA in trout, histone is displaced from the chromatin. Electrophoretic analyses of basic proteins extracted from developing trout testis chromatin, as well as nucleohistone and nucleoprotamine of trout testes, suggests that histone H1 is the last to be displaced from chromatin (223,233). Histone H4 is first displaced, followed by H2A, H2B, and H3, and lastly histone H1. This is surprising in light of data demonstrating that histone H1 is the histone most easily displaced by increasing ionic strength (240) or by direct addition of protamine to nucleohistone (223,241). However, a similar order of histone displacement has been reported for rat (239) and ram (242) spermiogenesis. Such results suggest the involvement of other processes, such as enzymatic modification and/or proteolysis, rather than direct displacement of histones by protamine (243,244).

Studies of histone modification during trout spermatogenesis have shown that phosphorylation of all histones occurs (237,245). However, histone phosphorylation appears to be coupled to histone synthesis during spermatogenesis in

trout (89). Protamine stage spermatids of trout are inactive in histone phosphorylation. These data suggest that histone phosphorylation is not involved in histone displacement from chromatin. In contrast, histones have been shown to be acetylated in protamine stage spermatids (101). A similar observation has been made in rat spermatids (246). This has prompted the suggestion that acetylation may be involved in the displacement of histones from chromatin (101,243-246). As yet, no direct evidence for this mechanism exists.

Chromatin structure has been examined in trout testes through analyses of micrococcal nuclease digests (247,248). Sedimentation analysis of the DNA fragments produced by digestion of nucleohistone and nucleoprotamine has demonstrated that the "limit digest" pattern typical of somatic cell chromatin persists only in nucleohistone. Nucleoprotamine is poorly digested (15-20%) by micrococcal nuclease (249). Evidently, histone displacement is accompanied by loss of the DNA fragments usually released by nuclease treatment. It is likely that protamines protect these fragments from digestion by the nuclease and do not induce nucleosome formation themselves.

This brief review and discussion emphasizes the availability in spermatogenic cells, of unique opportunities to study problems related to histone metabolism, histone variants, and chromatin structure. Data derived from such studies may be significant to the understanding of chromatin

structure and function in somatic cells. However, only the trout system has been examined in detail. The variability of the basic proteins in cells of the male germ line suggests that variability may also exist in histone metabolism and chromatin structure. Moreover, certain basic proteins may serve a common function in the spermatogenic cells of many organisms. Data from other spermatogenic systems is required before generalizations can be made.

The purpose of this thesis was threefold: (1) to develop methods for the dissociation and separation of spermatogenic cells from Xenopus laevis, (2) to examine the basic chromatin proteins of the spermatogenic cells of Xenopus laevis, and (3) to study the synthesis and enzymatic modifications of the basic chromatin proteins found in the spermatogenic cells. The South African clawed frog, Xenopus laevis, has been chosen because it belongs to a little studied category (Mytilus type) of sperm types.

Several interesting results have been obtained. First, histone displacement occurs in a sequence thus far unique to Xenopus laevis. Histone H1 is first to be displaced, followed by histones H2B and H2A. Histones H3 and H4 do not become fully displaced in testicular sperm. Second, several electrophoretic species of proteins (SP1-SP4) were found to be unique to spermatogenic cells. SP1 and SP2 were found to be electrophoretically related to histones. SP3 and SP4 appear to be similar to Mytilus edulis sperm proteins. Third, phosphorylation of histone H4 was found to occur in spermatids

that were inactive in the synthesis of histones. Fourth, the levels of acetylated forms of histones H3 and H4 were found to decrease in cells whose histones are being displaced. However, significant amounts of histone H4 remain acetylated even in testicular sperm.

The results of the studies described here demonstrate that histone displacement and certain aspects of histone metabolism in trout spermatids are different from those of Xenopus laevis spermatids. The significance is discussed within.

CHAPTER 2

THE DISSOCIATION AND SEPARATION OF SPERMATOGENIC CELLS

Introduction

The spermatogenic cells of the amphibian testes are organized into cysts of synchronously developing cell types (250). Each cyst is surrounded by sustentacular, Sertoli cells. The interstitial tissue is composed of Leydig cells, fibroblasts, and connective tissue elements. Detailed biochemical analyses of specific testis cells require methods which permit efficient dissociation and separation of the cell types of interest.

Cell separation techniques have, for the most part, relied upon differences in either cell size or cell density. Miller and Phillips (251) introduced the widely used STAPUT apparatus in which cells are separated on the basis of size. In this method, cells are loaded in a narrow band upon a linear or nonlinear gradient of bovine serum albumin and allowed to sediment at unit gravity. Cells sediment at a rate (mm/hr) approximating $r^2/4$, where r is the cell radius. A similar system has been described by Peterson and Evans (252). This method has been applied to the separation of testis cells from fish (253) and mammals (254-259).

Recently, Beckman Instruments has developed the Elutriator Rotor for use in cell separations. Cell separation in this apparatus is based upon floatation theory (260). A centripetally moving buffer elutes cells whose sedimentation rate is not equal to or greater than the "upward" velocity of the buffer. Separation of hamster and mouse testis cells has been achieved by centrifugal elutriation (260). This ap-

paratus has a greater capacity than the STAPUT apparatus and achieves separations in a much shorter time (2 hrs versus 4-5 hrs).

Separations achieved on the basis of size alone are often inadequate or require lengthy sedimentation periods due to the presence of cells of similar size in the testis. Rapid separation methods based upon parameters other than size may provide a means of achieving greater purity. Cell separations by equilibrium density gradient centrifugation in various supporting media have been reported (261-265). Recently, this approach has been extended to the separation of testis cells. Meistrich and Trostle (266) reported the separation of mouse testis cells in gradients of Renografin (66% methylglucamine, 10% sodium diatrizoate). Separations were achieved during a 20 min centrifugation. Colloidal silica gradients have been utilized to separate spermatid stages from ram testes (267) by isopycnic centrifugation.

Little has been done to extend cell dissociation and separation methodology to amphibian testis cells. In this study, collagenase (I.U.B. No. 3.4.4.19) has been utilized to prepare testis cell suspensions from the South African clawed frog, Xenopus laevis. The resulting cell suspensions are 99.9% composed of spermatogenic cells. A rapid separation of the spermatogenic cells has been achieved by isopycnic centrifugation in Metrizamide gradients. It is demonstrated that this separation technique can be utilized in conjunction with unit gravity sedimentation to obtain high-

ly enriched preparations of individual spermatogenic cell types. Preliminary observations from these studies have been published previously (268).

Materials

Adult male Xenopus laevis (50-70 g) were obtained from either the South African Snake Farm (Fishoek, S.A.) or Nasco (Port Atkinson, Wis.) Frogs were maintained in tanks of de-ionized water at room temperature and fed beef liver three times a week. HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, collagenase (Type 1) and bovine serum albumin (BSA-Fraction V) were purchased from Sigma Chem. Co. Metrizamide (2-(3-acetamido-5-N-methylacetamido-2,4,6-tri-iodobenzamido)-2-deoxy-D-glucose) was generously donated by Gallard Schlesinger Chemicals (Carle Place, N.Y.). Trypsin 1:250 was obtained from Nutritional Biochemicals (Cleveland, Ohio). Dialyzed, fetal calf serum was obtained from G.I.B.C.O. (Grand Island, N.Y.). Miracloth was purchased from Chicopee Mills, (New York, N.Y.). Other chemicals were reagent grade.

Methods

Cell Dissociation

Testes were dissected out of adult male frogs, rinsed twice at room temperature in TSS (testis salt solution: 75.8 mM NaCl, 24 mM KCl, 10 mM Na_2HPO_4 , 1 mM MgCl_2 , 1 mM

CaCl₂, 50 mM HEPES buffer, pH 7.6, 185±5 mOsm) and then minced with scissors to small (approx. 1 mm) fragments in TSS containing 0.15% (w/v) collagenase (195 U/mg). TSS is a modification of solution OR-2 which was devised for Xenopus oocytes (269). Approximately 10 ml of the collagenase solution were used per pair of testes. The testis fragments were teased with insect pins and then stirred at ambient temperature (22-25°C) for 1 to 1.5 hours. Dissociation times were found to be variable. Tissue fragments, undissociated cysts, and cell clumps were removed by filtration through two layers of Miracloth. Cells were pelleted by centrifugation at 650 x g (Sorvall Hb-4 rotor) for 5 mins (5°C), washed twice in cold TSS by repeated resuspension and centrifugation, and finally resuspended in 2 ml of cold TSS.

On specific occasions, collagenase dissociation was followed by trypsin treatment to achieve more complete dissociation. In such instances, trypsin 1:250 (0.1%, w/v) was added to the cell suspension following 30-40 mins of collagenase treatment. Stirring was continued for 15 mins whereupon dialyzed, heat inactivated, fetal calf serum was added to 5% to inactivate the trypsin. Filtration and washing were as described above.

Determination of Cell Number, Cell Viability, and Differential Cell Counts

Cell number was determined with a Neubauer hemocytometer using phase contrast optics. Viability was expressed as the percentage of cells capable of excluding 0.2% trypan

blue (270) 15 mins after addition of dye.

Differential cell counts were performed, with the aid of phase contrast optics, on wet mounts sealed with nail polish to prevent evaporation. Occasionally, a drop of 0.4% methyl green, 2 mM CaCl_2 , 6% acetic acid was added to the wet mounts to enhance nuclear detail and to facilitate the discrimination of primary spermatocyte stages.

The identification of specific cell types was based upon morphological criteria previously detailed in an autoradiographic study of the kinetics of spermatogenesis in Xenopus laevis (271).

Gradient Preparation

Metrizamide solutions were freshly prepared at room temperature prior to each separation. The ionic composition of all Metrizamide solutions was identical to that of PSS, with the exception of NaCl concentrations. NaCl concentrations in Metrizamide solutions were adjusted to offset the osmotic effects of Metrizamide and to approximate iso-osmotic conditions ($200 \pm 6 \text{ mOsm}$) throughout a 2-15% Metrizamide gradient. Osmolality was determined with a vapor pressure osmometer (Wescor, Inc.). Gradients were generated into 13 ml cellulose nitrate tubes at 4°C . The gradients consisted of a 1 ml underlayer of 50% Metrizamide (21 mM NaCl), 1 ml of 15% Metrizamide (21 mM NaCl), and a 10 ml, linear 2% (75.8 mM NaCl) to 15% Metrizamide gradient. Testis cells were introduced throughout the gradient by diluting a more

concentrated Metrizamide solution with the original cell suspension to prepare the 2% Metrizamide solution used in gradient preparation. All gradients were overlaid by approx. 1 ml of TSS. Up to 7×10^8 cells have been centrifuged in a single gradient without loss of resolution. Linear 30% (21 mM NaCl) to 50% Metrizamide gradients were prepared by similar methods. These gradients, however, are hyperosmotic.

Centrifugation and Gradient Collection

Metrizamide gradients were centrifuged to equilibrium at 13,200 x g in an SW41 rotor and a Beckman L2-65 B ultracentrifuge. Centrifugation was for 30 mins at 5°C. Deceleration occurred with the brake off. Approximately 0.4 ml fractions were collected from a puncture in the bottom of the tube at a rate (1.48 ml/min) controlled by pumping paraffin oil (ISCO Density Gradient Fractionator-Model 183) into the top of the sealed tube. Refractive indexes (n) were determined with an Abbe refractometer at 25°C. The density (ρ/cm^3) at 5°C for each fraction was determined from the following equation after correcting for salts:

$$\rho_{5^\circ\text{C}} = 3.559n_{25^\circ\text{C}} - 3.740$$

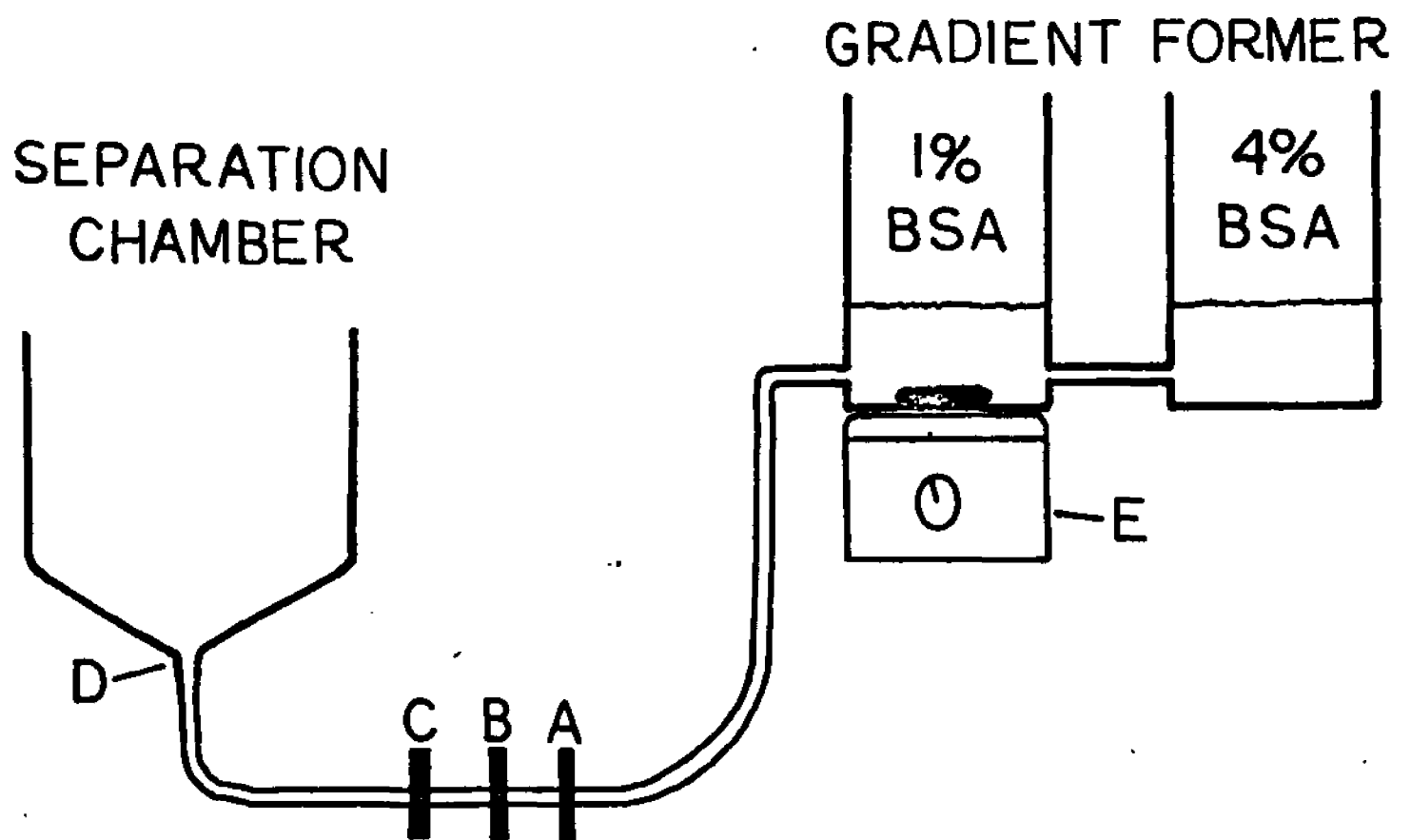
This equation was derived from least squares analysis (coeff. correl.=0.99) of refractive indexes of several Metrizamide concentrations at 25°C versus pycnometer derived density values at 5°C published by Nygaard and Co. (Oslo, Norway). The densities of specific cell types were deter-

mined from the average of the two half-maximal peak heights in plots of cell number versus density.

Unit Gravity Sedimentation

Separation of testis cells by velocity sedimentation at unit gravity was carried out at 4°C in a 600 ml linear gradient of 1 to 4% bovine serum albumin prepared in TSS. The apparatus (fig. 1) consisted of a siliconized glass separation chamber (i.d., 11.3 cm; cylindrical length, 8 cm), fitted with polyethylene tubing, which attached to a 2 liter gradient former. Screw clamps, located at A and B, were preset to permit flow rates of 3 ml/min and 10 ml/min respectively. The screw clamp at C was utilized to stop the flow of the gradient. The BSA gradient was permitted to flow to D at 3 ml/min whereupon flow was halted while cells ($5 \times 10^8/20$ ml) in 0.5% BSA in TSS were carefully layered on the gradient with a long tip, pasteur pipette. The cells were overlayed with 10 ml of TSS. The gradient was then loaded at 3 ml/min until the cell band reached the cylindrical portion of the chamber. The clamp at A was then gradually opened to achieve a flow rate of 10 ml/min (clamp B setting). Loading time required 1.5 hrs. Sedimentation followed for an additional 3.5 hrs prior to the collection of 10 ml fractions from the bottom of the gradient at 10 ml per min. Cells were pelleted in a clinical centrifuge (IEC, setting 5, 5 mins, 4°C) and resuspended in 0.5 ml of cold TSS for analysis.

Fig. 1. Diagram of the apparatus used for unit gravity separation of spermatogenic cells. A, screw clamp set for 3 ml/min flow rate; B, screw clamp set for 10 ml/min flow rate; C, screw clamp for on-off control of gradient flow; D, position at which cells are layered onto gradient; E, magnetic stirrer; BSA, bovine serum albumin.



Results

Morphological Characterization

Cell identifications were based upon morphological criteria such as cell and nuclear diameter, nucleolar size and number, and whole cell, as well as chromatin, morphology. Examination of sectioned testes has demonstrated that these characteristics are sufficient for discriminating most of the spermatogenic cell types of Xenopus laevis (271,272). Sertoli cells were identified by their large size (20-30 μ m dia.) while Leydig cells were identified by the presence of large numbers of lipid droplets in their cytoplasm. Sufficient detail of cellular structures persists in cell suspensions to permit cell identification using phase contrast optics (see fig. 2 A-K). This provided a rapid means to obtain differential cell counts relative to the time involved in preparing fixed and stained smears.

The characteristic morphologies of specific spermatogenic stages have been detailed previously (271,272). Identifying characteristics used here are briefly presented in table 1. Two distinctions should be made between previous reports and this one. The late spermatid stage described by Kalt (271) has been divided into two major types in this report: (1) slough stage spermatids, and (2) elongate stage spermatids.

Slough stage spermatids (fig. 2 J) are spermatids engaged in cytoplasmic shedding. These include very early

TABLE 1.--Morphological characteristics typical of specific spermatogenic cell types^a

Cell Type	Cell Diameter	Nuclear Diameter	Structural Characteristics
Primary spermatogonia	16-22	ND ^b	Convolutcd, diffuse nucleus; multiple (2-5), large nucleoli
Secondary spermatogonia and premeiotic S phase	9-15	6-9	Heterochromatic clumps and chromatin-clear areas; nucleoli reduced in number (1-2) and size relative to primary spermatogonia
Leptotene spermatocytes	11-13	8-10	Uniformly diffuse chromatin; 1 or 2 nucleoli
Zygotene spermatocytes	11-13	8-10	Chromatin is thinly stranded; 1 or 2 nucleoli
Pachytene spermatocytes	12-14	8-10	Thick "ribbons" of chromatin; nucleoli only occasionally visible
Diplotene spermatocytes	12-14	8-10	Condensed chromatin bivalents directly adjacent to nuclear membrane; no nucleoli apparent
Secondary spermatocytes	9-10	6-7	Chromatin organized into dense, heterochromatic patches; no nucleoli apparent
Round stage spermatids	6-8	4-5	Round, dense nucleus; occasional pre-acrosomal vesicle

TABLE 1 - continued

Cell Type	Cell Diameter	Nuclear Diameter	Structural Characteristics
Acrosome stage spermatids	7-9	4-5	Large acrosomal vesicle; dense nucleus indented adjacent to acrosome; often flagellated
Sloughing spermatids	4-5	2-5	Acrosome is condensed and no longer apparent; nuclei pass from a uniformly dense state (early) to an elliptical, highly condensed state (late); cell types range from those having a full complement of cytoplasm (early) to those actively shedding cytoplasm posteriorly (mid-late); all cells are flagellated
Elongate spermatids	7-13 ^c	1.5-3 ^d	Most cytoplasm cast off; nuclei are elongate, thin, and highly refractile
Mature spermatids and sperm	12-13 ^c	1.0-1.5 ^d	Helical (1.5 revolutions), highly refractile nuclei

^aCharacteristics visualized by phase contrast microscopy. Diameters given as μm .

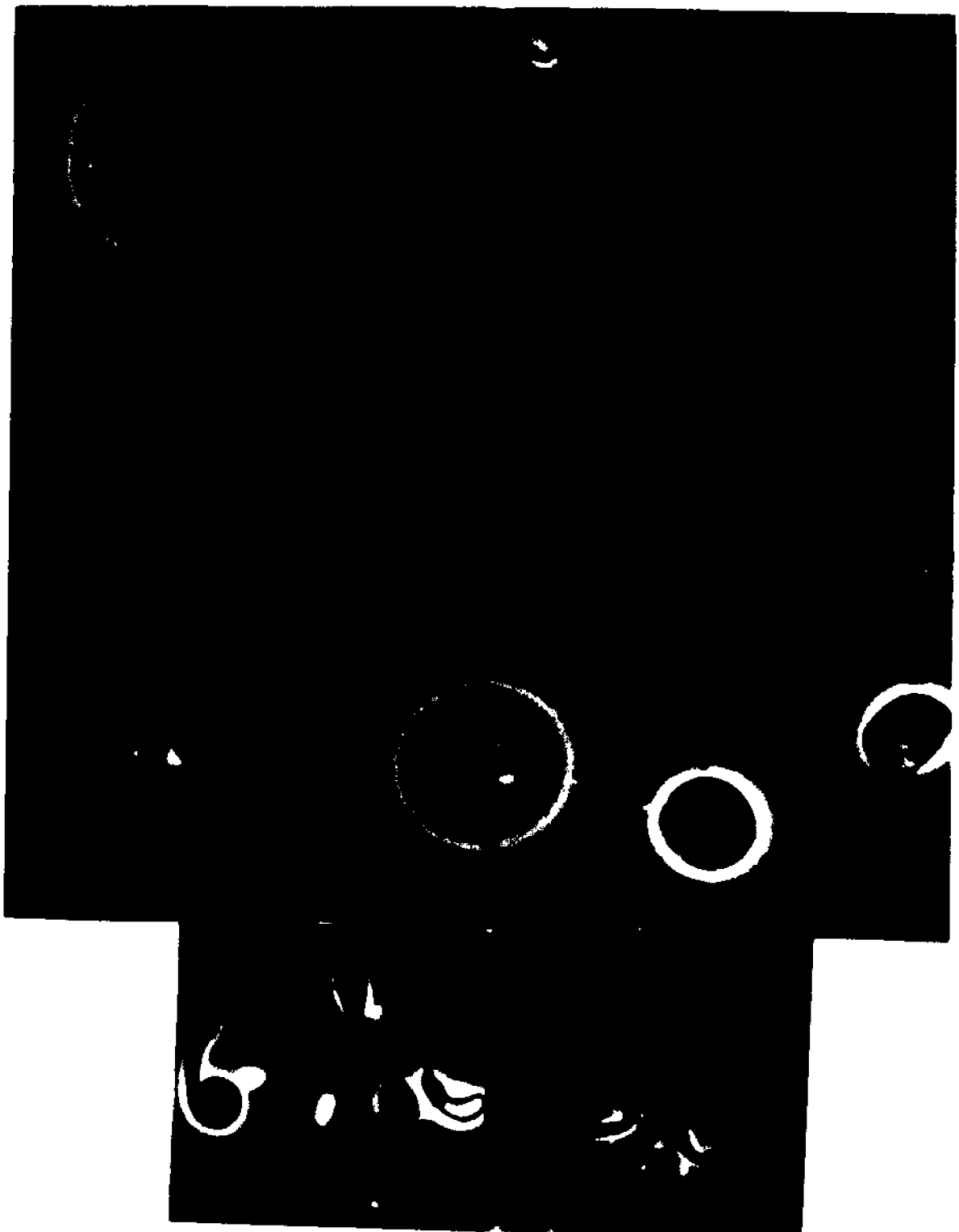
^bNuclear diameter not determined.

^cLength of cell body, excluding flagellum.

^dNuclear diameter measured over the narrow dimension.

Fig. 2. Phase contrast photomicrographs of spermatogenic cell types dissociated by collagenase treatment.

A, primary spermatogonium (1500x); B, early secondary spermatogonium (2240x); C, late secondary spermatogonium (1750x); D, leptotene spermatocyte (2240x); E, zygotene spermatocytes (2240x); F, pachytene spermatocyte (2240x); G, diplotene spermatocyte (2000x); H, secondary spermatocyte (2020x); I, round stage (1) and acrosome stage (2) spermatids (1440x); J, early-mid sloughing stage (1), late sloughing stage (2), and elongate stage (3) spermatids (1090x); K, mature spermatids and/or sperm (morphologically similar, 1040x).



slough stage spermatids which have somewhat elliptical, condensed nuclei and a condensed acrosome, which is no longer prominent as in the "acrosomal" spermatids. These cells still contain a full complement of cytoplasm. Mid-late slough stage spermatid nuclei are elliptical, highly condensed, and somewhat refractile. The latest slough stage cells have little cytoplasm and more extended nuclei than earlier stages.

Elongate spermatids (fig. 2J) have cast off most of their cytoplasm and contain highly condensed, refractile nuclei in various stages of elongation into the thin, helical shape typical of mature spermatids and sperm (fig. 2K).

While morphological criteria alone are sufficient to discriminate between most spermatogenic cell types in Xenopus laevis, certain limitations exist. As is often the case with sectioned material, the morphological delineation of leptotene (fig. 2D), zygotene (fig. 2E), pachytene (fig. 2F), and diplotene (fig. 2G) primary spermatocytes, using phase contrast optics, was somewhat arbitrary. This is due to the continuity of differentiation through these stages and the absence of absolute morphological boundaries. It is extremely difficult to distinguish late leptotene from early zygotene spermatocytes, as well as late zygotene from early pachytene primary spermatocytes. The same problem exists when assigning slough stage spermatids to mid- or late slough stage.

Cell Dissociation

Initial attempts to disperse testis cells by a rapid, mechanical means, such as gentle homogenization and repetitive pipetting, resulted in high yields of elongate spermatids, mature spermatids, and sperm. However, cells of earlier spermatogenic stages were severely damaged. Therefore, more gentle, enzymatic dissociation was attempted. Since trypsin had been shown to be effective in dissociating mammalian testis cells (55,257,265), attempts were made to trypsin dissociate Xenopus laevis testis cells. However, testis fragments stirred in 0.1% trypsin (1:250) for 30-60 min remained relatively undissociated. Basic histological differences between mammalian and anuran testes (250) may account for the difference in trypsin sensitivity.

Collagenase treatment of Xenopus laevis testes proved to be very effective in the preparation of spermatogenic cells. Testes fragments stirred in 0.15% collagenase became well dissociated in 1 to 1.75 hrs. The time required was variable and may be related to differences in the content of connective tissue in testes of different frogs (Kalt, personal communication). Dissociation progresses slowly for the first 30-40 min, with a period of rapid dispersal following this lag. The occurrence of this lag may be a function of the time required for adequate penetration of the tissue by collagenase.

The percentage of each cell type, the cell number (including residual bodies, the dense cytoplasmic bodies

cast off by spermatids), and the viability of cells obtained by collagenase treatment are presented in table 2. It is obvious from these data that collagenase treatment of Xenopus laevis testes produces a cell suspension highly enriched (99.9%) for spermatogenic cells. Few, if any, somatic cells are recovered in these preparations. Recently, a similar observation has been made following collagenase treatment of mammalian testes (259). Sertoli cells, when present, usually contain mature spermatids embedded in their cytoplasm. Most somatic cells remain in undissociated tissue fragments, which are removed by filtration.

The majority of the cells released by collagenase treatment are sperm and mature spermatids (morphologically similar). Unlike mammalian testes which store these cells in the epididymis, anurans store these cells intratesticularly. Secondary spermatocytes, and primary spermatocytes in diplotene, diakinesis, and metaphase (pooled due to the small numbers of these cells) represent a very small proportion of the cells released by collagenase treatment. The relative percentages of the individual primary spermatocyte stages, and secondary spermatocytes are consistent with those expected, considering the relative duration of each of these stages of spermatogenesis previously detailed (271). This observation suggests that spermatocysts containing these cell types are randomly dissociated by collagenase. However, deviations in total cell number and percentages of specific cell types may be expected because of

TABLE 2.--Percentages of cell types, cell number, and cell viability obtained following testis dissociation^{a, b}

Cell Type	Percentages of Cell Types	
	Collagenase Dissociate	Collagenase-Trypsin Dissociate
Primary spermatogonia . . .	0.3 [±] 0.2	0.5 [±] 0.2
Secondary spermatogonia + premeiotic S phase . .	0.6 [±] 0.2	1.0 [±] 0.1
Leptotene spermatocytes . .	1.4 [±] 0.7	1.2 [±] 0.2
Zygotene spermatocytes . .	1.2 [±] 0.4	1.2 [±] 0.3
Pachytene spermatocytes . .	2.0 [±] 0.4	2.1 [±] 0.3
Diplotene + metaphase spermatocytes	0.3 [±] 0.2	0.3 [±] 0.1
Secondary spermatocytes . .	0.2 [±] 0.1	0.3 [±] 0.1
Round stage spermatids . .	2.1 [±] 0.4	1.3 [±] 0.2
Acrosome stage spermatids .	3.0 [±] 0.5	2.3 [±] 0.2
Slough stage spermatids . .	4.4 [±] 1.3	2.6 [±] 0.2
Elongate stage spermatids .	5.8 [±] 0.6	5.9 [±] 0.6
Mature spermatids + sperm.	70.1 [±] 1.3	57.6 [±] 3.0
Sertoli cells	0.1 [±] 0.1	0.2 [±] 0.1
Leydig cells		0.2 [±] 0.1
Residual bodies	8.5 [±] 0.9 (n=4)	23.3 [±] 3.1 (n=3)
Cell Number/Testis (x 10 ⁻⁷)	8.5 [±] 0.9 (n=9)	7.5 [±] 1.2 (n=8)
Percent Viable Cells	98.7 [±] 0.4 (n=6)	94.0 [±] 2.0 (n=4)

^aErythrocytes not included in these analyses.^bAll data presented as means[±]standard errors of the means.^cCell numbers include residual bodies.

frog to frog and seasonal variations in the spermatogenic cell content of Xenopus laevis testes (271).

One disadvantage in the dissociation procedures described is the relatively lengthy periods required for adequate dissociation. The time of dissociation may be reduced if carried out at elevated temperatures (i.e., 30°C), if the viability of Xenopus spermatogenic cells is not significantly reduced at such temperatures. Potential adverse effects of lengthy dissociation periods may be avoided by carrying out the dissociations in a maintenance medium to prevent starvation for essential nutrients. Collagenase dissociation has been accomplished in modified DNOM (Defined Nutrient Oocyte Medium 273), lacking cysteine and cystine, with essentially the same yield of specific, viable spermatogenic cells.

In an attempt to increase the yields of spermatogonia and somatic cells, trypsin was added to suspensions containing testis fragments and spermatogenic cells which had been pre-treated with collagenase. Almost total dissociation of Xenopus laevis testes was achieved by this approach. Apparently, the resistance of this frogs testes to trypsin is removed by collagenase treatment. As table 2 demonstrates, spermatogonial and somatic cells were both increased in percent by this treatment. However, total cell number, percent sperm, and cell viability were all reduced in the cell suspensions obtained by the collagenase-trypsin method. An additional drawback to the use of trypsin is that it removes

the flagella from many spermatids and sperm.

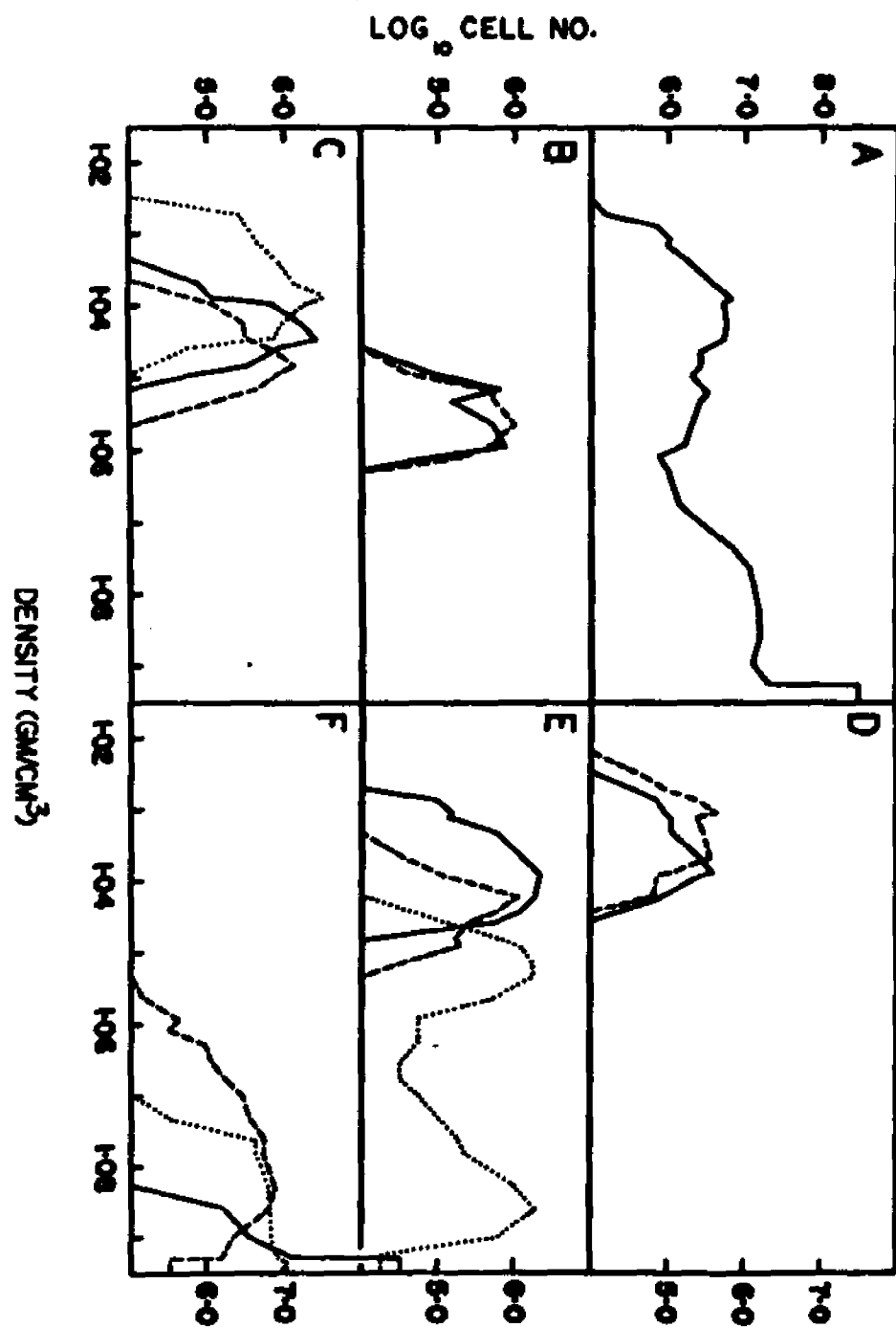
Cell Separation

Metrizamide, a nonionic, tri-iodinated carbohydrate, permits the preparation of solutions of relatively high density, low viscosity, and low osmolality (274). Moreover, Metrizamide appears to lack cytotoxic effects and does not irreversibly inhibit enzyme activity (275). Collagenase dissociated testis cells from Xenopus laevis have been incubated in 5% and 10% Metrizamide, at 4°C, without any appreciable reduction in cell number or cell viability (as measured by trypan blue exclusion) during a 4 hr period. These properties of Metrizamide permit its use as a density gradient material for the separation of testis cells by isopycnic centrifugation.

The distribution of spermatogenic cells following isopycnic centrifugation in a 2-15% Metrizamide gradient formed over 1 ml of 15% and 1 ml of 50% Metrizamide is depicted in figure 3(A-F). It is evident from figure 3A that the bulk of the cells form a dense band atop the 50% Metrizamide underlayer. This band is primarily composed of sperm and mature spermatids (23.2%), elongate spermatids (6.0%), and dense residual bodies (0.1%).

A relatively wide density range (1.065-1.092 g/cm³) is occupied by large numbers of residual bodies, mid-late slough stage spermatids and elongate spermatids. Another

Fig. 3. Distribution of spermatogenic cell types following isopycnic centrifugation in a 2-15% Metrizamide gradient. A, total cell distribution; B: ———, primary spermatogonia; -----, secondary spermatogonia; C: -----, leptotene; ———, zygotene;, pachytene; D: ———, secondary spermatocytes; -----, diplotene and metaphase; E: -----, round spermatids; ———, acrosomal spermatids;, sloughing spermatids; F: -----, residual bodies;, elongate spermatids; ———, mature spermatids and sperm. Density (g/cm^3) is relative to 5°C , the temperature of centrifugation.



relatively dense cell band occurs at $\bar{\rho}_{50C}=1.0403\pm.0008\text{ g/cm}^3$. This band is composed of primary spermatocytes, secondary spermatocytes, round stage spermatids, and acrosome stage spermatids.

As cells progress from spermatogonial, to diplotene, and metaphase stages their densities in Metrizamide appear to decrease. Primary spermatogonia (fig. 3B) band at two densities ($\bar{\rho}_{50C}=1.049\pm.002$ and $1.0578\pm.003\text{ g/cm}^3$). The reason for this is unknown. However, this distribution may be related to differences in DNA content created by S phase replication or gene amplification (276). Secondary spermatogonia and premeiotic S phase cells (fig. 3B) band at a density ($\bar{\rho}_{50C}=1.0553\pm.003\text{ g/cm}^3$) intermediate to those of primary spermatogonia. The distributions of leptotene ($\bar{\rho}_{50C}=1.047\pm.003\text{ g/cm}^3$), zygotene ($\bar{\rho}_{50C}=1.042\pm.001\text{ g/cm}^3$), pachytene ($\bar{\rho}_{50C}=1.0380\pm.005\text{ g/cm}^3$), and diplotene ($\bar{\rho}_{50C}=1.034\pm.001\text{ g/cm}^3$) primary spermatocytes (fig. 3 C-D) further demonstrate the tendency for spermatogenic cells to reduce in density as they prepare for cell division. Decreased densities of metaphase cells, relative to interphase cells, has been reported previously (263). Moreover, Meistrich and Trostle (266) have demonstrated a similar reduction in density as primary spermatocytes prepare for division during spermatogenesis in mice. This suggests that decreasing cell density during preparation for cell division is of general occurrence.

*Densities reported as mean $\pm$ standard error of the mean.

Secondary spermatocytes (fig. 3D) band at a position ($\bar{\rho}_{50C}=1.0378\pm.0003$ g/cm³) somewhat denser than the bulk of metaphase cells. This may be expected from the discussion above. The round stage spermatids (fig. 3E), produced following division of secondary spermatocytes, band at somewhat higher densities ($\bar{\rho}_{50C}=1.0423\pm.0008$ g/cm³). During the development of a large prominent acrosome, typical of acrosomal spermatids (fig. 2), spermatid density decreases .002g/cm³ ($\bar{\rho}_{50C}=1.0403\pm.003$ g/cm³).

Spermiogenesis in Xenopus laevis is accompanied by a gradual loss of cytoplasm from spermatids, condensation of the acrosomal vesicle, and condensation of the nucleus (271-272). All of these events may contribute to an increase in cell density. Slough stage spermatids exhibit two principal banding positions in these gradients (fig. 3E). Very early slough stage spermatids, which contain a full complement of cytoplasm, and a condensed nucleus and acrosome, band at higher densities ($\bar{\rho}_{50C}=1.050\pm.002$ g/cm³) than do the majority of acrosomal spermatids. As cytoplasm is cast off, these spermatids become further increased in density as exhibited by the mid-late slough stage spermatids ($\bar{\rho}_{50C}=1.0858\pm.0003$ g/cm³). The cytoplasmic remnants (residual bodies) produced by sloughing spermatids, may be found over a wide density range ($\bar{\rho}_{50C}=1.083\pm.0002$ g/cm³). Elongate spermatids and sperm are the densest spermatogenic cell types in Xenopus laevis (see below). Increasing cell density during spermiogenesis has also been demonstrated in

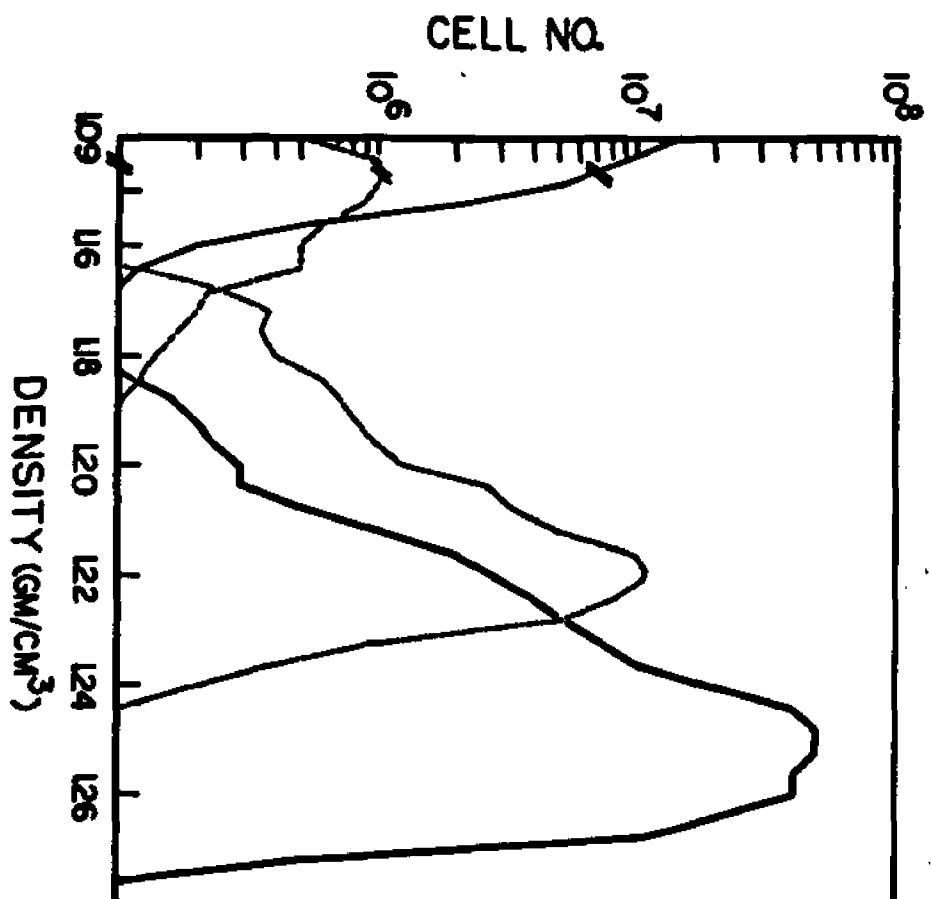
mouse (266), and ram (267).

A linear 30-50% Metrizamide gradient was used to separate cell types which form a dense band atop the 50% Metrizamide underlayer of the 2-15% Metrizamide gradients. Fractions with densities ranging from 1.0761 to 1.2743 g/cm^3 were pooled, diluted with 10 volumes of TSS, and the cells pelleted by centrifugation at 13,200 $\times$ g for 5 mins. These cells were resuspended in the 30% Metrizamide used to prepare the gradients. The methods of gradient formation, centrifugation, and gradient collection were the same as those for the 2-15% Metrizamide gradients. The cell type distribution in a 30-50% Metrizamide gradient is presented in figure 4.

Mature spermatids and sperm are the densest spermatogenic cell types in Xenopus laevis. These cells distribute over a relatively wide density region from 1.183 to 1.276 g/cm^3 . The majority of the mature spermatids and sperm band at $\rho_{50\text{C}} = 1.252 \text{ g/cm}^3$. Elongate spermatids were somewhat less dense than mature spermatids and sperm and banded at $\rho_{50\text{C}} = 1.219$. These spermatids were also distributed over a wide density range from 1.164 to 1.244 g/cm^3 . The distribution profiles of both elongate spermatids and mature spermatids plus sperm appeared skewed to lighter density regions. The reason for this is unknown as yet. However, it may be due to wall effects occurring during the collection of the gradients.

Residual bodies and mid-late slough stage spermatids

Fig. 4. Distribution of late spermatid and sperm cells following isopycnic centrifugation in a 30-50% Metrizamide gradient. —, residual bodies; ----, mid-late sloughing spermatids;, elongate spermatids; ———, mature spermatids and sperm. Density (g/cm^3) is relative to 5°C , the temperature of centrifugation.



form a band just beneath the layer of TSS placed on these gradients. However, both residual bodies and mid-late spermatids enter the gradients. Mid-late, slough stage spermatids banded at densities as high as 1.188 g/cm^3 .

Unlike the 2-15% Metrizamide gradients, it is not possible to counteract the osmotic strength of Metrizamide in 30-50% Metrizamide gradients. Therefore, the densities of the cell types reported above are most likely greater than their native densities. The hyperosmotic conditions in the 30-50% Metrizamide gradients (21 mM NaCl) did not alter the morphology of the spermatids described above. Moreover, many of the flagellated spermatids and sperm remained motile when they were recovered from the Metrizamide gradients and resuspended in 10% TSS.

Inclusion of the cells within Metrizamide gradients prior to centrifugation permits the rapid fractionation of a large number of cells (up to 7×10^8) in a single gradient. The average yield of intact, viable cells following isopycnic centrifugation was $79.1 \pm 4.9\%$. Similar distribution profiles to those presented in figures 3 and 4 have been obtained when cells were layered on a preformed gradient prior to centrifugation. However, the high concentration of cells in the starting cell band produced physical trapping of low density cell types by rapidly sedimenting high density cell types. The observation that the banding positions of individual cell types is independent of the starting position in

*Mean $\pm$ standard error of the mean.

the gradient demonstrates that this method is isopycnic. Further support for this comes from the observation that similar profiles are produced by 30, 45, or 60 mins of centrifugation.

Many of the cell types with overlapping density profiles in 2-15% Metrizamide gradients differ in size (see table 1). More refined separations of these cell types were achieved by a two-step purification procedure using unit gravity sedimentation and subsequent isopycnic centrifugation.

Spermatogenic cells obtained by collagenase dissociation were separated by unit gravity sedimentation in a 1-4% BSA gradient. The distribution of the spermatogenic cells following a 3.5 hr sedimentation period is presented in figure 5. The majority of the spermatogonia and primary spermatocytes sediment faster than 6.00 mm/hr while more advanced spermatogenic cells sediment slower. Cells sedimenting between 6.5 mm/hr and 13.0 mm/hr (130-360 ml) and cells sedimenting between 0.6 and 5.5 mm/hr (410-560 ml) were pooled. The composition of each of these pools is presented in table 3. It is evident that many of the cell types with similar densities have been separated by this procedure. Both pools from the unit gravity sedimentation were centrifuged to equilibrium in two separate 2-15% Metrizamide gradients (formed as described previously). The cell types which band atop the 50% Metrizamide underlayers were pooled and further separated in 30-50% Metrizamide gradients.

Fig. 5. Total spermatogenic cell distribution following unit gravity sedimentation through a 1-4% bovine serum albumin (BSA) gradient. Loading time was 90 mins. Sedimentation continued for 3.5 hrs, followed by a 90 min period of fractionation.

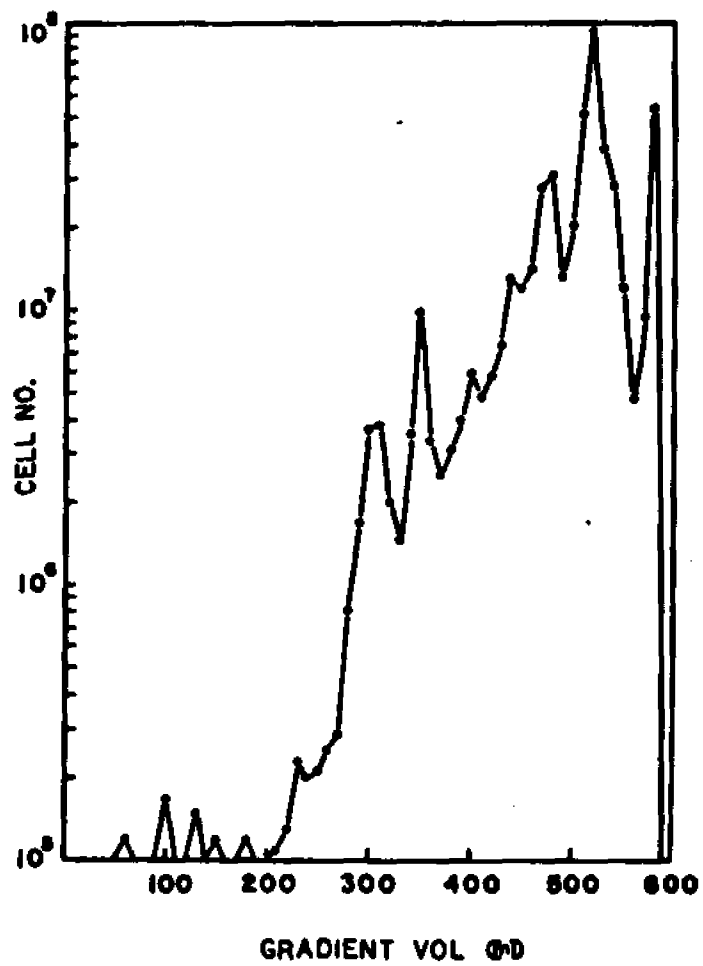


TABLE 3.--Percentages of cell types in pooled fractions obtained by unit gravity sedimentation

Cell Type	Cell Percents	
	6.5-13.0 mm/hr	0.6-5.5 mm/hr
Primary spermatogonia....	4.6	...
Secondary sperm-togonia + premeiotic S phase....	9.1	0.1
Leptotene spermatocytes..	15.0	0.2
Zygotene spermatocytes...	17.5	0.2
Pachytene spermatocytes..	29.8	0.3
Diplotene + metaphase spermatocytes.....	4.6	...
Secondary spermatocytes..	1.3	0.2
Round spermatids.....	1.6	2.4
Acrosomal spermatids.....	3.5	3.0
Sloughing spermatids.....	1.2	4.6
Elongate spermatids.....	0.2	5.0
Mature spermatids + sperm	0.8	74.8
Sertoli cells.....	0.3	...
Residual bodies.....	10.5	9.2

Table 4 presents the maximum purities of specific spermatogenic cell types that have been obtained by this two-step procedure. Obviously highly enriched fractions of most cell types were obtainable. However, certain cell types remain difficult to separate. Primary and secondary spermatogonia continue to be unseparated by this procedure. In addition, round spermatids are difficult to further purify because of the overlap of their density profiles and those of acrosome-stage spermatids and early slough stage spermatids. More discrete pooling of fractions obtained by unit gravity sedimentation would probably increase the enrichments obtainable for particular cell types.

The photomicrographs in figure 6 demonstrate that the morphological integrity of most cell types is retained during the separation procedure. The morphology of late spermatids is usually altered by the loss of flagella. This is most likely due to mechanical breakage of the flagella during testis dissociation and during gradient formation.

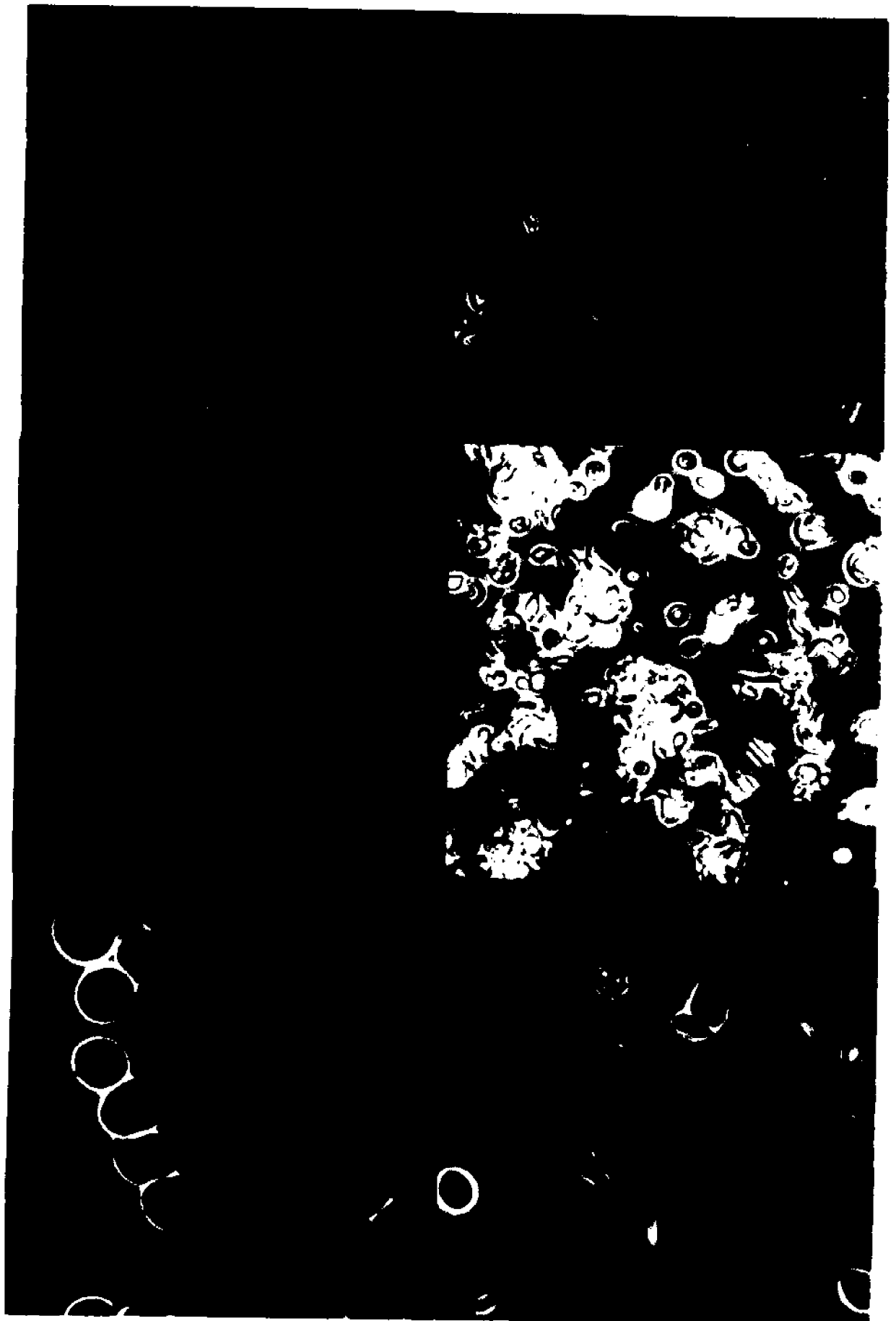
Discussion

Dissociation of Xenopus laevis testis cells with collagenase provides a cell suspension highly enriched for spermatogenic cells and deficient in somatic cell types. Somatic cells localized in the interstitial tissue remain relatively undissociated following collagenase treatment. Sertoli cells released by the action of collagenase invariably contained late-stage spermatids embedded in their cyto-

TABLE 4.--Maximum purities of spermatogenic cell types obtained by two-step purification

Cell Type	Maximum Purity (%)	Major Contaminating Cell Types
Primary spermatogonia	48.1	Secondary spermatogonia
Secondary spermatogonia + preneeliotic S phase	61.1	Primary spermatogonia
Leptotene spermatocytes . . .	79.4	Zygotene + secondary spermatogonia
Zygotene spermatocytes . . .	68.8	Leptotene + pachytene spermatocytes
Pachytene spermatocytes . . .	87.4	Zygotene + diplotene spermatocytes
Diplotene + metaphase sperma- tocytes	59.7	Pachytene spermatocytes
Secondary spermatocytes . . .	87.1	Acrosomal spermatids
Round spermatids	49.9	Acrosomal and sloughing spermatids
Acrosomal spermatids	81.4	Round spermatids
Early sloughing spermatids .	85.9	Mid-late sloughing spermatids
Mid-late sloughing spermatids	65.5	Residual bodies + elongate spermatids
Elongate spermatids	67.5	Mature spermatids + sperm
Mature spermatids + sperm . .	99.8	Elongate spermatids
Residual bodies	94.1	Mid-late sloughing spermatids

Fig. 6. Phase contrast photomicrographs of spermatogenic cells separated by unit gravity sedimentation and isopycnic centrifugation. A, leptotene spermatocytes (624 x); B, pachytene spermatocytes (580 x); C, acrosomal spermatids (720 x); D, mid-late sloughing spermatids (800 x); E, elongate spermatids (600 x); F, mature spermatids and sperm (560 x).



plasm. Sertoli cells which envelope spermatocysts containing earlier spermatogenic stages are apparently destroyed during the dissociation of the cysts and subsequently removed during filtration. Romrell et al., (259) have demonstrated that collagenase dissociation may also be used to prepare mouse spermatogenic cells free of somatic cells.

Cell viability as measured by trypan blue exclusion, was consistently high in cell suspensions prepared by collagenase dissociation. Measurements of radioactive amino acid and uridine incorporation into these cell suspensions have demonstrated that protein and RNA synthesis are continuous for at least 16 hours (see chapter 4). The ability to prepare a viable testis cell suspension, containing few somatic cells, offers obvious advantages when highly enriched spermatogenic cell types are required for study.

Unit gravity sedimentation has been utilized extensively to prepare fractions enriched for specific spermatogenic cells (253-259). However, similarities in the sizes of several spermatogenic cell types limits the resolution obtainable by this approach. Isopycnic centrifugation provides a means to separate cells with different densities. The sequential use of unit gravity sedimentation and isopycnic centrifugation takes advantage of differences in two distinct physical parameters and may be utilized to prepare highly enriched fractions of specific cell types.

Metrizamide offers several advantages over other den-

sity gradient materials for cell separations. Metrizamide is nonionic and may be used to prepare aqueous solutions of relatively high density, low viscosity, and low osmolality. Little change has been noted in the viability of Xenopus laevis spermatogenic cells following exposure to Metrizamide. The primary disadvantage in the use of Metrizamide is the necessity to prepare fresh solutions prior to each cell separation, due to the inability to store Metrizamide solutions longer than one week.

Isopycnic centrifugation in Metrizamide is a rapid means to achieve separation of Xenopus laevis testis cells. The centrifugation, collection, and pooling of fractions can be accomplished in 90 mins. Xenopus laevis spermatogenic cells display a wide variation in density (1.034 - 1.276 g/cm³), as measured in Metrizamide at 5°C. Mouse spermatogenic cells also display density differences following centrifugation in Renografin (266). However, specific mouse spermatogenic cells (minus sperm) band at higher densities (1.07 - 1.147 g/cm³) in Renografin than those of similar Xenopus laevis spermatogenic cells in Metrizamide. The higher densities of mouse spermatogenic cells in Renografin are most likely due to the hyperosmotic effect of Renografin rather than large inherent density differences between equivalent Xenopus laevis and mouse spermatogenic cells. The osmotic effects of Metrizamide have been compensated for by the adjustment of NaCl concentrations in the solutions used to prepare 2-15% Metrizamide gradients. Due to the lack of detectable osmotic

effects (morphological alterations) in the 2-15% Metrizamide gradients, the cell densities measured in these gradients most likely reflect the native densities of these cell types. However, it was not possible to totally compensate for the hyperosmotic condition in 30-50% Metrizamide gradients.

Separations of testis cells have been accomplished with as many as 7×10^8 cells in a single 2-15% gradient. This relatively large capacity could only be achieved by distributing the cells throughout the gradients prior to centrifugation. If such a large number of cells is layered on top of the gradients, physical trapping during centrifugation reduces the resolution obtainable. An additional factor affecting resolution is encountered during gradient collection. In early studies, it was difficult to avoid contamination of low density fractions with the large number of cells that sediment through 15% Metrizamide (elongate spermatids, sperm). Several modifications in the procedures helped overcome this problem. First, a 50% Metrizamide underlayer was introduced to prevent the cells from pelleting. Second, a 1 ml layer of 15% Metrizamide was layered on top of the 50% Metrizamide underlayer, prior to formation of the 2-15% linear gradient. This layer elutes late spermatids and sperm which may adhere to the walls of the centrifuge tube during gradient collection.

Many of the cells with overlapping density profiles differ in size. Highly enriched preparations of specific

cell types could be achieved by first separating cells with similar densities by unit gravity sedimentation, followed by density gradient centrifugation. The large numbers of sperm in Xenopus laevis testes make it difficult to obtain highly refined separations of spermatids during short (3-5 hrs) unit gravity sedimentations. However, the preparation of a rapidly sedimenting fraction (6.5-13.0 mm/hr) and a slowly sedimenting fraction (0.6-5.0 mm/hr) excluded most cells with similar densities from the same fraction. The cells in each fraction could then be separated by taking advantage of the density differences between those cells.

The time required to achieve dissociation and separation by the two-step procedure is approximately 9 hrs. The development of more rapid dissociation procedures could reduce the time involved. A more significant reduction in time might be achieved by the substituting sedimentation velocity in the Beckman Elutriator rotor (260) for unit gravity sedimentation. Improvements in the resolution of Xenopus laevis spermatogenic cells by velocity sedimentation would certainly increase the maximum enrichments obtainable by the two-step procedure.

CHAPTER 3

BASIC CHROMATIN PROTEIN CHANGES DURING SPERMATOGENESIS

Introduction

Block (213) has placed the sperm of Xenopus laevis in the "intermediate" or Mytilus category for sperm basic proteins. This classification was based upon cytochemical evidence which indicated that the arginine content of sperm basic proteins was intermediate to that of somatic histones and salmine. Cytochemical analyses by Bols and Kasinsky (277) have also demonstrated an increase in the arginine content of Xenopus laevis sperm basic proteins relative to somatic cell basic proteins.

Electrophoretic analyses of the basic chromatin proteins found in Xenopus laevis testis cells have demonstrated the presence of three electrophoretic bands (X₁, X₂, X₃) not found in somatic cells (278). These bands had electrophoretic mobilities intermediate to those of histones and salmon protamine. The histones were not well resolved in this study. As a result, little is known of the qualitative or quantitative changes which may occur in the histones during spermatogenesis in Xenopus laevis. As discussed in chapter 1, such variations in the histones are common in testis cells.

In this chapter, high resolution electrophoretic studies of the basic chromatin proteins on Xenopus laevis testis cells are described. The chromatin basic proteins of total testis cells, as well as specific spermatogenic cell types, have been studied and compared to somatic cell histones. The primary purpose of these studies was to determine

if qualitative and/or quantitative changes occur in the histones during spermatogenesis in this frog. An additional purpose was to determine the cell types in which such changes occur. Preliminary results from these studies have been presented elsewhere (279,280).

Materials

Tricaine methanesulfonate is a product of Ayerst (New York, N.Y.). Phenylmethylsulfonylfluoride was obtained from Sigma Chem. Co. Metrizamide was purchased from Gallard-Schlesinger Chem. Co. (Carle place, N.Y.). Acrylamide was obtained from Eastman Chemicals and recrystallized twice from chloroform, prior to use (29). N, N'-methylenebisacrylamide and ammonium persulfate were Bio-Rad electrophoretically pure reagents. Ultra-pure urea and guanidine chloride were purchased from Schwarz/Mann (Orangeburg, N.Y.). Miracloth was obtained from Chicopee Mills (New York, N.Y.). Electrophoresis was carried out in a Buchler apparatus equipped with a Buchler power supply. Dialysis tubing with a retention capacity of 3,500 daltons was obtained from A.H. Thomas Co., Philadelphia, Pa.

Methods

Tissue Preparation

Somatic histones were extracted from chromatin of liver, kidney, intestine, and erythrocytes. The histone composition of these tissues were examined in both adult male and

female Xenopus laevis and was found to be the same in both sexes. Tissue preparation and histone isolation were carried out at 4°C unless otherwise stated.

Tissues were taken from frogs anesthetized in Tricaine methanesulfonate, bled by heart puncture, and perfused with 0.14 M NaCl, 15 mM $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ (pH 7.0), 50 mM NaHSO_3 , 250 μM phenylmethylsulfonylfluoride (PMSF). On several occasions tissues were dissected out of doubly pithed frogs. In these instances, the blood clotted rapidly and was not collected. Tissues taken from pithed frogs were minced in perfusing solution and washed several times to remove most of the blood. Intestines and kidney were always dissected free of mesentery. Intestinal contents were removed if necessary. Blood cells were washed twice by repeating centrifugation and resuspension in perfusing solution. Centrifugation was at 450 x g for 5 mins in the Sorvall Ho-4 rotor. Supernatants and the buffy coat on the pellets were aspirated and discarded. All tissues were used fresh or immediately frozen in liquid nitrogen and stored on dry ice for no more than 2 days. Prolonged storage (greater than one week) of tissues at -20°C results in histone degradation.

Testis Cell Separations

Testis cells were dissociated by collagenase treatment and separated by unit gravity sedimentation followed by isopycnic centrifugation in Metrizamide (see chapter 2). Rapid separation of early spermatogenic stages (preceding

late sloughing spermatid stages) and late spermatid plus sperm cells was accomplished by centrifugation of dissociated germinal cells in 10 ml of 12% Metrizamide (in TSS adjusted to 21 mM NaCl). Cells were resuspended in the 12% Metrizamide and centrifuged at $13,200 \times g$ for 20 mins (5°C) in a Sorvall Hb-4 rotor. Pellets contained late spermatids and sperm. The supernatants, which contain spermatogonial to early slough stage spermatids, were diluted with 10 vols of TSS and pelleted at $13,200 \times g$ for 15 mins (Sorvall Hb-4 rotor, 4°C)

Nuclear Isolations

Method A: To obtain highly purified nuclei, a modification of the nuclear isolation method described by Panyim et al. (282) was used. All centrifugations were carried out in a Sorvall Hb-4 rotor at 4°C unless otherwise noted. Tissues were minced thoroughly (if applicable) in homogenizing solution (approximately 1 gm/10 ml) consisting of 0.25 M sucrose, 3 mM CaCl_2 , 3 mM Tris-HCL (ph 8.0), 50 mM NaHSO_3 , 250 μM PMSF. The minced tissues were homogenized in a Waring blender (micro-cup) equipped with a Variac transformer. Homogenization was carried out at 60 volts for 2 mins followed by 90 volts for 1 min. The homogenates of liver, kidney, and intestine were filtered through 4 layers of cheesecloth and then centrifuged at $650 \times g$ for 5 mins. The pellet of crude nuclei was resuspended in homogenizing solution containing 0.4% Triton X-100, and hand homogenized via

10 strokes in a Dounce homogenizer equipped with the loose-fitting pestle. The homogenate was filtered through two layers of Miracloth. Nuclei in the filtrate were pelleted as above. This step was repeated twice more. The final nuclear pellet was resuspended by hand homogenization (approx.) 1 gm per 10 ml) in 2.2 M sucrose, 0.4% Triton X-100 (otherwise identical to homogenizing solution). This was layered over 15 ml of 2.4 M sucrose, 0.4% Triton X-100 (otherwise identical to homogenizing solution) in an SW 25.2 centrifuge tube. Centrifugation was carried out at 24,000 rpm for 1.5 hrs in a Beckman L2-65B preparative ultracentrifuge. The pellets contained purified nuclei.

Phase contrast microscopy was utilized to assess nuclear purity throughout the purification procedure. While testis, erythrocyte, intestine, and kidney nuclei were essentially free of cytoplasmic tags and debris when purified by this procedure, liver nuclei remained contaminated by pigment granules.

Method B: The method to be described below is more rapid than Method A and more useful when histones are to be isolated from small amounts of tissue (see 228). Liver, intestine, and kidney were homogenized in the Waring blender as described in method A. However, 0.55 M sucrose was substituted for 0.25 M sucrose in the homogenizing solution. Erythrocytes and testes were homogenized in this solution with 10 strokes of a Dounce homogenizer equipped with the

loose-fitting pestle. Following filtration through cheese-cloth (liver, intestine, kidney) the nuclei were pelleted by centrifugation at 650 x g for 5 mins. The nuclei were then washed 4 times in 0.55 M sucrose, 0.4% Triton X-100 (otherwise identical to homogenizing solution) by homogenization with the Dounce homogenizer (5 strokes) and centrifugation at 650 x g for 10 mins. Each washing was followed by Miracloth filtration. Phase contrast microscopy indicated that nuclei prepared in this manner from testes and erythrocytes were essentially free of cytoplasmic tags and debris. However, liver, intestine, and kidney nuclear preparations remained contaminated by cytoplasmic components.

Chromatin Preparation and Histone Extraction

Nuclei prepared by either method were washed successively (twice in each solution, approximately 50 ml per ml of packed nuclei) in: (1) 0.14 M NaCl, 3 mM Tris-HCL (pH 8.0); (2) 0.30 M NaCl, 5 mM Tris-HCL (pH 8.0); and (3) 20 mM EDTA, 20 mM Tris-HCL (pH 8.0). All solutions contained 50 mM NaHSO₃ and 250 μ M PMSF. More than 2 washes of erythrocyte nuclei in 0.30 M NaCl, 5mM Tris-HCL (pH8.0) were occasionally required to remove hemoglobin from these nuclei. Washing was accomplished by homogenization (5 strokes) with the loose-fitting pestle in a Dounce homogenizer, followed by centrifugation at 450 x g for 5 mins. Chromatin was prepared by washing the final nuclear pellet two to three times with distilled H₂O containing 250 μ M PMSF. The swollen chromatin was

sheared at maximum speed for 3 mins (three, 1 min bursts) in a Virtis 45 homogenizer. The homogenate was centrifuged at 13,200 x g for 10 mins. Soluble nucleoprotein was made 0.4 N with H_2SO_4 and stirred for 2 hrs at 4°C. Acid insoluble material was removed by centrifugation at 13,200 x g for 20 mins. The acid-insoluble pellets were then re-extracted with 0.4 N H_2SO_4 and the extracts combined.

Late spermatid and sperm nuclei were found to be resistant to shear. Therefore, washed testis nuclei were first suspended (approximately 5 ml/10 testes) in 2 M guanidine chloride, 10 mM Tris-HCL (pH 8.0), 250 μ M PMSF, to disperse the chromatin, and then sheared in the Virtis 45 homogenizer as described above. The testis chromatin homogenate was made 0.4 N in H_2SO_4 and then dialyzed overnight against 200 volumes of 0.4 N H_2SO_4 , in dialysis membranes with a minimum retention capacity of 3500 daltons. Acid insoluble material was then removed by centrifugation as described above.

Basic proteins were precipitated from the 0.4 N H_2SO_4 extracts with 20% trichloroacetic acid or 5 vols of 95% ethanol (18 hrs at -20°C). Precipitates were collected by centrifugation at 13,200 x g for 10 mins. The pellets were washed twice each in cold acetone-HCL (0.1 ml conc. HCL/200 ml acetone) and cold acetone and then dried in vacuo.

Amberlite Chromatography

Erythrocyte H1 histones were fractionated by chroma-

tography on Amberlite CG-50. Amberlite CG-50 was prepared and poured in a 0.6 (i.d.) x 55 cm glass column as previously described (281). Six mg of histone H1 in 0.4 ml of 8% guanidine chloride (GuCl), 0.1 M sodium phosphate buffer (pH 6.8) were loaded on the column and then eluted with a 50 ml 8-13% gradient of GuCl in 0.1 M sodium phosphate buffer (pH 6.8), and then 8 ml of 40% GuCl in 0.1 M sodium phosphate buffer (pH 6.8). Fractions were collected at a flow rate of 2.9 ml/hr. Protein was determined by a Turbidimetric method (281). Percent GuCl was measured with an Abbe refractometer.

Electrophoresis

Histones were dissolved in 0.9 N acetic acid, 10% 2-mercaptoethanol, 15% sucrose (1 mg/ml). When working with very small amounts of histone, the 0.4 N H_2SO_4 extracts were dialyzed against 500 volumes of 0.9 N acetic acid, 10% 2-mercaptoethanol and then concentrated by dialysis against the same solution containing 25% sucrose. Short (5 mm, i.d. x 10 cm) gels were prepared, pre-electrophoresed and electrophoresed as described by Panyim and Chalkley (283, 284). Long gels (5.0 mm, i.d. x 25 cm) were prepared as previously described (282-284) except that the N, N-methylene bisacrylamide concentration was raised to 1% while acrylamide was lowered to 14%. This modification reduced the degree of contraction and swelling which occurs during polymerization, electrophoresis and staining. Generally, 20-30 μ g of histones were loaded on short gels and 40-50 μ g

on long gels. Electrophoresis was carried out with a Buchler apparatus and power supply at constant voltage. The conditions of electrophoresis are specified in the results section. Gels were stained in 1% fast green, 7% acetic acid, 20% ethanol for two hours. They were partially destained in an electrophoretic destainer (Canalco) and then diffusion destained (12-24 hrs in 7% acetic acid, 20% ethanol) to completion.

Gels were scanned at 630 nm with a Gilford 240 spectrophotometer equipped with a Model 2410 S linear transport device. Relative percents of individual histones were determined by cutting out and weighing histone peaks from photocopies of original gel scans. Paper was determined to be uniform in weight. Where peaks overlapped, equal contribution of each peak to the area of the other was assumed.

Several criteria were utilized to identify individual histone bands. Solubility in 5% perchloric acid was used to identify histone H1 (285). Histone H3 was readily identified by comparative electrophoresis of reduced and oxidized (histones incubated at 2 mg/ml in 0.4 M Tris-HCL, pH 8.0, at 25°C for 24 hrs) histones. Histone H3 is the only cysteine containing histone and forms disulfide containing dimers in amphibians (286,287). Histones H2B and H2A were differentiated by comparative electrophoresis of histones in gels of varying urea concentration. The relative electrophoretic mobility of amphibian histone H2A decreases with increasing urea concentration in polyacrylamide gels (282). Histone H4 was

identified by co-electrophoresis with calf thymus histone H4. Stained bands corresponding to histones H3 and H4 were used to align gels for comparative purposes. The protocol for identification of histone bands was developed by Panyim et al., (282).

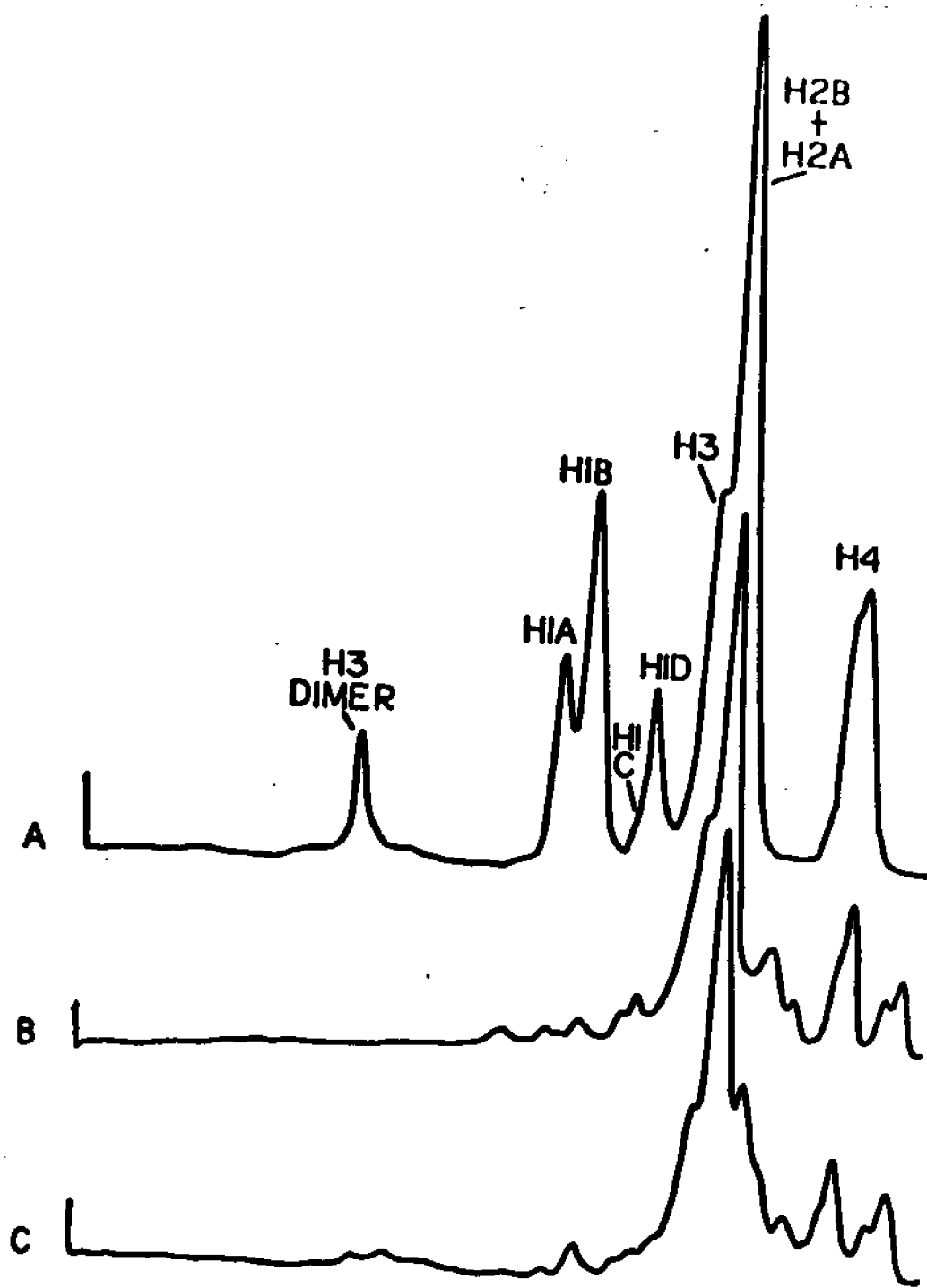
Results

Histone Degradation in Xenopus laevis

Previous studies have demonstrated the presence of highly active histone proteases on the chromatin of various tissues (288-293). These proteases were found to be effectively inhibited by 0.05 M NaHSO₃ (289,290). Initial attempts to isolate undegraded histones from Xenopus laevis (method A) with 0.05 M NaHSO₃ as the sole protease inhibitor were unsuccessful. As demonstrated in figure 7 (A-C), histone degradation was extensive during preparation from liver and kidney, whereas erythrocyte histones remained undegraded. Histone degradation is usually accompanied by the appearance of electrophoretic bands between histones H2A and H4 and leading histone H4 (282,289,290). Similar degradation was encountered during the preparation of intestine histones.

Panyim et al., (282,289) and Adler and Gorovsky (228) also encountered degradation problems while working with other cold blooded animals. These investigators noted a direct correspondence between histone integrity and animal health. Erythrocyte histones seemed to be very susceptible to degradation in Buffalo fish (282). Such is not the case

Fig. 7. Absorbance profiles of histones isolated by method A without phenylmethanesulfonylfluoride. A, erythrocyte histones (unreduced); B, kidney histones; C, liver histones. Degradation products appear as peaks between histones H2B+H2A and histone H4, as well as peaks which migrate somewhat faster than histone H4. Electrophoresis was on 10 cm, 2.5 M urea gels for 3.5 hrs at 130 volts and 25°C.



in Xenopus laevis. This suggests that the degradation observed may be due to highly active tissue specific proteases. Recently, Destree et al., (292) have shown that histone degradation in Xenopus laevis liver is due to cytoplasmic protease activity.

In an attempt to inhibit protease activity during histone isolation 250 μ M phenylmethylsulfonylfluoride (PMSF) has been included in all perfusing and nuclear isolation solutions. This compound has been shown to be an effective inhibitor of the chromatin bound histone protease of rat liver (293). Figures 8 and 9 present gel scans of histones isolated from nuclei prepared by method A in the presence of PMSF and NaHSO_3 . The lack of electrophoretic bands migrating faster than histone H4 or between histones H2A and H4 (see fig 9) suggests that histone degradation has been efficiently inhibited. Histone H1, which is most susceptible to degradation, remained intact when nuclei were prepared in the presence of PMSF.

Characterization of Basic Chromatin Proteins

Basic chromatin proteins extracted from various somatic tissues and testes by method A, were electrophoresed on 10 cm gels containing 2.5 M urea (fig. 8) and on 10 cm gels lacking urea (fig. 9). Basic proteins from somatic tissues of Xenopus laevis commonly exhibit 7 major bands on 2.5 M urea gels and 8 major bands on gels lacking urea. Histone H4 from Xenopus has been identified as the band with

Fig. 8. Absorbance profiles of histones prepared from various tissues and electrophoresed on 10 cm, 2.5 M urea gels. A, liver histones; B, intestine histones; C, kidney histones; D, erythrocyte histones; E, testis histones. Electrophoresis was for 4 hrs at 130 volts and 25°C.

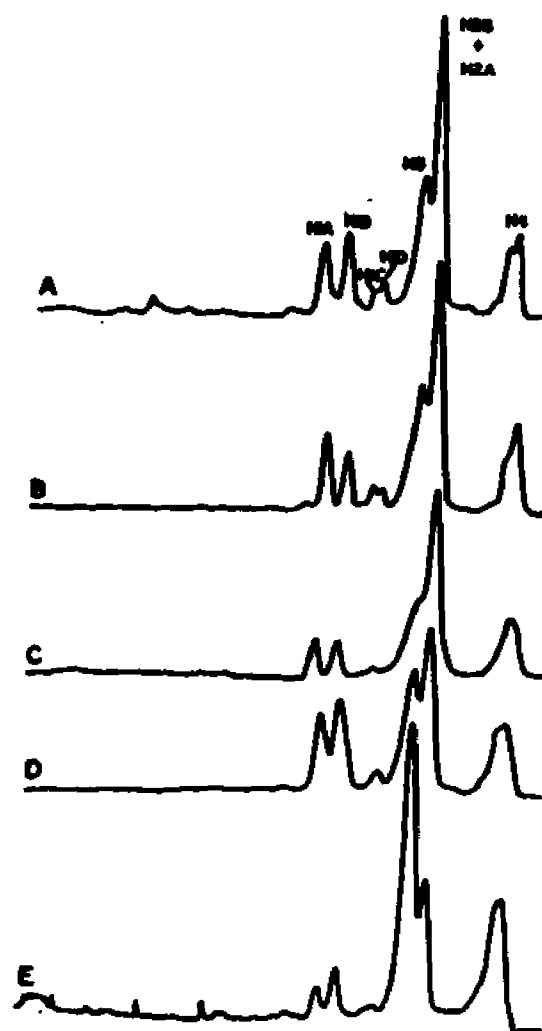
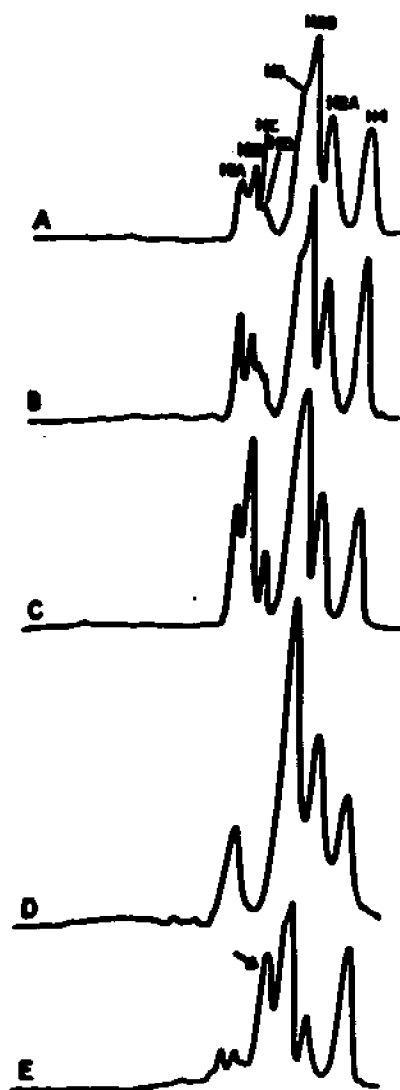


Fig. 9. Absorbance profiles of histones prepared from various tissues and electrophoresed on 10 cm gels lacking urea. A, liver histones; B, intestine histones; C, erythrocyte histones; D, calf thymus histones (a generous gift from N. A. Rubin); E, testis. Electrophoresis was for 3 hrs at 130 volts and 25°C.

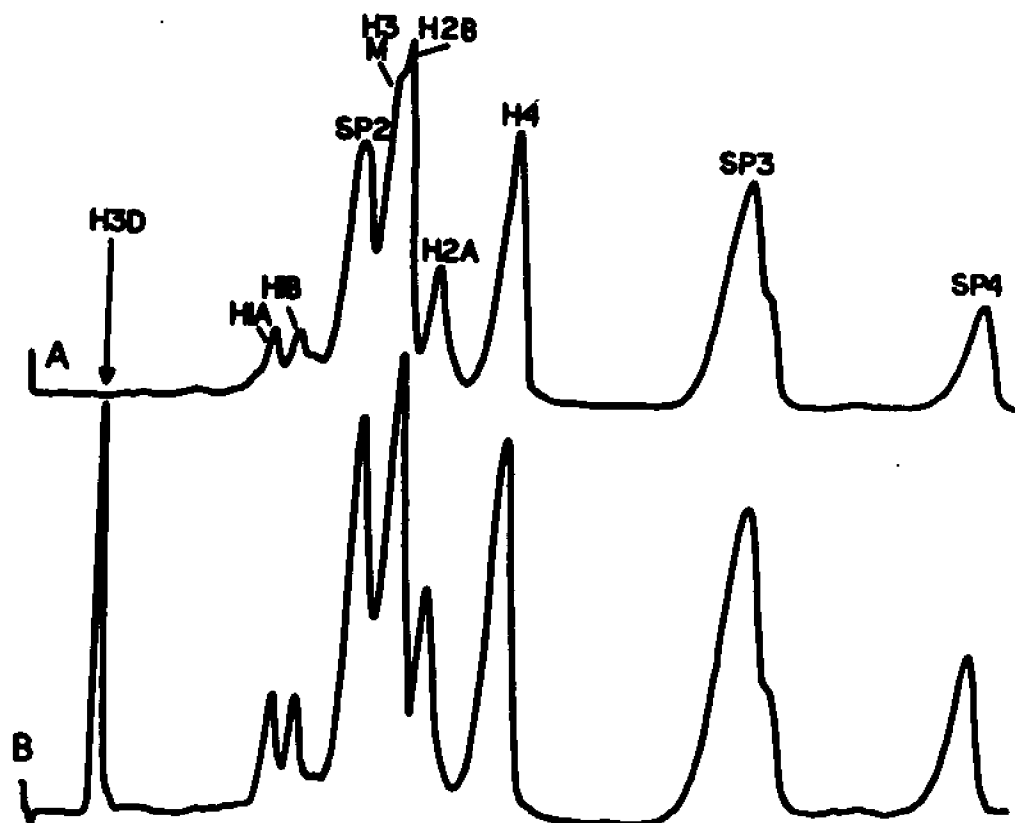


the greatest mobility on both types of gels. This band comigrates with calf thymus histone H4 and is often resolved into sub-fractions which typically correspond to unsubstituted, mono- and diacetylated species of histone H4 (see 282).

Calf thymus histone H3 comigrates with the third fastest band of Xenopus basic proteins on 2.5 M urea and with the fourth fastest on gels lacking urea. These bands become quantitatively reduced following incubation in 0.4 M Tris-HCL (pH 8.0) for 24 hrs. A band with approximately half of the electrophoretic mobility of these bands appears following incubation in Tris buffer (fig. 10). Previous studies have demonstrated that histone H3 alone contains cysteine and is capable of forming high molecular weight disulfide containing aggregates (286,287). These observations indicate that these bands are composed of Xenopus laevis histone H3. Since Xenopus histone H3 forms a single oxidation product it is likely that it contains a single cysteine residue. Amino acid analyses have confirmed this expectation (294,295).

Histones H2B and H2A from amphibians co-electrophorese on gels containing 2.5 M urea, while mammalian histones H2B and H2A are resolved into two distinct bands (282,283). A single band is found between histones H3 and H4 when Xenopus laevis histones are electrophoresed on gels containing 2.5 M urea (fig. 8). This band has a mobility similar to that of calf thymus histone H2B. Two electrophoretic bands appear between Xenopus histones H3 and H4 on gels lacking urea (fig. 9). These two electrophoretic species appear to have

Fig. 10. Electrophoretic comparison of oxidized and reduced testis basic proteins. A, testis basic proteins reduced with 10% 2-mercaptoethanol; B, testis basic proteins oxidized by incubation in 0.4 M tris-HCL (pH 8.0) at 25°C for 24 hrs; H3M, histone H3 monomer; H3D, histone H3 dimer. Electrophoresis was on 10 cm gels lacking urea for 2 hrs at 130 volts (25°C). The top (1 cm) of the gel was removed to permit scanning of the entire gel.



derived from components of the single band observed between histones H3 and H4 on 2.5 M urea gels. These bands are similar in mobility to calf thymus histones H2B and H2A and most likely represent the Xenopus counterparts of these two histones. The ability to distinguish amphibian histones H2B and H2A by these procedures is well documented (29,282,283). Increasing the gel urea concentration to 6.25 M results in the comigration of Xenopus histones H3 and H2A (fig. 11). This is indicated by the presence of a relatively large amount of absorbance in the H3 monomer position, even after oxidation of much of H3 to the dimer form (fig. 11). This observation agrees with previous reports (55,295,296).

The four electrophoretic bands with the slowest mobility have been designated as H1 histones. One of the criteria used for this assignment has been their selective solubility in 5% perchloric acid. Previous studies have shown that histone H1 alone is soluble in 5% perchloric acid (285). The 5% perchloric acid soluble histones from Xenopus laevis intestine and liver are shown in figure 11 (A,B). Only those bands designated as histones H1A, H1B, H1C, and H1D are soluble in 5% perchloric acid. That these bands are not artefacts created by proteolysis of a single major H1 histone is suggested by the observation that all four bands are present in 5% trichloroacetic acid extracts of a whole liver homogenate (fig. 11C). These 5% TCA soluble proteins were extracted from a liver homogenate prepared in the presence of 0.05 M NaHSO₃ and 250 μ M PMSF. De Noij and Westenbrink (297) have

Fig. 11. Absorbance profile of oxidized erythrocyte histones which had been electrophoresed on 10 cm, 6.25 M urea gels. H3M, histone H3 monomer; H3D, histone H3 dimer. Electrophoresis was for 4 hrs at 130 volts and 25°C.

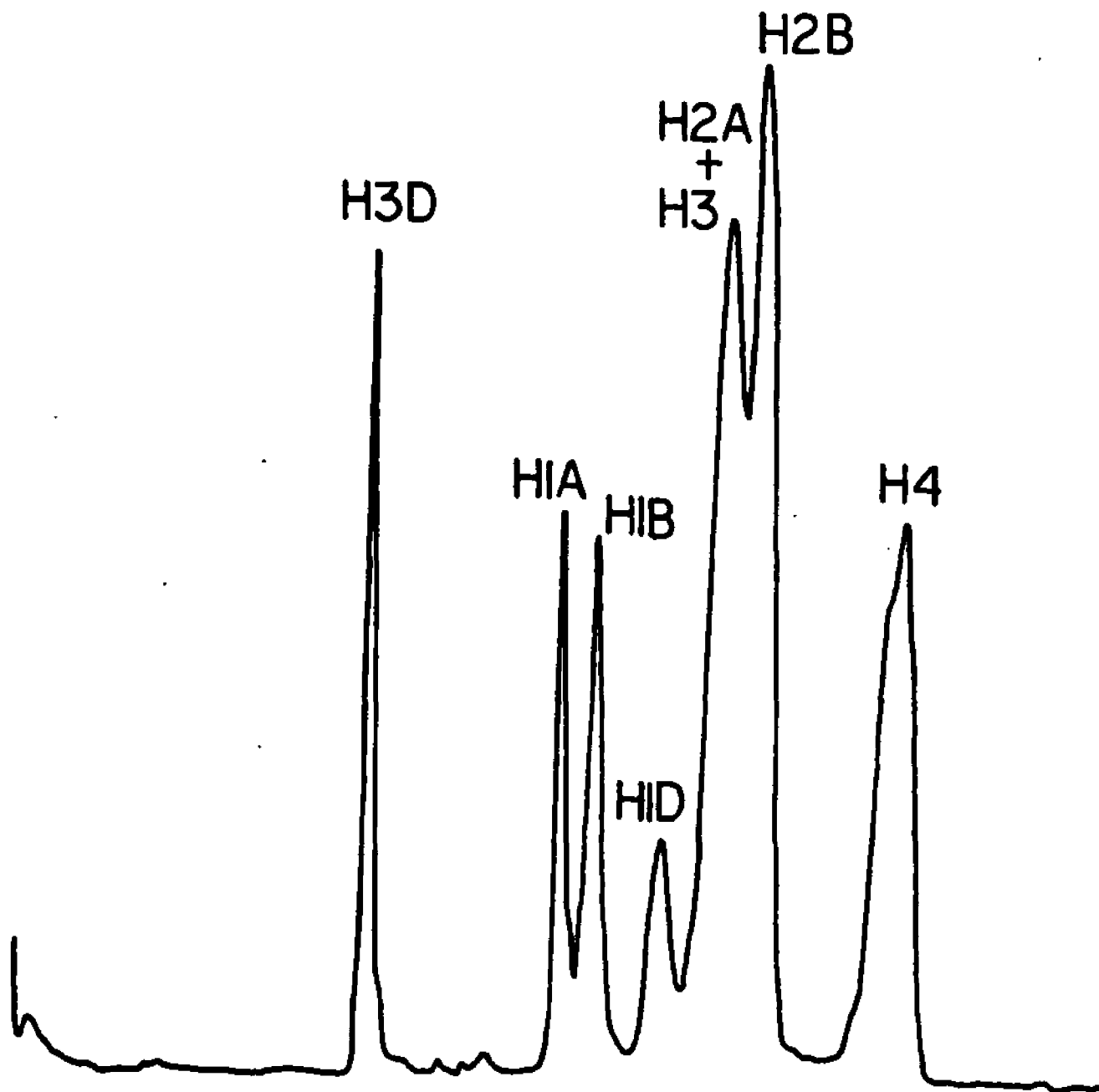
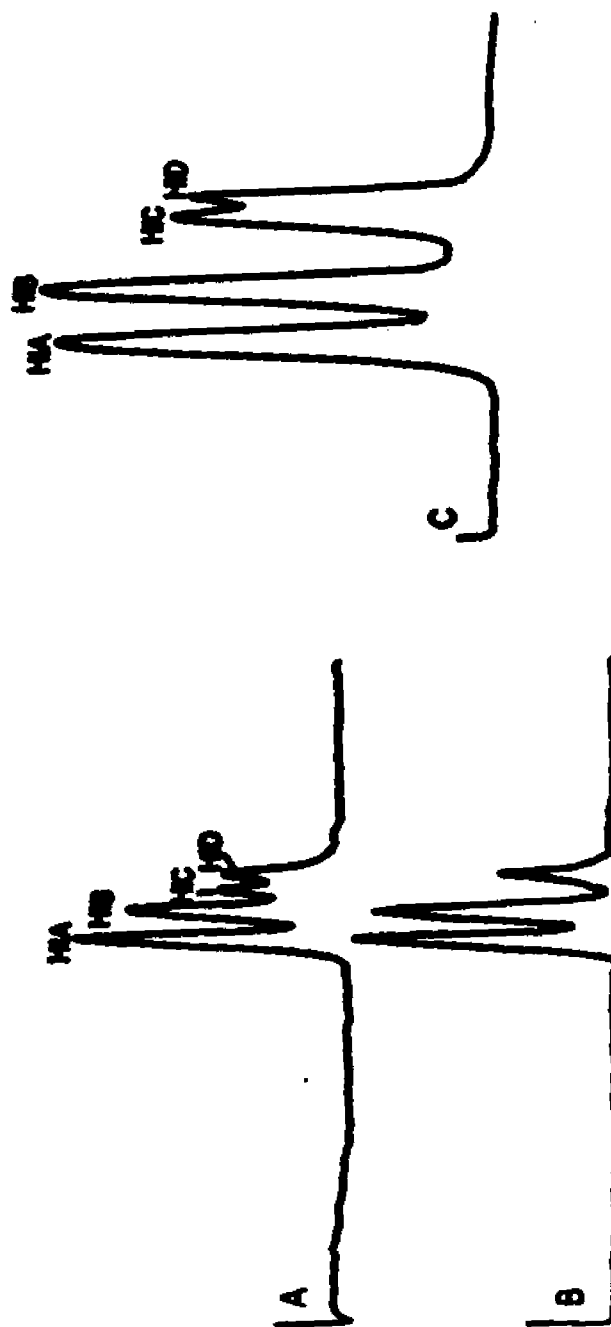


Fig. 12. Absorbance profiles of histones soluble in 5% perchloric acid and 5% trichloroacetic acid. A, intestine 5% PCA soluble histones; B, erythrocyte 5% PCA soluble histones; C, liver homogenate, 5% TCA soluble proteins. Electrophoresis was on 10 cm, 2.5 M urea gels for 4 hrs at 130 volts and 25°C in the case of the intestine and erythrocyte samples. The 5% TCA soluble liver proteins were electrophoresed on 25 cm, 2.5 M urea gels at 220 volts for 18 hrs (4°C).



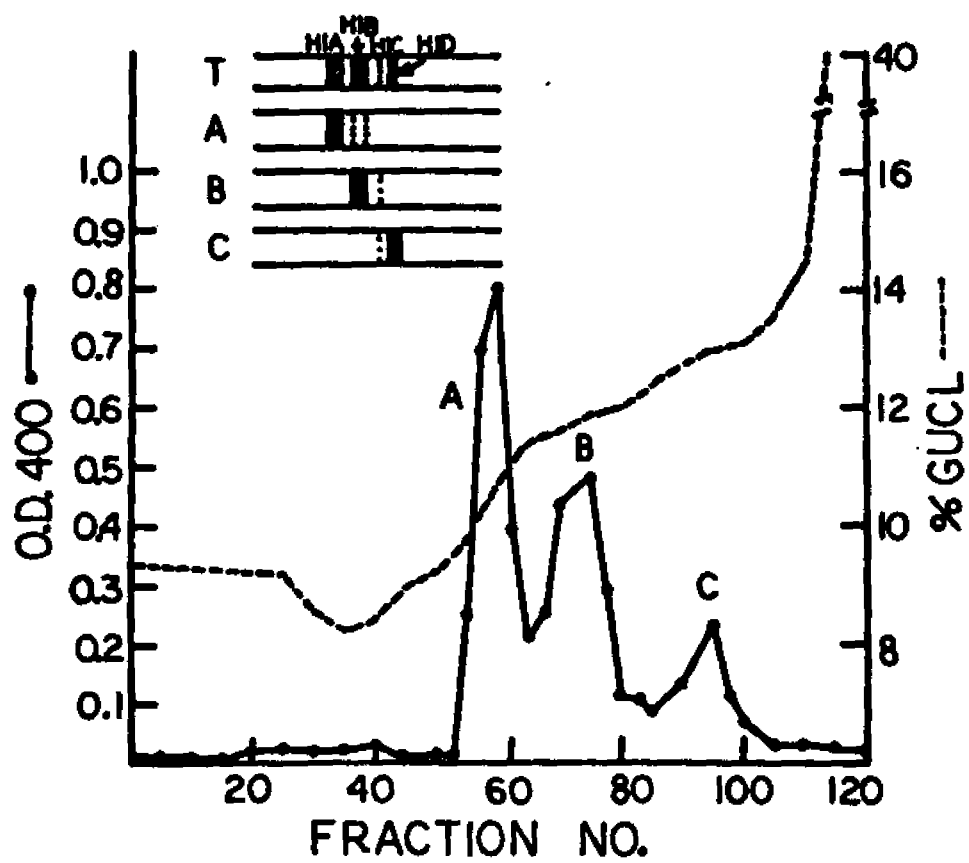
shown that only histone H1 is extracted from crude homogenates by 5% TCA.

Some of the bands designated as H1 histones may be 5% PCA or TCA soluble nonhistones. However, nonhistones can be removed from chromatin by treatment with 0.35 M NaCl (298, 299). The nuclear washes used in preparing purified nuclei include washes with 0.35 M sodium.

Additional evidence that these proteins are not non-histones has been obtained by chromatography of the 5% perchloric acid soluble proteins of Xenopus laevis erythrocytes on columns containing Amberlite CG-50. A linear 8-13% gradient of guanidine chloride in 0.1 M sodium phosphate buffer (pH 6.8) was used to elute these proteins (281). The order of elution of the four major 5% PCA soluble proteins was similar to the order in which these bands appear following electrophoresis on acid-urea gels (fig. 12). Nonhistone proteins usually elute from Amberlite CG-50 columns in 8% guanidine chloride (281).

In a study of embryonic Xenopus laevis histones, Destree et al., (296) did not find histones H1C and H1D. However, they did report the presence of a unique histone F2C (H5) in Xenopus laevis erythrocytes similar to the band designated here as H1D. The techniques for histone isolation described by Destree et al., (296) were used (with 250 μ M PMSF) to prepare histones from liver, kidney, and erythrocytes. With the exception of the presence of greater contamination by high molecular weight material, the histones so

Fig. 13. Chromatographic elution profile of erythrocyte histone H1 from a glass column containing Amberlite CG-50. Insert is a diagrammatic representation of erythrocyte histones electrophoresed on 10 cm, 2.5 M urea gels. Dotted lines have been used to indicate the presence of small amounts of a particular band. T, total erythrocyte H1 histones; A, proteins from column fractions 55-64; B, proteins from column fractions 68-80; C, proteins from column fractions 85-105; GUCL, guanidine chloride.



isolated were essentially identical to those presented in figures 8 and 9. No erythrocyte specific protein could be found in Xenopus laevis erythrocytes. This observation agrees with those of Panyim et al., (282) who were unable to find erythrocyte specific histones in fish, amphibians, and reptiles.

Comparison of testis histones and somatic histones electrophoresed on 2.5 M urea gels (fig. 8) suggests that the proportion of histone H3 is greatly increased in testes. However, electrophoresis of testis histones on gels lacking urea (fig. 9E) results in the separation of histone H3 and another species (arrow) which apparently coelectrophoresed with histone H3 on 2.5 M urea gels. This band appears to be unique to testis histones, since a corresponding band is not present in the other tissue histones. The change in the mobility of the testis band, as urea concentration changes, is similar to that exhibited by mammalian histone H2B (283,284).

Figure 20 presents gel scans of testis basic chromatin proteins electrophoresed on 10 cm gels lacking urea. In addition to the histone-like, testis specific band (SP2), a minimum of two (SP3,SP4) additional basic chromatin proteins are unique to testis. These proteins were previously described by Bols and Kasinsky (278) and designated as X2 (SP3) and X3 (SP4). Band X1, described by Bols and Kasinsky (278) as a protein with an electrophoretic mobility intermediate to those of histone H4 and X2 (SP3), has not been found in this study. It is evident that Xenopus laevis

testis basic chromatin proteins are qualitatively different from those of other tissues. The bands specific to testis will be referred to as SPs (spermatogenesis proteins) since biochemical analyses of these proteins have not been carried out.

The relative percentages of histones in the various tissues examined are presented in table 5. Significant ($P < .05$) differences between the means for individual histones from somatic cells were noted between: histone H1A of erythrocyte and kidney, histone H1B of erythrocytes and kidney, histone H1C of erythrocytes and all other tissues, histone H1D of erythrocytes and intestine, and histone H4 of erythrocytes and intestine. All of the significant differences are between the maximum and minimum values found, with the exception of the difference between the percentage of histone H1C ($0.5 \pm 0.1\%$) in erythrocytes and all other somatic tissues (2.8-3.1%). The meaning of these differences is unknown.

Comparison of the relative percentages of histones in testes to the average values for somatic cell histones reveals several significant differences. First, the relative percentages of H1 histones are reduced in testes. Second, histones H2B and H2A represent a smaller proportion of testis histones than somatic cell histones. Third, the percentages of histones H3 and H4 are increased in testes relative to other tissues. These results demonstrate that significant quantitative, as well as qualitative, differences

TABLE 5.--Percentages and ratios of histones extracted from various tissues of Xenopus laevis

Source	Percentages of Histone Fractions ^a							
	H1A	H1B	H1C	H1D	H3	H2A+H2B	H2A	H4
Erythrocyte	10.8 [±] 0.9	9.9 [±] 1.1	0.5 [±] 0.1	4.7 [±] 0.7	19.5 [±] 0.6	39.0 [±] 1.2	15.5 [±] 0.1	15.5 [±] 0.4
Liver	8.6 [±] 0.8	8.9 [±] 1.3	2.8 [±] 0.7	3.2 [±] 0.2	18.9 [±] 0.3	40.2 [±] 2.0	17.6 [±] 0.4	17.4 [±] 0.8
Intestine . .	8.9 [±] 0.5	8.0 [±] 0.6	2.8 [±] 0.2	2.4 [±] 0.2	20.2 [±] 0.6	38.6 [±] 1.4	16.6	19.1 [±] 1.0
Kidney. . . .	6.9 [±] 0.7	6.9 [±] 0.7	3.1 [±] 0.9	3.1 [±] 0.8	20.3 [±] 0.5	41.7 [±] 0.6		18.0 [±] 1.0
Average . . .	8.8 [±] 0.8	8.4 [±] 0.6	2.3 [±] 0.6	3.4 [±] 0.5	19.7 [±] 0.3	39.9 [±] 0.7	16.6 [±] 1.1	17.5 [±] 0.8
Testis. . . .	5.5 [±] 0.9	4.8 [±] 0.5	1.5 [±] 0.2		26.1 [±] 0.7	30.1 [±] 1.0	13.3 [±] 1.0	32.7 [±] 0.7

Tissue Type	Histone Ratios ^b				
	H1	H3	H2A+H2B	H2A	H4
Somatic	1.3	1.1	2.3	0.9	1.0
Testis	0.4	0.8	0.9	0.4	1.0

^aPercentages are presented as means[±]standard errors of the means.

^bHistone ratios relative to histone H4.

exist between the basic chromatin proteins of testis and those of other tissues in Xenopus laevis. Moreover, it is apparent that these differences are not restricted to high mobility, protamine-like proteins (i.e., SP3 and SP4) since the histones are also significantly different between somatic cells and testes.

Basic Chromatin Proteins of Spermatogenic Cells

To determine whether the testis specific basic proteins (SPs) are present in spermatogenic cells, basic proteins were prepared from collagenase dissociated spermatogenic cells (see chapter 2). In addition, basic chromatin proteins were extracted from spermatogenic cells which were separated by centrifugation in 12% Metrizamide into an early-mid spermatogenic cell fraction (supernatant) and a late spermatid plus sperm cell fraction. The percentages of specific spermatogenic cell types in each of these fractions are presented in table 6. Basic chromatin proteins were prepared by method A and electrophoresed on 25 cm, 6.25 M urea gels to obtain separation of the SPs from histones, and to resolve acetylated forms of histones H3 and H4 (282-284).

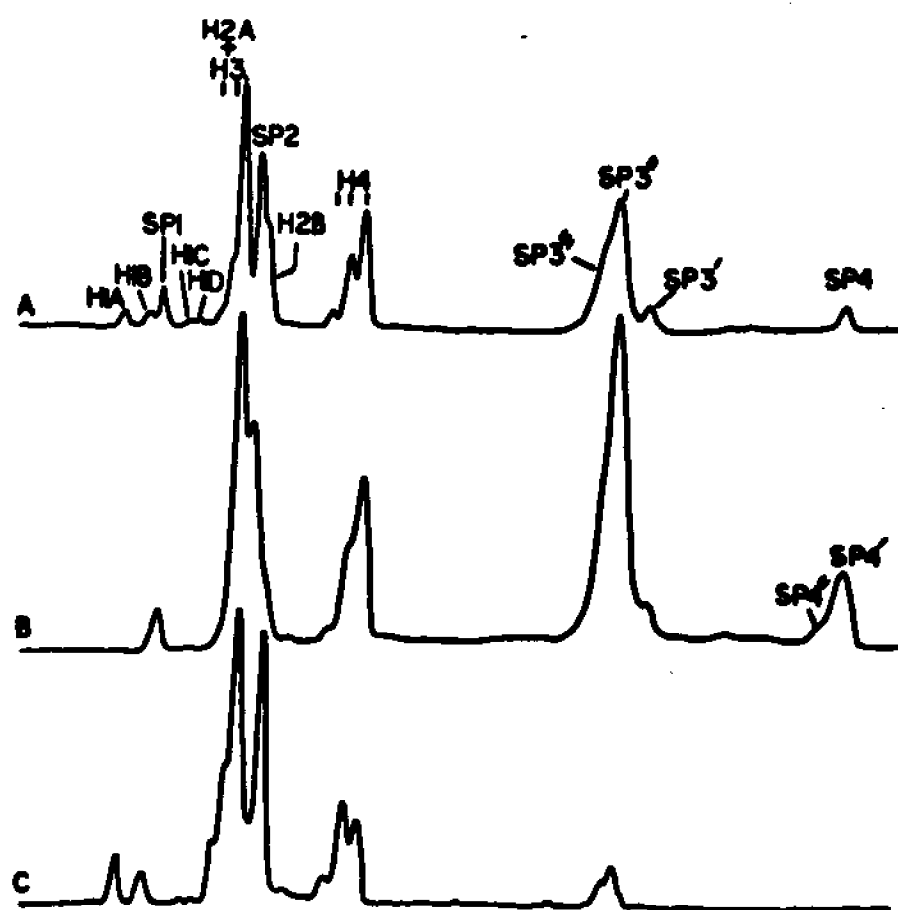
Figure 14A reveals the presence of SP2, SP3, and SP4 in the basic chromatin proteins of Xenopus laevis spermatogenic cells. SP2 has a mobility intermediate to those of histones H3 and H2B on 6.25 M urea gels. The increase in the electrophoretic mobility of the protein(s) in SP2, as gel urea concentration is increased from 0 to 6.25 M, is

TABLE 6.--Percentages of cell types in the early-mid spermatogenic cell and late spermatid plus sperm cell fractions prepared by centrifugation of spermatogenic cells in 12 % Metrizamide*

Early-Mid Spermatogenic Cell Fraction (A)	Percentages
Primary spermatogonia	0.2
Secondary spermatogonia + premeiotic S phase.	0.8
Primary spermatocytes	33.1
Secondary spermatocytes	3.2
Round spermatids.	17.3
Acrosomal spermatids.	24.4
Sloughing spermatids.	18.2
Elongate spermatids	1.2
Mature spermatids + sperm	1.6
Late Spermatid Plus Sperm Fraction (B)	
Primary spermatocytes + spermatogonia . .	0.1
Acrosomal spermatids.	0.4
Sloughing spermatids.	3.9
Elongate spermatids	13.7
Mature spermatids + sperm	81.2
Nuclei.	0.3
Erythrocytes.	0.4

* Residual bodies were excluded from these analyses.

Fig. 14. Absorbance profiles of basic chromatin proteins prepared from: (A) unseparated spermatogenic cells, (B) late spermatid and sperm cells, and (C) early-mid spermatogenic cells. Electrophoresis was on 25 cm, 6.25 M urea gels for 16 hrs at 220 volts (25°C).



similar to that exhibited by mammalian histone H2B (29, 282, 283). Another band (SP1), which comigrates with histone H1A on 0.75 M urea gels and H1B on 2.5 M urea gels, is resolved on these high urea gels. The results demonstrate the considerable degree of qualitative variance that exists between somatic and spermatogenic cell basic chromatin proteins in Xenopus laevis.

Figure 14B presents that absorbance pattern of basic chromatin proteins from late spermatids plus sperm (12% Metrizamide pellet). All of the SPs are apparently highly enriched in these cell types. Moreover, histone H2B is present in only small amounts, while histone H1 is absent. The early-mid spermatogenic cells contain histones typical of somatic cells and a small amount of SP3. SP2 may represent a small percentage of the basic proteins of the early-mid spermatogenic cells, yet not be resolved as a discrete band in the H3 and H2A region.

Quantitative analyses of the percentages of basic chromatin proteins extracted from total spermatogenic cells, early-mid spermatogenic cells, and late spermatids plus sperm reveal several interesting features (table 7). First, the ratios of histone H1 to H4 and histone H2B to H4 are reduced in total spermatogenic cells ($H1:H4=0.2$; $H2B:H4=0.3$) relative to whole testis cells ($H1:H4=0.4$; $H2B:H4=0.5$) or somatic cells ($H1:H4=1.3$; $H2B:H4=1.4$). These ratios are further reduced in late spermatids. Second, the ratio of histone H3 + H2A to H4 in total spermatogenic cells (1.3) is less than

TABLE 7.--Percentages of basic proteins and ratios of histones in the basic chromatin proteins extracted from total, early-mid, and late spermatogenic cells

Cell Types	Percentages of Basic Proteins ^a									
	H1A	H1B	H1C+D	H2A H3	H2B	H4	SP1	SP2	SP3	SP4
Total spermatogenic cells	1.4	1.4	1.3	22.5	5.6	17.4	2.4	12.9	31.1	4.0
Early-mid spermatogenic cells	3.2 ±0.2	3.0 ±0.2	1.2 ±0.5	41.2 ±1.6	27.9 ±1.5	16.4 ±1.2			7.1 ±0.9	
Late spermatids + sperm				19.8 ±3.3	3.6 ±1.4	16.0 ±1.8	2.3 ±0.3	14.4 ±1.4	27.4 ±1.0	7.0 ±1.4

Cell Types	Histone Ratios ^c			
	H1	H2A+H3	H2B	H4
Total spermatogenic cells	0.2	1.3	0.3	1.0
Early-mid spermatogenic cells	0.5	2.5	1.7	1.0
Late spermatids + sperm	...	1.2	0.2	1.0

^aData expressed as mean ± standard error of the mean.

^bData pertains to a single determination.

^cHistone ratios relative to histone H4.

that for somatic cells (2.0). Third, early-mid spermatogenic cells have a histone H3+H2A to H4 ratio slightly higher (2.5) than somatic cell and almost twice that in late spermatids plus sperm. The greater H3+H2A to H4 ratio may be due to the presence of a small amount of SP2 in this band. The ratio of histone H2B to H4 is somewhat higher (1.7) than that for somatic cells (1.4) and much greater than the H2B to H4 ratio in late spermatids plus sperm.

These results suggest that the proteins in the SP bands accumulate during spermiogenesis, particularly in late spermatids, and that certain histones become displaced from spermatid chromatin during maturation. It is evident from the data of figure 14 and table 7 that histones H1 and H2B become reduced to very low percentages of the basic proteins of spermatid chromatin. Moreover, histone H3 and/or histone H2A (which comigrates with histone H3 on 6.25 M urea gels) appear to be reduced in percentage in late spermatids.

Figure 14 also demonstrates heterogeneity in the SP3 and SP4 regions. SP3 is composed of 3 electrophoretic species, designated as SP3', SP3'', and SP3''' in order of decreasing electrophoretic mobility. SP4 is composed of two electrophoretic species, SP4' and SP4''. While these species are not always resolved as separate bands or distinct shoulders, they can be visually discriminated on 25 cm gels.

Previous studies have shown that histones H3 and H4 become resolved, on long gels, into subfractions corresponding to

the unacetylated and acetylated forms of these histones (74,95,282-284). Figure 14 suggests that the major acetylated forms of histone H3 (H3'' and H3''') and (H4'' and H4''') become reduced during spermiogenesis. This observation agrees with similar observations from sea urchins (117,226) and Rana pipiens (228).

To further study the basic protein changes and basic protein synthesis, the basic proteins of fractions enriched for specific spermatogenic cell types were examined on two separate occasions following incubation in ³H-labelled amino acids (5 hrs) and subsequent cell separation by unit gravity sedimentation and isopycnic centrifugation (see chapter 2). The basic proteins were prepared by method B. All incorporation data are presented in chapter 4.

In the first study, basic proteins of fractions enriched for specific cell types (table 8) were electrophoresed on 10 cm, 0.75 M urea gels. Figure 15 (A-F) presents the absorbance profiles, while table 9 lists the relative percentages of clearly resolved basic protein bands.

The SP bands obviously represent a greater proportion of the basic proteins in late spermatid stages relative to early spermatid stages and primary spermatocytes. SP1 (which comigrates with histone H1A on 0.75 M urea gels) and SP3 were present in the basic proteins of the earliest spermatid stage examined (acrosomal spermatids, fig. 15B). A small amount (1.5% of total basic proteins) of SP3 was also found in primary spermatocytes (fig. 15A). However, this

TABLE 8.--Percentages of cell types in spermatogenic cell fractions prepared by unit gravity sedimentation and isopycnic centrifugation^a

Fraction	Cell Type	Percentage
Primary spermatocytes	Secondary spermatogonia. . .	0.8
	Leptotene.	20.6
	Zygotene	25.8
	Pachytene.	35.1
	Diplotene + metaphase. . .	10.0
	Secondary spermatocytes. . .	1.0
	Round spermatids	3.7
	Acrosomal spermatids	3.0
Acrosomal spermatids	Primary spermatocytes. . . .	0.4
	Secondary spermatocytes. . .	4.3
	Round spermatids	23.1
	Acrosomal spermatids	71.1
	Sloughing spermatids	1.1
Early sloughing spermatids	Round spermatids	22.1
	Acrosomal spermatids	4.3
	Early sloughing spermatids .	46.5
	Mid-late sloughing spermatids	24.1
	Residual bodies.	4.0
Mid-late sloughing spermatids	Early sloughing spermatids .	9.1
	Mid-late sloughing spermatids	46.7
	Elongate spermatids.	35.2
	Mature spermatids + sperm. .	9.0
Elongate spermatids	Mid-late sloughing spermatids	15.3
	Elongate spermatids.	61.1
	Mature spermatids + sperm. .	24.6
Mature spermatids + sperm ^b	Elongate spermatids.	1.1
	Mature spermatids + sperm. .	98.9

^aCells used to prepare basic proteins for electrophoresis on 0.75 M urea gels.

^bSperm and mature spermatids are morphologically similar.

Fig. 15. Absorbance profiles of basic proteins prepared from fractions enriched for specific spermatogenic cell types and electrophoresed on 10 cm, 0.75 M urea gels. A, primary spermatocytes; B, acrosomal spermatids; C, early sloughing spermatids; D, mid-late sloughing spermatids; E, elongate spermatids; F, mature spermatids and sperm. Electrophoresis was for 2 hrs at 130 volts and 24°C.

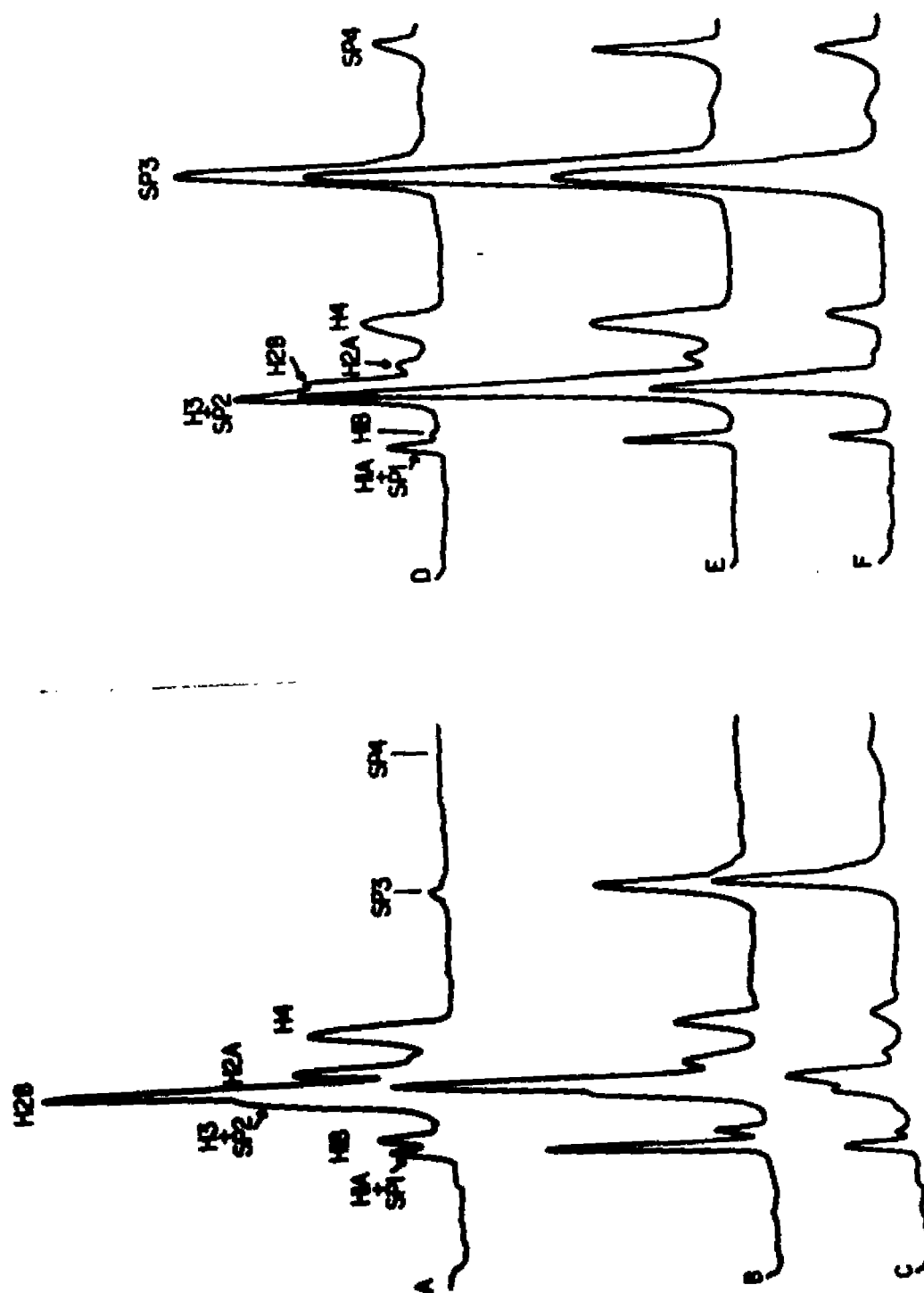


TABLE 9.--Percentages of basic protein fractions in separated spermatogenic cell fractions prepared by unit gravity sedimentation and isopycnic centrifugation ^a

Spermatogenic Cell Fraction	Basic Protein Fraction							
	SP1 + H1A	H1B	H1C + H1D	SP2+H3 + H2B	H2A	H4	SP3	SP4
Primary spermatocytes.	4.9	5.7	1.3	53.8	13.3	19.4	1.5	...
Acrosomal spermatids	11.7	3.1	0.9	43.5	8.8	10.4	21.9	...
Early sloughing spermatids	8.0	2.3	...	36.7	5.5	9.7	37.8	b
Mid-late sloughing spermatids. . .	3.3	1.0	...	30.1	4.6	13.9	38.7	8.4
Elongate spermatids.	3.1	0.6	...	31.8	2.7	12.2	42.6	6.8
Mature spermatids + sperm.	3.0	0.2	...	25.2	0.8	6.4	56.8	7.8

^aThese data pertain to the basic proteins electrophoresed on the 0.75 M urea gels.

^bSP4 was present in small amounts in this cell fraction, however the percentage was not determined because the band was not resolved well.

is most likely due to contamination of this fraction by spermatids, which represent 6.8% of the cells. The proportion of SP3 increases up to a maximum of 56.8% of the basic proteins in mature spermatids and sperm (fig. 15F). SP4 first appears in small amounts in the early sloughing spermatids (fig. 15C) and then rapidly increases in percentage (6.8-8.4%) during transition through the mid-late sloughing, elongate, and mature spermatid stages. SP2 was not resolved well enough to draw conclusions about the timing of its appearance during spermatogenesis.

Further inspection of figure 15 and the data in table 9 demonstrate that histones H1, H2A, and H2B are reduced in percentage during spermiogenesis. The decline in the percentage of histone H1 (as evidenced by the decline in H1B and H1C ~~plus~~ H1D) is accompanied by a decline in the histone H1 to H4 ratio. The most dramatic decline was noted between early (H1B:H4=0.2) and mid-late sloughing (H1B:H4=.01) spermatid stages.

The ratio of histone H2A to H4 declined from highs of 0.7 in primary spermatocytes (fig. 15A) and 0.7 in acrosomal spermatids (fig. 15B) to a low of 0.1 in mature spermatids plus sperm (fig. 15F). Earlier, it was observed that the ratio of histone H3 ~~plus~~ H2A to H4 on 6.25 M urea gels (fig. 14) reduced from a high of 2.5 in early-mid spermatogenic cells to 1.2 in late spermatids plus sperm. The 0.75 M urea gels shown in figure 15 clearly demonstrates that much of the reduction in the histone H3 and H2A band can be ac-

counted for by the loss of histone H2A.

The loss of histone H2B during spermiogenesis was clearly seen by the comparison of early-mid spermatogenic cell and late spermatid plus sperm cell basic proteins on 6.25 M urea (fig. 14). Figure 15 also demonstrates that the relative staining intensity in the histone H2B region declines during spermiogenesis.

To achieve greater separation of the SPs, and to examine the loss of histone H2B, basic proteins extracted from fractions enriched for specific cell types (table 10) were electrophoresed on 25 cm, 6.25 M urea gels. Absorbance profiles of the histones and SP3 regions of these gels are presented in figure 16 (A-C). SP4 was eluted from these gels. The percentages of individual basic proteins are given in table 11.

Good resolution of SP1, SP2, and most of the histones (H2A and H3 comigrate) is obtained on these gels. SP1 and SP3 first appear in the fraction enriched for round spermatids (fig. 16B). SP1 is noted only as a shoulder to H1B. Since acrosomal and early sloughing spermatids (which have a significant amount of SP1) contaminate this fraction, they may be the source of SP1. This may also be true for the SP3 in the round spermatid fraction.

However, the reduction in the percentage of acrosomal spermatids (71.1% to 29.0%) from the fraction enriched for these spermatids (table 9) to the round spermatid fraction is too large to account for the reduction in the percentage

TABLE 10.--Percentages of cell types in spermatogenic cell fractions prepared by unit gravity sedimentation and isopycnic centrifugation^a

Fraction	Cell Type	Percentage
Primary spermatocytes	Secondary spermatogonia.	1.4
	Leptotene.	24.4
	Zygotene	20.1
	Pachytene.	43.6
	Diplotene + metaphase.	9.0
	Secondary spermatocytes.	0.9
	Acrosomal spermatids	0.6
Round spermatids	Primary spermatocytes.	6.1
	Secondary spermatocytes.	7.1
	Round spermatids	49.9
	Acrosomal spermatids	29.0
	Early sloughing spermatids	7.9
Mid-late sloughing spermatids	Early sloughing spermatids	11.1
	Mid-late sloughing spermatids	52.0
	Residual bodies.	27.6
	Mature spermatids + sperm.	9.3
Elongate spermatids	Elongate spermatids.	34.6
	Mature spermatids + sperm.	64.3
	Residual bodies.	1.1
Mature spermatids + sperm ^b	Elongate spermatids.	0.2
	Mature spermatids + sperm.	99.8

^aCells used to prepare basic proteins for electrophoresis on 6.25 M urea gels.

^bSperm and mature spermatids are morphologically similar.

Fig. 16. Absorbance profiles of basic proteins prepared from fractions enriched for specific spermatogenic cell types and electrophoresed on 25 cm, 6.25 M urea gels. A, primary spermatocytes; B, round spermatids; C, mid-late sloughing spermatids; D, elongate spermatids, E, mature spermatids and sperm. Electrophoresis was for 18 hrs at 220 volts and 25°C. The regions of the gels between the origin and the histone region, and between histone H₄ and SP3, were removed prior to scanning.

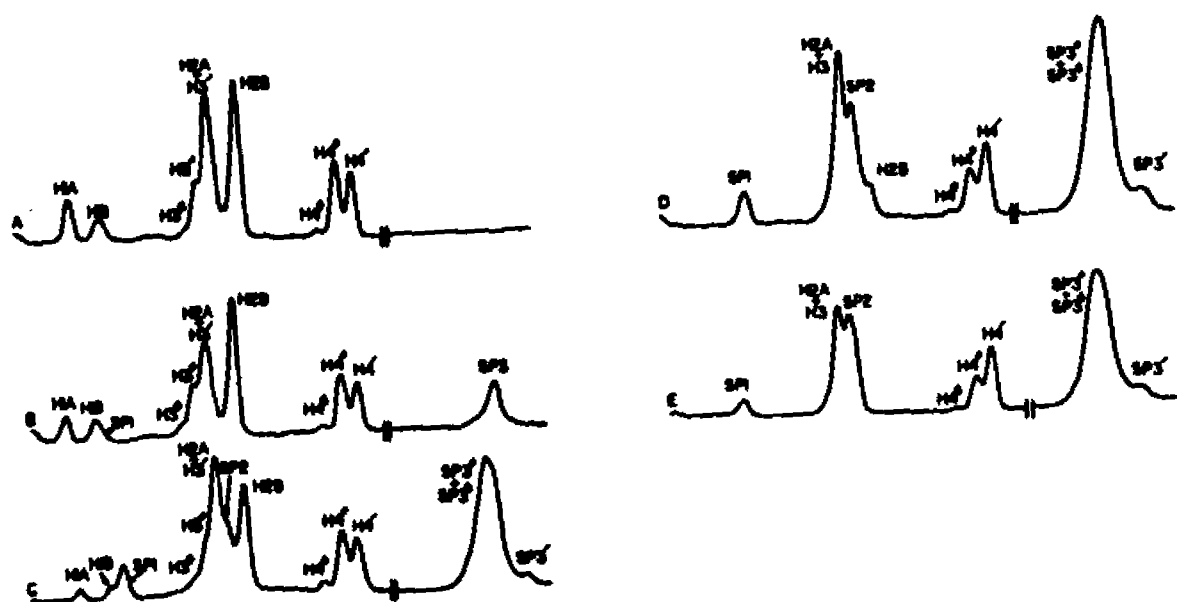


TABLE 11.--Percentages of basic protein fractions and ratios of histones in spermatogenic cell fractions prepared by unit gravity sedimentation and isopycnic centrifugation^a

Spermatogenic Cell Fraction	Basic Protein Fraction ^b								
	H1A	H1B	H1C + H1D	H2A + H3	H2B	H4	SP1	SP2	SP3
Primary spermatocytes. .	7.7	5.6	1.8	36.9	26.8	21.2	...		
Round spermatids	4.3	3.4	1.5	31.2	24.3	20.0	1.0		14.3
Mid-late sloughing spermatids.	1.2	1.2	...	22.1	12.5	13.8	3.9	6.8	38.5
Elongate spermatids. . . .	...	...	...	19.1	2.7	13.0	3.0	12.5	49.7
Mature spermatids + sperm	...	...	...	15.8		13.1	2.6	16.3	52.2

Spermatogenic Cell Fraction	Histone Ratios ^c			
	H1A	H3+H2A	H2B	H4
Primary spermatocytes. .	0.7	1.7	1.3	1.0
Round spermatids	0.5	1.6	1.2	1.0
Mid-late sloughing spermatids.	0.2	1.6	0.9	1.0
Elongate spermatids. . . .	...	1.5	0.2	1.0
Mature spermatids + sperm	...	1.2	...	1.0

^aThese data pertain to the basic proteins electrophoresed on 6.25 M urea gels.

^bSP4 was eluted from these gels.

^cHistone ratios relative to histone H4.

of SP3 between these two fractions (21.9% and 14.3%). It is probable that round spermatids contain a small percentage of SP3. Band SP2 becomes prominent in the mid-late sloughing spermatid fraction (fig. 16C). This agrees well with the observations made on the 0.75 M urea gels. However, the greater resolution obtainable on the 25 cm gels permits the detection of a small shoulder with a mobility similar to SP2 in round spermatids (fig. 16B).

In agreement with the observations from the 0.75 M urea gels, histones H1 and H2B become quantitatively reduced during spermiogenesis. Histone H1 is totally removed by the elongate spermatid stage, while histone H2B is only totally absent in mature spermatids and sperm. The ratios of histones H3 plus H2A to H4 also become reduced during spermiogenesis. Since histone H2A was shown to be reduced to very low levels during spermiogenesis (fig. 15), the reduction in the histone H3 plus H2A band is most likely due to the loss of histone H2A.

Some variation has been noted between frogs in the extent to which histones H2A or H2B are reduced in mature spermatids plus sperm. The complete absence of histone H2B and H2A has only been found in the mature spermatids and sperm of male Xenopus laevis having intense black pigmentation of the forelimbs. This is indicative of readiness for mating and suggests that these frogs have more mature sperm in their testes. It would be difficult to test this, since no discrete morphological criteria exist for differentiating be-

tween immature or mature sperm in Xenopus.

The pattern of basic protein changes accompanying spermatogenesis emerges as one in which specific histones are removed from chromatin and replaced by SPs. The order of histone replacement appears to occur in the following sequence: H1, H2B+H2A. Histones H3 and H4 remain associated with the DNA of mature spermatids and sperm in Xenopus testes.

Additional changes appear to involve alteration in the levels of acetylated forms of histones H3 and H4. Comparisons of early-mid spermatogenic cell and late spermatid plus sperm cell basic proteins on 25 cm, 6.35 M urea gels (fig. 14) suggested that acetylated forms of histone H3 and H4 become reduced during spermiogenesis. This is further demonstrated in the gel scans of fig. 16.

High levels of mono- and diacetylated forms of histone H3 (H3'' and H3''') and histone H4 (H4'' and H4''') persist throughout spermatogenesis (fig. 15A-C) until the elongate spermatid stage (fig. 15D). In these cells, histone H3 exists almost totally in the unacetylated form (H3'). Acetylated forms of histone H4 are also reduced in these cells. Histone H4'' and H4''' become reduced from 58% of the total H4 histone in earlier stages to 46% of the total in elongate spermatids. A further reduction (to 39% of the total H4) of the acetylated forms of histone H4 occurs in mature spermatids and sperm (fig. 15E). However, unlike histone H3, histone H4 has never been found in the totally unacetyl-

ated form.

The most dramatic alterations in basic chromatin proteins occurs during the maturation of spermatids through the sloughing spermatid stages to the elongate spermatid stages. During this time, three of the histones undergo displacement. SP2, SP3, and SP4 also become greatly increased in percentage during this time. Acetylated forms of histones H3 and H4 also become significantly reduced as spermatids pass through these stages. These events are correlated with the extreme condensation of chromatin which occurs as spermatids cast off cytoplasm and elongate (272).

Discussion

In Xenopus laevis at least four unique proteins (SP1-4) appear on the chromatin in the most mature spermatid stage examined. In addition to the occurrence of these unique SPs, somatic type histones undergo major qualitative and quantitative alterations during spermiogenesis. Histones H1, H2B, and H2A are lost from chromatin during spermiogenesis, resulting in testicular sperm which contain only histones H3 and H4 (in addition to the SPs). Moreover, a large proportion of histones H3 and H4 persist in acetylated forms throughout the stages preceding the final spermatid stages. In this stage the acetylated forms of histone H3 and H4 abruptly decline. However, acetylated species of histone H4 persist in the most mature spermatid stages of testes.

Further maturational changes in the basic chromatin protein complement may occur as testicular sperm are released and traverse the genital ducts. The sperm chromatin of several organisms, including mammals (221,231,232) and crickets (301), have been shown to attain their most mature state extratesticularly. It is entirely possible that the H3 and H4 histones, as well as any of the SPs, may be removed from Xenopus laevis sperm chromatin during such an extratesticular maturation. Initial attempts to obtain extratesticular sperm in quantities sufficient for basic protein analyses have been unsuccessful.

At present, the functions of the SP bands are not known. It seems probable that at least one of the SPs is an arginine rich protein, since cytochemical data (277) indicate that Xenopus laevis sperm have an arginine content greater than somatic nuclei. Bols and Kasinsky (278) have presented evidence for SP3 and SP4 as arginine-rich proteins. The accumulation of SP3 and SP4 would then account for the increased arginine content detected in sperm by cytochemical procedures. The extreme condensation of sperm chromatin in many species appears to be achieved through the action of arginine-rich protamines (see 213-215). SP3 and/or SP4 may be functionally analogous to protamines. Since SP2 also accumulates during spermiogenesis, it may also function in the condensation of sperm chromatin.

Basic chromatin proteins with electrophoretic properties similar to SP1 and SP2 have been reported in the sper-

matogenic cells of mammals (222,229), trout (223), and crickets (227). As is true for Xenopus laevis, these electrophoretically similar proteins were shown to be unique to spermatogenic cells. SP1 is present throughout spermiogenesis and represents only a small percent of the basic proteins of any cell type. For this reason, it is unlikely that SP1 directly acts to condense sperm chromatin in a manner analagous to protamine. Further conclusions about the function(s) of the SPs will have to await biochemical and synthetic analyses of each individual SP.

Studies of histone acetylation during trout (101) and rat (246) spermatogenesis have demonstrated that acetylation occurs in early-mid spermatogenic stages as well as late spermatid stages. These observations suggest that acetylation may be involved in histone displacement. Recently, it has been shown that chemical acetylation of calf thymus chromatin with acetic anhydride increases the susceptibility of histones H2B, H2A, and H3 to proteolytic degradation.(244). In Xenopus laevis substantial proportions of histones H3 and H4 persist in an acetylated state up to the latest spermatid stage examined (mature spermatid). Since acetate incorporation by individual cell types was not examined, previously acetylated and newly acetylated histone species cannot be distinguished. However, the presence of such large quantities of the acetylated forms of histones H3 and H4 in spermatids is consistent with a possible involvement of acetylation in histone displacement (101,

243-246).

The order of histone displacement which occurs during spermatogenesis in Xenopus laevis is very specific and perhaps unique. Studies of histone replacement during spermatogenesis in trout (223,233), rat (239), and ram (242), have suggested that histone H1 is the last histone to be displaced. However, Marushige and Dixon (223) and Wong and Marushige, (241) have demonstrated that direct addition of protamine to chromatin could displace histone H1. It seems reasonable to suspect that polycationic proteins, such as protamines, would bind DNA in the most accessible regions in chromatin. The DNA most accessible to nucleases (171,172, 184,185), methylation (302), and psoralen binding (303) appears to be associated with histone H1. Moreover, the histone most accessible and removable by salt (240) or proteases (166,189,288-291,304) is histone H1. The observation that histone H1 is the first histone to be displaced during spermatogenesis in Xenopus laevis is consistent with a mechanism of direct protamine (SP) displacement which may or may not involve proteolysis of H1.

Histones H2B and H2A are displaced from chromatin in a pairwise fashion, leaving histones H3 and H4 bound to the chromatin (and perhaps pairwise displaced extratesticularly). The removal of histones H2B and H2A together suggests that these histones interact strongly in chromatin and may be displaced cooperatively. Studies of histone-histone associations in solution (201-205,207) and in chromatin (206-210)

have demonstrated the strong, pairwise interactions which occur between histones H2B and H2A and histones H3 and H4. It appears that the displacement of these four histones occurs in a very specific sequence, reminiscent of the pairwise dissociation of histones H2B and H2A and histones H3 and H4 which occurs during NaCl dissociation of histones (240).

The mechanism of histone displacement from chromatin during spermiogenesis is as yet unknown. Histone acetylation (101,243-246), proteolysis (244), and protamine binding may all play a role in the removal of histones by altering histone-DNA and histone-histone associations. Protease treatment of chromatin results in the rapid and complete degradation of histone H1, followed by histones H3,H2B,H4, and H2A, in that order (166,189,288-291,304). It therefore seems unlikely that proteolysis alone could produce the sequence of histone displacement apparent during spermatogenesis.

Chemical acetylation of chromatin has been shown to markedly enhance the susceptibility of histones H3,H2B, and H2A to degradation (244). Here, it was demonstrated that acetylated forms of histones H3 and H4 were shown to persist through late spermatid stages in Xenopus laevis. Acetylation of histones H2B and H2A may also occur, producing increased susceptibility of these histones to proteolytic degradation. The acetylation of these histones may occur as a result of SP (protamine?) binding to chromatin and a subsequent increase

in the accessibility of sites available for acetylation in histones H2B and H2A. Li et al., (305,306) have demonstrated that histone binding to DNA may be weakened by the binding of polycationic proteins (i.e., polylysine) to chromatin. Wong and Marushige (241) have shown that addition of salmine to calf thymus chromatin also weakens the interactions of histone and DNA.

The order of histone displacement in Xenopus laevis is consistent with the following sequence of events. SPs (2,3, and/or 4) are synthesized by spermatids, transported to chromatin where they bind the most accessible regions of DNA in chromatin. These include regions bound to nonhistone proteins, the less basic regions of histones, and perhaps histone H1 (305,306). SP binding may result in direct displacement of histone H1. As a result of SP binding and/or acetylation, the histone-histone associations and/or histone-DNA associations typical of nucleosomes may become perturbed so that histone H2A or H2B becomes more susceptible to proteolysis. The pairwise removal of histones H2B and H2A may then occur cooperatively as a result of the strong interactions which occur between these histones (182,203, 205-207,210). Histones H3 and H4 may continue to interact strongly with one another and remain on chromatin or become displaced at a later time.

CHAPTER 4

THE SYNTHESIS AND PHOSPHORYLATION OF BASIC CHROMATIN PROTEINS DURING SPERMATOGENESIS

Introduction

Numerous electrophoretic analyses of changes in basic chromatin proteins during spermatogenesis have demonstrated the presence of unique proteins in spermatogenic cells (213-215). The results of the electrophoretic studies presented in chapter 3 suggested the existence of a minimum of 4 basic chromatin proteins (SP1-4), unique to the spermatogenic cells of Xenopus laevis. Such unique spermatid-sperm proteins are often assumed to be newly synthesized proteins which act to condense sperm chromatin. However, alternative explanations exist. First, such unique bands may represent unique enzymatic modifications of pre-existing basic chromatin proteins (i.e., histones). Second, unique bands may be degradation products of pre-existing proteins. Degradation products of histones may be expected to appear in spermatid cells which are in the process of histone replacement. Some portions of histones may be more resistant to complete proteolysis than other portions and remain bound to chromatin (166,189). These questions must be resolved in any attempt to assess the function of unique spermatid proteins.

In this chapter, the results of studies on basic protein synthesis and phosphorylation in spermatogenic cells are presented. The answers to several specific questions have been sought in these studies. Which spermatogenic cells synthesize histones? Are the proteins in the SP bands synthesized by spermatogenic cells, and if so, by which cell types? Do histones, and/or proteins of the SP bands become

phosphorylated during spermatogenesis? Phosphorylation has been examined to determine whether it may play any role in SP binding to DNA or histone replacement.

Materials

Amino acids, oxaloacetate, pyruvate (used in media preparation), and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, Mo.). Vitamins, antibiotic-antimycotic (100 X), glutamine, and dialyzed fetal calf serum were obtained from Grand Island Biological Co. (Grand Island, N.Y.). New England Nuclear (Boston, Mass.) supplied L-leucine-4,5-³H (42 Ci/mmole), L-arginine-³H (33 Ci/mmole), carrier free H₃³²PO₄, Aquasol scintillation cocktail, and Protosol. L-lysine-4,5-³H (53 Ci/mmole), L-valine-³H (7 Ci per mmole), and uridine-5-³H (27 Ci/mmole) were purchased from Schwarz/Mann (Orangeburg, N.Y.). The scintillants, 2,5-Diphenyloxazole (PPO) and 1,4-bis 2-(5-phenyloxazolyl) benzene were scintanализed products of Fisher Scientific Co. (Springfield, N.J.). Alkaline phosphatase (BAPC, 39 Un/mg) was obtained from Worthington Biochemicals (Freehold, N.J.).

Methods

Preparation of Cell Suspensions

Testes were aseptically dissected from frogs and rinsed twice in sterile TSS containing 1% antibiotic-antimycotic (10⁴ units penicillin, 10⁴ mcg streptomycin, and 25 mcg Fungi-zone per ml). The testes were then minced and dissociated in

either sterile TSS, containing 0.15% collagenase, or sterile, modified DNOM (Defined Nutrient Oocyte Medium, 273), containing 0.15% collagenase. The medium was modified from the original as follows: NaCl was lowered from 48.2 mM to 41.6 mM, polyvinylpyrrolidone, cysteine, and cystine were omitted. All other operations were as described in chapter 2.

Cell Incubations

All incubations were carried out in sterile DNOM, modified as follows: NaCl was lowered to 41.6 mM, polyvinylpyrrolidone was omitted, and dialyzed, heat-inactivated (55°C for 30 mins) fetal calf serum was added to 10% (v/v). Spermatogenic cells were incubated at 24°C at approximately 5×10^7 cells/ml. When radiolabelled amino acids were included in the incubants, the corresponding unlabelled amino acids were excluded from the medium. Disodium phosphate was excluded from the medium when carrier-free $\text{H}_3^{32}\text{PO}_4$ was included. All isotopes were neutralized with NaOH prior to addition to the medium. The exact conditions of incubation are described in the results section.

Following incubations in radiolabelled amino acids and/or $\text{H}_3^{32}\text{PO}_4$, incubants were diluted with 5 volumes of ice cold TSS and pelleted (Sorvall, Hb-4 rotor) at 650 x g for 10 mins (5°C). The cell pellets were washed twice more in ice cold TSS prior to cell separation.

Analysis of Incorporated Radioactivity

The kinetics of incorporation of radiolabelled amino acids into protein, and radiolabelled uridine into RNA, were examined by measuring acid-precipitable radioactivity. Aliquots were removed at intervals ranging from 0 to 16 hrs following the start of incubations. After washing the cells, as described above, the cells were sonicated (maximum voltage for 3 mins in a Biosonik IV sonicator) in 5 ml TSS. The sonicate was adjusted to 20% trichloroacetic acid. Acid precipitable material was pelleted (13,000 x g, 10 mins, Sorvall Hb-4 rotor), resuspended in 20% TCA by sonication and then pelleted again. The final pellets were resuspended in 0.5 ml Protosol. The samples were incubated at 55°C for 6 hrs and then added to 5 ml Aquasol for scintillation spectrometry.

Radioactivity in acrylamide gel slices was released by hydrolyzing gel slices in 0.4 ml of 30% H_2O_2 at 60°C for 4 hrs. Gels were sliced with a parallel razor blade device (Bio-Rad, Inc.) into slices which averaged 1.1 mm (10 cm gels) or 2.2 mm (25 cm gels) in thickness. Five ml of Triton-X-100, toluene-PPO, POPOP (33% Triton-X-100, 67% toluene containing 0.5% PPO and 0.01% POPOP) were added to each hydrolysate. Following dark storage (12 hrs) radioactivity was determined in a Beckman LS-150 scintillation spectrometer at an efficiency of 17%. All samples were corrected for background and spillover (dual isotope counting). Radioactivity was superimposed over absorbance profiles by aligning specific stained protein bands with the gel slices which contained them.

Specific activities were determined by summing the radioactivity in the region of interest and dividing by the area under the O.D.₆₃₀ profile (normalized to a scale of 0-3.00 OD₆₃₀) created by scanning the stained protein bands in this region.

Alkaline Phosphatase Treatments

Basic proteins were dissolved (1 mg/ml) in 0.4 N Tris-HCL (pH 7.8). E. coli alkaline phosphatase (3 Un/ml) was added and the solution incubated at 37°C for 2 or 15 hrs. Proteins were precipitated with 20% TCA, washed in acetone-HCL and acetone, then dried in vacuo.

Results

Cell Viability During Incubation

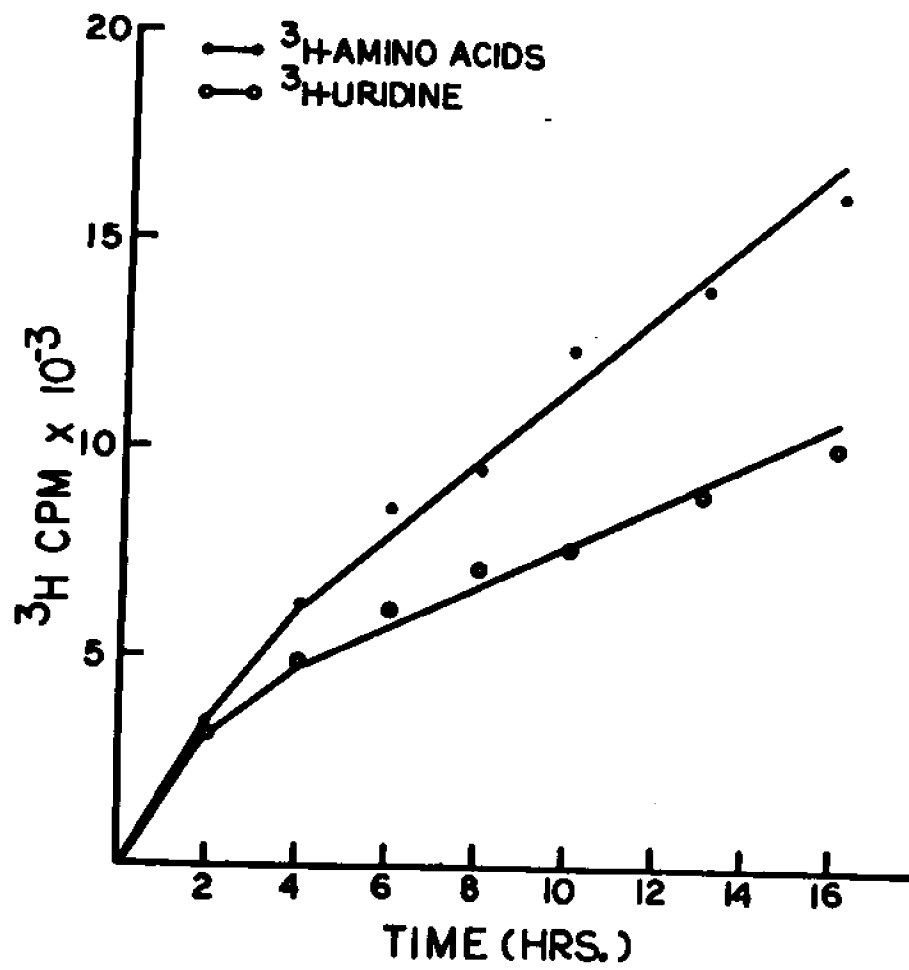
Most of the studies to be discussed in this chapter were in vitro experiments. In vitro studies of radiolabel incorporation permit more sensitive and controlled experiments than in vivo studies. The basic medium chosen for the in vitro maintenance of Xenopus laevis spermatogenic cells was developed for the long term, in vitro maintenance of Xenopus laevis oocytes (273). Due to plasmolysis of primary spermatocytes in DNOM, the original recipe had to be modified to reduce the osmotic strength (from 220 to 205[±]5 mOsm). Dialyzed, heat inactivated, fetal calf serum was added (10%) to the basic medium, as suggested by Dr. M. Kalt (personal

communication).

Spermatogenic cells released by collagenase treatment exhibited a high level of viability (98.7%) when viability was assayed by trypan blue exclusion. The ability of collagenase dissociated spermatogenic cells to incorporate radio-labelled amino acids and uridine into acid precipitable material was also examined, during incubation of these cells in DNOM as an estimate of their viability. The testes of 2 frogs were collagenase dissociated in TSS, and then divided into two groups. One group was incubated in modified DNOM (without lysine, arginine, valine, and leucine) containing 5 Ci/ml each of ^3H -labelled lysine, valine, arginine, and leucine. The second group was incubated in complete modified DNOM containing 5 Ci/ml of ^3H -labelled uridine. Aliquots were removed from each incubant during a 16 hr period. Figure 17 presents the time course of radiolabel incorporation into acid precipitable material during 16 hrs of incubation. Following an initial rapid rate (0-2 hrs), incorporation of labelled amino acids and uridine slowed (2-4 hrs) to a constant rate, which was maintained for the remainder of the incubation period (4-16 hrs).

At present, the basis for the nonlinear kinetics shown in figure 17 is unknown. Interpretation is difficult because of the heterogeneity in the spermatogenic cell population. However, the nonlinearity may be related to altering permeability properties of the cells during incubation. Collagenase treatment may cause the cell membranes to exhibit an ini-

Fig. 17. Incorporation of ^3H -labelled amino acids and uridine into acid precipitable material (20% TCA) by spermatogenic cells maintained in vitro in modified DNOM.



tial high permeability to the precursors used here. The permeability may then decline following membrane repair in DNOM, and remain constant thereafter. Regardless, figure 17 demonstrates that spermatogenic cell protein synthesis and RNA synthesis continue for at least 16 hrs during maintenance in modified DNOM. It will also be shown in this chapter, that all of the spermatogenic cell types used in this study are actively synthesizing basic chromatin proteins during incubation in modified DNOM. All incubations in these studies have been limited to a 5 hr period.

Basic Protein Synthesis by Spermatogenic Cells

Spermatogenic cells were incubated for 5 hrs in modified DNOM containing 5 Ci/ml each of L-lysine-4,5- ^3H (53 Ci/mole), L-arginine- ^3H (33 Ci/mole), L-leucine-4,5- ^3H (42 Ci/mole) and L-valine- ^3H (7 Ci/mole). Cells were separated by unit gravity sedimentation and isopycnic centrifugation in Metrizamide (see chapter 2) and basic proteins prepared (method B).

Figure 18(A-E) presents the radioactivity profiles (disintegrations per min) for the basic proteins electrophoresed on 0.75 M urea (10 cm) gels. Relative specific activities (dpm/A₆₃₀) are presented in table 12. Comparisons of the radioactivity profiles and specific activities reveal that primary spermatocytes (fig. 18A) are most active in histone synthesis (89 dpm/A₆₃₀). Moreover, it is evident that each of the histones are synthesized by primary spermatocytes.

Fig. 18. Absorbance and radioactivity profiles for basic proteins prepared from separated spermatogenic cells which had been incubated in the presence of ^3H -labelled amino acids. A, primary spermatocytes; B, acrosomal spermatids; C, early sloughing spermatids; D, mid-late sloughing spermatids; E, mature spermatids and sperm. Electrophoretic conditions were as described for figure 15.

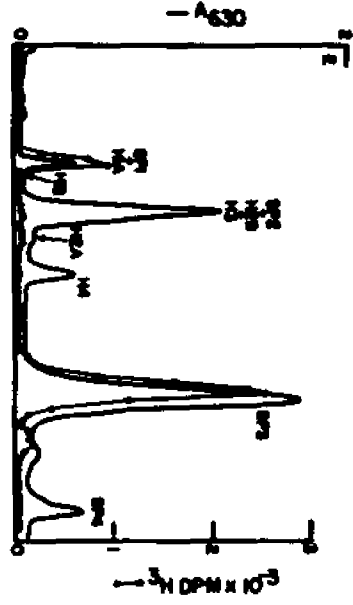
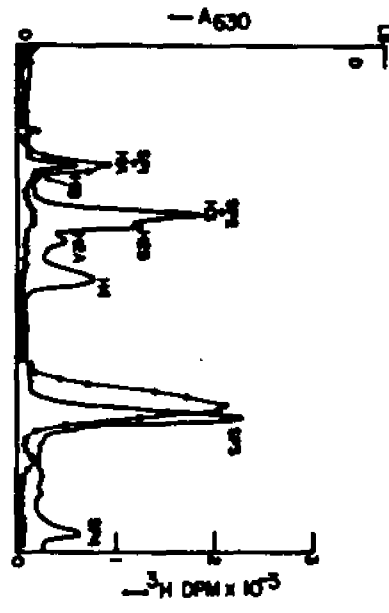
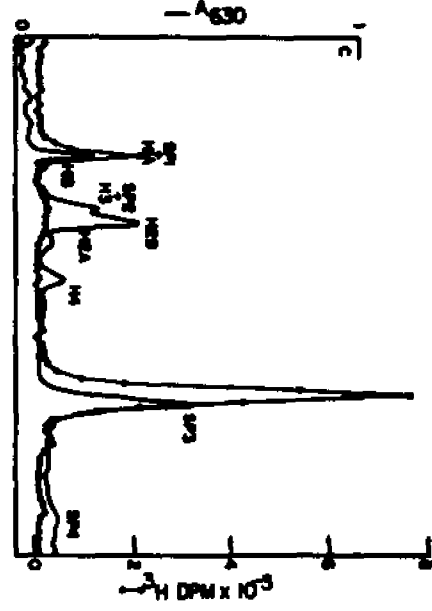
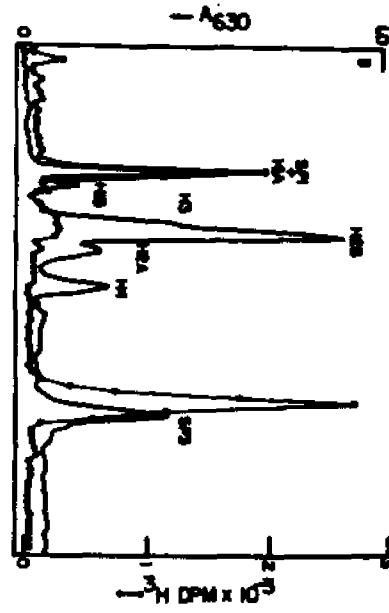
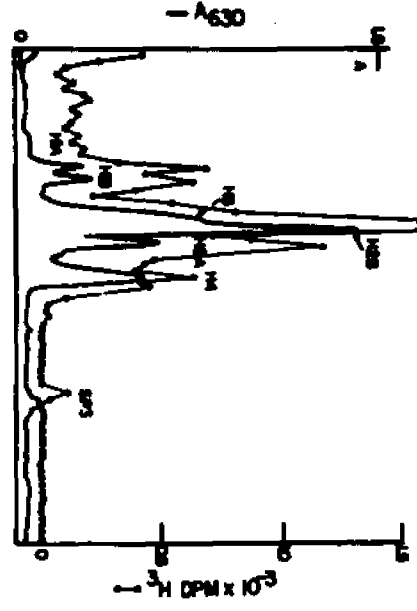


TABLE 12.--Relative specific activities of basic proteins extracted from separated spermatogenic cells and electrophoresed on 10 cm, 0.75 M urea gels

Spermatogenic Cell Fraction	$^3\text{H-DPM}/A_{630}^a$			
	Histone + SP2 ^b	SP1 + H1A	SP3	SP4 ^c
Primary spermatocytes. . . .	90	...	108	
Acrosomal spermatids	36	346	379	
Early sloughing spermatids .	69	960	1282	(442) ..
Mid-late sloughing spermatids	17	431	181	(125) 9
Mature spermatids + sperm. .	8	237	64	(124) 6

^aAll specific activities normalized to a 0-3.00 OD₆₃₀ scale.

^bTotal histone minus SP1 and H1A.

^cNumbers in parentheses refer to the total DPM in SP4.

The specific activity of histones declines in more advanced spermatogenic cell types until it reaches a minimum value (8 dpm/A₆₃₀) in mature spermatids plus sperm. It should be emphasized that histone H3 comigrates with SP2 on this gel system. This makes it difficult to distinguish between radioactivity incorporated into histone H3 and that which may be incorporated into SP2. However, the observation that radioactivity can be localized over all of the histones in the acrosome and slough stage spermatids, suggests that the radioactivity in the band containing histones H3 is incorporated into the H3 histone. This point remains to be resolved.

Figure 18 clearly demonstrates that all of the spermatid stages examined (acrosome through mature spermatids plus sperm) synthesize the proteins in bands SP1 and SP3. While the radioactivity associated with SP1 is completely coincidental with the stained band, this is not the case for SP3.. Most of the radioactivity in the SP3 region trails the major portion of the stained band. This is highly reproducible, and can be seen to be true for each spermatid stage examined. This lack of coincidence may be expected if newly synthesized SP3 becomes highly phosphorylated, as does trout and rat protamine (89,237-239), and/or acetylated, as is the case for histone H4 (74,102).

Radioactivity was found to be associated with SP4 in early sloughing spermatids (fig. 18C), mid-late sloughing spermatids (fig. 18D), and mature spermatids plus sperm (fig. 18E). However, the number of dpm in this band are very low,

relative to the number of dpm in the other newly synthesized basic proteins extracted from these cell types. Early sloughing spermatids contained the greatest number of dpm in SP4 (442), followed by mid-late sloughing spermatids (125), and mature spermatids plus sperm (124). The low levels of radioactivity associated with SP4 suggest that most of the protein(s) in this band is synthesized during a relatively brief period of spermatogenesis (i.e., during conversion of early to mid-late sloughing spermatids). Alternatively, SP4 may be composed of processing or degradation products of one of the other newly synthesized SPs and/or histones.

During the transition of spermatids through the sloughing stages, the chromatin becomes very dense, and refractile (see chapter 1). At the same time, the relative percents of SP3 and SP4 become greatly increased (see chapter 3). Additionally, SP3 seems to be most actively synthesized by sloughing (early) spermatids since the specific activity of the proteins in this band are by far the greatest in these cells (table 12). These correlations suggest that the components of SP3, and possibly SP4, are newly synthesized proteins which play a role in the condensation of Xenopus laevis sperm chromatin.

In another study, spermatogenic cells were incubated for 5 hrs in modified DNOM containing 5 Ci/ml each of l-lysine-4,5-³H (53 Ci/mmol) and l-arginine-³H (33 Ci/mmol). Following cell separation, basic proteins were extracted (method B) and then electrophoresed on 25 cm, 6.25 M urea gels to obtain high resolution. Figure 19 (A-E) exhibits the

absorbance profiles, and the distribution of ^3H -cpm. Relative specific activities are presented in table 13.

Histone synthesis was most prevalent in primary spermatocytes (17 cpm/ A_{630}). The cell fraction enriched for round spermatids (fig. 19B) also appeared to be active in the synthesis of histones, albeit at a lower level (13 cpm/ A_{630}) than primary spermatocytes. The radioactivity comigrated with most of the individual histone absorbance peaks in synthetic cells. However, most of the radioactivity over histone H4 comigrated with bands which correspond to acetylated forms of histone H4. This observation agrees well with studies which have demonstrated that histone H4 rapidly becomes acetylated following synthesis (74,102).

The specific activity in the histones of late sloughing spermatids (fig. 19C) represents only 6% of that of primary spermatocytes. This value is too low to rule out contaminating cells as the source of histone ^3H -cpm in this cell fraction. Elongate spermatids and mature spermatids were essentially inactive in histone synthesis. Taken together with the observations from the 0.75 M urea gels, it would seem that histone synthesis does not take place in any spermatogenic stage following the early-mid sloughing spermatid stages.

Due to the greater resolving power of the long gels, and the high gel urea concentration (6.25M), SP1 and SP2 become separated from the histones (fig. 19). As demonstrated

Fig. 19. Absorbance and radioactivity profiles for basic proteins prepared from separated spermatogenic cells which had been incubated in the presence of ^3H -labelled amino acids. A, primary spermatocytes; B, round spermatids; C, mid-late sloughing spermatids; D, elongate spermatids; E, mature spermatids and sperm. Electrophoretic conditions were as described for figure 16.

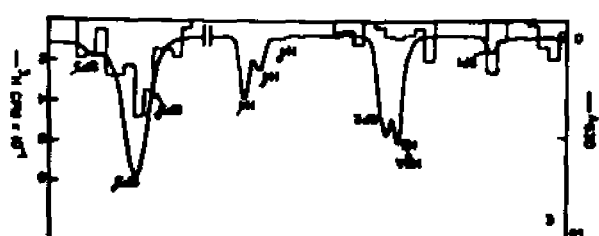
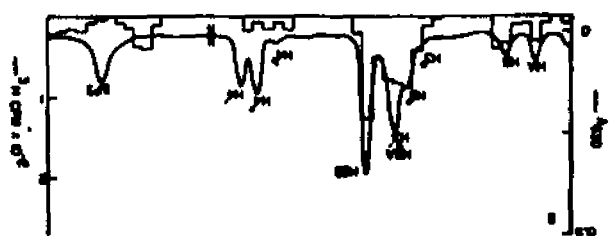
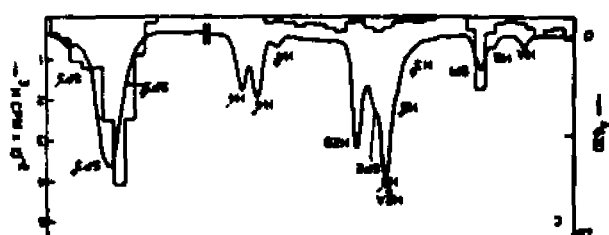


TABLE 13.--Relative specific activities of basic proteins extracted from separated spermatogenic cells and electrophoresed on 25 cm, 6.25 M urea gels

Spermatogenic Cell Fraction	$^3\text{H-CPM}/A_{630}^a$			
	Histone + SP2	SP1	SP2 + H3	SP3
Primary spermatocytes. . . .	17	..	16	..
Round spermatids	9	65	13	9
Mid-late sloughing spermatids	<1	17	<1	10
Elongate spermatids.	<1	6	<1	1
Mature spermatids + sperm. .	<1	5	<1	2

^aAll specific activities normalized to a 0-3.00 OD₆₃₀ scale.

on the 0.75 m urea gels, SP1 is synthesized by mid-late sloughing, elongate, and mature spermatids. It also appears that some synthesis of SP1 may occur in round spermatids (fig. 19B). However, the acrosomal spermatids which contaminate the round spermatid fraction may be the source of the radioactivity which appears associated with SP1. It was previously shown (fig. 18B) that acrosomal spermatids are very active in the synthesis of SP1. Figures 18 and 19 clearly demonstrate that SP1 is a newly synthesized protein which is synthesized by all spermatid stage cells (with the possible exception of round spermatids).

SP2 is resolved as a band or shoulder with a mobility somewhat greater than histone H3. Very low levels of radioactivity were associated with this band in late spermatid stages (fig. 19C-E). This was surprising since it is in these spermatogenic stages that SP2 becomes a major component of chromatin. SP2 may be synthesized by earlier spermatogenic stage cells. Figure 18 (B and C) demonstrated that radioactivity was associated with the histone H3+SP2 band in acrosomal and early sloughing spermatids. Moreover, round spermatids (fig. 19B) appear to incorporate radioactive amino acids into the SP2 region. However, the presence of radioactivity in the histones of these cells prevents a clear evaluation of whether these cells synthesize SP2.

SP3 was synthesized by all of the spermatids examined on 6.25 m urea gels. The specific activity of this complex band was greatest in the sloughing spermatids (table 13).

Taken together with the observations from the 0.75 m urea gels it seems that SP3 is synthesized throughout spermiogenesis with a peak of synthesis in sloughing spermatids. This pattern is what may be expected if SP3 plays a major role in the condensation of spermatid chromatin.

On the 25 cm (6.25m urea) gels the SP3 components are better resolved. SP3' is clearly resolved from the major peak of staining, which contains SP3" and SP3'''. As noted previously (fig. 17), the majority of ^3H -label appears to coincide with the trailing portion of the major absorbance band (SPE'''). Lower levels of radioactivity may also be found in SP3" and SP3'. The lack of complete coincidence between the peak of absorbance and the majority of radioactivity suggests that SP3 is either composed of several different proteins with very different specific activities or modified forms of a single protein.

Basic Protein Phosphorylation

Incorporation of ^{32}P into the basic chromatin proteins of spermatogenic cells has been examined. These studies were instituted to determine whether the complexity of SP3 may be due to phosphorylation and to determine whether any of the other SP's or histones were phosphorylated by early or late stage spermatogenic cells. Cycloheximide was employed to ascertain whether phosphorylation is associated with newly synthesized or "old" basic proteins.

Spermatogenic cells were prepared by collagenase dissociation. The cell suspension divided into two approximately equal portions. Each portion was incubated for 5 hrs in modified DNOM containing 5 Ci/ml each of L-lysine-4,5- ^3H (53 Ci/mmol) and L-arginine- ^3H (33 Ci/mmol), and 100 $\mu\text{Ci/ml}$ of carrier-free $\text{H}_3^{32}\text{PO}_4$. However, one incubant also contained 100 $\mu\text{g/ml}$ of cycloheximide. Following incubation, the spermatogenic cells of each incubant were separated into two fractions (A and B) by centrifugation in 12% Metrizamide. The fractions consisted of: (A) early spermatogenic stages preceding mid-late sloughing spermatids, and (B) mid-late sloughing spermatids, elongate spermatids, mature spermatids, and sperm. Basic proteins were extracted and electrophoresed on 25 cm, 6.25 M urea gels. The histone, SP3, and SP4 regions of the gels were cut out, scanned, and then sliced into 2.2 mm slices.

Figure 20 presents the absorbance and radioactivity profiles for the basic proteins extracted from the cells in fractions A and B of the incubant lacking cycloheximide. Relative specific activities are presented in table 14. Comparisons of the ^3H -cpm profiles between figure 20A and 20B clearly demonstrate that the cells of fraction A synthesize histones, while the cells of fraction B synthesize SP1 and SP3, but not histones. This result was expected from observations of basic protein synthesis in particular separated spermatogenic cell types (figs. 18 and 19). It is apparent that the bulk of the ^3H -cpm in histone H4 are associated with its acetylated forms.

Fig. 20. Absorbance and radioactivity profiles for basic proteins prepared from (A) early-mid spermatogenic cells and (B) late spermatid plus sperm cells, which had been incubated with ^3H -labelled amino acids and $\text{H}_3^{32}\text{PO}_4$. Electrophoresis was for 16 hrs on 25 cm, 6.25 M urea gels, at 220 volts (24°C). Gels were cut and then scanned in the histone region (A and B) and the SP3 and SP4 (B only) regions.

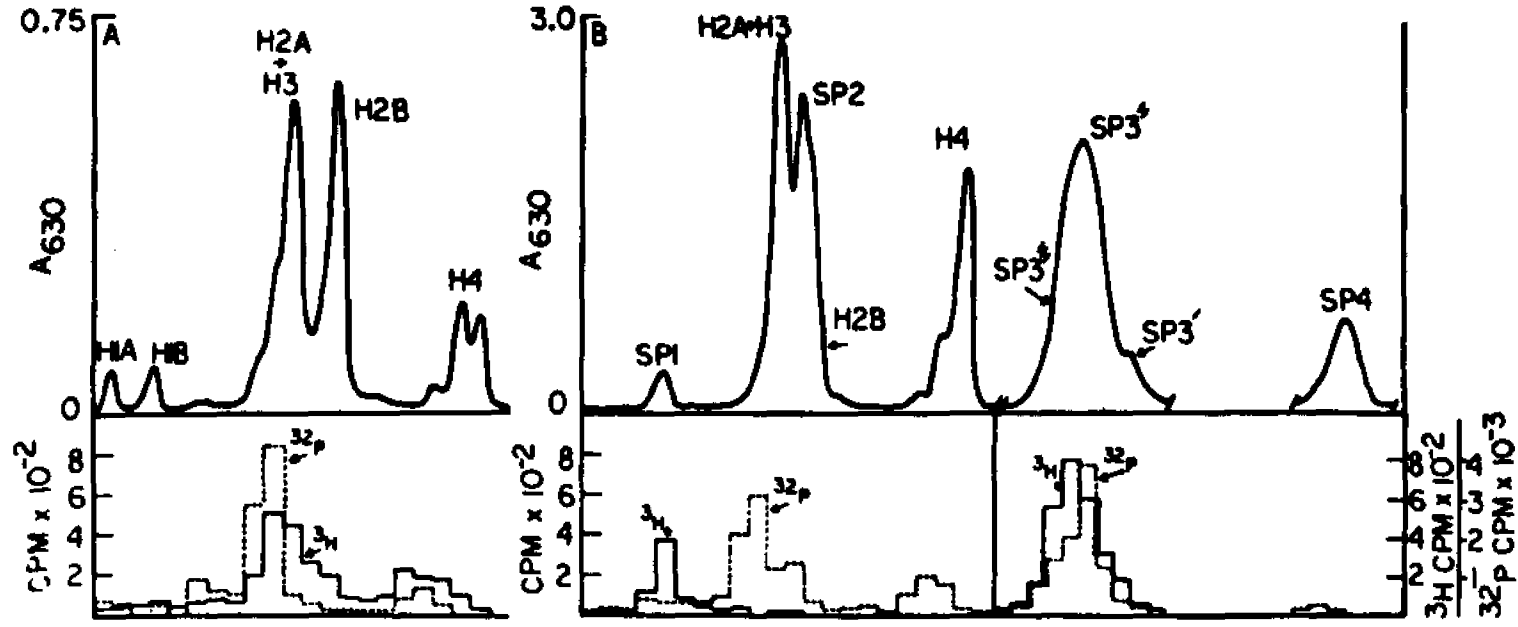


TABLE 14.--Relative ^3H and ^{32}P specific activities of basic proteins extracted from early-mid spermatogenic cells and late spermatid plus sperm cells which had been incubated in the presence and absence of cycloheximide

Spermatogenic Cell Fraction	^3H CPM/A ₆₃₀ ^a							
	H1	H2A+H3	H2B	H4	SP1	SP2	SP3	SP4
Early-mid spermatogenic cells								
Control.	21.7	28.2	11.1	32.6		...		...
+ 100 $\mu\text{g/ml}$ cycloheximide ^b . .	4.6	1.1	1.0	2.2		...		...
Late spermatids + sperm cells								
Control.		0.6	0.2	0.3	38.9	0.2	8.5	0.1
+ 100 $\mu\text{g/ml}$ cycloheximide ^c . .		0.2	...	0.1	0.3	...	1.2	...
^{32}P CPM/A ₆₃₀								
Early-mid spermatogenic cells								
Control.	20.8	33.3	1.6	14.0		...		...
+ 100 $\mu\text{g/ml}$ cycloheximide . .	21.5	17.6	2.4	3.7		...		...
Late spermatids + sperm cells								
Control.		8.1	...	3.9	7.9	2.7	31.0	4.4
+ 100 $\mu\text{g/ml}$ cycloheximide . .		10.7	...	5.9	9.8	2.0	24.7	6.4

^aAll specific activities normalized to a 0-3.00 OD₆₃₀ scale.

^bSpecific activity decreased by 90.5% relative to control.

^cSpecific activity decreased by 96.3% relative to control.

In agreement with earlier observations the ^3H -cpm in SP3 migrated more slowly than the major absorbance peak (fig. 20B).

The highest level of phosphorylation ($33.3 \text{ cpm}/A_{630}$) was associated with the histone H3+H2A band of the basic proteins from early-mid spermatogenic stage cells (fig. 20A). This was also the band with the highest specific activity. ($8.1 \text{ cpm per } A_{630}$) in the late spermatids plus sperm cells. Histone H2A is typically a highly phosphorylated histone (90,91). Moreover, the bulk of the ^{32}P -cpm in the histone region comigrates with histone H2B on gels containing 0.75 M urea (see fig. 21). The relative mobility of histone H2A also becomes increased as gel urea concentration is reduced (see chapter 3). Previous studies have demonstrated that the phosphorylated form of histone H2A comigrates with histone H2B (90). These observations suggest that much of the ^{32}P in the histone H3+H2A band (fig. 20) is associated with histone H2A.

Lower levels of phosphorylation are also associated with histones H1 ($20.8 \text{ cpm}/A_{630}$) and histone H4 ($14 \text{ cpm}/A_{630}$) in the cells from fraction A. The ^{32}P -cpm in the histone H4 bands comigrate with the acetylated species of this histone. The acetylated forms of histone H4 are also phosphorylated in the late spermatid plus sperm cell fraction (fig. 20B), albeit at a level ($3.9 \text{ cpm}/A_{630}$) which is 28% of that in the cells of Fraction A.

All of the SPs appear to be phosphorylated in the cells from B (fig. 20B). By far, SP3 exhibited the highest level ($31 \text{ cpm}/A_{630}$) of phosphorylation. The peak of phosphorylation

occurred over the peak of SP3 absorbance (SP3''), with another region of phosphorylation which comigrated with the peak of ^3H -cpm in SP3''. Little ^{32}P is associated with SP3'.

Phosphorylation in spermatogenic cells incubated without cycloheximide was also examined on 10 cm, 0.75 M urea gels. Spermatogenic cells were incubated for 5 hrs in modified DNOM containing 200 Ci/ml and then separated by centrifugation in 12% Metrizanide. As can be seen in fig. 21, the peak of histone ^{32}P comigrated with histone H2B. This is most likely due to comigration of histone H2B and phosphorylated histone H2A (see discussion above). Phosphorylation in the histone H4 region of the gel, appears to be associated with acetylated forms of histone H4. In addition, the presence of two discrete regions of phosphorylation in SP3 is confirmed in the radioactivity profile of fig. 21B.

Previous studies (74,90,91) have demonstrated that phosphorylation of histone H4 is associated with its synthesis. Louie and Dixon (89) have shown that histone phosphorylation is absent in trout spermatids that don't synthesize histones. Here, it was demonstrated that Xenopus laevis spermatids, which do not synthesize histones, still phosphorylate the histones. That this was not due to contamination of the spermatids by a small fraction of synthetic cells is shown in the radioactivity profiles of cycloheximide treated spermatogenic cells (fig. 22).

Cycloheximide (100 g/ml) treatment resulted in a reduction of basic protein synthesis by 90.5% in early-mid

Fig. 21. Absorbance and radioactivity profiles for basic proteins prepared from (A) early-mid spermatogenic cells and (B) late spermatid plus sperm cells, which had been incubated with $H_3^{32}PO_4$ in DNOM. Electrophoresis was for 2.5 hrs at 130 volts on 10 cm, 0.75 M urea gels (25°C).

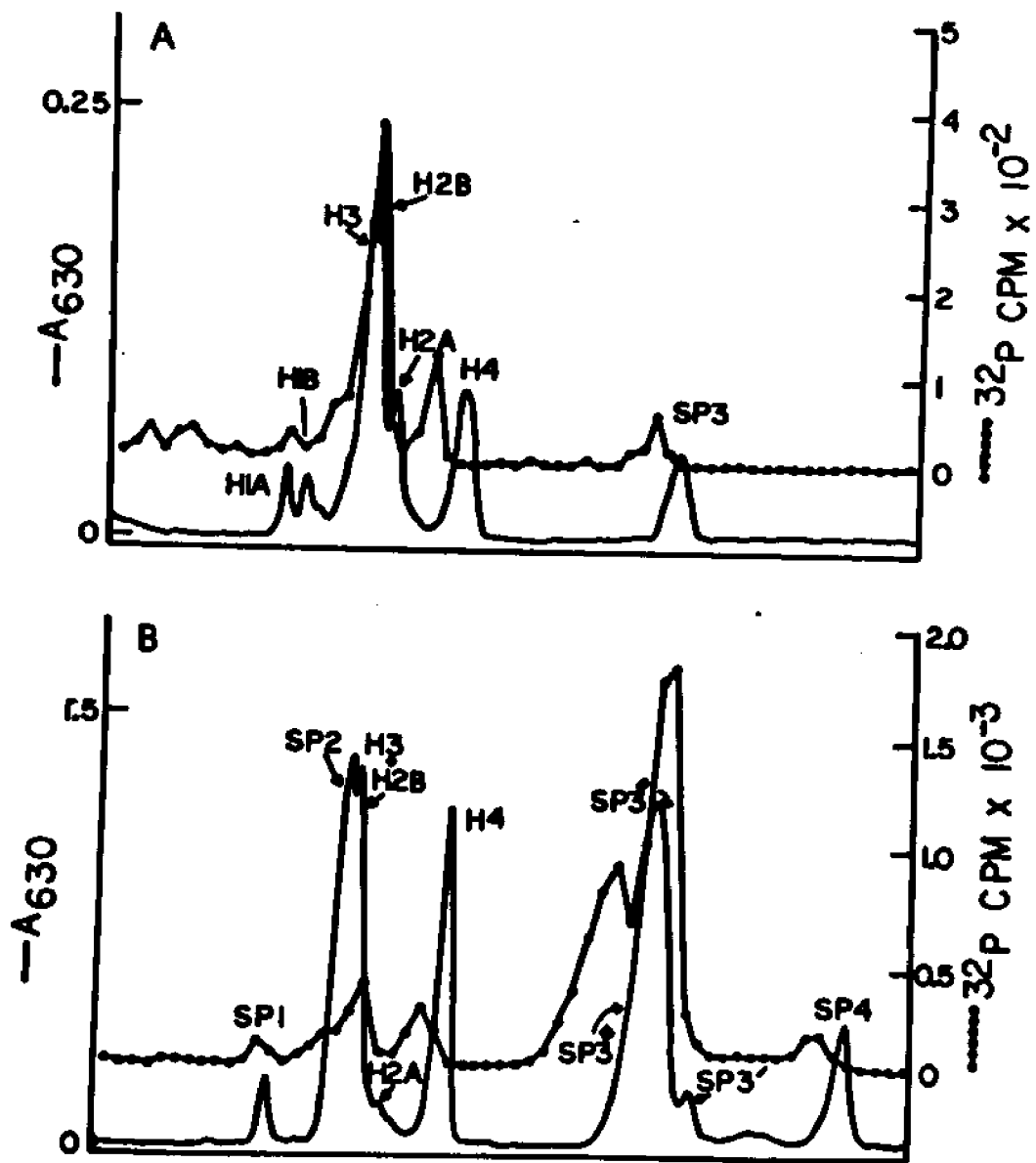
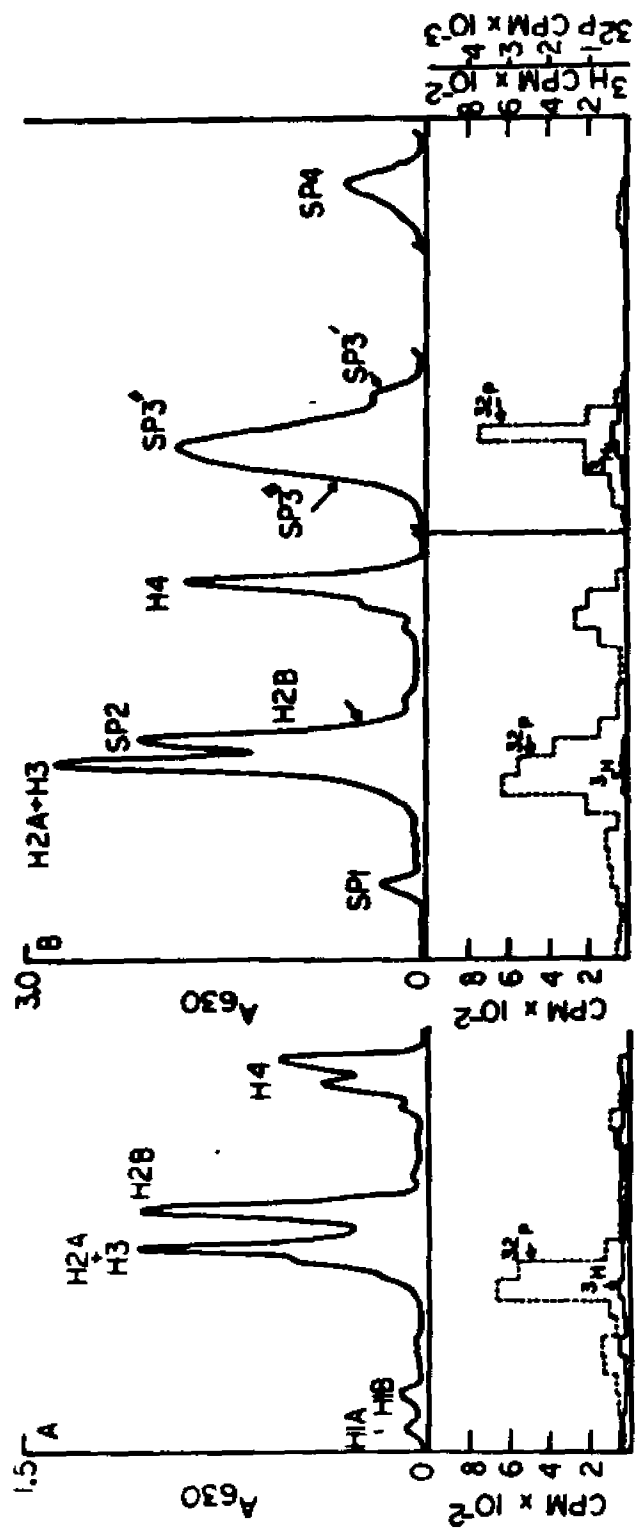


Fig. 22. Absorbance and radioactivity profiles for basic proteins prepared from (A) early-mid spermatogenic cells and (B) late spermatid plus sperm cells, which had been incubated in the presence of ^3H -labelled amino acids, $\text{H}_3^{32}\text{PO}_4$, and cycloheximide. Electrophoretic conditions were as described for figure 20. Gels were cut, scanned, and sliced in the histone regions (A and B) and the SP3 and SP4 regions (B only).



spermatogenic cells and 96.3% in late spermatids plus sperm (see table 14). The levels of ^{32}P incorporation were reduced by 47% in histones H3+H2A and by 74% in histone H4. The phosphorylation of histone H1 appeared to be unaffected by cycloheximide treatment. These observations suggest that phosphorylation of histone H1 is not associated with its synthesis, while as much as 74% of histone H4 phosphorylation and 47% of phosphorylation in histone H3+H2A are occurring on newly synthesized molecules, in the early-mid spermatogenic cells.

Histone phosphorylation in the late spermatid plus sperm fraction was essentially unaffected by cycloheximide treatment. Therefore, the incorporation of ^{32}P into the histones of these cells occurs on "old" histone molecules. Similar conclusions may be drawn for phosphorylation of SP1, SP2, and SP4. However, ^{32}P incorporation into SP3 was reduced by 20% in cycloheximide treated cells. It appears that the incorporation into the trailing region of SP3 (SP3''') was most affected by cycloheximide treatment.

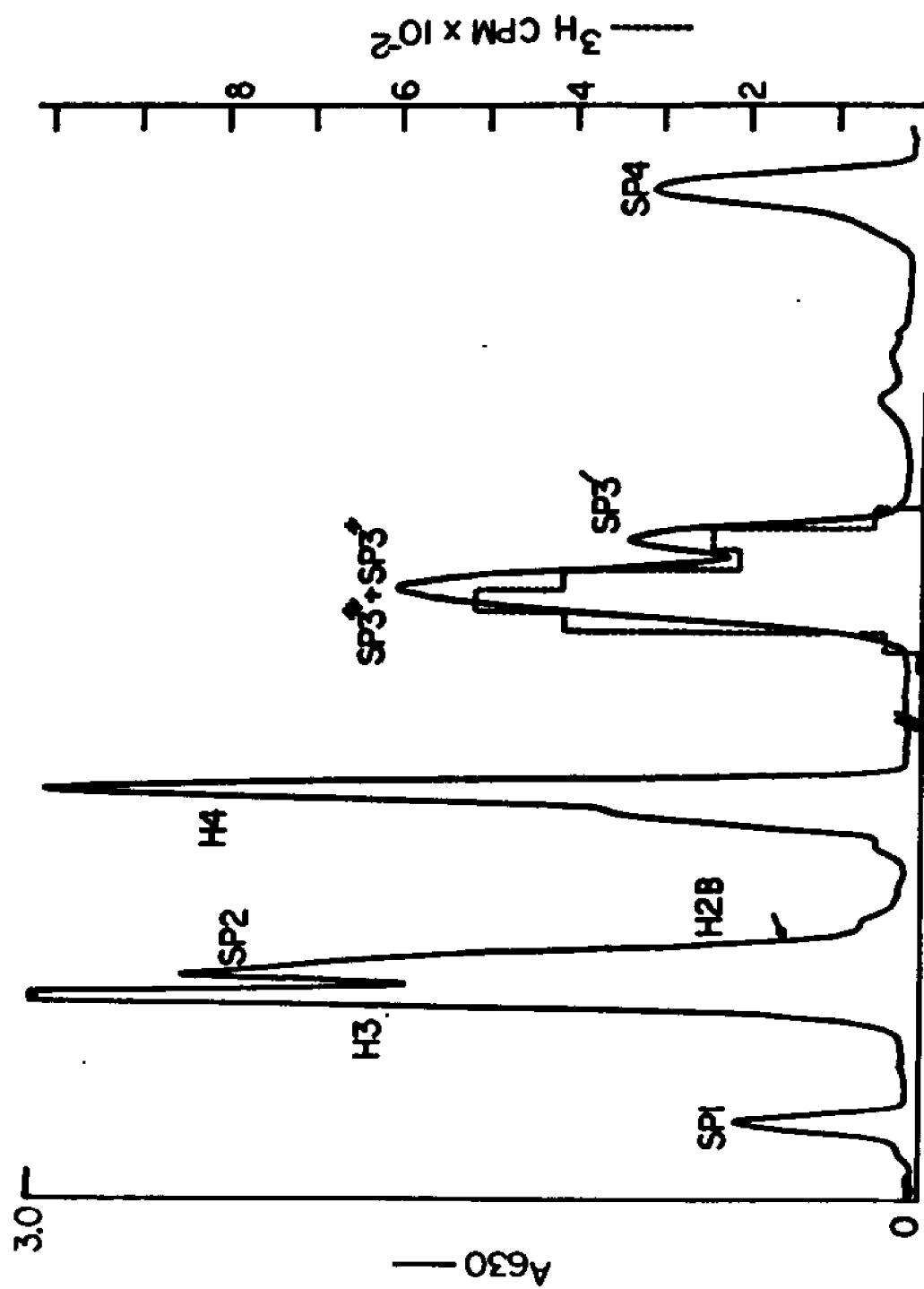
These experiments demonstrate that histone phosphorylation is occurring in spermatids which are undergoing histone displacement. This is clearly different from trout spermatids (89). While phosphorylation was ruled out as a mechanism involved in histone displacement in trout spermatids, such cannot be done for Xenopus laevis spermatids. Variability in the basic chromatin proteins associated with spermatogenesis seems to extend to the level of histone metabolism (phosphorylation). Moreover, the studies suggest that phosphoryla-

tion of SP3 may play a major role in producing the heterogeneity in this region.

That the ^{32}P bound to the basic proteins is covalently bonded is indicated by the observation that greater than 97% of the ^{32}P -cpm can be removed by treatment of the basic proteins with E. coli alkaline phosphatase. This was not due to protein degradation during the incubation because less than 2% of ^3H -amino acid labelled proteins became solubilized. Figure 23 presents a gel scan of late spermatid plus sperm basic proteins (labelled with ^3H -amino acids and ^{32}P) which had been treated with alkaline phosphatase for 2 hrs. Little difference is noted between the absorbance profiles of phosphatase treated and untreated basic proteins in the histone region of the gel. This indicates that the SP bands and the multiple bands of histone H4 are not solely phosphorylated derivatives of histones. However, a significant change in the absorbance profile of the SP3 region of the gel occurred as a result of phosphatase treatment. The relative amount of SP3' increased to 22% of the total protein in the SP3 bands. The ^3H specific activities of SP3' (8.4 cpm/ A_{630} unit) and SP3''+SP3''' (8.8/ A_{630} unit) were found to be similar to each other and to the specific activity of the entire SP3 region (8.5 cpm/ A_{630}) of untreated basic proteins (see table 14). Similar results have been obtained following 15 hrs of alkaline phosphatase treatment.

These results demonstrate that the heterogeneity of the SP3 region is in part due to the presence of phosphorylated

Fig. 23.. Absorbance and radioactivity profiles of late spermatid plus sperm basic proteins (labelled with ^3H amino acids and $^{32}\text{PO}_4$) which had been treated for 2 hrs with E. coli alkaline phosphatase. Electrophoretic conditions were as described for figure 20. The gel was cut, scanned, and sliced in the histone and the SP3-SP4 regions only.



derivatives of a single parent molecule (SP3'). Phosphorylation cannot be the sole cause of heterogeneity, since the absorbance of SP3'-SP3''' could not be totally converted to absorbance in SP3'. Old, as well as newly synthesized SP3s, are both phosphorylated since 5 hrs of cycloheximide treatment only reduced phosphorylation by 20%. At present, the underlying basis for the remaining heterogeneity is unknown. It may be due to the presence of more than one protein in the SP3 region. Alternatively, SP3'' and SP3''' may be derivatives of SP3', acetylated and phosphorylated to different extents.

Discussion

Basic protein synthesis in a differentiating system, such as spermatogenesis, may best be studied in vitro, where the environment may be rigorously controlled. However, it is essential to demonstrate that the cells remain viable during the incubation periods. Data were presented in this chapter to indicate that the spermatogenic cells of Xenopus laevis may be maintained in vitro for at least 16 hrs. Longer in vitro maintenance periods may be possible, however more detailed analyses of cell survival are required to evaluate the maximum length of time that these cells may be maintained. The ability to maintain spermatogenic cells in vitro permits detailed analyses of chromatin protein synthesis and modification in these cells. These data may then be compared to the large literature on these subjects in somatic, tissue culture cells.

Evidently, histone synthesis is restricted to early spermatogenic cells in Xenopus laevis. Histone synthesis is greatest in primary spermatocytes, and then declines until the late sloughing spermatid stage in which it ceases. Undoubtedly spermatogonial cells (not studied here) are also very active in histone synthesis since they are actively replicating DNA. The close relationship between histone and DNA synthesis has been documented previously (36,37,52). These results agree well with those of other studies of basic protein synthesis during spermatogenesis in trout (89) and rat (306). One intriguing question unanswered as yet is why histones are synthesized at all in stages succeeding spermatogonia and early spermatocytes which synthesize DNA. Recent studies (55) have demonstrated that amphibian germ cells (oocytes) synthesize histones in the absence of DNA synthesis. However, this histone is apparently stored for use during the early stages of development. Histone storage in sperm is highly improbable. Uncoupled histone and DNA synthesis has also been reported in primary spermatocytes of crickets (307), newt (308), and lily (309).

Louie and Dixon (102) have demonstrated that histone H4 synthesis in trout spermatogenic cells is followed by a gradual process of acetylation and deacetylation which requires approximately one day. Shortly, after synthesis (4 hrs) most of the ³H-amino acid label was found over the diacetylated form of histone H4. In trout, unsubstituted and monoacetylated forms of histone H4 did not become labelled until 16 hrs of incuba-

tion.

In Xenopus most of the ^3H -amino acid label was found over the diacetylated and triacetylated forms of histone H4 following 5 hrs of incorporation (see fig. 19). Label was found over histone H4 unacetylated and monoacetylated forms following 5 hrs of incubation and 12 hrs of cell separation at 5°C (see fig. 18). It is apparent that newly synthesized histone H4 (which was acetylated during 5 hrs of incubation) had become deacetylated during the cell separation procedure at 5°C . Therefore, the time required for unsubstituted histone H4 to appear in Xenopus spermatogenic cells may be less than that in trout spermatogenic cells. This may represent a difference in the organisms or differences in incubation conditions. Species specific differences may be expected however, because of specificity in terms of the time required for the process of spermatogenesis. Similar to the situation in trout, the maturation of newly synthesized histone H4 in Xenopus spermatogenic cells appears to require a much longer time than that in dividing cells (74,310).

Phosphorylation of histone H4 appears to occur shortly after synthesis in erythroblasts (74,90) and hepatoma tissue culture cells (310) and has a short half life (74). Trout spermatogenic cells do not phosphorylate newly synthesized histone H4 for 12 hrs (102). Moreover, phosphorylation of histone H4 appears to be limited to trout spermatogenic cells active in histone synthesis (89). In Xenopus spermatogenic cells, as in trout spermatogenic cells, histone H4 phosphory-

lation is greatest in early stages active in histone synthesis. However, late spermatids, inactive in histone synthesis, also phosphorylate histone H4. While 74% of the phosphorylation of histone H4 in early spermatogenic cells could be inhibited by cycloheximide, no such effect was noted in late spermatids. These observations suggest that histone H4 becomes phosphorylated shortly after its synthesis in Xenopus early spermatogenic cells, rather than 12 hrs later as noted in trout spermatogenic cells. Histone H4 is also phosphorylated in the absence of its synthesis in the late spermatids of Xenopus laevis.

The SP bands may all be newly synthesized proteins, since radioactivity was associated with each of these bands. However, some caution must be maintained as yet. The very low levels of radioactivity noted in SP4 do not permit the exclusion of the possibility that a minor quantity of SP4 is a degradation product of another newly synthesized SP. The bulk of the protein in this band may then be a degradation product of one of the histones. Phelan et al., (311) have demonstrated the presence of a Mytilus sperm protein ($\phi 311$) with an amino acid composition very similar to the carboxyl region of lysine-rich histone. This protein is electrophoretically similar to Xenopus laevis SP4 and may represent a degradation product of one (H1) of the Mytilus histones (311,312). Since histones H1, H2B, and H2A undergo displacement during the appearance of SP4 it is conceivable that it represents a stable degradation product of one of these histones.

Interestingly, the SP's appear to be synthesized at different times during spermatogenesis. SP1 is synthesized by all of the spermatid stages. SP2 appears to be synthesized predominantly by early-mid stage spermatids and perhaps primary spermatocytes. SP3 is synthesized in all of the spermatid stages. However, the major synthesis of SP3 seems to occur in sloughing spermatids, a relatively late stage of spermiogenesis. The synthesis of SP3, a protamine-like protein, by mid-late stage spermatids is similar to the timing of protamine synthesis during trout (89) spermatogenesis.

All of the SP's are phosphorylated to some extent. The highest level of phosphorylation is associated with SP3. Phosphorylation of SP3 occurs in two major peaks. The minor peak trails the peak of newly synthesized SP3 (SP3'') while the major peak migrates slightly ahead of the ^3H -amino acid peak. Cyclohexamide treatment reduces the level of phosphorylation in the minor peak without influencing the level of phosphorylation in the major peak. Previous studies have demonstrated that the protamines of trout (89,237,238) and rat (239) undergo extensive phosphorylation following synthesis and then become slowly dephosphorylated. Treatment of Xenopus SP3 with alkaline phosphatase demonstrated that the heterogeneity of this region of the gel is partially due to phosphorylation, but not due to phosphorylation alone. The complexity may be due to the presence of several (3) different proteins in this electrophoretic region. Multiple protamine species have been demonstrated previously (215-221).

An alternative explanation for the staining and labeling heterogeneity of SP3 may be obtained by assuming that SP3 is enzymatically modified by some other mechanism than phosphorylation. Thus, SP3' may be a final unmodified form of a protein which is acetylated and phosphorylated shortly after synthesis. SP3' may be acetylated at two sites following synthesis and also phosphorylated at one or two sites. The two acetates and one or two phosphates would then give SP3' a mobility similar to SP3'''. Following synthesis and acetylation, SP3''' may then become deacetylated and dephosphorylated stepwise to a monoacetylated-phosphorylated form (SP3'') and an unacetylated-unphosphorylated form (SP3'). The failure to convert all of the SP3s to SP3' with alkaline phosphatase would then be due to the presence of the acetate groups on SP3''' and SP3''. A similar sequence of events occurs following synthesis of histone H4 (74,102). Resolution of the basis of heterogeneity in SP3 may be accomplished through pulse-chase studies of SP3 synthesis, acetylation, and phosphorylation and through biochemical characterizations of the SP3 components.

CHAPTER 5

SUMMARY AND CONCLUSION

The histones of many organisms undergo extensive modifications in composition and/or enzymatic group substitution (phosphorylation, acetylation, methylation) during sperm maturation (see chapter 1). These observations suggest that spermiogenesis is an excellent system for examining the mechanisms and controls underlying the interactions of these fundamental, eukaryotic proteins. To date, only the spermiogenic system of trout has been extensively studied relative to these problems. Since large variation in the basic chromatin proteins of sperm is known to exist, it is not unreasonable to assume that variation may extend to the mechanisms responsible for modification. Prior to generalizing from the events of spermiogenesis to those which occur in somatic cells, other spermiogenic systems should be examined in detail.

The purpose of this thesis was threefold. First, to develop methods whereby Xenopus laevis spermatogenic cells may be mildly dissociated, maintained in vitro, and then separated. Second, to examine the basic chromatin protein composition of spermatogenic cells by electrophoretic means. This was initiated to determine the extent of qualitative and quantitative changes which occur in the basic chromatin proteins during spermiogenesis. Third, to examine the synthesis and phosphorylation of basic chromatin proteins in an attempt to discern which proteins were newly synthesized and which were phosphorylated and whether these processes were related to the process of chromatin maturation during sper-

miogenesis.

In chapter 2, it was demonstrated that collagenase (in salt solution or maintenance medium) could be used to prepare cell suspensions highly enriched for spermatogenic cells. Since somatic cells, such as Leydig or Sertoli cells, presumably contain histones, the ability to separate them from spermatogenic cells in a single step is of obvious advantage. Data in chapters 2 and 4 were presented to demonstrate that spermatogenic cells so dissociated remain viable and may be maintained in vitro for as long as 16 hrs. These developments permit the study of basic chromatin protein synthesis, enzymatic modification, and displacement under strictly controlled conditions.

Spermatogenic cells were separated by unit gravity sedimentation and subsequent isopycnic centrifugation. Data presented in chapter 2 demonstrate that many of the spermatogenic cell types may be separated into highly enriched preparations by these techniques. The introduction of isopycnic centrifugation in Metrizamide permits more refined separations of spermatogenic cells than can be achieved by unit gravity sedimentation alone. The coupling of rapid, velocity sedimentation techniques (i.e., Beckman L5utritator) to isopycnic centrifugation would provide a means for the preparation of highly purified spermatogenic cell types in relatively little time. The ability to do this may allow detailed in vitro kinetic analyses to be performed on specific spermatogenic cell types from a variety of organisms.

Techniques developed in chapter 2 were applied to an electrophoretic analysis of basic chromatin proteins in separated spermatogenic cells. It was demonstrated in chapter 3, that extensive qualitative and quantitative changes occurred in the basic proteins during spermatogenesis in Xenopus laevis. At least 4 unique electrophoretic bands were shown to appear in the chromatin of spermatogenic cells. In addition to the two protamine-like electrophoretic bands, SP3 and SP4, it was shown that 2 unique bands (SP1 and SP2) appeared in the histone region of the polyacrylamide gels. Furthermore, it was demonstrated that SP3 and SP4 were composed of 3 and 2 electrophoretic components, respectively.

The SP bands are spermatogenic cell specific as well as testis specific. SP3 and SP4 were shown to be limited to spermatid stage cells. Moreover, these proteins do not appear in chromatin simultaneously. SP3 was present in the earliest spermatid stage examined (round spermatids) and increased in proportion during spermiogenesis. SP4 first appeared in the chromatin of sloughing spermatids (predominantly late sloughing spermatids) and increased in proportion in later stages.

The multiple components of SP3 were shown to be synthesized to the greatest extent by sloughing spermatids. Very little radioactivity was found in the SP4 of most spermatids with the exception of sloughing spermatids. This observation raises the possibility that SP4 is synthesized during a brief period in spermiogenesis or is composed of

degradation products of other proteins which contribute to both the absorbance and the radioactivity in this band.

SP1 was shown to be a protein present in and synthesized by all spermatid stages examined. Synthesis of this protein appeared to be greatest in early-mid spermatid stage cells. Since this protein never represents more than 3% of the total basic protein, it may be a cytoplasmic contaminant. SP1 may be acrosomal or cytoplasmic basic protein which enters the nucleus following cell breakage and then binds to DNA.

SP2 is composed of a protein(s) synthesized primarily by early-mid spermatogenic cells. Synthesis of this protein(s) appears to occur from the early round spermatid stage through the early sloughing spermatid stage. Primary spermatocytes may also be actively engaged in the synthesis of SP2. Unfortunately, the electrophoretic conditions employed did not permit a conclusive resolution of this problem.

The data presented in chapter 4 demonstrated that all of the SPs are phosphorylated proteins. By far, SP3 is phosphorylated to the greatest extent. The levels of phosphorylation in SP3 could be reduced by approximately 20% by inhibiting protein synthesis for 5 hrs with cycloheximide. This observation suggests that some of the phosphorylation is coupled to the synthesis of the SP3 protein(s). Incorporation of ^{32}P into SP1, SP2 and SP4 was essentially unaffected by cycloheximide treatment.

SP3 is a heterogeneous electrophoretic region composed of 3 bands (SP3', SP3'', SP3'''). It was shown that most of the newly synthesized protein in this region co-electrophoresed with SP3''', with smaller amounts in SP3'' and SP3'. Phosphorylation occurred in two major peaks which co-electrophoresed with SP3'' and SP3'''. Alkaline phosphatase treatment demonstrated that the heterogeneity in the SP3 region is only partially due to the presence of phosphorylated species of SP3'.

Two alternative explanations exist for the heterogeneity of SP3. First, SP3 may be composed of several distinct proteins. The complexity of the synthesis and phosphorylation data would then be due to the additive effects of the labelling patterns for each distinct protein. Second, the multiple components of SP3 may represent a single protein species enzymatically modified to different extents. Since the modification cannot be phosphorylation alone, it is possible that this protein is also acetylated to varying degrees. The complexity would then be due to the interconversion of SP3 into the various acetylated and phosphorylated states following synthesis.

The results of the studies on spermatogenic cell specific proteins (SPs) have revealed a considerable degree of complexity in terms of cell type specificity, time of synthesis, and degree of phosphorylation. Proteins with similar electrophoretic properties to SP1 and SP2 have been reported in mammalian testes (11,222,229) and cricket sper-

matids (227). Little is known of their comparative biochemical properties and their relationships to histones. It would be interesting to biochemically compare these proteins from mammals, crickets, and Xenopus to determine the degree of structural similarity that exists.

SP3 and SP4 are closely related (electrophoretically) to the sperm specific proteins of Mytilus edulis (312,313). Proteins such as the TP proteins of mammals (232) and histone T (H6) described in trout spermatids (224) have similar electrophoretic properties to SP3 in Xenopus. Whether one or all of the SP3 or SP4 components is biochemically similar to any of these proteins is unknown.

The most significant result of the studies described in chapter 3 was that the sequence of histone replacement during spermiogenesis is unlike that reported for trout (223,232) or rat (239). It was shown that the H1 histones were first to be totally displaced, followed by histones H2B and H2A. Histones H3 and H4 remain associated with the chromatin of the mature spermatids and sperm in significant amounts. Histone replacement during spermiogenesis in trout (223,232) and rat (239) suggested that histone H1 was the first histone to be displaced. It is apparent that the sequence of histone replacement is not constant and may be as variable as the composition of basic proteins present in sperm. Studies of basic proteins in cricket spermatids have demonstrated that all five histones were concomitantly replaced (227). This further demonstrates that the sequence

of histone replacement may be species specific and related to the types of proteins which may replace the histones.

Interestingly, it was noted that the order of histone replacement during spermiogenesis in Xenopus is essentially the same as that which occurs during displacement with increasing amounts of NaCl (240). Moreover, the displacement occurs in a pairwise and possibly cooperative manner. Thus, histones H2A and H2B are removed together. Histones H3 and H4 may also be removed together, yet at a slower rate or later time. Studies of histone-histone interactions in solution (201-205,207) and in chromatin (206-210) have demonstrated that strong associations are formed between histones H2A and H2B, H2B and H4, and H3 and H4. Removal of one of the histone pairs could result in the displacement of the other member of the pair or the exposure of that member to proteolytic degradation.

Previous studies have demonstrated that histone H1 is most susceptible to proteolytic degradation in chromatin (166,189,288-291,304). Histones H3, H2B, H4 and H2A are degraded by proteases in the order listed. This led to the conclusion that histone H2A forms the core of the nucleosome complex (166,189,304). It seems that the usual distribution of histones in the nucleosome core is perturbed in Xenopus spermatids. Thus, histones H2B and H2A are displaced prior to histones H3 and H4. Such a perturbation may occur as a result of histone acetylation and SP binding to DNA.

Marushige et al., (244) have shown that chromatin saturated with protamine and then chemically acetylated does not lack any of the histones. However, proteolysis produced a rapid degradation of histones H3, H2B, and H2A in this chromatin relative to controls. It was concluded that all three processes must be involved in histone displacement.

Acetylated forms of histones H3 and H4 persist through the spermatid stages in which histones H2B and H2A begin to be displaced. This is consistent with a mechanism involving histone acetylation with histone displacement during spermiogenesis (101,245,246). During removal of histones H2B and H2A, the acetylated forms of histones H3 and H4 become significantly reduced. However, acetylated forms of histone H4 were present in the latest spermatid stage examined.

Phosphorylation of histones may also be involved in histone replacement during spermiogenesis in Xenopus laevis. Studies of histone phosphorylation during spermiogenesis in trout (89) demonstrated that this process does not occur in spermatids undergoing histone displacement. This led to the conclusion that histone phosphorylation was not involved in histone displacement. However, data presented in chapter 4 suggests that phosphorylation of histones H2A and H4 occurs in spermatids which are replacing their histones. Moreover, the phosphorylations occurred in the absence of histone synthesis. Therefore, the possibility that phosphorylation of histones plays a role in their removal from DNA cannot be ruled out for Xenopus laevis.

Conclusion

Much of the work presented here represents the results of a series of studies designed to characterize the changes which occur in the basic chromatin proteins during spermatogenesis in Xenopus laevis and to adapt this system for highly refined, detailed analyses of chromatin structure during alterations in the basic protein complement. Several studies should be carried out to obtain the data required for a more definitive analysis of the basic chromatin protein changes. First, basic protein:DNA ratios should be determined in several spermatogenic cell types. This information would permit calculation of the absolute amounts of individual proteins in chromatin rather than relative amounts. Second, the basic chromatin proteins of sperm which have traversed the male genital tract should be examined to determine the final pattern of basic chromatin proteins. Third, the electrophoretic studies presented herein should be repeated by two dimensional gel electrophoresis (i.e., acid-urea gels in one dimension and SDS gels in the second dimension). The use of a high resolution system may permit resolution of the SP2, H2A, H3 region and subsequently more precise studies of synthesis and phosphorylation. Fourth, each of the basic proteins should be fractionated, purified, and characterized biochemically to determine the extent of primary sequence heterogeneity in particular electrophoretic bands and the degree of relatedness of the SPs of Xenopus to the SPs of other organisms.

In addition to the above studies, chromatin structure may be examined during spermiogenesis in Xenopus laevis. Comparisons of the susceptibility of individual histones to exogenous protease treatment and chemical acetylation in different spermatid stages may reveal whether histone displacement is accompanied by an abrupt change in the susceptibility of particular histones to modification or proteolysis. Crosslinking studies may also be carried out to determine whether histone-histone proximities become grossly altered during displacement. Moreover, a variety of probes, such as psoralen (303), may be employed to determine whether the accessibility of DNA becomes either qualitatively or quantitatively altered during histone replacement. The information obtained by these studies should reveal several interesting features of the roles of histones H2B and H2A, or H3 and H4 in the maintenance of chromatin and nucleosome structure.

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