ELECTROPHORETIC AND AMINO ACID ANALYSIS

OF AMPHIBIAN AND REPTILIAN HISTONES

by

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ABSTRACT

Small amounts of amphibian and reptilian histones, basic proteins associated with DNA, can be characterized by a combination of electrophoresis and subsequent amino acid analysis of the stained bands. A simple method has been devised for obtaining histones for such analysis from Xenopus laevis, the South African clawed toad. Xenopus somatic histones analyzed both by starch and acrylamide gel electrophoresis show microheterogeneity for the very lysine-rich Hl histones of heart and lung, but no tissue-specificity for this group of basic nuclear proteins. Xenopus embryonic histones prepared by the method of Destrée et al. (1972) can also be characterized by starch gel electrophoresis. In this procedure, the possibility of contamination of nuclei by yolk platelets and ribosomes is reduced. No change is observed in the starch gel electrophoretic profiles of Xenopus histones throughout early embryogenesis. This confirms the original observation of Destrée et al. (1973) using polyacrylamide gel electrophoresis.

Histones can be extracted from amphibian and reptilian testis cell suspensions and analyzed by starch and polyacrylamide gel electrophoresis. Since only one animal is required in this method, the analysis can be extended to include different species for which many representatives are difficult to obtain. In addition, using Houston's (1971) method of hydrolyzing amidoblack-stained protein bands on polyacrylamide gels for amino acid analysis, one can obtain the chemical composition of testis-specific histones from a number of amphibians and reptiles. Amphibian testis-specific histones show entirely different patterns from each other, while the reptilian histones show remarkable similarity to each other both in their electrophoretic properties and amino acid composition.

Although the present survey examines only a limited number of species, the data do point to the reptiles as one place in vertebrate phylogeny where the diversity of testis-specific histones in fish and amphibians gives way to a relative constancy of such proteins.

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To My Parents

and

My Husband

ACKNOWLEDGEMENT

Part I

ELECTROPHORETIC ANALYSIS

INTRODUCTION

In order to gain a better understanding of the molecular bio-
logy of spermiogenesis and embryogenesis in the vertebrates, it is
necessary to develop micromethods to measure the important macro-
molecular constituents in the cells. At present we use biochemistry
to characterize rather large amounts of material and histochemistry
to analyze very small amounts of tissue components. Biochemistry
gives us a great deal of information about the chemistry of the con-
stituents and their interaction but tells little about the spatial
orientation of such components in cells or tissues. Histology and
histochemistry depend on a limited number of reactions and often
do not define the chemical components in an adequate manner. These
problems are particularly acute in the study of histones, their con-
tent, synthesis and modification in vertebrate spermiogenesis and
embryogenesis, particularly in amphibians and reptiles. In these
organisms, the amount of tissue available for study is small and
problems exist with respect to contamination with basic proteins,
such as those present in ribosomes and yolk platelets.

In this thesis, I will examine the electrophoretic properties
and amino acid composition of amphibian and reptilian histones
in order to demonstrate that useful biochemical data can be
obtained from small amounts of material when these methods
are applied to somatic cells, testis and embryos. Furthermore, I
have chosen to look at tissues in these lower vertebrates in
order to contrast the diversity of testis-specific histones in
these animals with the greater degree of constancy of histones

TABLE 1

Principal Components of Calf Thymus Histones and Commonly Used Nomenclature Systems

Class	Nomenclature	Lys/Arg Ratio	Total Residues	Molecular Weight	N terminal	C terminal
Very lysine-rich	H1(I, f1)	22.0	215	21,500	Ac-Ser*	Lys
Lysine-rich	H2a(IIb1, f2a2)	1.17	129	14,004	Ac-Ser	Lys
	H2b(IIb2, f2b)	2.50	125	13,774	Pro	Lys
Arginine-rich	H3(III, f3)	0.72	135	15,324	Ala	Ala
	H4(IV, f2a1)	0.79	102	11,282	Ac-Ser	Gly

All data compiled from Elgin et al., (1971) and DeLange and Smith, (1974)

* Ac-Ser = N-acetylated-serine

from somatic and embryonic tissues.

Histones are basic proteins closely associated with DNA at some point in their life history. They fall into five categories: H1, H2a, H2b, H3 and H4 (Table 1). Histones H2a, H2b, H3 and H4 appear to be aggregated with DNA to form a nucleosome structure (Kornberg, 1977; Kornberg and Thomas, 1974; Bradbury et al., 1972; Richards and Pardon, 1970) looking like beads on a string (Olins and Olins, 1974). These histones show little tissue-or species-specificity. Histones H4 in fact is the most evolutionarily conservative protein known (De Lange and Smith, 1975). Lysine-rich histone H1 is thought to crosslink the chromosome structure in a higher order of chromosome complexity (Baldwin, et al., 1975). H1 generally shows both tissue-and species-specificity (Stellwagen and Cole, 1969).

The high resolving power of zone electrophoresis in starch or polyacrylamide gels has been utilized extensively in studies on tissue specificity of histones from various sources. Using starch gel electrophoresis, Vendrely et al. (1965) observed a remarkable similarity in histone patterns from calf thymus, liver and lung as well as from rat thymus and liver. Starch gel electrophoretic patterns of histones from domestic fowl revealed the tissue specificity of the erythrocyte-specific histone (Neelin and Butler, 1961), an observation which led to the isolation of chicken erythrocyte specific histone H5. Similarly, polyacrylamide gel electrophoresis of histones from rat spleen, thymus, liver, kidney, heart and brain showed only minor variations from the general pattern known for mammalian histones (Hnilica, 1972).

The most extensive and careful study on electrophoretic similarities of histones from numerous vertebrate species was undertaken by Panyim et al. (1970, 1971a) using a system of long polyacrylamide gels (Panyim and Chalkley, 1969a; 1969b). The authors separated histones into protein bands corresponding to fractions H1, H2a, H2b, H3 and H4. In all species analyzed, the arginine-rich histones H3 and H4 had a constant electrophoretic mobility, indicating considerable conservation of these fractions during the evolution of vertebrates. Only small variations were observed for histones H2a and H2b, while the lysine-rich histone H1 varied considerably in its electrophoretic heterogeneity and mobility, indicating substantial changes in the primary structure of this fraction during evolution. These findings were in excellent agreement with the chemical analyses of the arginine-rich and slightly lysine-rich histone fractions (Hnilica, 1972; De Lange and Smith, 1974), whereas the H1 Histones were found to be both tissue and species specific (Bustin and Cole, 1968; De Lange and Smith, 1974).

The testis is a specialized somatic tissue containing germ cells: the male gametes. Both starch and polyacrylamide gel electrophoresis have shown that amphibian and reptilian testis containing sperm have fast-moving bands migrating faster than the somatic histone region (Kasinsky et al., 1977; Bols et al., 1976; Bols and Kasinsky, 1973 and Bloch, 1962). These testis-specific histones may be sperm-specific as well, as suggested by Kasinsky et al., (1977) and Bols et al. (1976). During spermiogenesis there is generally a replacement of histones by more arginine-

rich proteins in many species. Although there is an ample amount of cytochemical evidence about this transition, the best bio-chemically documented system is the appearance of protamines during the terminal stages of sperm maturation in many species of fish (Louie et al, 1973). The components typical for spermatozoa are generally more basic than somatic histones, usually with a high arginine and serine content (5 to 18 mole%). Also, tyrosine and cysteine are amino acids common to many highly basic sperm histones (Subirana, 1975). This group of proteins shows extreme non-conservation amongst vertebrates (Kasinsky et al., 1977; Bloch, 1969). These diverse proteins have been cataloged into 5 types by Bloch (1969) (Fig. 1) based in part on the original scheme of Kossel (1928). Class 1 is Salmo type; Class 2, mammalian type; Class 3, Mytilus type; Class 4, Rana type and Class 5, crab type (no histones). All 5 types of sperm-specific histones have been observed cytochemically and biochemically in different fish and amphibian species (Bloch, 1976). In reptiles, only two species have been examined and both have been grouped as Type 1 (Bloch, 1976). However, a question mark is indicated for reptilian sperm histones in Fig. 1. This is due to the fact that these data are based on Bloch's (1969) unpublished observation and on a single positive Sakaguchi test on a single animal (Sud, 1961). By itself, the Sakaguchi test is not specific for Type 1 sperm histones (Bols and Kasinsky, 1972). In mammals these proteins generally fall into Type 2 (Bloch, 1976). As for birds, there has only been one species studied thus far, Gallus domesticus. The rooster sperm histone has been placed in the Type 1 category

Fig. 1. Cytochemical classification of sperm-specific histones in vertebrate phylogeny.

Type	Name	Representative animal
1	Salmo type (protamine, monoprotamine)	Trout
2	Mammalian type (stable protamine, basic keratin)	Rat
3	Mytilus type (intermediate, di-, triprotamine)	Mussel
4	Rana type (somatic like)	Frog
5	Crab type (no histones present)	Crab

Based on data of Bloch (1969, 1976), Bols and Kasinsky (1972, 1973, 1974, 1976). Alder and Gorovsky (1975), and Picheral (1970). Arrow indicates evolutionary trend from relative diversity of sperm-histone type in fish and amphibians to relative constancy in reptiles birds and mammals. (2) indicates newt spermatid-specific histones.

SPERM HISTONES

Fish	Amphi-bians	Reptiles	Birds	Mammals
4	4			
3	3			2
2	(2)	?		
1	1		1	(1)
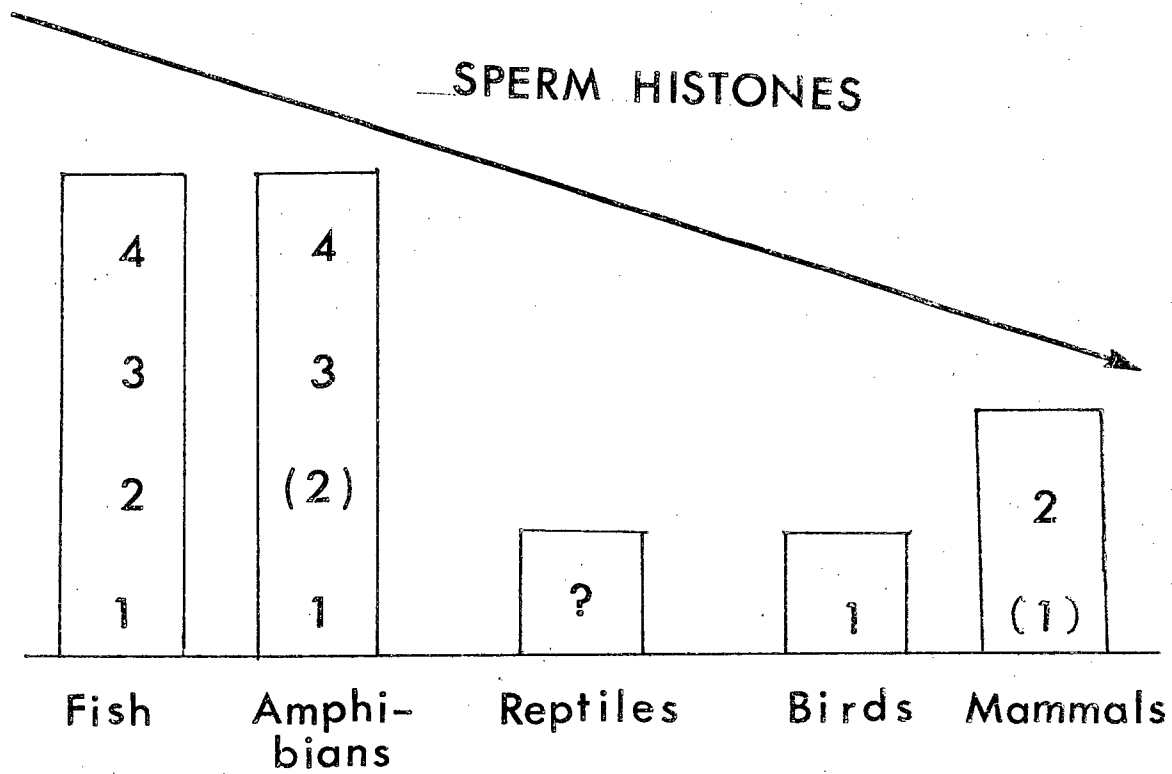
on the basis of its primary structure (Nakano et al., 1976). It
is not known if this observation holds true for other species in
the class Aves. However, there does appear to be an evolutionary
trend from diversity of sperm-specific histones in fish and
amphibians to a relative constancy of such proteins in reptiles
and mammals (Kasinsky et al., 1977).

Although the reason for the replacement of histones by
arginine-rich protamines or similar proteins during spermiogenesis
is unknown, it has been suggested that the more basic arginine-
rich proteins may be more efficient than histones in genetic
inactivation and packing of the DNA in mature sperm (Marushige
and Dixon, 1969). As the DNA is packed tighter, the probability
of its being damaged decreases since water, enzymes and bacteria
have a smaller chance of reaching it (Subirana and Puigjaner,
1973). The appearance of cystine bridges should reinforce this
protection (Bedford and Calvin, 1974). The role of tyrosine
is not clear, although it may be involved in energy transfer
processes which would protect DNA against radiation damage
(Subirana, 1972). It is likely that these basic proteins also
are involved in other biochemical processes which take place
during spermiogenesis and perhaps during fertilization.

Early embryogenesis is characterized by rapid cleavages,
fast growth and differentiation. Since histones are closely
associated with DNA, it is interesting to know if there are changes
in the content or type of histones concurrent with these complex
processes. Most investigators agree that typical histones are
present in the nuclei of blastula and gastrula embryos depending

on the particular animal species examined. Analytical studies
show that in advanced embryos, the histones are similar to those
in adult tissue (Hnilica, 1967). No detectable changes in the
chemical composition and electrophoretic patterns of histones
were observed during chick embryogenesis (Kischer et al., 1966;
Kischer and Hnilica, 1967). The electrophoretic profiles of
this group of proteins are essentially the same during early
embryogenesis in Xenopus (Destrèe et al., 1973) and in the devel-
opment of frog tadpole (Stenroos and Reichard, 1970). Using a
$^{14}CO_2$ labelling method, Byrd and Kasinsky (1973a, b) showed that
there was extensive synthesis of each of the major classes of
histones prior to gastrulation in Xenopus laevis. These newly
synthesized embryonic histones were similar to those in adult
liver when compared by gel electrophoresis and amino acid analysis.
Adamson and Woodland (1974), using ^{3}H-lysine, labelling, two-
dimensional gel electrophoresis and peptide mapping of arginine-
rich histone H4, found that the four main histone fractions other
than histone H1 were synthesized at all stages of Xenopus develop-
ment. H1 histone (adult type) synthesis was first detected at
the late blastula stage. Similar results were also obtained in
sea urchin embryos. Changes in the proportion of histone classes
synthesized during development have been established (Easton and
Chalkley, 1972; Johnson and Hnilica, 1971; Seale and Aronson, 1970).
Aside from this, synthesis of an H1 characteristic of the morula
stage gives way to synthesis of a new and electrophoretically
distinct H1 at the gastrula stage (Ruderman et al., 1974) Also,
Cohen et al. (1976) observed microheterogeneity of slightly lysine-

rich histones H2a and H2b during early sea urchin embryogenesis. The synthesis of the complete complement of adult type histones first appears during blastulation in sea urchin and in Xenopus and even later in other animal species. The synthesis of arginine-rich histones appears first, then the synthesis of lysine-rich histones. Once established, the qualitative pattern of histones in differentiated tissues remains unchanged, with the exception of some specialized cells, such as nucleated erythrocytes or spermatozoa. What is the significance of the late synthesis of the entire complement of histones during embryogenesis? Is there a maternal pool of histones stored to compensate for this late synthesis? (Adamson and Woodland, 1977; Woodland and Adamson, 1977). The answers to these questions remain to be determined.

MATERIALS AND METHODS

MATERIALS

Eggs and embryos of <u>Xenopus laevis</u> were obtained from a laboratory breeding stock as described by Gurdon (1967) and were dejellied in 2% (w/v) cysteine hydrochloride dissolved in Brown's solution (0.035% NaCl, 0.0005% KCl, 0.001% $CaCl_2$, 0.002% $MgCl_2 \cdot 6H_2O$), adjusted to pH 8.0 with 10 N sodium hydroxide. Other amphibians and reptiles were purchased from Camosun Aquaria, Vancouver, B.C. and obtained from dealers throughout North America. They were identified by reference to standard sources (Conant, 1975; Stebbins, 1966), and dissected after a brief period at room temperature. Organs were examined when fresh or stored at $-70^{\circ}C$.

METHODS

A. <u>Isolation of Histones</u>

<u>Cell Suspension (Kasinsky et al., 1977)</u>

Somatic tissues (hearts), testes or swimming tadpoles were homogenized in PBS solution (phosphate buffered saline: 1.6% NaCl, 0.04% KCl, 0.23% Na_2HPO_4, 0.1% $MgCl_2$ and 0.1% $CaCl_2$), and filtered onto the 3mm glass fiber filter paper. Histones were extracted directly from the cells trapped on the filter paper using 0.4 N H_2SO_4 for 15 minutes.

<u>Method of Byrd (1974)</u>

Somatic tissues (hearts), testes or swimming tadpoles were homogenized in 0.14 M NaCl, 0.024 M EDTA, 1% triton-X-100 with 0.05 M $NaHSO_3$ as a proteolytic inhibitor at pH 8.0. Nuclei were

pelleted by centrifuging for 15 minutes at 1,500 g. Histones were
extracted directly by stirring the nuclear pellets in 0.4 N H_2SO_4
for 1 hour.

Method of Destrée et al. (1972)

Somatic tissues (hearts), testes and embryos were homogenized
in 2.4 M sucrose containing 3mM $CaCl_2$, 5 mM tris-HCl buffer, pH 7.5,
0.05M $NaHSO_3$ and 0.5% triton-X-100. Nuclei were pelleted by cen-
trifuging for 2 hours in Beckman model L-type 50 rotor at 108,000 g.
The nuclear pellet was washed twice in 0.02 M EDTA, 0.01 M tris-
HCl buffer, pH 8.0, 0.05 M $NaHSO_3$ and was centrifuged at 4,300 g
for 10 minutes. The resulting pellets were washed twice with water
and centrifuged for 10 minutes at 12,000 g (Destrée et al. 1972).
Histones were extracted from this chromatin pellet with 0.4 N
H_2SO_4 for one hour.

Modified Method of Bonner et al. (1968)

Somatic tissues (hearts), testes and embryos were homogenized
in saline-EDTA solution (0.075 M NaCl, 0.024 M EDTA, 0.05 M NaHSO_3,
pH 8.0), and then strained through 4 layers of cheesecloth. The
modification of the original method of Bonner et al. (1968) was the
addition of sodium bisulfite to prevent proteolysis (Destrée et al.,
1972). The homogenate was centrifuged at 1,500 g for 15 minutes.
The pellets were washed successively with saline-EDTA solution and
tris buffer (0.05 M tris, 0.05 M $NaHSO_3$, pH 8.0). The pellets thus
obtained were homogenized by hand in tris buffer and then centri-
fuged at 10,000 g for 15 minutes. This step was repeated. Histones
were extracted from the final pellet with 0.4 N H_2SO_4 for 1 hour.
The histones were then precipitated with 4 volumes of 95% ethanol.

The precipitate was washed 3 times with 95% ethanol and then dried in a vacuum desiccator.

Isolation of Lysine-rich Histone H1 (John, 1976)

Xenopus laevis hearts, lungs or embryos (5 g) was washed with 0.14 M NaCl, then homogenized with 20 ml of 5% $HClO_4$. The homogenate was centrifuged for 30 minutes at 1,100 g. The sediment was extracted once more in the same manner with 10 ml of 5% $HClO_4$. The combined supernatant fluids were clarified by filtering them through 4-layered cheesecloth, and trichloroacetic acid was added to a final concentration of 18% (w/v). The precipitate was recovered by low-speed centrifugation, washed once in acidic acetone (200 ml of acetone containing 0.1 ml of concentrated HCl), then three times in acetone and finally dried under vacuum.

B. Isolation of Ribosomal Proteins

Ribosomes were obtained by the procedure of Hallberg et al. (1973, 1975). 100 Xenopus laevis eggs were homogenized in 6-8 volumes of RS buffer (0.01 M tris, pH 7.5, 0.0015 M $MgCl_2$) and the homogenate was spun at 27,000 g (15,000 rpm) using the Sorvall SS-34 rotor for 15 minutes. The supernatant was spun under the same condition once again and then at 125,000 g for 1.25 hour (or 42,500 rpm for 1.65 hour). The ribosomal pellets were homogenized in RS buffer and were centrifuged again. The ribosomal basic proteins were then extracted from the ribosome pellets with 0.4 N H_2SO_4 for one hour. For Xenopus embryos, the technique was the same except for the addition of 0.5% sodium deoxycholate to the initial homogenization medium.

C. Isolation of Yolk Basic Proteins

Method of Wallace and Karasaki (1963)

100 Xenopus eggs or embryos were homogenized in 10 ml of

0.25 M sucrose solution containing 5% polyvinyl-pyrrolidone (PVP)

pH 7.8. 20 ml of the homogenate were overlayered on 15 ml of 1.0

M sucrose-5% PVP and centrifuged at 1,000 g for 20 minutes. The

pellets were washed with homogenation medium and centrifuged as ab

above for 3 times. The basic proteins were extracted from the fi-

nal pellet with 0.4 N H_2SO_4 for one hour.

Method of Masui (1968)

100 Xenopus eggs or embryos were homogenized in 10 ml of 0.25

M sucrose solution containing 2.5% ficoll and 1.2 mM $CaCl_2$ and the

homogenate was centrifuged at 1,000 g for 10 minutes to sediment

the bulk of yolk granules and nuclei together with a small amount

of pigment granules. The sediment was resuspended, layered on a

70% sucrose solution containing 0.5mM $CaCl_2$ and centrifuged at

50,000 g for one hour. Nuclei and pigment granules sedimented,

but yolk granules always remained at the interface and were collected

with a pipette. The collected yolk granules were resuspended in

homogenation medium and centrifuged at 10,000 g for 10 minutes.

Basic proteins were extracted from this pellet with 0.4 N H_2SO_4

for one hour.

D. Electrophoresis

Starch Gel Electrophoresis

The basic proteins were electrophoresed on vertical starch gel

slabs at 250 volts (4^oC) in the upwards direction, using the method

of Louie and Dixon (1972) (12x25x0.6 cm., pH 3.4, 4 M urea, 15 hrs).

The gels were bisected and stained by the sensitive procedure of

Sung and Smithies (1969). In this procedure each half of the gel

was placed for 30 minutes in a tray containing 200 ml of 0.125%

amidoblack in 1% acetic acid to which 0.6 ml of 1 M cobalt nitrate

had just been added. The final concentration of cobalt nitrate was

0.003 M. The gel was then destained in 1 N sulfuric acid. Accor-

ding to Sung and Smithies (1969) the sensitivity of the method is

based on the differential behavior of free and protein-bound amido-

black stain. In the presence of Co^{++}, free dye molecules condense

into small micelles and therefore no longer absorb much of the

incident light. On the other hand, protein-bound dye does not con-

dense and continues to absorb the light. They indicate that the

detailed mechanism of this peculiar behavior is not known but can

be discerned from microscopic examination of the gels. The presence

of heavy metal is indispensable in their opinion. However, accor-

ding to Wray and Stubblefield (1970), a similarly sensitive staining

of basic proteins can be accomplished in polyacrylamide disc gel

using amidoblack without cobalt nitrate, provided that the gels were

destained in sulfuric acid rather than the more conventional 7%

acetic acid. These authors therefore feel that the results do not

depend on the presence of heavy metal but rather that free dye is

oxidized to a colorless leuco form in the presence of a strong

chemical oxidizing agent like sulfuric acid, whereas protein-bound

dye is not. Whichever explanation is the correct one for starch

gels, the fact remains that Sung and Smithies (1969) have observed

a hundred-fold increase in sensitivity in the detection of histones

by amidoblack using their staining procedure. We have therefore

utilized the cobalt nitrate-amidoblack-acetic acid mixture to obtain

our starch gel electrophoretograms.

Two-dimensional starch slab gel electrophoresis was based on the aluminum lactate-urea system of Sung and Smithies (1969) in the first-dimension and a modification of the triton-X-100 polyacrylamide gel system of Spiker et al. (1976) in the second dimension. After the first dimensional separation, the histone regions were cut and were placed on the top of the second starch gel for the second-dimensional separation (7 M urea, 0.03 M aluminum lactate, 1% triton X-100). The electrophoresis was carried out in the same manner as in the first dimension run except that the tray buffer contained 1% triton-X-100.

Polyacrylamide Gel Electrophoresis

a) Method of Bonner et al. (1968).

Stock solutions (suitable for 3 months when stored refrigerated in amber glass bottle):

1. TEMED solution: 48 ml of 1N KOH, 17.2 ml of glacial acetic acid, 4 ml of TEMED (N, N, N', N''-tetra-methylenediamine), distilled water up to 100 ml.

2. Acrylamide solution: 60 g of acrylamide, 0.4 g N, N'-methylene-bisacrylamide, distilled water up to 100 ml.

3. 0.2% (w/v) ammonium persulfate: in freshly deionized 10 M aqueous urea solution.

For preparation of 15% acrylamide gels, one part of TEMED solution and two parts of acrylamide solution were added to five parts of persulfate-urea solution. The mixture was then pipetted into each electrophoresis tube (0.6 cm x 6.0 cm). This was carefully overlayered with 3 M urea solution to allow anaerobic polymerization of acrylamide. Polymerization was complete after 30

minutes.

The histone samples, usually saturated with urea, were placed on the top of the gels. The gels were then electrophoresed in new tray buffer (31.2 g of β-alanine, 8 ml of glacial acetic acid, distilled water to 1 liter), at 4-5 ma/gel for 90 minutes at room temperature. After electrophoresis, the gels were stained with 1% amidoblack in 20% (v/v) ethanol, 7% glacial acetic acid, aqueous solution for 20-30 minutes, and were destained in the same solution in charcoal diffusion destainer (Hoeffer Scientific Co.,). For photography, Polaroid black/white film (Type 107) was employed.

b) Method of Panyim and Chalkley (1969b)

Stock solutions

1. 4% TEMED (w/v) in distilled water.

2. 60% acrylamide (w/v) and 0.4% N, N'- methylene-bis-acrylamide in distilled water.

3. 0.2% ammonium persulfate (w/v) in 10 M urea solution freshly prepared.

15% polyacrylamide gel was prepared by mixing three stock solutions in ratios: one part of solution 1, two parts of solution 2 and five parts of solution 3. Electrophoresis was performed at room temperature, 2 ma/gel for 6 hours, using 0.6 x7.5 cm gel tubes. The tray buffer was 0.9 N acetic acid. The gels were stained for 2 hours with 0.1% amidoblack in 20% ethanol, 7% acetic acid and water, then destained in the same solution without amidoblack.

RESULTS

A. Isolation and Characterization of Xenopus Somatic Histones

Histones were extracted from Xenopus heart by different methods and electrophoresed on starch gels and polyacrylamide gels. They showed a characteristic pattern on the gels (Fig.2, 3). The tentative identification of the bands on the polyacrylamide gel in Fig. 2 is consistent with the results of Spiker et al. on pea histones (1976), Fambrough and Bonner on pea histones (1969), Panyim and Chalkley on calf thymus histones (1969) and Felden et al. on calf thymus histones (1976), as shown in Fig. 4A. For starch gels, the tentative identification of individual histones shown in Fig. 3 is consistent with the results of Felden et al. on calf thymus histones (1976), Louie on trout testis histones (1968) and Sung et al. on rat liver histones (1971), as shown in Fig. 4B. Histones from highly differentiated cells such as trout red blood cells were used as a marker since they contain relatively few cytoplasmic organelles which might yield contaminating problems that interfere with analysis of histones. Calf thymus H4 (a gift from Drs. D. Fambrough and J. Bonner), the most evolutionarily conservative histone, was the marker for the fast-moving histones in the somatic region.

It has been reported that very lysine-rich H1 histone could be extracted with 5% perchloric acid (Johns and Butler, 1962), 5% trichloracetic acid (De Nooij and Westerbrink, 1962), or 0.1M citric acid at pH 2.0 (Setterfield and Neelin, 1972). These methods were employed to identify this group of nuclear proteins. After selective extraction with 5% perchloric acid, heart H1

Fig. 2 Electrophoretic profiles of Xenopus heart histones on polyacrylamide disc gels. Histones were prepared by extracting chromatin with 0.4 N H_2SO_4 (Bonner et al., 1968). The chromatin was prepared with the addition of sodium bisulfite to prevent proteolysis of the histones.

A. The gel system of Bonner et al. (15% polyacrylamide gel containing 6.25 M urea, electrophoresed for 90 minutes at 5 ma/gel).

B. The gel system of Panyim and Chalkley (15% polyacrylamide gel containing 6.25 M urea, pH 3.2, electrophoresed for 240 minutes at 2 ma/gel).

In these gels and all subsequent ones, the origin is at the top of the gel and positively charged basic proteins migrate towards the negative pole at the bottom. Channels from the same gel run are grouped together.
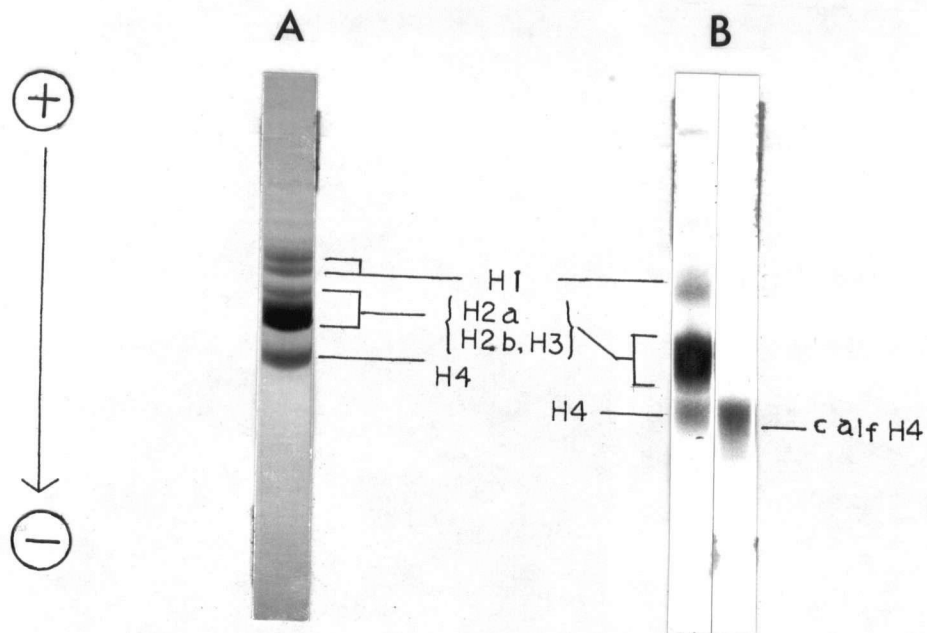
Fig. 3   Electrophoretic profiles of <u>Xenopus</u> histone H1 after selective extraction by 5% perchloric acid (Johns, 1976).

A. Starch gel (containing 4 M urea, 0.02 M aluminum lactate, pH 3.4; electrophoresed for 16 hours at 250 mamp, 250 volts)

    a) Calf thymus histone H4.

    b) <u>Xenopus</u> lung histone H1.

    c) <u>Xenopus</u> heart histone H1.

    d) <u>Xenopus</u> heart histones extracted by 0.4 N H_2SO_4 from chromatin preparation.

    e) Trout red blood cell histones extracted by 0.4 N H_2SO_4 using the method of Neelin <u>et</u> <u>al</u>. (1968) in a separate experiment.

B. Polyacrylamide gel (Bonner <u>et</u> <u>al</u>., 1968)

    a) Heart histone H1.

    b) Heart whole histones.

    c) Calf thymus histone H4.

H4(-AC)= deacetylated histone H4

H3(d)= dimer of histone H3

H3(m)= monomer of histone H3

A

B

H3(d)

H1,H2
H2b,H3(m)

H4

H4(-Ac)

H1

calf H4

H2a
H2b, H3

calf H4

a b c d

e
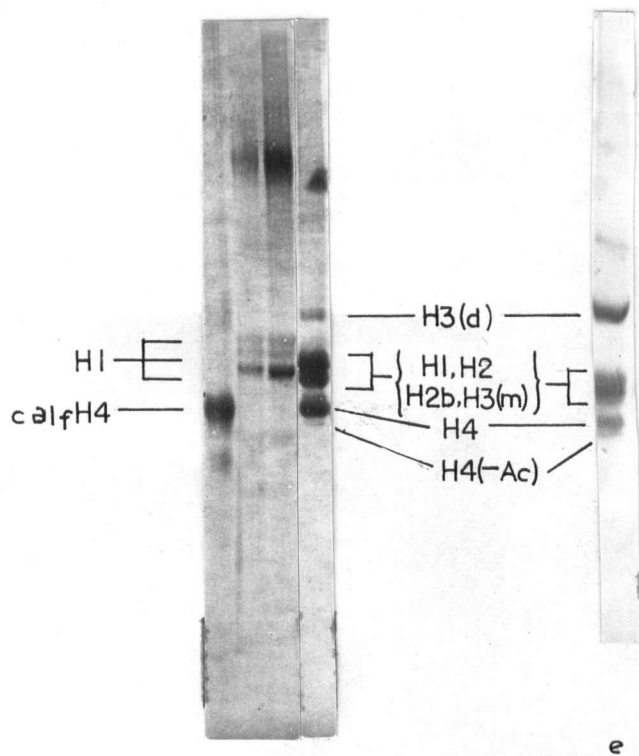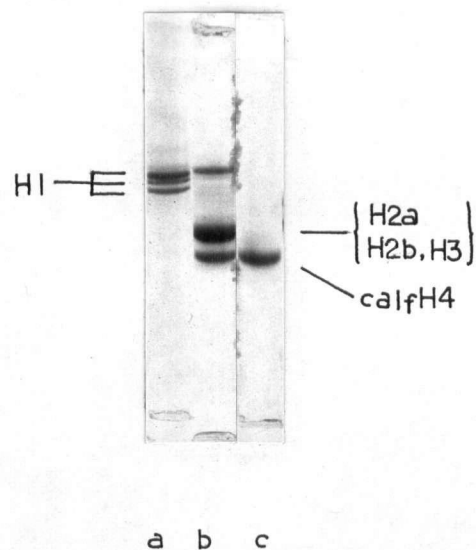
a b c

Fig. 4   Diagram of electrophoretic comparison of _Xenopus_ heart histones with literature data using both polyacrylamide and starch gels.

A. Polyacrylamide gel electrophoresis

   a) Pea whole histones on the gel system of Panyim and Chalkley (1969a). Data of Spiker and Wakim (1976).

   b) Pea-bud histones on the gel system of Bonner et al. (1968) Data of Fambrough and Bonner (1969).

   c) Calf thymus histones on the gel system of Panyim and Chalkley (1969a). Data of Felden et al. (1976).

   d) Same. Data of Panyim and Chalkley (1969a).

   e) _Xenopus_ heart histones (extracted in our laboratory) on the gel system of Bonner et al. (1968). Experimental data.

B. Starch gel electrophoresis

   a) Calf thymus histones. Data of Felden et al. (1976).

   b) Somatic histone region of trout testis. Data of Louie (1968) H2a (+p) = phosphorylated histone H2a.

   c) Rat liver histones. Data of Sung et al. (1971). Electrophoresis runs a-c were performed on the gel system of Sung and Smithies (1969).

   d) _Xenopus_ heart histones (extracted in our laboratory) on the gel system of Sung and Smithies (1969). Experimental data.
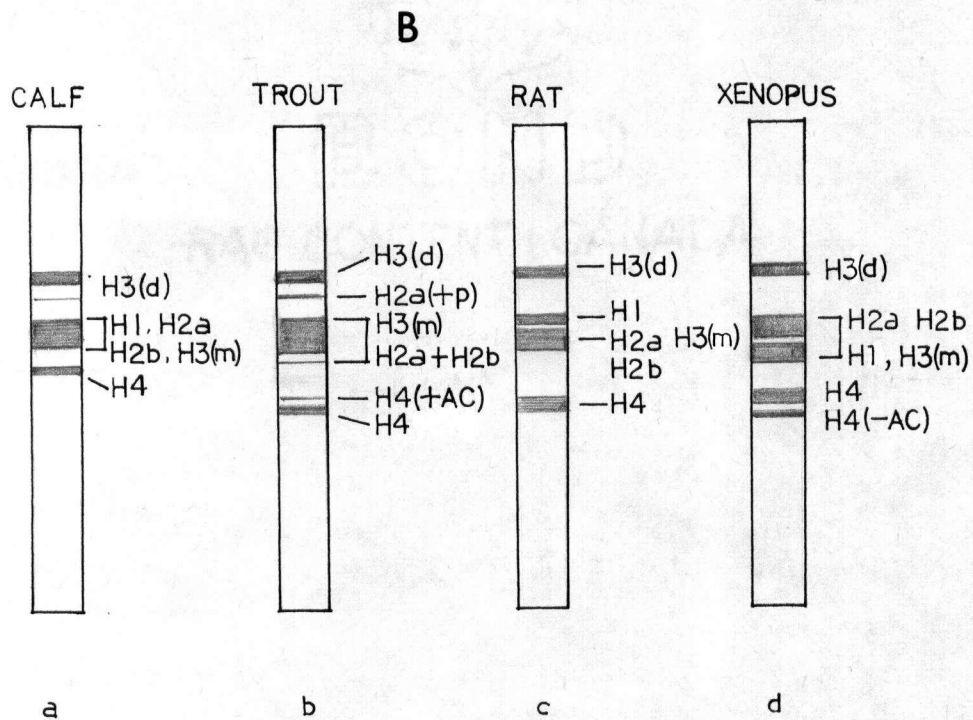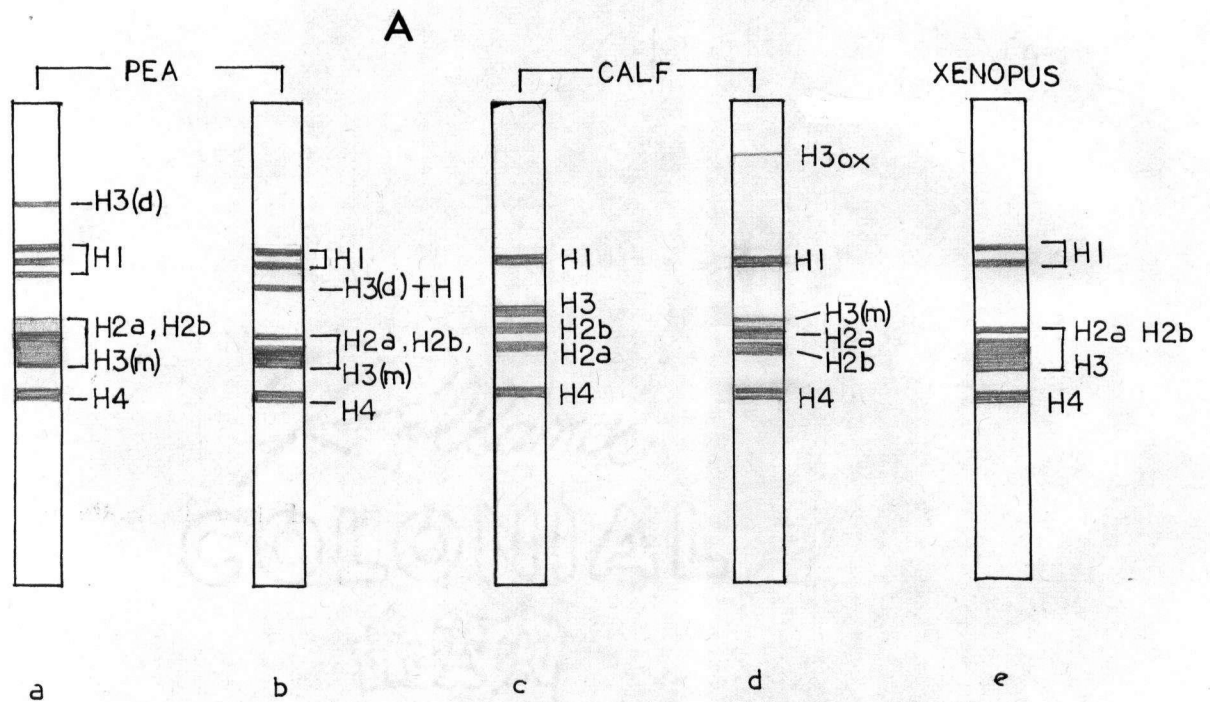
histones appeared as three bands on both starch gel (Fig. 3A, channel C) and polyacrylamide gel (Fig. 3B, channel a), while only one band was observed when whole histones were prepared with $0.4N$ H_2SO_4. This might have been due to the preferential extraction of H1 histones by 5% TCA. In whole histone preparations, it appeared that H1 separated more readily from bulk histones on polyacrylamide gel (Fig. 3B, channel b) than on starch gel (Fig. 3A, channel d).

In most organisms, histone H3 contains one or more cysteine residues and tends to form aggregates that move more slowly than H3 monomer upon electrophoresis (Panyim et al., 1970; 1971a; 1971b). Xenopus and Rana histone H3 contains one cysteine (Byrd and Kasinsky, 1973; and Panyim et al., 1970) and readily forms dimers. The band with lowest mobility in the somatic histone region on starch gels (Fig. 3, channel d) in our preparations is likely to be the dimer of histone H3. This is confirmed in a comparison with literature data (Fig. 4B). The mobility of histone H3 dimer on polyacrylamide gels as compared to that of histone H1 varies, as we can see that H3 dimer of pea moves slower than H1 on the gel system of Panyim and Chalkley (1969a) (Fig. 4A, channel a) while it migrates faster on the gel system of Bonner et al. (1968) (Fig. 4A, channel b). Presumably histone H3 in either the dimer or monomer form in my preparation should migrate between H1 and H4 since the gel system of Bonner et al. (1968) was employed (Fig. 3A, channel e). Histone H4 was identified by rapid mobility, co-electrophoresis with marker calf thymus H4 and amino acid analysis (as indicated in Part II of this thesis). A band moving slightly

faster than histone H4 was often observed both in trout blood cell histones (Fig. 3A, channel e) and in _Xenopus_ heart histones (Fig. 3A, 5A). Since red blood cell histones are less likely to be contaminated by basic ribosomal proteins this extra band could be the deacetylated form of histone H4 (Adamson and Woodland, 1974; Panyim and Chalkley, 1969a; Pogo _et al._, 1968).

Different histone extraction methods were employed in the hope of discovering a relatively simple one for isolating histones from a small amount of somatic tissues. A method was devised by Dr. E. W. Byrd, Jr. (1974) that involves preparing nuclei and acid-extracting them to obtain histones. This differs from the methods of Bonner _et al._ (1968) and Destrée _et al._ (1972), which rely more heavily on chromatin preparations from which the histones are acid-extracted. As shown in Fig. 5A, heart histones isolated from fresh organs either by nuclei preparation or chromatin extraction appear to yield similar results. This finding was further confirmed by using stored organs. It was found, however, that histones extracted from organs stored at -70°C showed proteolytic degradation (Fig. 5C). Of the three different preparations employed, it appears that Byrd's method yielded the least degraded histone preparation from stored organs (Fig. 5B, channels c and d). This was probably due to the fact that after thawing, proteolytic enzymes were released. Less proteolysis should have taken place if the extraction procedure had been performed more rapidly. In Byrd's method the entire extraction of histones took place in about two hours. This is less than half the time required for the other procedures.

Fig. 5   Starch gel electrophoresis of <u>Xenopus</u> heart histones from fresh and stored organs.

A. Fresh organs

 a) Modified method of Bonner <u>et</u> <u>al</u>. (1968).

 b) Byrd's method (1974).

B. Stored organs (-70°C, 1 month)

 a) Method of Destrèe <u>et</u> <u>al</u>. (1972)

 b) Modified method of Bonner <u>et</u> <u>al</u>.(1968)

 c) Byrd's method. (1974).

 d) Repeat.

C. Comparison of heart histones prepared from fresh and stored organs by modified method of Bonner <u>et</u> <u>al</u>. (1968)

 a) Fresh organs.

 b) Stored organs.

SH= somatic histones

The dotted arrows correspond to products of proteolytic degradation.
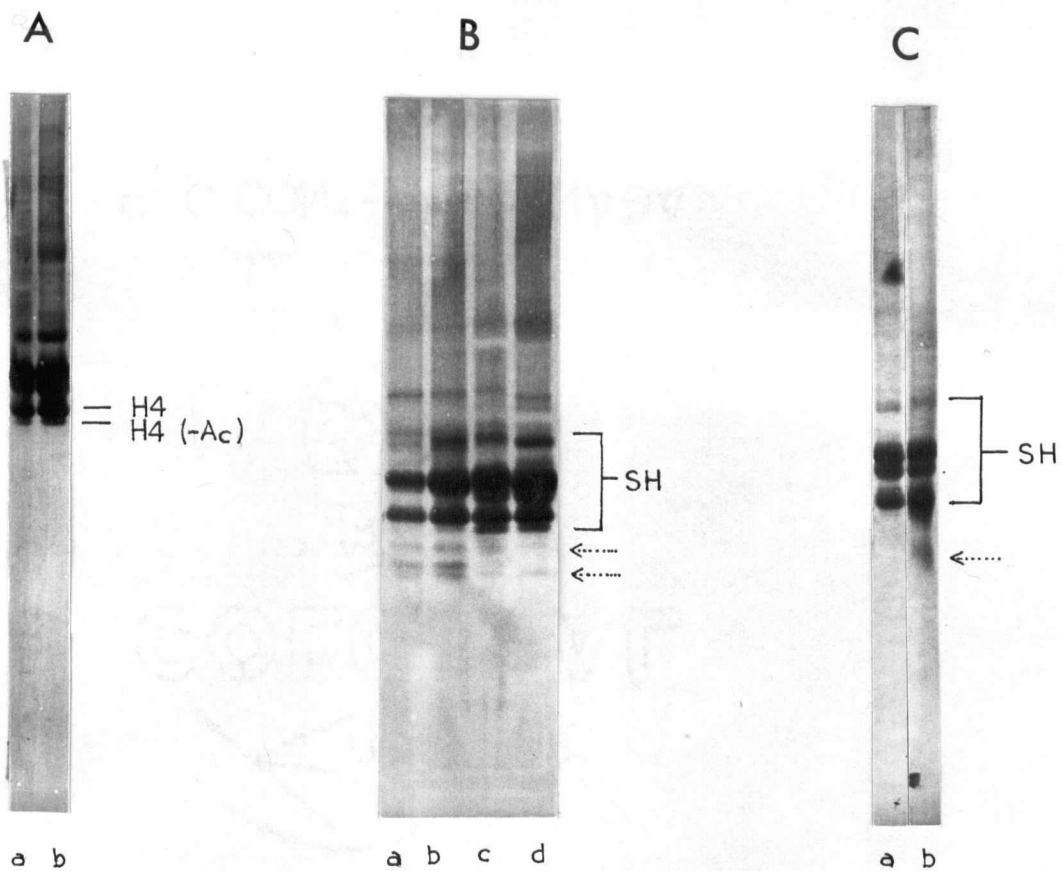
Two-dimensional gel electrophoresis has been attempted on polyacrylamide gels with the addition of triton-X-100 (Alfageme et al., 1974 and Spiker et al., 1976) in the hope of obtaining better resolution of histones. We applied this method to histone separation on starch gels. In Fig. 6 we see the effect of 1% triton-X-100 treatment on the separation. The bulky doublet bands (H1 + H2a + H2b + H3 reduced) split into three spots in the second dimension in the presence of triton-X-100 (Fig. 6B), while the doublet bands still remained as two spots without detergent treatment (Fig. 6A). Separation on triton-X-100 - containing gels is based on the hydrophobic character of the histones rather than charge properties. According to Spiker et al. (1976) the order of calf thymus histone mobility in triton-containing polyacrylamide gels was H1 > H2b > H4 > H3 > H2a. This does not seem to be the case for starch gel where the mobility of histone H4 is still greater than histone H1 in the presence of detergent. More work has to be done on the ident-ification of the three spots in Fig. 6B. In addition, the experimental conditions have to be tested in order to obtain as satisfactory resolution on two dimensional starch gel as on polyacrylamide gels.

B. Isolation and Characterization of Testis-Specific Histones
    in Amphibians and Reptiles

Different methods were employed to isolate testicular histones from Xenopus laevis. In Fig. 7, the electrophoretograms showed that Xenopus testis contained two fast-moving bands, X_s and X_f, on both starch gel (Fig. 5A) and polyacrylamide gel (Fig.

Fig. 6   Two-dimensional starch gel electrophoretogram of <u>Xenopus</u> heart histones.

A. The first dimension (left to right) is an aluminum lactate-urea gel system (4 M urea, 0.02 M aluminum lactate, pH 3.4). The second dimension (top to bottom) is a similar gel system (7 M urea, 0.03 M aluminum lactate, pH 2.7).

B. The first dimension (right to left) and the second dimension are the same as those in A except that in the second dimension run 1% triton-X-100 is added.

For reference, a diagram of each of the two gel channels of the first dimension is pictured above the photograph and a diagram of the two-dimensional gel is shown below.
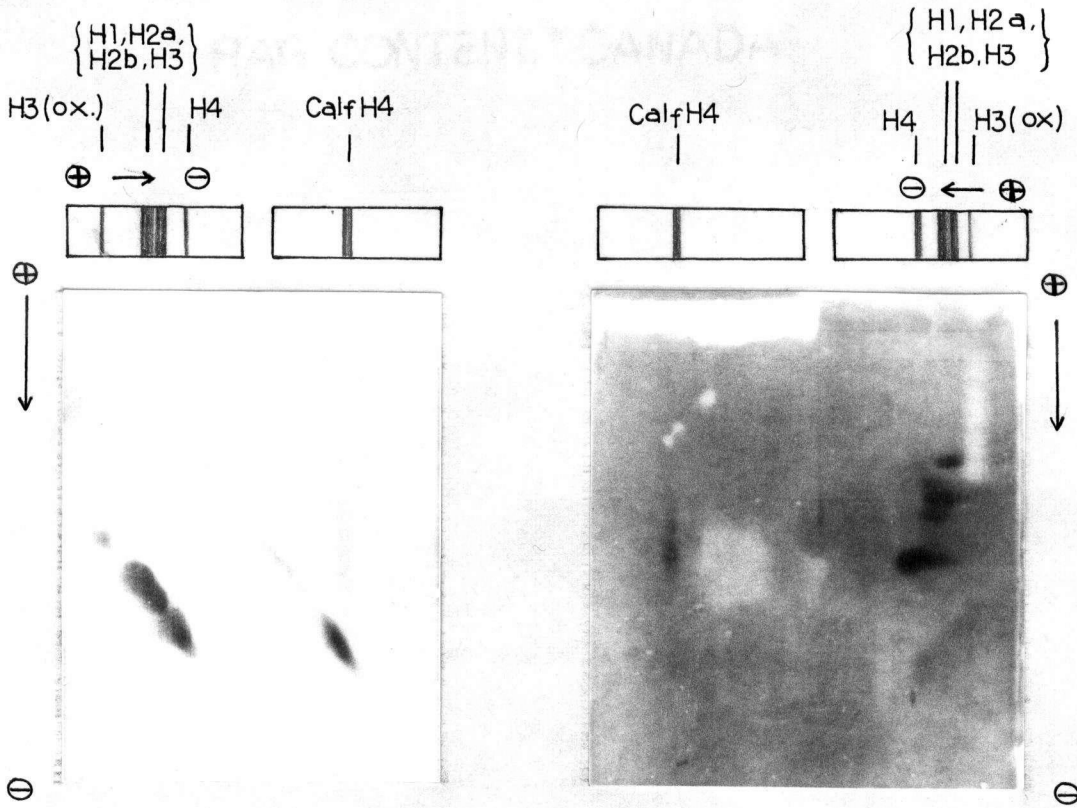
A

B
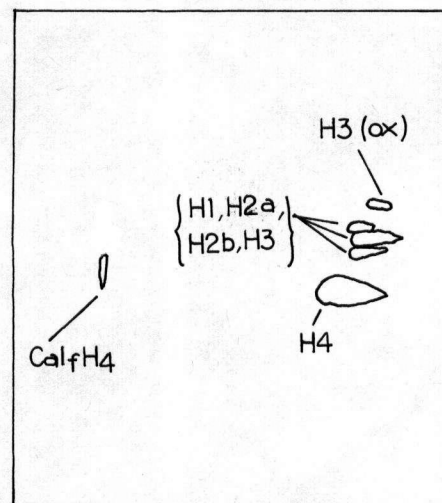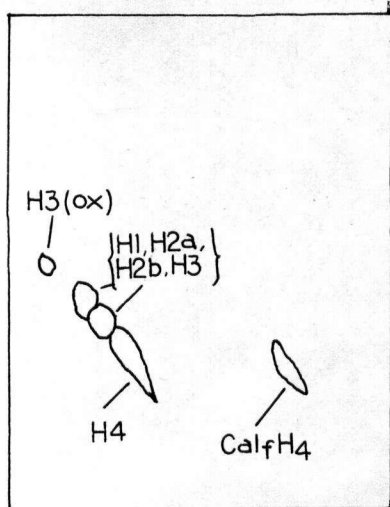
+ Triton-X-100

7B). Comparing the different extraction procedures used, it appears that the testicular cell suspension is as good as more careful purification procedures in characterizing the testis-specific histones of Xenopus. Because it is a much simpler procedure requiring only small amounts of material, a single testis from one small animal being sufficient, we have been able to examine the testis-specific histones from several species amongst amphibians and reptiles. The comparison of amphibian testicular histones is shown in Fig. 8. Sigma protamine prepared from herring was used as a marker (channel g). Testis-specific histones of three anuran species (channels b, d and f) and urodeles species (channel c) showed marked diversity upon polyacrylamide gel electrophoretogram. Rana had only somatic histones (channel b); Xenopus had two bands moving more rapidly than H4 (channels d and e); Bufo showed a single band that moved somewhat faster than Sigma protamine (channel f); Cynops, the Japanese fire-belly newt, also showed a single band that moved to the region between Xenopus X_f and Sigma protamine (channel c).

The testis-specific histones of reptiles also have been examined. As indicated in Fig. 9, Anolis (channel c); Terrapene, the Florida box turtle (channel f) and Sceloporous, the desert spiny lizard (channel h), each had a single band migrating in the region of the gel close to Sigma protamine (channels a, e and g), as did the ductus-deferens-specific histone of Thamnophis (channel b) and the semen-specific histone of Elaphe (channel d).

Amphibian testis-specific histones were also characterized by starch gel electrophoresis (Fig. 10). Rana showed no bands

Fig. 7   <u>Xenopus</u> testis-specific histones extracted by different

methods.

A. Starch gel electrophoretogram

    a,b) Cell suspension.

    c) Byrd's method (1974).

    d) Modified method of Bonner <u>et</u> <u>al</u>. (1968).

    e) Method of Destrée <u>et</u> <u>al</u>. (1972).

    f) Xenopus heart histones from chromatin preparation (modified

       method of Bonner <u>et</u> <u>al</u>. (1968).

B. Polyacrylamide gels (Bonner <u>et</u> <u>al</u>., 1968)

    a) Modified method of Bonner <u>et</u> <u>al</u>. (1968).

    b) Byrd's method (1974).

    c) Cell suspension.

    d) Repeat.

    e) Calf thymus histone H4.

    f) Xenopus heart histones extracted from cell suspension.

X_s and X_f = slow and fast testis-specific <u>Xenopus</u> histones.

A

B
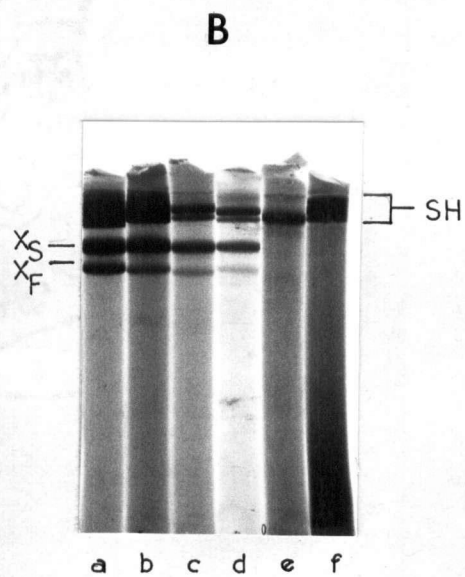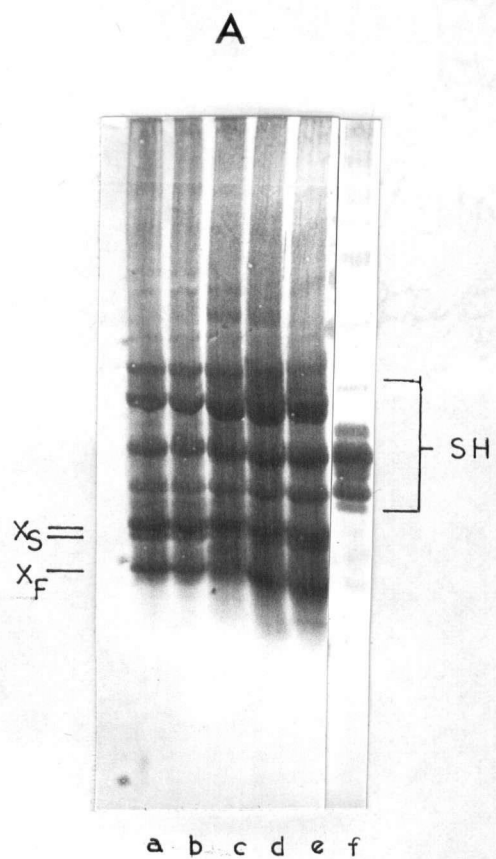
Fig. 8   Electrophoretic comparison of testis-specific histones

from amphibian cell suspension on polyacrylamide gels. Gel system

of Bonner et al. (1968) was employed.

    a) Calf thymus H4.

    b) Rana pipiens (frog) testis.

    c) Cynops pyrrhogaster (newt) testis.

    d) Xenopus laevis (toad) testis.

    e) Xenopus laevis (toad) testis.

    f) Bufo marinus (toad) testis.

    g) Sigma protamine (herring).

P= Sigma protamine prepared from herring.
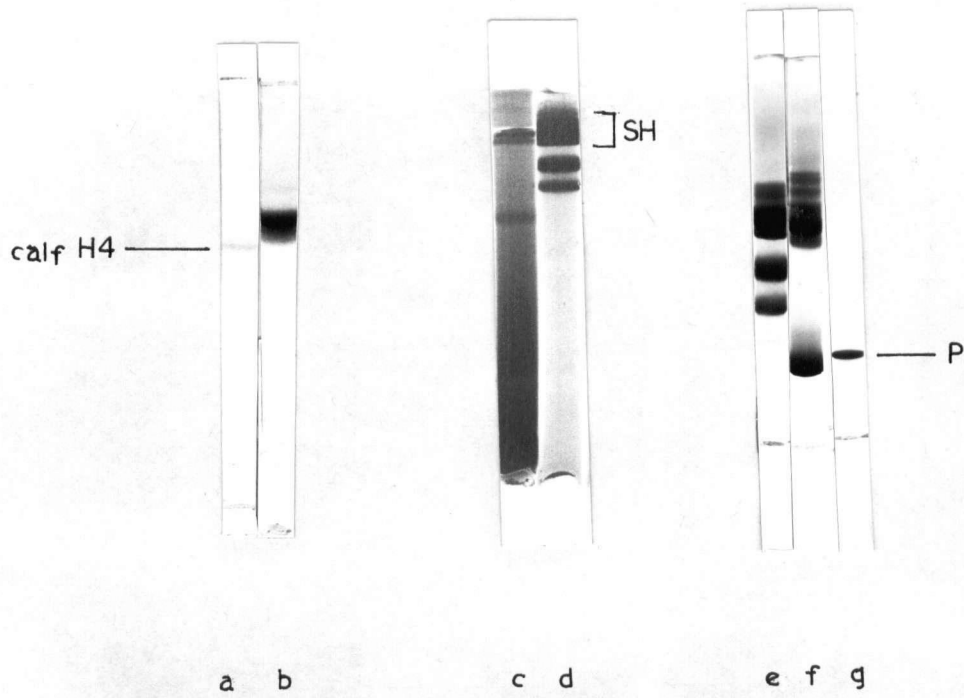
Fig. 9. Electrophoretic comparison of testis-specific histones from reptilian cell suspension on polyacrylamide gels. The gel system of Bonner et al. (1968) was employed.

a) Sigma protamine (herring).

b) Thamnophis Sirtalis (snake ductus deferens.

c) Anolis carolinensis (lizard) testis.

d) Elaphe guttata guttata (snake) semen.

e) Sigma protamine (herring).

f) Terrapene corolina bauri (turtle) testis.

g) Sigma protamine (herring).

h) Sceloporous magister (lizard) testis.

i) Anolis carolinensis (lizard) testis.
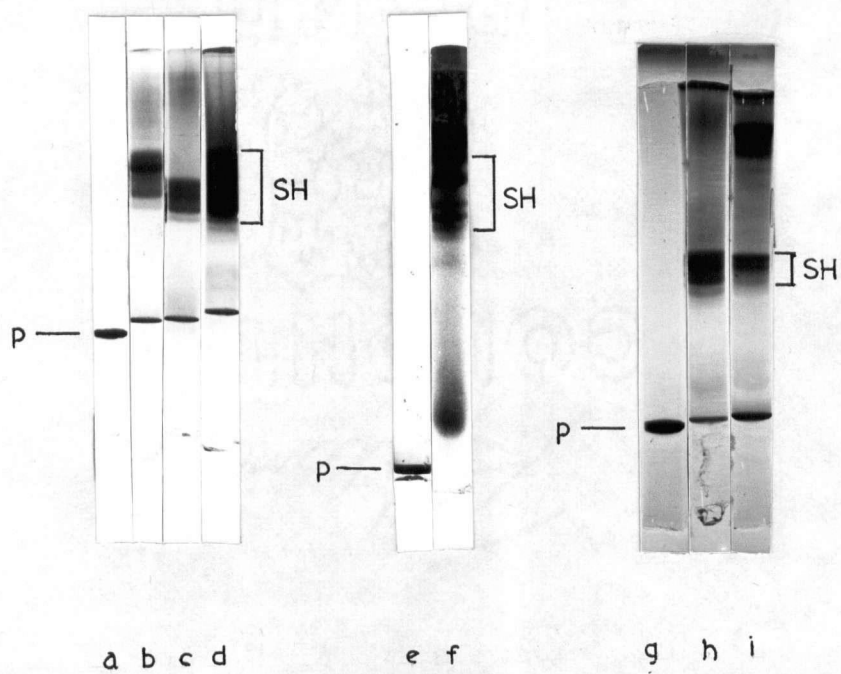
Fig. 10   Electrophoretic comparison of testis-specific histones from amphibian cell suspensions on starch gels.

    a)   <u>Cynops pyrrhogaster</u> (newt).

    b)   <u>Xenopus</u> <u>laevis</u> (toad).

    c)   Calf thymus H4.

    d)   <u>Rana</u> <u>pipiens</u> (frog).

Cp = newt testis-specific histones.
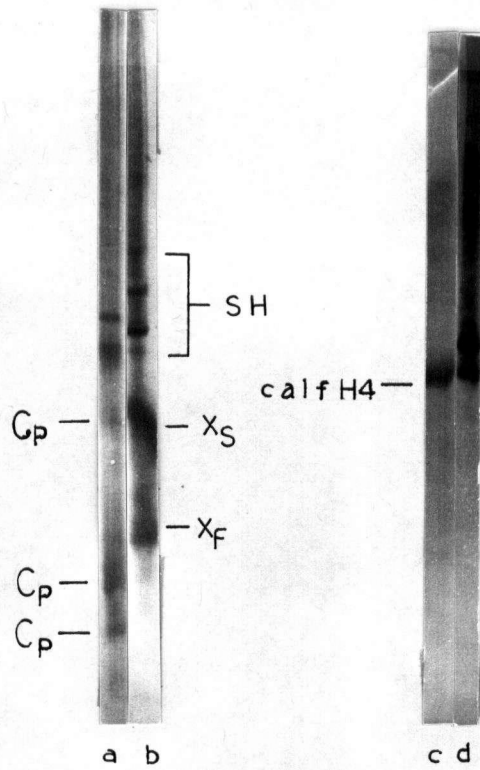
moving faster than H4 (channel d); <u>Xenopus</u> had two faster-moving

bands (channel b). Those were the same as bands on polyacrylamide

gels. Instead of showing only one band as on polyacrylamide gel

(Fig. 8, channel c), a testis cell suspension from <u>Cynops</u> showed

a doublet moving more rapidly than X_f and a third band migrating

in the region of X_s. (Fig. 10, channel a). A variety of banding

patterns due to different gel systems employed was also observed

by Bols <u>et</u> <u>al</u>. (1976). They found that the testis-specific

histones from Notophthalmus, the eastern red spotted newt, showed

one band on the polyacrylamide gel system of Panyim and Chalkley

(1969), but 3 bands on the polyacrylamide gel system of Bonner <u>et</u>

<u>al</u>. (1968), and the starch gel system of Sung and Smithies (1969).

It seems that starch gel yielded greater separation than did

polyacrylamide gel for testis-specific histones when the sample

was relatively unpurified. This phenomenon could also be observed

for Sigma protamine extracted from herring (Fig. 19). However,

the urodele bands usually were more diffuse on starch than on

polyacrylamide gels (Kasinsky, Byrd, Kwauk and Yee, unpublished

data).

C. <u>Isolation and Characterization of Xenopus Embryonic Histones</u>

Histones isolated from <u>Xenopus</u> swimming tadpoles (stage 50)

showed the same electrophoretic pattern as did those from adult

heart upon starch gel electrophoresis (Fig. 11). Whether the his-

tones were obtained by Byrd's (1974) nuclear preparation (channel

b) or by the chromatin preparation of Destrée <u>et</u> <u>al</u>. (1972).

(channel a), they showed adequate resolution. However, for early

embryos before stage 40, Byrd's method was not applicable because

of the presence of a large amount of yolk proteins as well as

Fig. 11   Starch gel electrophoretic profiles of histones from

Xenopus swimming tadpoles (stage 50) (Nieuwkoop and Faber,1967).

    a) Method of Destrèe et al. (1972).

    b) Byrd's method (10 tadpoles).

    c) Calf thymus H4.
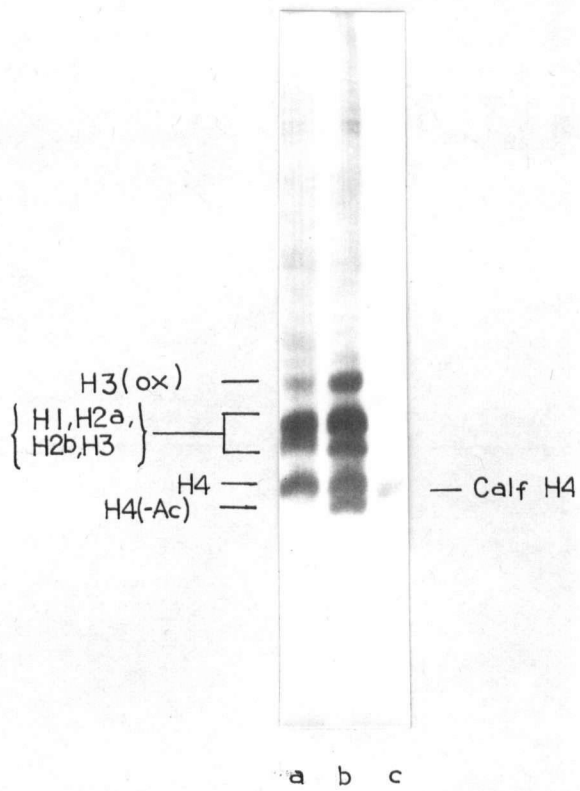
newly-made ribosomal proteins (Hallberg and Brown, 1973). Therefore, the method of Destrée et al. (1972) was employed to extract histones from Xenopus gastrula and neurula. In the method of Destrée et al. (1972), 2.4 M sucrose centrifugation was capable of separating the bulk of the yolk granules and cytoplasmic organelles from the nuclei, which were pelleted by centrifugation. Yet yolk granules in the early embryos were not completely removed. This possible contamination makes the histone banding pattern of the early embryos difficult to characterize. However, the method of Destrée et al. (1972) appears to be the best method known so far. The other advantage of using this method, combined with starch gel electrophoresis, is that only a small number of embryos (ca. 150) are required to obtain clear resolution.

The banding patterns were essentially similar for Xenopus gastrula, neurula and swimming tadpoles (Fig. 12). In order to completely characterize histones from early embryos, amino acid analysis of the bands will be required. The lysine-rich histone H1 from tadpoles also showed three bands (Fig. 12, channel g) that were similar to those of somatic histone H1 (Fig. 3A, channels b and c). Histones extracted with 0.1 M citric acid at pH 2.0 showed two bands on starch electrophoretogram (channel i), possibly lysine-rich histones H1, H2a+H2b. Since the order of release of histone fractions from DNA is a function of pH and involves the breakdown of electrostatic linkages between histone molecules and DNA, histone H1 and H2a+H2b were more readily extracted than H3 and H4 at pH 2.0 (Murray, 1966). It is assumed that the first band was H1 and the second band was H2a+H2b when channels e,g and i are compared.

Because histones from early embryos could be assayed by starch

Fig. 12.   Electrophoretic profiles of Xenopus embryonic histones

on starch gels.

   a) Gastrula (stage 9), method of Destrèe et al. (1972). (100-

      150 embryos).

   b) Neurula (stage 13), method of Destrèe et al. (1972). (100-

      150 embryos).

   c) Swimming tadpoles (stage 50), Byrd's (1974) method (10 embryos).

   d) Xenopus heart histones from chromatin, method of modified

      method of Bonner et al. (1968).

   e) Swimming tadpoles (stage 50), Byrd's (1974) method.

   f) Gastrula (stage 9), method of Destrèe et al. (1972).

   g) Swimming tadpoles (stage 50), 5% perchloric acid extraction.

   h) Swimming tadpoles (stage 50).

   i) Lysine-rich histones selectively extracted with 0.1 M citric

      acid at pH 2.0. (Setterfield and Neelin, 1972)

   j) Calf thymus H4.

H3(ox)

H4
H4(-Ac)

H3(ox.)

H1
H2a, H2b

H4
H4(-Ac)

a b   c d   e f g   h i j
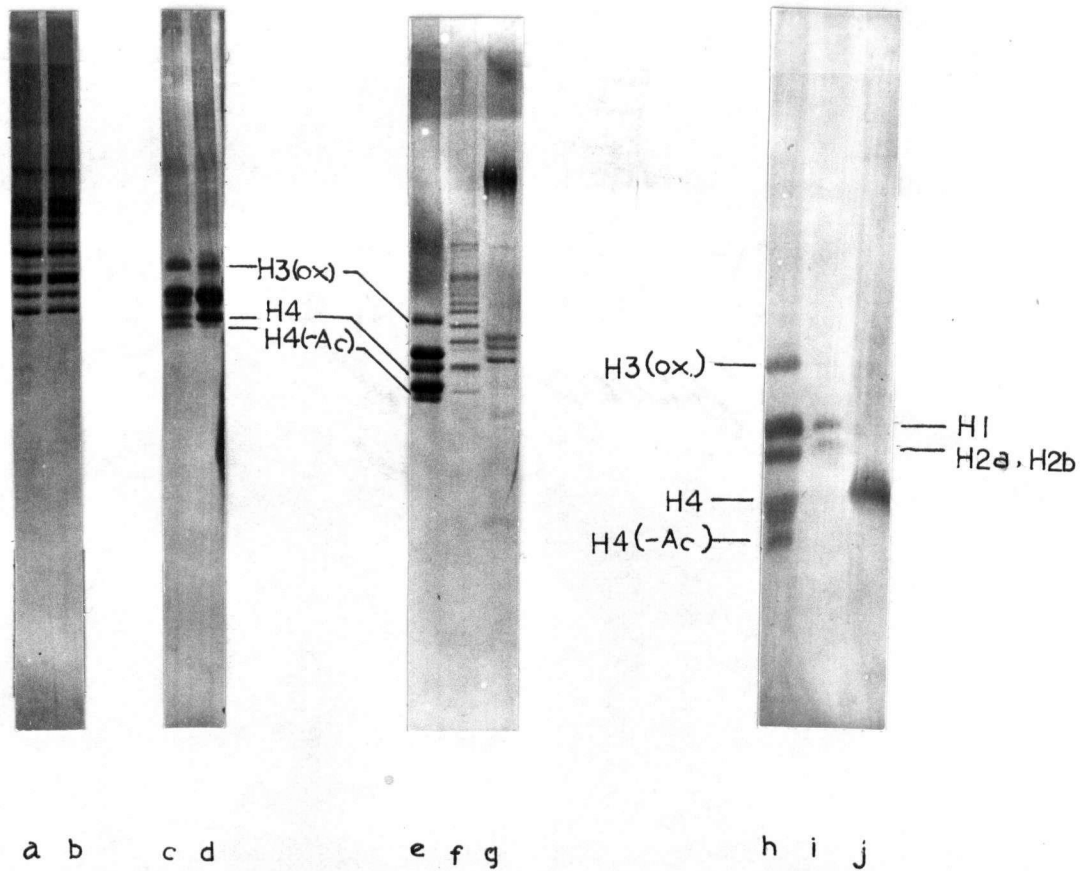
gel electrophoresis using small numbers of animals using the method of Destrée et al. (1972), a study of histone changes during Xenopus embryogenesis was undertaken. The results (Fig. 13.) indicated the electrophoretic profiles of histones remained the same during early embryogenesis, as previously noted by Destrée et al. (1973) using polyacrylamide gel electrophoresis. The bands slower than histone H3 (oxidized form) and the band moving between H3 and the rest of the somatic histones were probably yolk proteins (Fig. 13C). These bands disappeared after the embryos started to feed (stage 41) when the yolk pool was depleted.

In order to check the possible contamination of embryonic histones by ribosomal and yolk basic proteins, the latter two classes of proteins were isolated and characterized electrophoretically on starch and polyacrylamide gels. Yolk basic proteins were extracted from yolk granules by two methods. Masui's method (1968) could not be repeated because of the substantial contamination of yolk granules by nuclei. It has been reported that Ca^{++}, Na^+, etc, would cause the breakdown and the extensive aggregation of yolk granules (Greenhouse and Morrisey, 1974; Essner, 1954). Therefore, a simple sucrose solution containing 5% polyvinyl-pyrrolidone (PVP) without cations was used. From Fig. 14, it appears that the major yolk basic proteins ran slower than somatic histones on starch gel with one band running between H3 (oxidized form) and H1+H2+H3(m) (Fig. 14B). A similar result has been obtained upon polyacrylamide gel electrophoresis (Fig. 14A).

In Fig. 15, we see that ribosomal proteins overlapped the histones in some regions of the gel. Because the ribosomes were

Fig. 13   Electrophoretic profiles of <u>Xenopus</u> embryonic histones on starch gels during embryogenesis. Histones were extracted according to the method of Destrée <u>et</u> <u>al</u>. (1972).

A. a)   Calf thymus histone H4.

  b-d)   Stages 33-34.

  e-h)   Stages 8-11.

    i)   Stages 1-4.

B. a)   Calf thymus H4.

    b)   Swimming tadpoles after stage 45.

  c-d)   Stage 40

  e-f)   Stages 22-26.

  g-h)   Stage 13.

  i-j)   Stages 10½-11.

C. Diagram of <u>Xenopus</u> histones during embryogenesis.

    a)   Calf thymus H4.

    b)   Swimming tadpoles after stage 45.

    c)   Stages 33-34.

    d)   Stages 22-26.

    e)   Stages 13-15.

    f)   Stages 9-10½.

    g)   Stages 1-4.

About 100 embryos were used in each channel. The location of yolk basic proteins is indicated in C.

A

B

calf H4 —

calf —
H4

a b c d c d e f g h i j

a b c d e f g h i

C

H3 (OX) —

{H1, H2a, H2b, H3} —

H4 —

H4 {-Ac} —

Yolk
basic
Protein

a b c d e f g
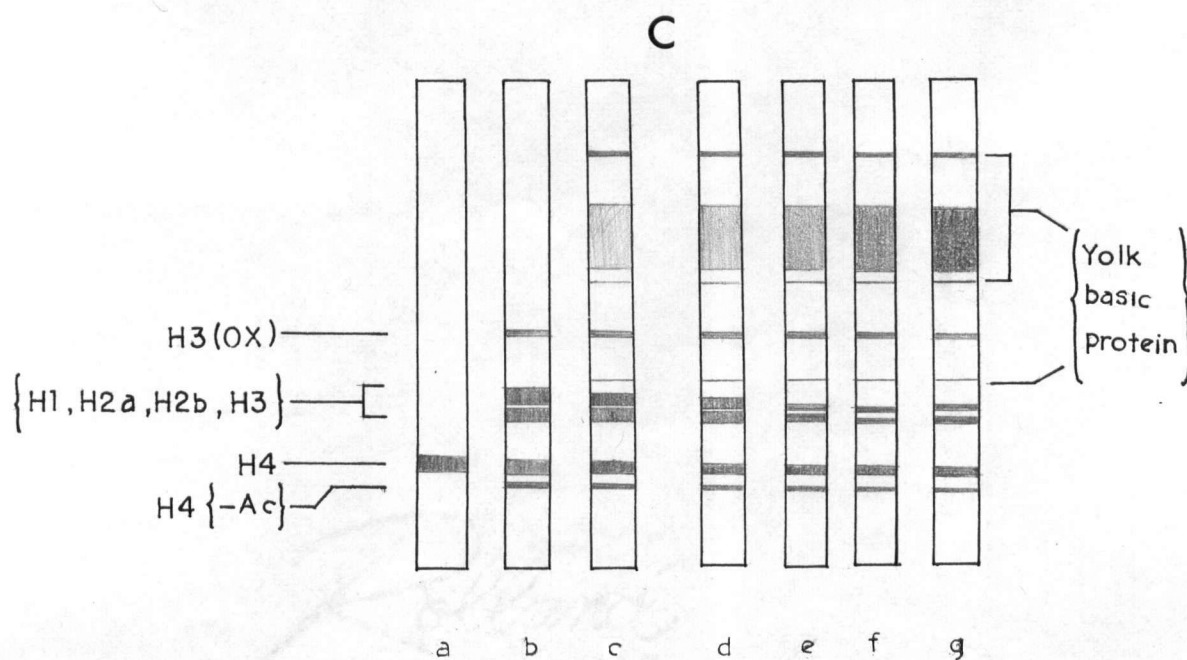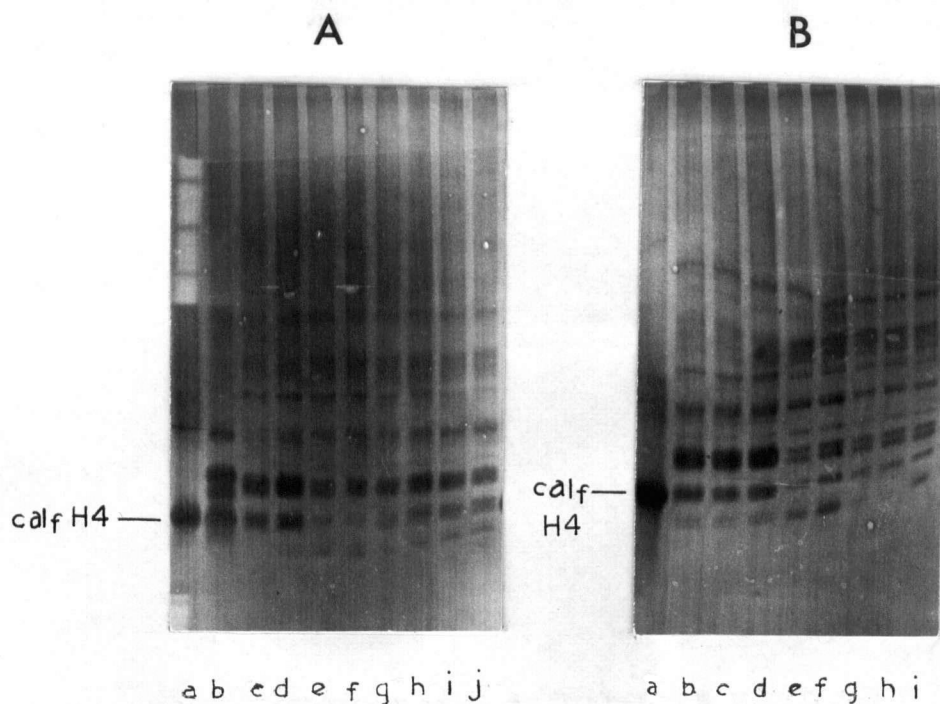
Fig. 14   Electrophoretic comparison of <u>Xenopus</u> embryonic histones
and yolk basic proteins.

A. Polyacrylamide gel (Bonner <u>et</u> <u>al</u>., 1968)

    a) Swimming tadpole histones (stage 50).

    b) Early embryo (fertilization to stage 12) yolk basic proteins
       (solid lines)

B. Starch gel (Sung and Smithies, 1969)

    a) Tadpole histones.

    b) Early embryo yolk basic proteins. (solid lines).
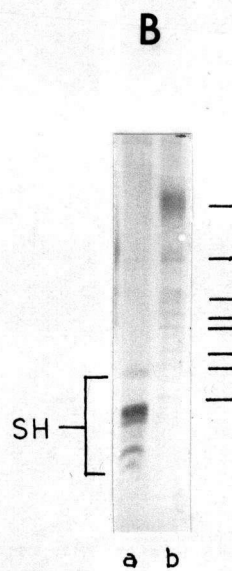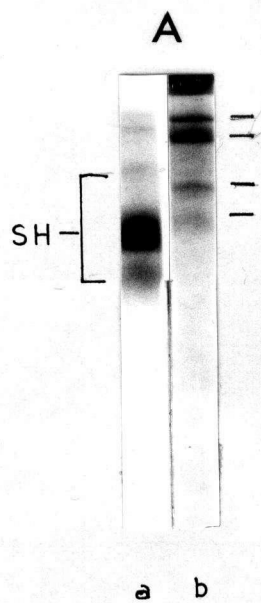
Fig. 15   Electrophoretic comparison of <u>Xenopus</u> embryonic

histones and ribosomal basic proteins.

A. Starch gel (Sung and Smithies, 1969)

    a) Swimming tadpole histones.

    b) Gastrula ribosomal basic proteins extracted without 0.5%

       sodium deoxycholate.

    c) Gastrula ribosomal basic proteins extracted in the presence

       of 0.5% sodium deoxycholate.

B. Polyacrylamide gel (Panyim and Chalkley, 1969)

    a) Gastrula ribosomal basic proteins.

    b) Swimming tadpole histones.

    c) Calf thymus histone H4.

The dotted arrow indicates an artifact due to a loose piece of gel.
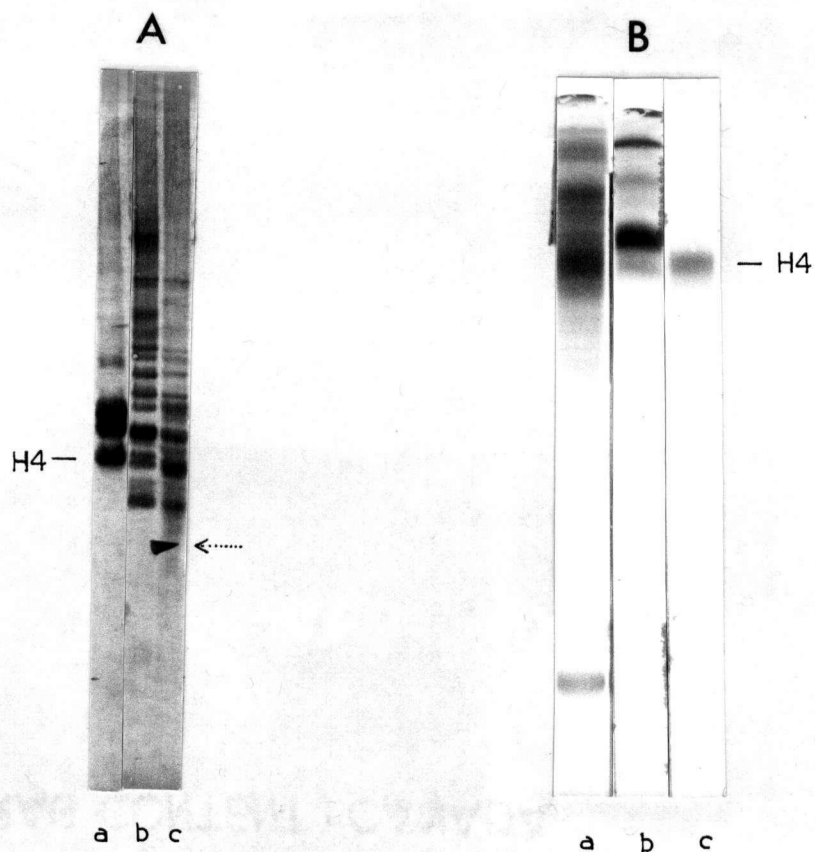
spun down from a post-nuclear supernatant which was centrifuged at high speed twice, it was unlikely that they were contaminated by nuclei. In addition, ribosomal basic proteins showed bands moving faster than those of the somatic histones. Whether these faster bands represented degraded proteins is not known at this time. However, this result seems to be consistent with the results of Neelin and Vidali (1968) on starch gel and of Felden et al. (1976) on polyacrylamide gel in that both show overlapping and fast moving bands for ribosomal proteins in birds and fungi. Histones extracted from aspermatogenic testis cell suspensions showed no bands moving faster than histone H4 upon electrophoresis (Fig. 16) while those from spermatogenic testis showed fast-moving bands (Fig. 8c, Fig. 9c). This observation supports the view that the fast moving testis-specific bands appearing upon electrophoresis are not due to possible contamination of testicular histones by ribosomal basic proteins. Otherwise, one would expect the same pattern in both aspermatogenic and spermatogenic testes.

Fig. 16   Electrophoretic profiles of aspermatogenic testis cell suspensions from lizard and newt.

a) Sigma protamine (herring)

b) Anolis carolinensis (lizard) testis, no sperm observed by light microscopy.

c) Cynops pyrrhogaster (newt) testis, no sperm observed by light microscopy.

d) Sigma protamine (herring)

a   b   c d
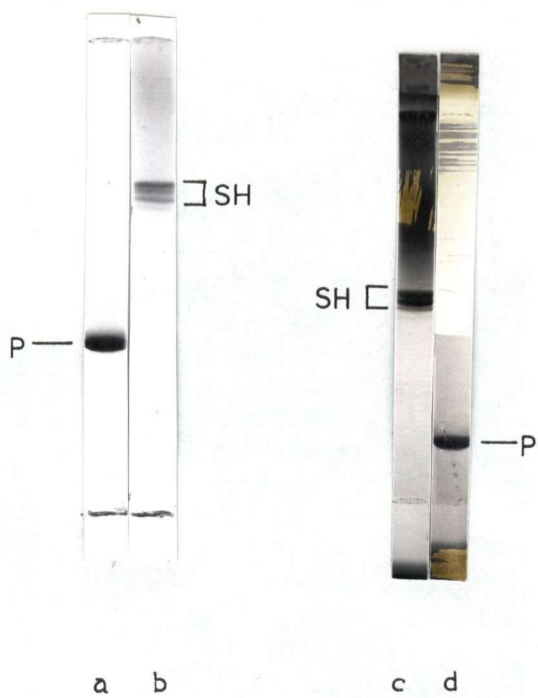
DISCUSSION

Xenopus heart histones have been extracted and characterized

on both starch gels and polyacrylamide gels. The order of mobility

of the five histone fractions are H3 (dimer) > H1 + H2a + H2b + H3

(monomer) > H4 on starch gel and H1 > H2a + H2b + H3 (d,m) > H4 on

polyacrylamide gel. The tentative identification of the banding

patterns on gels needs further confirmation by amino acid analysis.

Different methods of histone isolation have been tried in order

to find a simple method to ensure the minimal loss and degradation

of material during the preparation procedures from a small amount

of material. The method of Bonner et al. (1968) involves a "dire-

ct" chromatin preparation and that of Destrèe et al. (1972) invo-

lves an "indirect" chromatin preparation from isolated nuclei.

It has been reported (Furlan et al., Garrels, et al.,1972) that

there are nuclear histone proteases in calf thymus nuclei, chromatin

and rat liver chromatin. Destrèe et al. (1975) and Heinrich et al.

(1976) observed the subcellular distribution of histone-degrading

enzyme activities from Xenopus liver and rat liver. These facts

suggest that the time-consuming chromatin preparations, either

prepared directly or indirectly from isolated nuclei, are likely

to be accessible to proteolytic degradation. Histones extracted

from a nuclear preparation (suitable for heart) and from a cell

suspension (suitable for testis) seem to undergo little proteo-

lysis, if any, as revealed by our experimental results. For em-

bryonic histones, because of the serious cytoplasmic contamination

(Destrèe et al., 1972; Asao et al., 1969), the chromatin prepara-

tion from isolated nuclei using the method of Destrèe et al. (1972) is required.

Electrophoretic characterization indicates the existence of microheterogeneity in histone H1. This is consistent with the general agreement that there are subfractions separable by electrophoretic and chromatographic means within this histone class (Stellwagen and Cole, 1969; Sherod et al., 1974). Different Xenopus tissues, including lung and heart have been examined and each one shows all the H1 histone bands. This reveals no tissue specifity, although Panyim et al. (1971) have suggested that the relative intensity of different H1 bands might be tissue-dependent. It is not known whether the microheterogeneity is caused by such factors as phosphorylation or acetylation of the same molecule or by molecules with slightly different sequences. A contribution from all three possibilities is likely. Swimming tadpole histone H1 also shows three bands upon starch gel electrophoresis. This is consistent with the previous observation of synthesis of three H1 histones in Xenopus swimming tadpole as revealed by chromatography on amberlite (Byrd and Kasinsky, 1973). It appears that the microheterogeneity of histone H1 in adult tissues seems to have its origin in early development. Similar results (subfractionation of histone H1) have also been reported by Imoh and Minamidani (1973) in newt embryos and Sevaljević (1973) in sea urchin embryos.

It can be seen from our electrophoretic data that during the development of Xenopus the histone complement remains qualitatively unaltered. It is difficult to discern changes in relative quantities of different histone fractions because of the differences

in histone extractability from chromatin during successive development stages. For instance, structural changes in the nuclei of intact embryos have been observed with the light microscope (Immers, 1972) and with the electron microscope (Runnstrom, 1967) and these changes might be correlated with differences in the extractability of histones during development.

Adamson and Woodland (1974) have found four of the five main histone fractions in Xenopus being synthesized at all stages of early development, except for histone H1, which was first detected at the late blastula stage. Byrd and Kasinsky (1973a) have found that major classes of histones are synthesized both in cleavage and swimming Xenopus embryos and appear to be qualitatively the same. However, Adamson and Woodland (1974) were able to examine several embryos from each stage using microinjection techniques to label the histones with H^3-lysine, while Byrd and Kasinsky (1973a) had to average their data from the two-cell stage to gastrula embryos as their $^{14}CO_2$ label was much less sensitive in labeling the histones. In sea urchin embryos, an increase in H1 or its subfractions and a decrease in slightly lysine-rich histones during early development have been observed by Benttinen et al. (1971) in Lytechinus pictus and by Easton et al. (1972) in Arbacia punctulata, respectively. Ševaljević (1973) has found an increase in the synthesis of relative amounts of H1 and H3 histones compared to that of the H2a + H2b, and a qualitative change of H1 histone related to the transition of embryos from blastula to gastrula stage in Paracentrotus lividus. Ruderman et al. (1974) also have observed that in Lytechinus and Arbacia, the synthesis of different kinds

and amounts of hiatones do in fact differ characteristically from one stage of development to the next. Such a pattern of histone changes during development of the surf clam, Spisula solidissima, has also been reported by Gabrielli and Baglioni (1975). The discrepancy between observations reported here and those cited literature studies might be due to the fact that the cited works emphasize "synthesis" of histones while the present work concerns mainly the "content" of histones. Differences in extractability and/or degradation of histones could influence the results cited in the above literature. In addition, the maternal pool of histones accumulated during oogenesis to provide enough histones for subsequent development (Adamson and Woodland, 1974, 1977; Woodland and Adamson, 1977; Gagnetti, 1974) might play a role in redering the content of each histone fraction qualitatively constant. The present results indicate that there is no dramatic change in the electrophoretic profiles of histones during early embryogenesis in Xenopus. The results are entirely consistent with the earlier observations of Destrĕe et al. (1973) on the histone content of Xenopus embryos. However, employing a detergent-polyacrylamide gel system might reveal differences in H2a and H2b subfractions as Cohen et al. (1976) observed in sea urchin embryogenesis.

Before going into the discussion of electrophoretic properties of testis-specific histones, I would like to point out that "testis-specific" are possibly "sperm-specific" as well because they only show up electrophoretically when the testis contains sperm. However, cell separation techniques, such as those of Eckhardt and Risley (1976) will be needed to distinquish histones in spermatids from those in

sperm. Initial experiments (Kasinsky et al. 1974) using STAPUT

cell separation in bovine serum albumin gradients (Lam et al. 1970)

indicate that sperm contain the two main Xenopus bands and the

multiple Bufo bands upon starch gel electrophoresis of acid-ex-

tracted sperm histones.

The starch and polyacrylamide electrophoretic comparisons of

nuclear basic proteins present in Rana, Xenopus, Bufo, and Cynops

testes reveal a broad spectrum of histones in these amphibians.

Rana testis contains only the somatic complement of histones.

This is in agreement with several earlier studies. Vendrely (1957)

found that the amino acid composition of histones extracted from

Rana sperm and from somatic tissues were similar, while Bloch (1962),

and Bols and Kasinsky (1972) found no cytochemical differences

between histones from testes and somatic tissues. Bols and Kasinsky

(1973) saw similar electrophoretic patterns in testis and somatic

tissue but Alder and Gorovsky (1975) did find a new lysine-rich

histone H1 in Rana sperm by electrophoresis on long polyacrylamide

gels.

Unlike Rana pipiens, electrophoresis of histones from Xenopus

laevis testis reveals two fast-moving bands, X_s and X_f , which are

probably sperm-specific (Kasinsky et al ., 1974; Eckhardt and Risley,

1976). Bands X_s and X_f demonstrate mobilities intermediate between

somatic histones and the very arginine-rich protamines. Thus the

Mytilus (mussel) class of sperm histone to which Xenopus sperm

histone belongs is not only an "intermediate" type cytologically

(Bloch, 1969) but also appears to be "intermediate" with respect

to basicity and molecular weight upon electrophoresis.

One fast-moving band on polyacrylamide gel is specific to the
testis of Bufo and migrates in the vicinity of Sigma protamine.
It is probably also sperm-specific (Kasinsky et al. 1974). Bols
and Kasinsky (1972) tentatively suggested that Bufo sperm might be
classified as a Rana type rather than a Salmo protamine based on the
unusual cytochemical data. Bloch (1969), on the other hand, classi-
fied the sperm histones of Bufo vulgaris as the Mytilus type on
the basis of his unpublished cytochemical data. The present elec-
trophoretic experiments favor the Bufo sperm histone being similar
to the salmon type. However, since the electrophoretic characteri-
zation is based on charge, molecular size and shape, it is difficult
to reconcile the cytochemical classification with the electrophoretic
properties.

Electrophoresis of Cynops pyrrhogaster histones extracted from
cell suspensions reveals that the pattern of testis-specific basic
proteins in this urodele is distinct from that in the anurans,
Rana, Bufo and Xenopus. Cynops testis-specific histones show a
single band migrating a bit slower than herring protamine on the
polyacrylamide gel system of Bonner et al. (1968). On starch gels
it shows three bands in the region between histone H4 and Sigma
protamine. This is consistent with the electrophoretic results
obtained with testes from newt Notophthalmus (Bols and Kasinsky,
1976). It has been reported (Picheral, 1970; Bols and Kasinsky,
1976) that during spermiogenesis in the newts Pleurodeles waltlii
and Notophthalmus viridescens cells undergo a progressive shift
from somatic histones →"stable protamines"→protamines in the mature
sperm. Thus, the three-banded patern of sperm-specific histones in

the starch gel might reflect molecular species in different tran-
sition stages or might represent heterogeneity in the same sperm
nucleus, as predicted by Bedford and Calvin (1974). Cytochemically,
the newt's testis-specific histones are classified as the "salmon
type" (Bols and Kasinsky, 1976; Picheral, 1970). However, the
electrophoretic results indicate that they are in fact distinct
from those in salmon. Thus, the cytochemical classification of newt
sperm as the salmon type in Bloch's scheme (1969) probably indicates
that a distinct range exists for possible basic proteins falling
into this cytochemical category.

In contrast to the amphibian story, the polyacrylamide gel
electrophoretic comparison of testis-, ductus deferens- and semen-
specific histones present in Thamnophis, Anolis, Elaphe, Terrapene
and Sceloporous reveals a marked similarity in the mobility of these
basic proteins. They all show a single band that migrates in the
vicinity of Sigma protamine. Our earlier observations by starch
gel electrophoresis (Kasinsky et al., 1977) indicated that the testis-
specific histones of lizards (Lacertilia) and snakes (Sauria) also
showed a remarkably similar two-banded pattern in this medium, one
band moving close to the fast-moving band of Xenopus and the other
close to that of trout protamine. The results from both gel systems
show the similarity in the testis- and sperm -specific histones of
the reptiles thus far examined.

In general, then, both electrophoretic and cytochemical pro-
perties show the similarity among reptilian testis-specific histones
and the diversity among amphibian ones. This is also true of the
chemical data thus far obtained, as we will see when we discuss

the amino acid analysis of these basic proteins in Part II of the

Discussion.

Part II

AMINO ACID ANALYSIS

MATERIALS AND METHODS

The amino acid analyses were performed on the amidoblack-stained bands from polyacrylamide gels in tubes or from starch gel slabs. Methods for the electrophoresis of these gels have been described in Part I.

The analysis of bands on polyacrylamide gel was based mainly on the methods of Houston (1971), and Pallotta and Tessier (1976). The individual stained histone bands were carefully cut out using a new single edge razor blade and transferred into a clean test tube (no. 9800, Pyrex). Hydrolysis was carried out in 0.5 ml of 6 N HCl at 110°C for 24 hours or at 145°C for 4 hours (Roach and Gehrke, 1970). To each tube 10 μl of 0.8 M β- mercaptoethanol was added. Following the hydrolysis, the tubes were allowed to cool before they were placed in an ice bath for 1 hour. To eliminate the acrylamide residue, the tubes were centrifuged at 3,000 g for 10 minutes in a Sorval centrifuge equipped with a SS-34 rotor. The resulting supernatants were dried in a vacuum desicator and the material was dissolved in 0.4 ml of 0.2 N sodium acetate dilution buffer, PH 2.2. After filtration, the sample was ready for amino acid analysis.

For starch gels, the individual stained histone bands were carefully cut out and the protein in the bands was eluted with 0.5 ml of 6 N HCl overnight at 4°C. The eluate was collected by filtration through a millipore filter and hydrolyzed at 110°C for 24 hours (Durgo, 1977). After hydrolysis the material was dried and then dissolved in the same dilution buffer. The samples were

filtered before being applied to the column if charred residues we were present.

The amino acid analyses were performed on a Beckman Model 118C Amino Acid Analyzer, using a single 6 x 510 mm column, 3 hours run. The total flow rate was 52.5 ml/hr. The sensitivity of recording was maximal on the 0.1 O.D. scale. The time settings were as follows: buffer 1, 51 minutes; buffer 2, 16 minutes; buffer 3, 88 minutes and NaOH wash, 12 minutes. To quantitate amino acids appearing in the chromatogram of a particular sample, the area under the peak was integrated by weighing IBM Copier traces of the chromatogram on a Mettler semimicro-balance. Nanomoles of each amino acid were calculated by comparison with the standard curve obtained using the Beckman Amino Acid Calibration Standard.

$$K = \frac{\text{weight of peak area of standard amino acid}}{\text{known nanomoles of standard amino acid}}$$

$$\text{nanomoles of amino acid in unknown} = \frac{\text{weight of peak area of unknown}}{K}$$

K is a constant specific for each amino acid. To obtain a confident K value, 10 runs of 6.25 nmoles of Beckman Amino Acid Calibration Standard were done and the mean of 10 runs was taken for the above calculation.

Two runs were performed on each stained gel band examined. The average of two results is shown in the table in the Results.

RESULTS

Houston (1971) as well as Pallotta and Tessier (1976) have
previously described a method for analyzing the amino acid compo-
sition of proteins directly from stained polyacrylamide gel slices.
The technique proved successful with serum albumin and ovalbumin
using the gel system of Ornstein and Davis (Houston, 1971), as well
as with somatic histones using the acrylamide-urea gel system of
Panyim and Chalkley (Pallotta and Tessier, 1976). However, amino
acid analysis of histones which are separated on the acrylamide
gel system of Bonner et al. (1968) has never been attempted. For
this reason we did several control experiments using protamine
(herring) prepared by the Sigma Chemical Co. and calf thymus H4
(a gift from Drs. D. Fambrough and J. Bonner) before analyzing
the testis-specific histones of amphibians and reptiles.

Standard Amino Acid Chromatography

A mixture of standard amino acids (6.25 nmoles of each amino
acid, 3.125 nmoles cystine, Beckman Co.,) were run on a Beckman
model 118C Amino Acid analyzer using a 6 x 510 mm column for 3 hours.
The chromatogram is shown in Fig. 17. The original recording is
a series of dots which we have redrawn for clearer representation.
A shift upwards in the baseline after the second buffer is sometimes
observed. This is probably due to the storage of ninhydrin. It
does not affect the actual area under each peak.

The integration of each amino acid is obtained by weighing an
IBM photocopy of the peak on a Mettler semi-micro analytical balance.
This method has the shortcoming of involving many variables, such

Fig. 17   Chromatogram of Beckman amino acid calibration standard on the Beckman Model 118C Amino Acid Analyzer using a 6 x 510 mm column, 3 hours run. 6.25 nmole per amino acid except for cystine (3.125 nmole). Total flow rate: 52.5 ml/hr. Sensitivity: 0.1 O.D. scale. The readout is presented as dots by the recorder; we have drawn a continuous trace by connecting the dots.
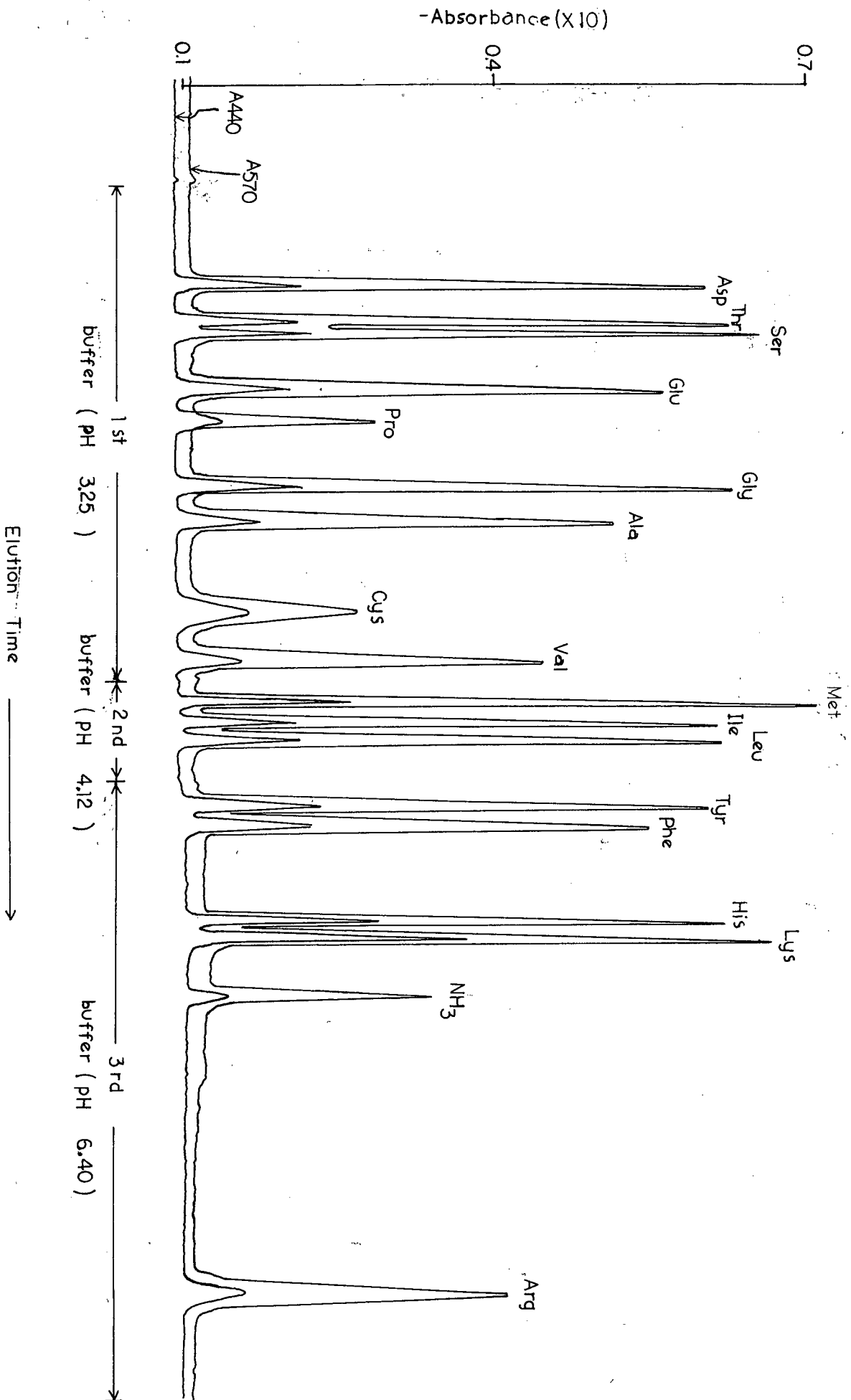
as inconsistency in cutting the paper by hand and inconsistency in
the sensitivity of the balance. However, according to Table 2,
which shows the K value and its 95% confidence interval of each
amino acid, K values calculated for weighing method are reliable
since their ranges of the 95% confidence interval are very small.
Therefore, to deal with a considerably large set of data, the weighing
method has proved to be the most convenient and time-saving.

Residual Polyacrylamide Gel Particles Effect

After acid hydrolysis, the polyacrylamide gel pieces in the
sample have to be removed completely. If the sample contains re-
sidual material from hydrelyzed gel, the peaks on the chromatogram
will show shoulder and tailing effects. In Fig. 18, we see a portion
of the chromatogram of Xenopus testis-specific histone X_S in a
clarified solution (Fig.18A) and in the solution with residual
material from hydrolyzed gels still present due to incomplete cen-
trifugation (Fig. 18B). The reason for the tailing is not known;
it is possibly due to the interaction of hydrolyzed polyacrylamide
with amino acids.

Amino Acid Analysis Before And After Gel Electrophoresis

Sigma protamine was run on polyacrylamide gels containing 6.25
M urea (Fig. 19). The stained band was cut out and hydrolyzed in
6 N HCl in the presence of β-mercaptoethanol. The results are
presented in Table 3 (column D) and can be compared with those of
Sigma protamine powder hydrolyzed directly (Column B). There is
no significant difference between these two amino acid analyses

Table 2

K values And Their 95% Confidence Intervals for Weighing Method.

Amino Acid	K ($\times 10^3$)	C.I. ($\times 10^3$)
Lys	23.42	$\pm$ 3.14
His	18.28	$\pm$ 2.97
Arg	25.51	$\pm$ 1.24
Asp	24.61	$\pm$ 0.90
Thr	15.53	$\pm$ 0.71
Ser	16.21	$\pm$ 1.34
Glu	29.54	$\pm$ 1.27
Pro	7.45	$\pm$ 0.30
Gly	26.55	$\pm$ 1.05
Ala	22.83	$\pm$ 0.93
Cys/2	14.07	$\pm$ 0.48
Val	22.53	$\pm$ 0.87
Met	30.58	$\pm$ 1.00
Ile	22.79	$\pm$ 0.67
Leu	26.01	$\pm$ 1.35
Tyr	23.92	$\pm$ 1.04
Phe	20.33	$\pm$ 1.12

K is a constant, specific for each amino acid. (see Materials And Methods, Part II). The K value indicated above is the mean of 10 runs. C.I. is the 95% confidence interval of K.

Fig. 18   Effect of residual polyacrylamide gel particles on
the amino acid profile from Xenopus testis-specific histones.

A. Clarified solution.

B. Residual polyacrylamide gel particles present.

A and B are from the same preparation.
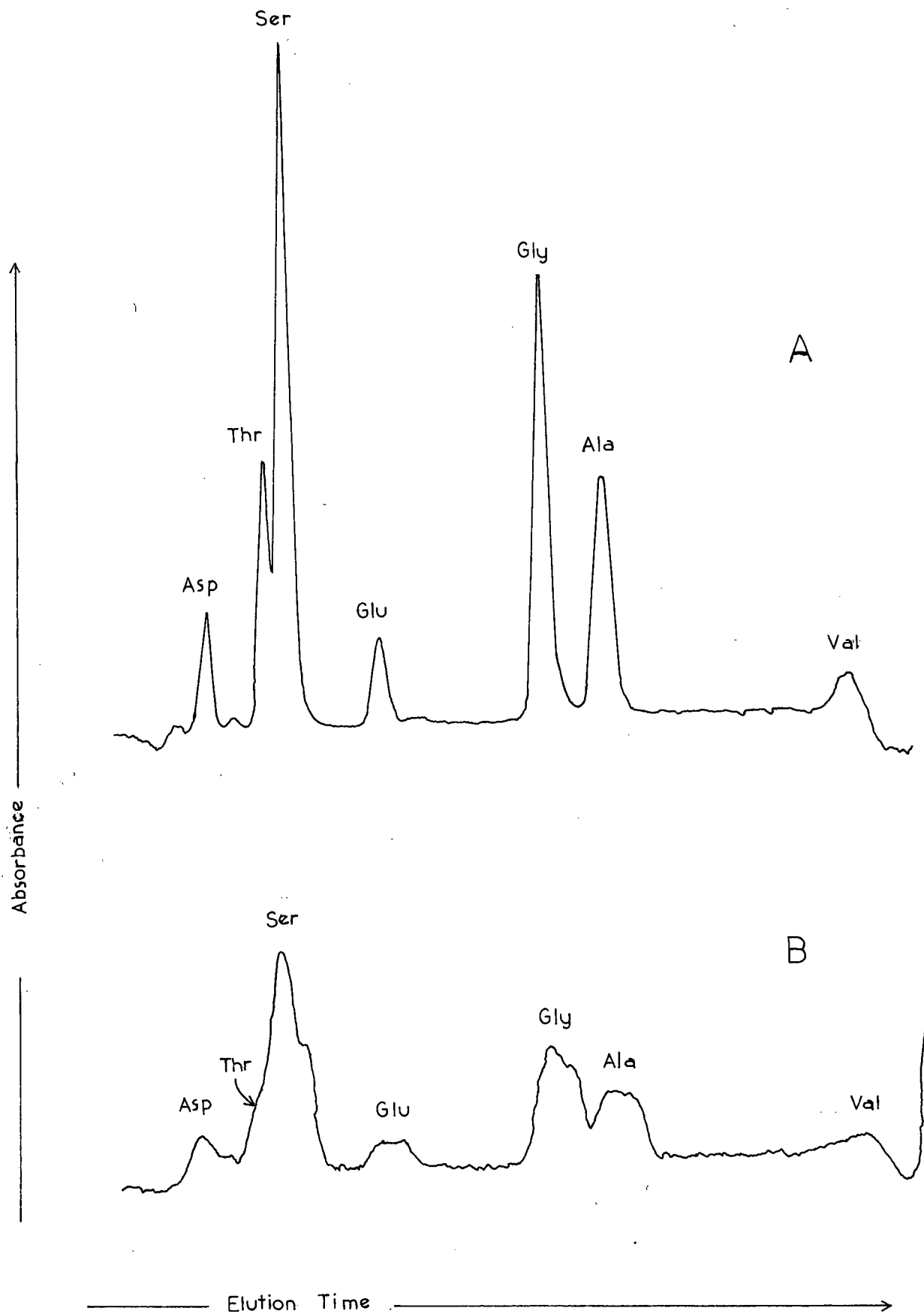
Fig. 19   Electrophoretic profiles of Sigma protamine (herring).

A.   Starch gel (Sung and Smithies, 1969). Dotted arrows indicate

possible impurities.

B.   Polyacrylamide gel (Bonner et al., 1968).

A   B

except that Sigma protamine on the gel has a bit higher percentage of serine and glycine. This might be due to bacterial contamination in the distilled water. When distilled water itself was analyzed, it showed very small peaks in the histidine, serine and glycine regions of the chromatogram.

The amino acid composition of Sigma protamine (Table 3, Column A and B) was compared to the results of Ando and Suzuki (1967) (column F) based on the primary structure of clupeine (herring protamine) preparation. The difference between these two sets of data might due to impurities in the Sigma protamine. This can be seen when the Sigma protamine is electrophoresed on starch gels (Fig. 19B). Although it only shows a single band (Fig. 19A) on the polyacrylamide gel system of Bonner et al. (1968), impurities are clearly evident on the starch gel. The bands moving faster than P on the starch gel might represent modified forms of the protamine, as seen in trout protamine (Louie and Dixon, 1972).

Hydrolysis Time and Temperature Effect

Different hydrolysis conditions were examined in order to find suitable condition for the amino acid analysis. Hydrolysis at 145°C for 4 hours (Roach and Gehrke, 1970) and hydrolysis at 110°C for 24 hours (Moore and Stein, 1963) were studied since these two conditions were reported to be the best. The results, shown in Table 3 (column A and B), show no significant differences in the data obtained by these two methods. However, hydrolysis at 110°C for 24 hours is preferable if time permits since fluctuations in the total time of hydrolysis will not be so critical for the longer 24-hour period (145°C).

TABLE 3

Amino Acid Composition of Sigma Protamine (Herring)

Amino acid	A (mole %)	B (mole %)	C (mole %)	D (mole %)	E (mole %)	F (mole %)
Lys	Tr	Tr	1.7	Tr	2.3	–
His	2.2	1.2	Tr	1.7	Tr	–
Arg	56.1	57.4	70.6	57.3	58.8	64.5
Asp	1.2	1.1	Tr	Tr	Tr	–
Thr	2.6	2.5	3.6	2.5	4.7	6.5
Ser	9.3	9.4	7.6	11.6	10.1	9.7
Glu	Tr	1.3	Tr	1.1	1.2	–
Pro	6.6	6.3	Tr	5.4	Tr	6.5
Gly	3.0	2.6	2.2	4.2	4.1	3.2
Ala	8.5	8.3	8.3	8.3	11.3	6.5
Cys/2	–	–	–	–	–	–
Val	7.4	7.3	5.0	5.9	6.5	6.5*
Met	–	–	–	–	–	–
Ile	1.5	1.6	–	1.1	–	(3.2)**
Leu	Tr	Tr	–	Tr	–	–
Tyr	–	–	–	–	–	–
Phe	–	–	–	–	–	–
Lys/Arg	–	–	0.02	–	0.04	–
Basic/Acidic	29.94	24.94	84.28	34.07	31.78	–

A. Sigma protamine powder hydrolyzed at 145°C for 4 hours (no-NaOH treatment) and chromatographed on a Beckman 118C Amino Acid Analyzer, single 51 cm column, 3 hour run, 0.1 O.D. scale. No correction is made for hydrolytic losses.

B. Sigma protamine powder hydrolyzed at 110°C for 24 hours (no-NaOH treatment). 118C Analyzer.

C. Sigma protamine powder hydrolyzed at 145°C for 4 hours (NaOH treated) analyzed on a Beckman 120B Amino Acid Analyzer modified by Dr. E. A. Boeker for a single 60 cm column, 5 hour run. Proline is present only in trace amounts.

D. Sigma protamine on polyacrylamide gel hydrolyzed at 110°C for 24 hours (no-NaOH treatment) according to Houston (1971). 118C Analyzer.

E. Sigma protamine on polyacrylamide gel hydrolyzed at 145°C for 4 hours (NaOH treated) according to Pallota and Tessier (1976). 120B modified Analyzer. Only a trace of proline is seen.

F. Clupeus harengus Y (Ando and Suzuki, 1967). Data based on primary structure of protein.

* only in YI   ** (only in YII)

▲ Basic/Acidic = (Lys + His + Arg) / (Asp + Glu). No correction is made for hydrolytic losses.

Effect of Sodium Hydroxide on Amino Acid Analysis

A large amount of ammonia is released during the hydrolysis of the stained gel pieces as the polyacrylamide is converted to polyacrylic acid (Houston, 1971). It has been suggested that the addition of 10 N NaOH eliminates much of the ammonia when basic amino acids are analyzed on the short column of a two-column Beckman model 120C analyzer (pollotta and Tessier, 1976). However, the analyzer used in this study is a single column type (Beckman 118C) that readily separates ammonia from basic amino acids. Therefore, studies of the effect of sodium hydroxide treatment were undertaken. Comparing the results in Table 3, column A and column B with those of column C, or comparing column D with column E, one can see that sodium hydroxide treatment interferes with the analysis of proline and isoleucine. Pallotta and Tessier (1976) also observed the destruction of proline, phenylalanine and tyrosine by this treatment. Therefore, sodium hydroxide treatment was not employed in subsequent amino acid analysis on the 118C analyzer in order to obtain the true composition of the proteins.

β-Mercaptoethanol Effect on Amino Acid Analysis

It has been reported that tyrosine and histidine are destroyed during protein hydrolysis without the protection of β-mercaptoethanol (Houston, 1971). When Xenopus testis-specific histone X_S was examined without β-mercaptoethanol, it was found (Table 4) that methionine and tyrosine were degraded, but not histidine. This destruction may result from oxidation since it is difficult to remove all trapped oxygen within the gel piece.

TABLE 4

β-mercaptoethanol Effect on Acid Hydrolysis of <u>Xenopus</u> Testis-Specific
Histone X_s

Amino acid	A (mole %)	B (mole %)
Lys	1.3	1.8
His	2.5	3.4
Arg	30.1	30.4
Asp	4.9	5.0
Thr	10.2	9.8
Ser	15.6	16.6
Glu	4.3	4.2
Pro	1.1	1.1
Gly	9.3	9.3
Ala	12.2	11.6
Cys/2	-	-
Val	2.8	2.6
Met	<u>2.2</u>	Tr
Ile	Tr	Tr
Leu	1.4	1.9
Tyr	<u>1.8</u>	Tr
Phe	Tr	Tr

A. X_s from polyacrylamide gel hydrolyzed with 0.8 M β-mercaptoethanol.

   X_s was prepared from a testis chromatin preparation using the

   modified method of Bonner <u>et al.</u> (1968). Methionine and tyrosine

   are present it 0.8 M -mercaptoethanol, prepared f   t

B. Same, without 0.8 M β-mercaptoethanol, prepared from a testis cell

   suspension.

Testicular Histone Preparation Effect

Table 5 and 6 show the amino acid composition of Xenopus testis-
specific histones X_s and X_f using different preparation methods.
The overall compositions are basically similar amongst the histones
obtained from a cell suspension preparation (Fig. 7B, channel c),
a nuclear preparation (Fig. 7B, channel b) and from a chromatin
preparation (Fig. 7B channel a), with an exception for X_f in the
latter instance. The X_f band from a chromatin preparation (Table 6,
column A) shows a high content of arginine and a lower content of
histidine than X_f obtained in the two other methods (Table 6. co-
lumns B and C). Xenopus testis-specific histones have been reported
to show microheterogeneity on starch gels (Bols et al., 1976) and
on polyacrylamide gels (Risley and Eckhardt, 1975). It is possible
that the chromatin preparation selectively extracts a particular
species of X_f. This could explain the differences between columns
B and C in Table 6.

Amino acid Composition of Histone H4 from Calf Thymus And Xenopus

Table 7 shows the amino acid composition of the evolutionarily
conservative histone H4 from Xenopus and from calf thymus. Calf
thymus histone H4, a relatively purified powder, was electrophoresed
on polyacrylamide gel (Fig. 7, channel e). Xenopus histone H4
from a cell suspension preparation was also electrophoresed in the
same manner (Fig. 7, channels c and d). The results shown in Table
7, columns A and B, are compared to those of John's (1971) for calf
thymus H4 histone and Byrd and Kasinsky (1973) for Xenopus liver
histone H4 purified from chromatin by selective extraction and chro-

TABLE 5

Comparison of Amino Acid Composition of Xenopus Testis-Specific Histone

X_s Prepared by Different Methods

Amino acid	A (mole %)	B (mole %)	C (mole %)
Lys	1.3	2.0	1.9
His	2.5	3.4	3.8
Arg	30.1	25.9	25.8
Asp	4.9	5.7	5.5
Thr	10.2	8.7	9.4
Ser	15.6	17.1	16.5
Glu	4.3	5.2	4.1
Pro	1.1	1.1	1.4
Gly	9.3	11.2	9.6
Ala	12.2	11.0	11.9
Cys/2	-	-	-
Val	2.8	2.8	2.9
Met	2.2	1.4	2.1
Ile	Tr	Tr	Tr
Leu	1.4	1.9	1.9
Tyr	1.8	1.8	2.6
Phe	Tr	Tr	Tr
Lys/Arg	0.04	0.08	0.07
Basic/Acidic	3.68	2.87	3.29

A. X_s extracted from a testicular chromatin preparation by the modified

method of Bonner et al. (1968) (gel).

B. X_s extracted from a testis nuclear preparation by Byrd's method

(gel).

C. X_s extracted from a testis cell suspension (gel).

TABLE 6

Comparison of Amino Acid Composition of <u>Xenopus</u> Testis-Specific Histone X_f Prepared by Different Methods

Amino acid	A (mole %)	B (mole %)	C (mole %)
Lys	2.4	3.0	2.1
His	2.6	7.4	10.7
Arg	28.1	19.4	16.6
Asp	3.5	4.5	3.9
Thr	10.4	5.3	8.3
Ser	17.5	23.4	22.5
Glu	4.7	4.5	3.6
Pro	2.3	Tr	1.3
Gly	9.5	16.9	11.7
Ala	13.7	10.0	10.2
Cys/2	–	–	–
Val	4.5	5.1	4.2
Met	Tr	Tr	Tr
Ile	Tr	Tr	1.3
Leu	Tr	Tr	1.8
Tyr	Tr	Tr	Tr
Phe	Tr	Tr	1.3
Lys/Arg	0.09	0.16	0.10
Basic/Acidic	4.05	3.32	4.91

A. X_f extracted from a testicular chromatin preparation by the modified method of Bonner et al. (1968) (gel).

B. X_f extracted from a testis nuclear preparation by Byrd's method (gel).

C. X_f extracted from a testis cell suspension (gel).

TABLE 7

Comparison of the Amino Acid Composition of Evolutionarily Conservative
Histone H4 in Xenopus Tissues and Calf Thymus

Amino acid	A (mole %)	B (mole %)	C (mole %)	D (mole %)
Lys	9.4	11.9	10.2	13.4
His	5.0	2.3	2.2	2.2
Arg	11.2	12.8	12.8	13.2
Asp	6.0	5.3	5.2	6.2
Thr	7.1	6.9	6.3	4.7
Ser	7.4	2.2	2.2	2.7
Glu	6.6	7.3	6.9	6.4
Pro	1.5	Tr	1.5	2.6
Gly	16.4	16.6	14.9	12.0
Ala	8.0	8.1	7.7	7.1
Cys/2	–	–	–	–
Val	7.7	10.0	8.2	6.5
Met	Tr	Tr	1.0	1.2
Ile	3.9	4.3	5.7	5.9
Leu	7.0	7.3	8.2	8.6
Tyr	Tr	3.0	3.8	3.9
Phe	2.2	1.4	2.1	3.6
Lys/Arg	0.84	0.93	0.80	1.02
Basic/Acidic	2.04	2.14	2.08	2.28

A. Histone H4 from Xenopus testis cell suspension (gel).

B. Histone H4 from calf thymus, a gift from Drs. D. Fambrough and
J. Bonner (gel).

C. Histone H4 from calf thymus (Johns, 1971).

D. Histone H4 from Xenopus liver chromatin (Byrd and Kasinsky, 1973a).

matography. They show considerable similarity. The higher histidine, serine and glycine content in the <u>Xenopus</u> testis histone H4 might be due to slight contamination of distilled water with bacteria, as mentioned earlier.

<u>Amino Acid Analysis of Testis-Specific Histones from Amphibians And Reptiles</u>

Testis-specific histones extracted from cell suspensions of the South African clawed toad <u>Xenopus laevis</u>, the toad <u>Bufo marinus</u>, the Japanese fire-belly newt <u>Cynops pyrrhogaster</u>, the snakes <u>Thamnophis sirtalis</u> and <u>Elaphe guttata guttata</u> and the lizard <u>Anolis carolinensis</u>, were electrophoresed on polyacrylamide gels (Fig.8 and 9) and bands were cut out for amino acid analysis.

Table 8 indicates the amino acid composition of fast-moving testis-specific histones from three amphibian species (<u>Xenopus</u>, <u>Cynops</u> and <u>Bufo</u>). They show considerable diversity in their amino acid composition. The basic/acidic ratio ranges from 1.58-10.3. <u>Bufo</u> shows a very high content of arginine (45 mole %) and a very low content of serine and glycine; <u>Xenopus</u> shows an intermediate content of arginine (20 mole%) and a high content of serine and glycine; <u>Cynops</u> has only about 10 mole% arginine but shows a high content of serine and glycine. <u>Rana</u> does not have any fast-migrating band and the amino acid composition of its testicular histones has been shown to be similar to that of somatic histones (Vendrely, 1957). This diversity in amino acid composition is consistent with the diversity previously observed in the electrophoretic properties of these testis-specific histones from amphibians. (see Part I).

TABLE 8

Amino Acid Composition of Testis-Specific Histones from Amphibian
Cell Suspensions

Amino acid	A (mole %)	B (mole %)	C (mole %)	D (mole %)
Lys	1.9	2.1	4.8	1.8
His	3.8	10.7	7.8	9.5
Arg	25.8	16.6	45.5	10.7
Asp	5.5	3.9	Tr	6.5
Thr	9.4	8.3	6.4	2.6
Ser	16.5	22.5	8.5	23.0
Glu	4.1	3.6	5.4	7.3
Pro	1.4	1.3	6.1	2.6
Gly	9.6	11.7	1.5	16.6
Ala	11.9	10.2	6.1	7.9
Cys/2	-	-	-	-
Val	2.9	4.2	5.4	5.3
Met	2.1	Tr	Tr	Tr
Ile	Tr	1.3	Tr	Tr
Leu	1.9	1.8	Tr	2.7
Tyr	2.6	Tr	1.2	Tr
Phe	Tr	1.3	Tr	1.7
Lys/Arg	0.07	0.13	0.11	0.16
Basic/Acidic	3.29	4.91	10.30	1.58

A. First fast-moving band (X_s) of testis-specific histones of Xenopus
   (gel).

B. Second fast-moving band (X_f) of testis-specific histones of Xenopus
   (gel).

C. Testis-specific histone of the toad, Bufo marinus (gel).

D. Testis-specific histone of the Japanese fire-belly newt, Cynops
   pyrrhogaster (gel).

Table 9 shows the results of amino acid analysis of fast-moving histones from testis, ductus deferens and semen of three reptilian species (Thamnophis, Elaphe and Anolis). Unlike the amphibian species the reptiles show a remarkable similarity in their testis- or semen-specific histones. The arginine contents are in the range of 23 mole% to 29 mole%; serine contents range from 12 to 16 mole% and glycine contents are in the range of 18-24 mole%. This confirms the similarity previously observed in the electrophoretic properties of the testis-specific histones in these snakes and lizards (see Part I). However, it should be noted that Bufo, Anolis, and snake bands show microheterogeneity on starch gels (Kasinsky et al., 1977) so that these analyses might represent either an averaging of micro-heterogeneous proteins with similar amino acid compositions, as has been observed in trout protamine (Ling, et al., 1971), or one protein with different degrees of phosphorylation of serine residues or acetylation of lysine residues (Louie and Dixon, 1972; Sung et al., 1977).

Amino Acid Analysis of Stained Bands on Starch Gels

For amino acid analysis of starch gel bands, Durgo's method (1977) was employed. Instead of hydrolyzing the whole band as in the case of polyacrylamide gels, the proteins were first eluted from the stained band before hydrolysis, since it has been reported by Tristan (1939), Bailey (1937) that in the presence of carbohy-drates arginine and methionine were destroyed extensively. The amount of degradation was proportional to the concentration of carbohydrates. Johns et al. (1961) extracted histones from stained

TABLE 9

Amino Acid Composition of Testis-Ductus Deferens-and Semen-specific Histones
from Reptilian Cell Suspensions

Amino acid	A (mole %)	B (mole %)	C (mole %)
Lys	4.4	7.0	9.8
His	7.0	7.7	9.3
Arg	23.0	29.5	26.3
Asp	5.4	4.3	4.2
Thr	1.9	1.9	Tr
Ser	16.2	14.6	12.3
Glu	4.1	4.2	3.6
Pro	2.1	1.0	Tr
Gly	18.4	18.8	24.5
Ala	5.9	3.8	4.2
Cys/2	–	–	–
Val	5.2	3.5	2.7
Met	–	–	–
Ile	1.3	1.7	Tr
Leu	2.7	1.6	1.2
Tyr	Tr	Tr	Tr
Phe	1.6	Tr	Tr
Lys/Arg	0.19	0.25	0.38
Basic/Acidic	3.61	4.84	5.77

A. Ductus deferens-specific histone of the snake Thamnophis sirtalis
   (gel).

B. Semen-specific histone of the snake Elaphe guttata guttata (gel).

C. Testis-specific histone of the lizard Anolis carolinensis (gel).
   The histones in A and B are probably sperm-specific as this is the
   predominant cell type.

starch gels with 0.1 N HCl by freezing and thawing methods. In our experiments, water, 0.4 N HCl and 6N HCl were used to extract histones from stained bands. The results (not shown) indicated that 6 N HCl was much better than the other two eluting agents. However, unknown peaks also appeared in the chromatogram, and accurate results could not be obtained. This was probably due to the fact that starch gel itself was a good medium for bacterial growth, even at low pH. The unknown peaks picked in the analysis were probably specific to contaminating bacteria. Further experiments are required to bring the amino acid analysis of starch gel bands up to the accuracy of the polyacrylamide gel band analysis.

DISCUSSION

Starting from the methods of Houston (1971) and Pallotta and
Tessier (1976), I was able to analyze the amino acid composition
of the protein in a single band on the polyacrylamide gel system
of Bonner et al. (1968). Both control experiments on Sigma pro-
tamine and calf thymus histone H4 show the same amino acid analysis
before and after gel electrophoresis. That is to say, the gel and
the dye used, amidoblack, do not affect the amino acid analysis if
the gel residue after hydrolysis is completely removed. Therefore,
one does not have to rely on matching an unstained gel with a
stained gel, and then trying to excise the corresponding band and
eluting the protein out of it for further analysis. One can hydro-
lyze the amidoblack-stained protein directly in the gel for amino
acid analysis.

Testis-specific histones from different preparations, i.e.,
chromatin, nuclei preparations and cell suspensions show a similar
composotion. Combining the ease of preparing histones from cell
suspensions and analyzing a single band on polyacrylamide gel, I
was able to examine the testis-specific histones from a single
animal of different amphibian and reptilian species.

Using Bloch's categories (1969), we find that the testis- ,
or sperm-specific histones of the three amphibians examined,
Xenopus, Bufo and Cynops and the three reptiles, Thamnophis, Elaphe
and Anolis, all fall into the Mytilus or intermediate type (Type
3) since they contain more than one dibasic amino acid and no
cysteine. However, when one examines the amino acid composition

of each one, one would see that the relative quantity of the three basic amino acids varies markedly from an arginine content of 45 mole% to one of only 10 mole% and a lysine content of 1.3 mole% to 9.8 mole%. Literature data show that Loligo pealeii sperm histone, which is in the Mytilus category cytochemically, has an arginine content as high as 75 mole% while its lysine and histidine contents are as low as 0.8 mole% and 0.1 mole% respectively (Subirana et al., 1973). Felix (1952) classified Salmo fontinalis sperm histones as the Salmo type cytochemically. However he found 2 mole% lysine content in the amino acid composition of the protein. Bols and Kasinsky (1973) classified Bufo americanus sperm histone as either the Salmo or the Mytilus type. Bufo boreus sperm histone, which was thought to be the Rana type cytochemically (Bols and Kasinsky, 1972), actually showed a fast-moving band migrating faster than somatic histones (Bols and Kasinsky, 1973; Kasinsky et al.,1977). All these cited data point out that the cytochemical classification of sperm-specific histones is a very loose one, especially with regard to the Mytilus type of sperm histones. This is probably due to the limitations and the relative insensitivity of the cytochemical reactions in situ. The cytochemical approach, although promising, is frustrated by the lack of information on basic protein composition of most sperm, by the problems of comparing conclusions from biochemical work with those drawn from a cytochemical approach. A more exacting classification based on the amino acid composition is recommended, such as that indicated in Table 10. This scheme continues to be a modification of Kossel's (1928)

Table 10

A Brief History of Sperm Histone Classification.

Date	Investigator	Classification scheme devised	Methods used
1928	Kossel	monoprotamine (1)[*] diprotamine (2)[*] triprotamine (3)[*]	chemical
1950's	Felix	same	chemical
1960's	Bloch and Alfert	Class 1-Salmo (mono-) type Class 2-mammalian type Class 3-Mytilus (di-, tri-) type Class 4-Rana (somatic) type Class 5-crab type (non-histone)	chemical and cytochemical
1977	Huang and Kasinsky	Proposed scheme Type 1-monoprotamine Type 2-basic keratin (cysteine containing) Type 3A - < 40% Arg Type 3B - > 40% Arg Type 4-Rana type Type 5-Crab type	cytochemical, electrophoretic and amino acid analysis

[*] (1), (2), (3) = the number of different kinds of basic amino acid

residues (arg, lys or his) in proteins.

original classification of sperm histones into mono-, di-, or
triprotamines depending on the presence of arginine (mono-),
arinine+lysine (di-), arginine+histidine (di-), or arginine+lysine+
histidine (triprotamine) in the protein. In this scheme, categories
1, 2 and 4 of sperm histones remain unaltered, as does category 5 of
sperm lacking histones. Type 3, by Bloch's definition, includes the
di- and triprotamines of Kossel's classification scheme. Examining
the amino acid compositions of amphibian and reptilian sperm histones
actually obtained in this thesis, one finds that they encompass a
wide range of arginine contentswithin two broad categories. There-
fore, a content of 40 mole% arginine has been set as an arbitary
but realistic line to separate Type 3 into low-arginine Type 3A
(arginine content less than 40 mole%) and high-arginine Type 3B
(arginine content greater than 40 mole%) subclasses. In this sense,
amongst the amphibians, Bufo should be categoried as Type 3B and
Xenopus and Cynops should be placed in Type 3A. Amongst the reptiles,
Thamnophis, Elaphe and Anolis all fall into the Type 3A category.

Comparing the amino acid composition of these testis- (sperm-)
specific histones, one can see the marked diversity amongst amphibian
and the similarity amongst reptilian proteins. This is also reflected
in their electrophoretic properties. Both cytochemical and biochemical
studies show that there is marked diversity in fish sperm histones
(Bloch, 1976); that mammalian sperm histones show conservation and
fall into either Type 1 or Type 2 categories (Bloch, 1976). If
one can generalize from the limited number of representatives
examined so far, it would appear that the relative conservation of

testis-or sperm-specific histones in reptiles represents a crossover
point from the diversity of such proteins in fish and amphibians to
relative constancy of such protein type in mammals (Fig. 20). As
the only bird sperm histone that has been examined thus far is the
rooster protamine (Nakano, et al., 1976), we cannot say whether this
relative constancy will also include the sperm histones of birds.

Fig. 20   Classification of Sperm-Specific Histones in Vertebrate

Phylogeny by Amino Acid Composition

Based on data of Bloch (1976), Nakano et al. (1976), Vendreley

(1957) and present chemical analyses. Parentheses denote sperm

histone type determined only by cytochemical means. As in Figure

1 arrow denotes evolutionary trend towards relative constancy of

sperm histone type in vertebrate phylogeny.
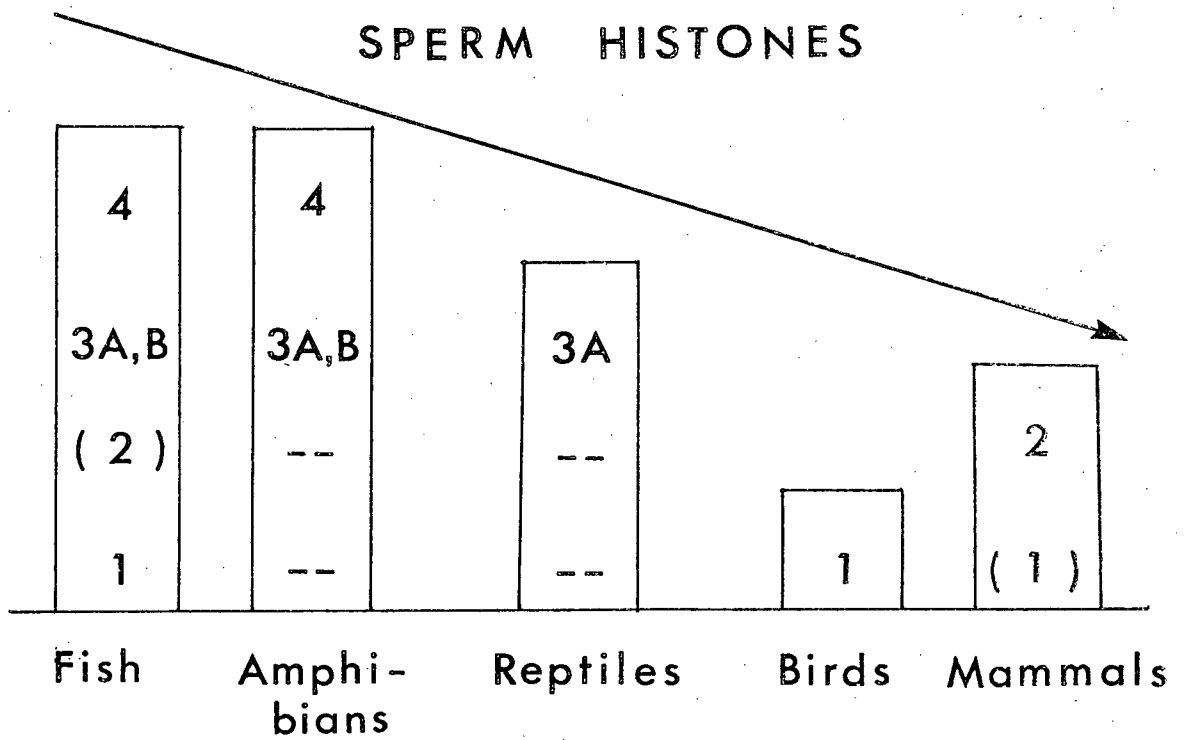
CONCLUSION

Combining the use of the polyacrylamide electrophoresis with amino acid analysis of the stained bands has proved to be an appropriate method to analyze small amounts of histones. In this study, I have demonstrated biochemically the contrast between the diversity of testis-specific histones and the constancy of somatic and embryonic histones by these methods of gel electrophoresis and amino acid analysis.

Whether redefinition of the intermediate class of sperm histones into high-arginine and low-arginine subgroups will prove to be valid for other vertebrates awaits a great deal more experimental work on these proteins. Although we have made some progress since Kossel's original observations on sperm histones more than 50 years ago, the mystery of sperm histone diversity is still not fully understood.

REFERENCES

Adamson, E.D., and Woodland, H.R., 1977. Changes in the rate of histone synthesis during oocyte maturation and very early development of Xenopus laevis. Devel. Biol. 57: 136-149.

Adamson, E.D., and Woodland, H.R., 1974. Histone synthesis in early amphibian development: Histone and DNA synthesis are not co-ordinated. J. Mol. Biol. 88: 263-285.

Alder, D., and Gorovsky, M.A., 1975. Electrophoretic analysis of liver and testis histones of the frog Rana pipiens. J. Cell. Biol. 64: 389-397.

Alfageme, G.R., Zweidler, A., and Cohen, L.H., 1974. Histones of Drosophila embryos. J. Biol. Chem. 249: 3729-3736.

Alfert, M., 1956. Chemical differentiation of nuclear proteins during spermatogenesis in the salmon. J. Biophys. Biochem. Cytology. 2: 109-114.

Alfert, M., 1958. Cytochemische Unter schung an basischen Kernproteinen warend der Gametenbildung. Befruchtung. und Entwicklung. Ges. physiol. Chem. Colloq. 9: 73-84.

Ando, T., and Suzuki, K., 1967. The amino acid sequence of the component of clupeine. Biochim. Biophys. Acta. 10: 375-377.

Bailey, K., 1973. The sulphur distribution of proteins. Biochem. J. 31: 1396-1405.

Baldwin, J.P., Bosley, P.G., Bradbury, E.M., and Ibel, K., 1975. The subunit structure of the eukaryotic chromosome. Nature, 253: 245-249.

Bedford, J.M., and Calvin, H.I., 1974. The occurrence and possible functional significance of the S--S crosslinks in sperm heads with particular reference to eutherian mammals. J. Exptl. Zool. 188: 137-156.

Bettinen, L.C., and Comb, D.G., 1971. Early and late histones during sea urchin development. J. Mol. Biol. 57: 355-358.

Bloch, D.P., 1976. Sperm histones. In Handbook of Genetics. Vol. V. (R.C. King, ed.) Plenum press, London. pp. 139-167.

Bloch, D.P., 1969. A catalog of sperm histones. Genetics. (Supplement) 61: 93-111.

Bloch, D.P., 1962. Synthetic process in the cell nucleus. I. Histone synthesis in non-replicating chromosome. J. Histochem. Cytochem. 10: 137-144.

Bols, N.C., and Kasinsky, H.E., 1976. On the diversity of sperm histones in the vertebrates. I. Changes in the basic proteins during spermiogenesis in the newt Notophthalmus viridescens. Differentiation. 7: 31-38.

Bols, N.C., and Kasinsky, H.E., 1973. An electrophoretic comparison of histones in anuran testis. Can. J. Zool. 51: 203-208.

Bols, N.C., and Kasinsky, H.E., 1972. Basic protien composition of anuran sperm: a cytochemical study. Can. J. Zool. 50: 171-177.

Bonner, J., Chalkley, G.R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R.C., Huberman, J., Jeusen, R., Marushige, K., Ohlenbusch, H., Olivera, B., and Widholm, J., 1968. Isolation and Characterization of chromosomal nucleoproteins. In Methods in Enzymology. Vol. 12B. Grossman, L. and Moldave, K., eds., Academic Press, New York.

Bradbury, E.M., Molgaard, H.V., Stephens, R.M., Bolund, L.A., and Johns, E.W., 1972. X-ray studies of nucleoproteins depleted of lysine-rich histone. Eur. J. Biochem. 31:474-482.

Bustin, M., and Cole, R.D., 1968. Species and organ specificity in very lysine-rich histones. J. Biol. Chem. 243: 4500-4511.

Byrd, Jr. E.W., 1974. Personal communication.

Byrd, Jr. E.W., and Kasinsky, H.E., 1973a. Nuclear accumulation of newly synthesized histones in early Xenopus development. Biochim. Biophys. Acta. 331: 430-441.

Byrd, JR. E.W., and Kasinsky, H.E., 1973b. Histone synthesis during early embryogenesis in Xenopus laevis. Biochem. 12:246-253.

Cognetti, G., Spinelli, G., and Vivoli, A., 1974. Synthesis of histones during sea urchin oogenesis. Biochim. Biophys. Acta. 349: 447-455.

Cohen, L.H. Newrock, K.M., and Zweidler, A., 1976. Stage-specific switches in histone synthesis during embryogenesis of sea urchin. Science. 190: 994-997.

Conant, R., 1975. A Field Guide to Reptiles and Amphibians of Eastern and Central North America. second edition. Houghton Mifflin Co., Boston.

DeLange, R.J., and Smith, E.L., 1975. Histone function and evolution as viewed by sequence studies. Ciba. Found. Symp. 28: 59-76.

DeLange, R.J., and Smith, E.L., 1974. Histones: Structure and function. Ann. Rev. Biochem. 40: 279-314.

De Nooij, E.H., and Westenbrink, H.G.K., 1962. Isolation of a homo-

geneous lysine-rich histone from calf thymus. <u>Biochim</u>. <u>Biophys</u>. <u>Acta</u>.
62: 608-609.

Destrèe, O.H.J., d'Adelhart-Toorop, H.A., and Charles, R., 1975. Cyto-
plasmic origin of the so-called nuclear neutral histone protease. <u>Bio</u>-
<u>chim</u>. <u>Biophys</u>. <u>Acta</u>. 378: 450-458.

Destrèe, O.H.J., d'Adelhart-Toorop, H.A., and Charles, R., 1973. Ana-
lysis of histones from different tissues and embryos of <u>Xenopus</u> <u>laevis</u>
(Daudin). II. Qualitative and quantitative aspects of nuclear histones
during early stage of development. <u>Cell</u>. <u>Differ</u>. 2: 229-242.

Destrèe, O.H.J., d'Adelhart-Toorop, H.A., and Charles, R., 1972. Ana-
lysis of histones from different tissues and embryos of <u>Xenopus</u> <u>laevis</u>
(Daudin). I. Technical problems in the purification of undegraded
native total histone preparation. <u>Acta</u>. <u>Morph</u>. <u>Neerl</u>. <u>Scand</u>. 10:
232-248.

Durgo, J., 1977. Personal communication.

Easton, D., and Chalkley, R., 1972. High-resolution electrophoretic
analysis of the histones from embryos and sperm of <u>Arbacia</u> <u>punctulata</u>.
<u>Exptl</u>. <u>Cell</u>. <u>Res</u>. 72: 502-508.

Eckhard, R.A., and Risley, M.S., 1976. Histone replacement during sper-
miogenesis in <u>Xenopus</u> <u>laevis</u>. <u>J</u>. <u>Cell</u>. <u>Biol</u>. 70: 785a.

Elgin, S.C.R., and Weintraub, H., 1975. Chromosomal proteins and chroma-
tin structure. <u>Ann</u>. <u>Rev</u>. <u>Biochim</u>. 44: 726-773.

Elgin, S.C.R., Frochner, S.C., Smart, J.E., and Bonner, J., 1971. The
biology and chemistry of chromosomal proteins. <u>Adv</u>. <u>Cell</u>. <u>Mol</u>. <u>Biol</u>. 1:
1-57.

Essner, E.S., 1954. The breakdown of isolated yolk granules by cations.

Protoplasma. 43: 79-89.

Fambrough, D.M., and Bonner, J., 1969. Limited molucular heterogeneity of plant histones. Biochim. Biophys. Acta. 175: 113-122.

Felden, R.A.,Sander, M.M., and Morris, N.R., 1976. Presence of histones in Aspergillus nidulans. J. Cell. Biol. 68: 430-439.

Furlan, M., Jericijo, M., and Suhar, A., 1968. Purification and properties of a neutral protease from calf thymus nucleus. Biochim. Biophys. Acta. 167: 154-160.

Felix, V.K.F., 1952. Zur chemie des Zellkerns. Experientia. 8: 312-317.

Gabrielli, F., and Baglioni, C., 1975. Maternal messenger RNA and histone synthesis in embryos of surf clam Spisula solidissima. Exptl. Biol. 43: 254-263.

Garrels, J.J., Elgin, S.C.R., and Bonner, J., 1972. A histone protease of rat liver chromatin. Biochem. Biophys. Res. Commun. 46: 545-551.

Greenhouse, G., and Morrisey, J.H. 1974. Resolution of acid-soluble protein component of yolk platelets from Xenopus laevis eggs on polyacrylamide gels. Devel. Biol. 39: 168-171.

Gurdon, J. B., 1967. African clawed frogs. In Methods in Developmental Biology. (Wilt, F.H., and Wessells, N.K., eds), T.Y. Crowell Co., N.Y. pp. 75-84.

Hallberg, R.L., and Brown, D.D., 1973. Co-ordinated synthesis of some ribosomal proteins and ribosomal RNA in embryos of Xenopus laevis. Mol. Biol. Amphi. Devel. MSS Information Corporation. N.Y. pp. 145-164.

Hallberg, R.L., and Smith, D.C., 1975. Ribosomal protein synthesis

in Xenopus laevis oocytes. Devel. Biol. 42: 40-52.

Heinrich, P.C., Raydt, G., Puschendorf, B., and Jusić, M., 1976. Subcelluar distribution of histone-degrading enzyme activities from rat liver. Eur. J. Biochem. 62: 37-43.

Hnilica, L.S., 1972. In Structure and Biological Function of Histones. Weast, R.C. ed. CRC press. Ohio. pp. 3-78.

Hnilica, L.S., 1967. Proteins of cell nucleus. Progr. Nucl. Acid. Res. Mol. Biol. 7:25

Hnilica, L.S., Ewards, L.J., and Hey, A.E., 1966. Studies on nuclear proteins. II. Quantitative distribution of histone fractions in various tissues. Biochem. Biophys. Acta. 124:109-117.

Houston, L.L., 1971. Amino acid analysis of stained bands from poly-acrylamide gels. Anal. Biochem. 44: 81-88

Immers, J. 1972. Changes within the chromatin during sea urchin embryo-genesis. Exptl. Cell. Res. 72: 150-156.

Imoh, H., and Minamidani, T., 1973. Changes in protein during develop-ment of Triturus embryos. I. Contents and synthesis of soluble or basic protein of cell components. J. Embryol. Exptl. Morph. 30: 649-659.

Johns, E.W., 1976. Fractionation and isolation of histones. In Sub-nuclear Components Preparation and Fractionation, Birnie, G.D., ed., Butterworths, London. pp. 187-208.

Johns, E.W., 1971. The preparation and characterization of histones. In Histones and Nucleohistones. Phillips, D.M.P., ed., Plenum Publish-ing Co., Ltd., N.Y. pp. 1-45.

Johns, E.W., and Butler, J.A.V., 1962. Further fractionation of histones from calf thymus. Biochem. J. 82: 15-18.

Johns, E.W., Phillips, D.M.P., Simson, P., and Butler, J.A.V., 1961. The electrophoresis of histones and histone fractiona on starch gel. Biochem. J. 80: 189-193.

Johnson, A.W., and Hnilica, L.S., 1971. Cytoplasmic and nuclear basic protein synthesis during early sea urchin development. Biochim. Biophys. Acta. 246: 141-154.

Kasinsky, H.E., Bols, N.C., Byrd, Jr. E.W., Huang, S.Y., Kwauk, S., and Sweeney, M.A.J., 1975. On the diversity of sperm histones in the verte- brates. J. Cell. Biol, 67: 202a.

Kasinsky, H.E., Bols, N.C., Byrd, Jr. E.W., and Sweeney, M.A.J., 1974. Comparative aspects of testicular histones in anura spermatogenesis. J. Cell. Biol. 63:163a.

Kasinsky, H.E., Huang, S.Y., Kwauk, S., Mann, M., Sweeney, M.A.J., and Yee, B., 1977. On the diversity of sperm histones in the vertebrates: III. Electrophoretic variability of testis-specific histone patterns in Anura contrasts with relative constancy in Squamata. J. Exptl. Zool. in press.

Kischer, C.W., and Hnilica, L.S., 1967. Analysis of histones during organogenesis. Exptl. Cell. Res. 48: 424-429.

Kischer, C.W., Gurley, L.R., and Shephard, G.R., 1966. Nuclear histones and early embryogenesis of chick. Nature, 212: 304-309.

Kornberg, R.D., 1977. Structure of chromatin. Ann. Rev. Biochem. 46: 931-954.

Kornberg, R.D., and Thomas, J.O., 1974. Chromatin structure: oligomers

of histones. Science, 184: 865-867.

Kossel, A., 1928. In the Protamines and Histones. Longmans Green Co., London.

Ling, V., Jergil, B., and Dixon, G.H., 1971. The biosynthesis of protamine in trout testis. III. Characterization of protamine components and their synthesis during testis development. J. Biol. Chem. 246: 1168-1176.

Louie, A.J., 1968. Biology of the histones and protamines. A Ph.D. thesis. University of British Columbia, Vancouver, B.C.

Louie, A.J., and Dixon, G.H., 1972. Trout testis cells. I. Characterization by deoxyribonucleic acid and protein analysis of cells separated by velocity sedimentation. J. Biol. Chem. 247: 5490-5497.

Louie, A.J., Candido, E.P.M., and Dixon, G.H., 1973. Enzymatic modifications and their possible roles in regulating the binding of basic proteins to DNA and in controlling chromosomal structure. Cold. Spring. Habor. Symposium. 38: 803-819.

Marushige, K., and Dixon, G.H., 1969. Developmental changes in chromosomal composition and template activity during spermatogenesis in trout testis. Devel. Biol. 19: 397-414.

Masui, Y., 1968. Analysis of amphibian yolk protein by starch gel electrophoresis. SABCO. J. 4:44-54.

Moore, S., and Stein, W.H., 1963. Chromatographic determination of amino acids by the use of automatic recording equipment. Methods Enzymol. 6:819-931.

Murray, K., 1966. The acid extraction of histones from calf thymus deoxyribonucleoprotein. J. Mol.Biol. 15: 409-419.

Nakano, M., Tobita, T., and Ando, T., 1976. Studies on a protamine (Galline) from fowl sperm. 3. The total amino acid sequence of intact galline molecule. Int. J. Peptid. Prot. Res. 8: 565-578.

Neelin, J.M., and Butler, G.C., 1961. A composition of histones from chicken tissues by zone electrophoresis in starch gel. Can. J. Biochem. Physiol. 39: 485-489.

Neelin, J.M., and Vadali, G., 1968. Ribosomal proteins from goose reticulocytes are not histones. Can. J. Biochem. 46: 1507-1514.

Nieuwkoop, P.D., and Faber, J., 1967. Normal Table of Xenopus Laevis (Daudin). North-Holland Publishing Co., Amsterdam.

Olins, A.J., and Olins, D.E., 1974. Spheroid chromatin units (ν bodies). Science. 183: 330-332.

Pallotta, D., and Tessier, A., 1976. Amino acid composition of sperm histones in the house cricket (Acheta domesticus). Can. J. Biochem. 54: 56-88.

Panyim, S., and Chalkley, R,, 1969a. High resolution gel electro-phoresis of histones. Arch. Biochem. Biophys. 130: 337-346.

Panyim, S., and Chalkley, R., 1969b. The heterogeneity of histones. I. A quantitative analysis of calf histones in very long poly-acrylamide gels. Biochem. 8: 3972-3975.

Panyim, S., Bilek, D., and Chalkley, R., 1971a. An electrophoretic comparison of vertebrate histones. J. Biol. Chem. 246: 4206-4215.

Panyim, S., Sommer, K.R., and Chalkley, R., 1971b. Oxidation of the cysteine-containing histone F3. Detection of an evolutionary mu-tation in a conservative histone. Biochem. 10: 3911-3917.

Panyim, S., Chalkley, R., Spiker, S., and Oliver, D., 1970. Cons-tant electrophoretic mobility of the cysteine-containing histone in plants and animals. Biochim. B iophys. Acta. 214: 216-221.

Picheral, E., 1970. Nature et évolution des proteins basique au cours de la spermiogeneses chez Pleurodeles waltlii Michah., amphibien urodele. Histochemie. 23: 189-206.

Pogo, B.G.T. , Pogo, A.O., Allfrey, V.G., and Mirskey, A.E., 1968. Changing pattern of histone acetylation and RNA synthesis in regeneration of the liver. Proc. Natl. Acad. Sci. U.S. 59: 1337-1344.

Richards, B.M., and Pardon, J.F., 1970. The molecular structure of nucleohistone. Exptl. Cell. Res. 62: 184-196.

Risley, M.S., and Eckhardt, R.A., 1975. Basic protein changes during spermiogenesis in Xenopus laevis. J. Cell. Biol. 67: 362a.

Roach, D., and Gehrke, C.W., 1970. The hydrolysis of proteins. J. Chromatog. 52: 393-404.

Ruderman, J.V., Baglioni, C., and Gross, P.R., 1974. Histone mRNA and histone synthesis during embryogenesis. Nature, 247: 36-38.

Searl, R., and Aronson, A., 1973. Chromatin associated proteins of developing sea urchin embryo. II. Acid-soluble proteins. J. Mol. Biol. 75: 647-658.

Setterfield, G., and Neelin, J.M.,1972. Effects of sequential extraction of histone proteins on structural organization of avian erythrocyte and liver nuclei. Exptl. Cell. Res. 74: 27-41.

Ševaljević, L., 1973. Developmental changes of sea urchin histones. Wilhelm. Roux. Archiv. 174: 210-214.

Sherod, D., Johnson, G., and Chalkley, R., 1974. Studies on the heterogeneity of lysine-rich histones in dividing cells. J. Biol. Chem. 249: 3923-3931.

Spiker, S., Key, J.L., and Wakim, B., 1976. Identification and fractionation of plant histones. Arch. Biochem. Biophys. 176: 510-518.

Stebbins, R.C., 1966. A Field Guide to Western Reptiles and Amphibians, Houghton Mifflin Co.,Boston.

Stellwagen, R.H., and Cole, R.D., 1969. Chromosomal proteins. Ann. Rev. Biochem. 38: 951-992.

Stenroos, O.O., and Reichard, S.M., 1970. Metamorphic alternations in nuclear RNA and histones. Am. Zool. 10: 321-328.

Subirana, J.A., 1975. On the biological role of basic proteins in spermatozoa and during spermiogenesis. In The Biology of Male Gamete. (Duckett, J.G. and Racey, D.A.,eds.) . Academic Press. London. pp. 239-244.

Subirana, J.A., 1973. Studies on the thermal denaturation of nucleohistones. J. Mol. Biol. 74: 363-386.

Subirana, J.A., and Puigjaner, L.C., 1973. X-ray diffration studies of nucleoprotamines from molluscs. In Conformation of Biological Molecules and Polymers. The Jerusalem Symposia on Quantum Chemistry and Biochemistry. Bergman, E.D. and Pullman, eds. The Israel Academy of Science and Humalities. Jerusalem. Vol. V. pp. 645-653.

Subirana, J.A., Cozcolluela, C., Palau, J., and Unzeta, M., 1973. Protamines and other basic proteins from spermatozoa of molluscs. Biochim. Biophys. Acta. 317: 364-379.

Sud, B.N., 1961. Morphological and histochemical studies of chromatois body in the grass snake, Natrix natrix. Quart. J. Microscop. 102:51-58.

Sung, M.T., Dixon, G.H., and Smithies, O., 1971. Phosphorylation and synthesis of histones in regenerating rat liver. J. Biol. Chem. 246: 1358-1364.

Sung, M.T., Harford, H., Bundaman, M., and Vidalakas, G., 1977. Metabolism of histones in avian erythroid cells. Biochem. 16: 279-285.

Sung, M.T., and Smithies, O., 1969. Differential elution of histones from gel-trapped nuclei. <u>Biopolymers</u>. 7: 39-58.

Tristran, G.R., 1939. The basic amino acids of leaf proteins with a discussion of various methods of analysis. <u>Biochem</u>. <u>J</u>. 33: 1271-1281.

Vendrely, R., 1957. Données récentes sur la chimie de l'ADN et des deoxyribonucleoproteins. <u>Archiv</u>. <u>der</u> <u>Julius</u> <u>Klaus-Stiff</u>. <u>Vererbungsforsch</u>., <u>Sozialanthropol</u>. <u>u</u>. <u>Rassenhyg</u>. 32: 538-553.

Vendreley, R., Genty, N., and Coirault, Y., 1965. Étude comparée d'histones d'èrythrocytes chez diverses espèces animales. <u>Bull</u>. <u>Soc</u>. <u>Chim</u>. <u>Biol</u>. 47: 2233-2237.

Wallace, R.A., and Karasaki, S., 1963. Studies on amphibian yolk. J. <u>Cell</u>. <u>Biol</u>. 18: 153-166.

Woodcock, C.Z.F., 1973. Ultrastructure of intact chromatin. <u>J</u>. <u>Cell</u>. <u>Biol</u>. 59: 368a.

Woodland, H.R., and Adamson, E.D., 1977. The synthesis and storage of histones during the oogenesis of Xenopus laevis. <u>Devel</u>. <u>Biol</u>. 57: 118-135.

Wray, W., and Stubblefield, E., 1970. A highly sensitive procedure for detection of histones in polyacrylamide gels. <u>Anal</u>. <u>Biochem</u>. 38: 454-460.