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PERMANENT ADDRESS:

.. 25. Parkway. Forest. Drive..

.. Apartment. 1712.....

.. Willowdale. Ontario.....

DATED... November. 4 1976

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Supervisor: J.M. Neelin

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THE HISTONES OF FISH ERYTHROCYTES

by

BRIAN LEO ALLAN MIKI, B.Sc.

A thesis submitted to the Faculty of
Graduate Studies in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Carleton University

Ottawa, Ontario

September, 1976

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The undersigned hereby recommend to the Faculty
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fulfilment of the requirements for the degree of
Doctor of Philosophy.

.....
Chairman, Department of Biology

.....
Supervisor

.....
External Examiner

To my parents and my wife .

ABSTRACT

Erythrocyte-specific histone (H5) has been extensively studied among several bird species and has been established as a prominent and universal feature of these nucleated erythrocytes but the existence of H5-like proteins in nucleated erythrocytes of other vertebrates has not been settled. It has been suggested that H5 in birds functions in chromatin condensation and genome inactivation during avian erythroid cell maturation. Since the fish erythrocytes mature like the avian erythrocytes, the role of this histone in general erythropoiesis can be assessed in these primitive and diverse vertebrate species.

The erythrocyte histones of carp (Cyprinus carpio), trout (Salmo gairdnerii), perch (Perca flavescens), black crappie (Pomoxis nigromaculatus) and white sucker (Catostomus commersoni) were extracted under conditions which minimize proteolysis, and individual histones were examined by polyacrylamide gel electrophoresis. Each fraction was isolated by cation-exchange chromatography followed by exclusion chromatography or selective precipitation before final identifications and further characterizations were made by amino acid analysis.

Histones H3 and H4 of carp and trout were indistinguishable and in no cases displayed species specificity, even when compared to chicken erythrocyte H3 and H4. In contrast, H2A and H2B of carp, trout, and white sucker differed in electrophoretic mobilities and as a group could be distinguished from avian H2A and H2B. However, no differences among these fish histones were evident in amino acid compositions and the relative proportions of H2A and H2B appeared to be uniform among the fish species.

A protein homologous to H5 was isolated and identified among the erythrocyte histones from carp, trout, perch and black crappie, but not from white sucker. Densitometric profiles of polyacrylamide gels revealed extensive differences in the relative proportions of H5 in the various species. Since the relative levels of H5 were generally inverse to those of H1, functional similarities may exist between these two proteins. The apparent absence of H5 in the typical erythrocytes of sucker and minute levels in those of carp implies that H5 is not required for normal erythropoiesis. In these particular cells, however, a particular subfraction of H1 may perform H5-like functions despite their highly different amino acid compositions.

Although the fish H1 displayed the most divergent amino acid compositions, the fish H5 were also highly variable and exhibited greater divergence than the avian H5 homologues reported to date. The fish H5 as a group also differed from avian H5 in their generally greater relative mobilities in polyacrylamide gels, their easier elution from cation-exchange columns and their amino acid compositions. Threonine is somewhat elevated but serine, arginine, glutamic acid and histidine tend to be lower than in avian H5. The fish H5 examined here resemble cell-specific homologues of H5 from extremely diverse species. In consideration of a broad spectrum of H5-like proteins, the avian H5 are the most extreme.

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LIST OF ABBREVIATIONS

deoxyribonucleic acid	(DNA)
ethylenediamine tetraacetate	(EDTA)
guanidinium chloride	(GuCl)
histone 1	(H1, I, f1)
histone 5	(H5, V, f2c)
histone T	(H6)
histone 2A	(H2A, IIb1, f2a2)
histone 2B	(H2B, IIb2, f2b)
histone 3	(H3, III, f3)
histone 3 dimer	(H3d)
histone 3 monomer	(H3m)
histone 4	(H4, IV, f2a1)
hydrochloric acid	(HCl)
optical density	(OD)
phenylmethanesulfonyl fluoride	(PMSF)
ticainemethanesulfonate	(MS-222)
trichloroacetic acid	(TCA)

I. INTRODUCTION

Histones are a group of small, basic structural proteins, other than protamines, which are associated with the DNA of eukaryotic cells, usually in equal amounts by weight. Among proteins, histones are distinctive in their high content of basic amino acids, lysine and arginine, which account for their alkaline isoelectric points. They are often identified by their lack of tryptophan. The histones range in size from 102 to 212 amino acid residues (Hnilica 1972).

Generally, there are five common molecular species of histones, H1, H2A, H2B, H3 and H4 (Bradbury 1975), differing in primary sequence, size, overall charge and distribution of basic amino acids (Hnilica 1972, Johns 1971). The evolutionary stability of the histones and their maintainance in stoichiometric proportions to DNA is well documented. The H4 fraction from pea and calf differ only in two of 102 total amino acids and both are conservative replacements (DeLange et al. 1969). H3 from these species differs by only 4 of 135 total amino acids (Patthy et al. 1973) and carp H3 differs from calf by only one amino acid (Hooper et al. 1973).

Histone H2A displays slightly more divergence than H3 or H4. Between trout testis H2A (Bailey and Dixon 1973) and calf thymus H2A (Sautière et al. 1975), there are four substitutions, two deletions and one insertion in 129 total amino acids. Although the sequence of sea

urchin H2A is only partially completed, enough differences are revealed to suggest further divergence (Wouters-Tyrou et al. 1974). The lysine-rich histone (H1) differs markedly from the other histones in that it displays partial species and tissue specificity in the expression of genetically distinct subfractions (Bustin and Cole 1968, Kinkade and Cole 1966a,b, Bustin and Stollar 1973, Sluyser and Bustin 1974). Homologous subfractions of rabbit and calf thymus H1 display 10-20% differences within the first 73 residues of the amino terminus (DeLange and Smith 1975). Extreme selective pressures have probably maintained the precise sequence of amino acids in the arginine-rich histones H3 and H4. This suggests a crucial function for every portion of these molecules. Less stringent compositional requirements have resulted in more changes in H2A and H2B (DeLange and Smith 1975) and especially in H1.

Histones H2A, H2B, H3 and H4 appear to function in the packaging of DNA into structural subunits (ie. nucleosomes) of chromatin (Thomas and Kornberg 1975, Olins and Olins 1974, Olins et al. 1975, Oudet et al. 1975, Baldwin et al. 1975, Honda et al. 1975). The construction of these subunits must depend on the conserved sequences of the histones and the proper spatial arrangements of their interacting surfaces.

Certainly, the information for assembly is inherent in the histones rather than the DNA sequences, since the source of DNA is not important in reconstituted subunits (Olins et al. 1975). The above investigations revealed that H1 binds to regions of DNA outside of the nucleosomes and may act to cross-link them into a higher order of chromatin organization (Bradbury et al. 1973). Whether this function requires less sequence conservation due to less intimate interactions is not known. The occurrence of H1 subfractions may suggest different and evolving regulatory and structural functions for these proteins, however, no specificity in H1 subfraction distribution along chromatin is noticeable (Zick et al. 1975).

How the histones evolved to meet the challenge of eukaryote DNA packaging is difficult to ascertain, since the histones from primitive eukaryotes, such as ciliates and yeasts are similar to those of the higher animals (Caplan 1975, Gorovsky et al. 1974, Goff 1976). Furthermore, rather typical chromatin subunits exist in the yeasts, *Neurospora crassa* (Noll 1976) and *Aspergillus nidulans* (Morris 1976). Sequence homologies among H2A, H3 and H4 perhaps imply that these proteins share a common ancestor (Bauer 1975). The discovery of cysteine in sea urchin H4 as well as further sequence homologies between this and calf H4 and H3 from calf and chicken,

reinforce this conclusion (Strickland et al. 1974). Likewise, limited sequence homologies between H1 and H2B may imply their common origin (DeLange and Smith, 1975). Divergence, followed by parallel evolution, must have been a very early event.

The non-histone proteins have been known for many years as a complex mixture of structural and enzymic proteins, among which specific regulators of gene activity are believed to occur (Paul and Gilmour 1975, O'Malley and Means 1974). Lately, some of these proteins were found to bear similarities to histones. A group of non-histone proteins co-extracted with H1 and designated HMG1 and HMG2 are composed of 26% basic amino acids and 25% acidic amino acids. They weigh about 26,000 daltons. The distribution of acidic amino acids is weighted to the carboxyl-terminus and the protein will bind nonspecifically to DNA (Johns et al. 1975, Walker et al. 1976a,b). Although no common sequences between these proteins and histones have been noted (Johns et al. 1975), the proteins are similar to H1 in their high content of lysine. A nucleolar protein designated A24 (Orrick et al. 1973) contains all the tryptic and chymotryptic peptides of histone 2A but is almost double its size (Goldknopf and Busch 1975). Whether A24 is an independent protein with H2A sequences over half its length or a histone 2A precursor

is undecided, but metabolic studies have not revealed a simple precursor-product relationship (Goldknopf and Busch 1975). These scanty examples of non-histone proteins resembling histones may indicate the existence of a pool of genes for chromosomal proteins, outside of the normal histone coding sequences (Kedes 1976), which are continuously evolving but which escape detection among the complex array of non-histone proteins and histone degradative peptides.

The demonstration of only five universal histones was very significant since the initial illustration of histone heterogeneity by the Stedmans' implied that histones may function in specific gene expression (Stedman and Stedman 1950). Such a function would require considerable tissue specificity among the histones. The replacement of spermatozoan histone by protamine, known since 1895 (Luck 1964), provided an extreme example of such specificity. Further evidence was found in chicken erythrocytes which contained an arginine-rich component not common to other tissues such as thymocytes or lymphocytes (Stedman and Stedman 1950). The results of starch gel electrophoresis supported the hypothesis of histone heterogeneity and suggested again that a tissue-specific histone might be associated with chicken erythrocytes (Neelin and Connell 1959, Neelin and Neelin 1960, Neelin and Butler 1961). Finally, the isolation

of different histone fractions was achieved by cation-exchange chromatography on Amberlite IRC-50 (Crampton et al. 1950) with guanidinium chloride as eluant (Luck et al. 1956). Generally, these techniques revealed only five common histones which alone were insufficient for specific regulation of genes; yet it clearly demonstrated the existence of an additional cell-specific histone (H5) in avian erythrocytes (Neelin 1964).

H5 was isolated by cation-exchange chromatography on Amberlite IRC-50 eluted with GuCl (Neelin 1964; Neelin et al. 1964) or by carboxymethylcellulose followed by Sephadex G-75 exclusion chromatography (Hnilica 1964). This protein had a distinctive amino acid composition, containing high levels of lysine, arginine, alanine and serine. On the basis of its characteristic electrophoretic mobility on starch gels H5 was recognized among the erythrocyte histones of duck, goose, turkey, pigeon and gulls but not in spleens or marrows of duck, goose and turkey (Neelin 1968). Similar results were obtained with polyacrylamide gel electrophoresis (Purkayastha and Neelin 1966). The presence of H5 only in the terminally differentiated nucleated erythrocytes of birds implied a specific function for this histone in erythroid cell maturation.

The erythrocytes of lower vertebrates, unlike mammals, retain their nuclei, even beyond the final stages

of terminal differentiation. In birds, this process has been well characterized. The series of erythropoietic events, after the determination of the stem cells, includes a cessation of DNA synthesis in blast cells, followed by a progressive inhibition of general RNA synthesis, leaving a selective transcription of globin mRNA, which ceases prior to the dwindling of protein synthesis. This wave of events is accompanied by a progressive aggregation of condensed chromatin and shrinkage in nuclear volume, a loss of cytoplasmic organelles and changes in cell shape (Sinclair and Brasch 1975, Lucas and Jamroz 1961, Cameron and Prescott 1963, Cameron and Kastberg 1964, Williams 1972, Brasch et al. 1974, Sanders et al. 1973, Ruiz-Carrillo et al. 1974, Lucas and Jamroz 1961).

The detailed involvement of H5 in these erythropoietic events has not been elaborated, but the correlation of its appearance with particular changes is important for its functional interpretation. H5 exists in the earliest erythroid cells yet obtained. Erythroblast-enriched populations of birds suffering from erythroblastosis (Sotirov and Johns 1972), mixed immature populations of cells from phenylhydrazine treated birds (Dick and Johns 1969, Mazen and Champagne 1972) or fractions of erythroblast enriched cells obtained by buoyant density centrifugation (Billet and Hindley 1972, Appels et al. 1972, Adams and Neelin 1972) exhibit the presence of

H5. Similarly, H5 has been demonstrated in the early stages of primitive erythropoiesis (Moss et al. 1973). Among these cells DNA, RNA and protein synthesis still continue. Also, condensed chromatin aggregation and hemoglobin accumulation have not been completed, therefore the presence of H5 alone cannot account for these events.

Several models may account for the ineffectiveness of the early H5 in immature cells. It is generally agreed that a lower level of H5 exists in immature cells and that net addition of H5 occurs during maturation. Biochemical studies reported the selective incorporation of radioactive amino acids into H5 in maturing erythrocytes (Lunn and Neelin 1968, Appels and Wells 1972). Other reports, however, showed an accumulation of radioactive amino acid into erythroblast H5 (Seligy et al. 1973). In either case, H5 synthesis could not be tightly coupled to DNA synthesis like the other histones (Seligy et al. 1973) and this accumulation of H5 may contribute to subsequent chromatin condensation and genome inactivation.

Not only has it been illustrated that H5 synthesis occurs in maturing cells by isolation of the H5 mRNA (Scott and Wells 1975), but it has been proposed that H5 alone is turning over (Appels and Wells 1972). It is possible that maturation may be the consequence of

turnover deceleration which results in the template blockage and chromatin condensation.

H5 is modified by phosphorylation of specific serine residues (Seligy and Neelin 1971, Adams et al. 1970). Presumably the added phosphates counteract the basicity of the H5, thus reducing the strength of its binding to DNA. Since the extent of phosphorylation may be inversely related to the degree of erythrocyte maturity, then binding of H5 to DNA may become progressively stronger thus accounting for chromatin condensation and transcriptional blockage (Brasch et al. 1974). Acetylation may act similarly since H1 and H5 are the only histones not acetylated internally in mature duck erythrocytes (Sanders et al. 1973).

Although changes in nuclear acidic proteins are known to accompany erythrocyte maturation (Dingman and Sporn 1964, Harlow and Wells 1975, Ruiz-Carrillo et al. 1974, Vidali et al. 1973, Shelton and Neelin 1971), no specific protein has been shown to alter H5 binding in immature cells, therefore the participation of non-histone proteins in the maturation events is not understood.

Of particular interest is the suggestion that the amount of condensed chromatin of primitive line erythrocytes does not change during maturation (Dardick and Setterfield 1976). Morphometric measurements revealed

40
that nuclear shrinkage and reduction of regions between condensed chromatin creates the illusion of progressive condensation. In fact this process reflects the aggregation of condensed chromatin. If condensation has thus begun very early, a direct relationship between H5 presence and degree of condensation may indeed exist and the above models which account for the lack of early condensation may not be required.

The direct participation of H5 in chromatin condensation has been demonstrated several times. The selective removal of H5 from erythrocyte nuclei visibly loosens the supercoiled 200 Å chromatin fibrils (Brasch et al. 1972, Brasch et al. 1974). Similarly H1 and H5 were assigned the responsibility for maintaining condensed chromatin, as revealed by its precipitability, after selective extraction and reassociation of H1 and H5 with chromatin (Billet and Barry 1974). Condensation, indicated by the degree of DNA base tilting, was studied by circular dichroism of DNA and DNA intercalating dye, ethidium bromide (Williams et al. 1972). H5 removal from chromatin contributed most significantly to the drop in ellipticity. Similarly, the release of ethidium bromide and actinomycin D during thermal denaturation of chromatin revealed that H1 and H5, but not H1 alone, determined the thermal transitions distinguishing the mature erythrocyte chromatin from those of liver, kidney

or immature erythrocytes (Lurquin and Seligy 1976). The evidence indicates the direct involvement in H5 in chromatin condensation.

Although H5 is not a component of the chromatin subunits (Olins et al. 1975, Oudet et al. 1975, Kornberg et al. 1975, Seligy et al. 1976, Weintraub et al. 1975, Ramsay Shaw et al., 1976), it may supplant or intensify the cross-linking function of H1 in erythrocyte chromatin, rather than functioning in the gross distribution of chromatin (Brasch et al. 1972).

The importance of H5 in restricting the template capacity of erythrocyte chromatin with exogenous polymerase has been illustrated with acid-extracted chromatin (Seligy and Neelin 1970, Williams et al. 1972) and salt-extracted chromatin (Gasaryan and Andreeva 1972, Seligy and Miyagi 1974). In both cases H5 removal resulted in a dramatic increase in template capacity. Reconstitution of H5 to partially deproteinized chromatin showed H5 to be a stronger template inhibitor than H1 (Gasaryan et al. 1976).

Whether the specificity of transcription is maintained by isolated chromatin transcribed by exogenous polymerase is important for studies in regulation of gene expression. Complementary DNA prepared against globin mRNA provides a sensitive probe for measuring the specific transcription of the globin genes. Evidence

is accumulating that erythrocyte chromatin maintains its specific pattern of gene expression compared to liver chromatin (Axel et al. 1973, Barrett et al. 1974, Paul and Gilmour 1975), and that reconstituted chromatin also maintains the specificity dictated by the source of the non-histone proteins rather than the histones. (Barrett et al. 1974, Paul and Gilmour 1975). Although the removal of H5 increases the number of polymerase binding sites in reticulocyte chromatin, there apparently results a tenfold decrease in the concentration of globin mRNA transcript (Felsenfeld et al. 1975). Histone 5, therefore, does not confer any recognizable specificity with its template restriction.

The appearance of H5 in erythrocytes represents an unusual deviation from the other common histones, but it is not alone in the categorie of cell-specific or species-specific histones (Hnilica 1972). A similar lysine-rich histone has been found in sea urchin sperm which also experiences nuclear inactivation and chromatin condensation without replacement by protamines (Palau et al. 1969). This $\phi 1$ histone from various marine invertebrates is homologous to calf thymus H1 in charge distribution and conformation, but exists in higher quantities. It contains variably elevated arginine levels (Puigdomènech et al. 1975), directly related to the respective contracted states of the chromatins (Puigdomènech et al. 1976) in the various species.

Such parallels in similar cell systems might indicate that these specialized histones have common ancestors with H1, or may have evolved from H1 subfractions with higher arginine contents. The presence of H1 subfractions may have removed the selective pressures from the H1 proteins and allowed them to evolve specialized functions, which have become stabilized in particular tissues only. The erythrocyte-specific H1 subfraction, Flb, of sea turtle, may be the intermediate of such a process (Tsai and Hnilica 1975). Since the other histones are so crucial to subunit assembly and show no subfractions, selective pressures have restricted their evolution.

Several similarities between H1 and H5 have already been noted (Greenaway and Murray 1971). H5 has relatively large molecular weight of 21,450 daltons (Garel et al. 1975) which is comparable to 22,000 daltons for H1 (Hnilica (1972)). Both have a similar distribution of basic amino acids in the amino terminus (Garel et al. 1975). Long segments of sequence homologies are also apparent (Sautière et al. 1975) and there apparently exists several similar tryptic peptides between H5 and Flb (Tsai and Hnilica 1975). Both H1 and H5 are lysine-rich, have internal phosphoserines, and are not acetylated internally (Sanders et al. 1973). Both have at least partially masked acetylated amino terminal residues (Seligy et al. 1976b). They appear to function similarly

by cross-linking chromatin subunits and within chromatin they are similarly accessible to attack by exogenous proteases (Weintraub et al. 1975). Given these and other similarities, the proposition that H5 is functionally replacing H1 in erythrocytes (Greenaway and Murray 1971) is plausible. If the two proteins have a common origin, then divergence must have occurred early since H1 and H5 are immunospecifically distinct (Mura et al. 1974) and have different primary sequences (Seligy et al. 1976b, Sautière et al. 1975).

The presence of H5 appears to be an essential feature of erythrocyte differentiation and maturation in birds; however, nucleated erythrocytes are common to all the lower vertebrates, and are even found in the cartilaginous hagfish (Sekhon and Maxwell 1970) and the invertebrate sipunculid worms (Mazen and Champagne 1976). The metabolic events involved in progressive inactivation of amphibian erythrocyte nuclei has been characterized in detail in newts (Grasso and Woodard 1967, Grasso and Woodard 1966) and frogs (Hilder et al. 1975, Thomas and McLean 1975, DeWitt et al. 1972). They parallel very closely the events described in avian erythrocytes. Histological and cytological studies illustrate also that fish erythrocytes develop similarly and that nuclear shrinkage and condensed chromatin aggregation also characterize the later stages of erythrocyte maturation (Sekhon and Beams 1969, Catton 1951,

Jakowska 1956, Weinreb 1963, Watson et al. 1962). If H5 is essential for the maturation of nucleated erythrocytes, its presence should be universal among them.

Early surveys by starch gel electrophoresis of erythrocyte histones of three bird species, two reptilian species, two amphibian species and three fish species revealed bands analogous to H5 (Vendrely et al. 1965). Later analysis of lysine-rich histone extracts of chicken and trout erythrocyte histone demonstrated an elevated arginine content suggesting the presence of H5, but in snake arginine levels were low (Vendrely et Picaud 1968). Since independent analyses of trout liver histone did not reveal high arginine levels in H1 preparations (Palau and Butler 1966), H5 presence in trout erythrocytes was likely. Eventually, H5 fractions which bear strong resemblances to chick H5 were isolated from fish (*Caranx hippos*) and frog (*Rana catesbeiana*) erythrocytes. They yielded similar amino acid compositions, with variability in levels of lysine, arginine and serine (Edwards and Hnilica 1968).

In contrast, an extensive survey of histones using high resolution polyacrylamide gel electrophoresis revealed H5 bands among avian erythrocyte histone but not in erythrocytes of several species of reptiles, amphibians or fish including buffalo fish, pike and carp

(Panyim et al. 1971). Fractionation of buffalo fish histone did not uncover H5 but the action of an active protease generated bands which migrated with electrophoretic mobilities comparable to that of avian H5. The activity of this protease was directly correlated with the state of health of the fish (Panyim et al. 1971). The authenticity of H5 from erythrocytes of species other than birds was thus suspect.

Central to the interpretation of the biological importance of H5 is its universality in the nucleated erythrocytes of the lower vertebrates. If H5 is indeed specific to avian erythrocytes, its mere presence may not be important to the events of general erythroid cell differentiation or maturation. Its function may be substituted by other proteins or related to subtle physiological differences between the birds and other lower vertebrates. For these reasons, an investigation of H5 occurrence among the erythrocytes of fish, which are phylogenetically the most primitive vertebrates, was undertaken. This report describes the results of this investigation.

II. MATERIALS AND METHODS

Rainbow trout (*Salmo gairdnerii*) were obtained from Normandelle and Shanandoe trout hatcheries and Thistle Springs Trout Farm in Ontario. Carp (*Cyprinus carpio*), perch (*Perca flavescens*), black crappie (*Pomoxis nigromaculatus*) and white sucker (*Catostomus commersoni*) were captured in the Rideau Canal, Ottawa, Ontario. All animals were maintained in large holding tanks with a continuous flow of water and nourished with Purina Trout Chow (Ralston Purina Co., St. Louis, Mo.).

The fish were handled under the influence of MS-222 (Sandoz Pharmaceuticals, Dorval, P.Q.) (Bell 1964, Blaxhall and Daisley 1973).

A. Blood Collection

Fish blood was collected directly into ice cold Alsevers solution (Bukantz et al. 1946) from the caudal artery by severing the caudal peduncle. Isotonic saline supplemented with sodium citrate or ethylenediamine tetraacetate (EDTA) resulted in hemolysis, as previously noted (Sanders 1970), but Cortlands solution (Wolf 1963) with EDTA (5.0 mg./ml) provided an effective alternative to Alsevers solution for collecting and washing blood cells.

When 50 mM sodium bisulfite was used to inhibit proteolytic enzymes, it was dissolved in the media just before use (Panyim et al. 1971). When 1.0 mM

phenylmethanesulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, Mo) was used as enzyme inhibitor, a 50 mM stock solution in isopropanol was diluted before chilling (Chong et al. 1974).

Geese and chicken were anaesthetized with a subcutaneous injection of nembutol and blood was collected from the jugular vein into cold isotonic saline with sodium citrate as anticoagulant (Purkayastha and Neelin 1966).

Fresh blood smears were taken immediately after bleeding, air dried, then stained with May-Grünwald and Giemsa stains (Lucas and Jamroz 1961).

Blood cells were washed two or three times in a large excess of cold collecting medium, by centrifugation at 755 X g for 10 minutes, followed by cell resuspension. The buffy coat was removed from the surface of the cell pellets by aspiration during the successive cell washes.

B. Isolation of Cell Nuclei

The washed fish erythrocytes were lysed by resuspending them in excess 0.6% saponin (British Drug Houses) in 0.32 M sucrose-10 mM calcium chloride, pH 6.0 and stirring for 30-40 minutes at 0°C, followed by 10 full passes of a tight-fitting B pestle in a Dounce-type homogenizer (Kontes Glass Co., Vineland, N.J.). The appropriate protease inhibitors were employed in the media

as described previously. The nuclei were sedimented by centrifugation at 12,100 X g for 10 minutes, then subsequently washed by three successive resuspensions into 0.32 M sucrose-10 mM calcium chloride with a loose-fitting Dounce homogenizer. Each step of the procedure was monitored by phase-contrast microscopy. The length of time required to attain clean nuclei starting from the sacrifice of the animals seldom exceeded 4.5 hours.

Goose and chicken nuclei were prepared according to established protocol (Purkayastha and Neelin 1966). Erythrocytes were stirred at 0°C for 30 minutes in 0.3% saponin in isotonic saline, then the nuclei washed in isotonic saline as described above. Protease inhibitors were not necessary when isolating avian erythrocyte nuclei.

Following blood collection, livers were dissected from the fish and placed in ice-cold Cortlands solution containing 0.32 M sucrose and 1.0 mM PMSF. They were trimmed, minced, washed through cheesecloth to remove residual erythrocytes, then homogenized for 45 seconds at medium speed on a Virtis "23" homogenizer, in the above medium. The homogenates were filtered through four layers of cheesecloth and the filtrate centrifuged at 270 X g, for 10 minutes. The nuclei were washed

repeatedly by gentle resuspension into excess Cortlands solution supplemented with 0.32 M sucrose, 1.0 mM PMSF, and 0.05% Triton X-100 (British Drug Houses).

C. Histone Extraction and Modification

Initially, trout and carp nuclei were washed with 10 mM citric acid-0.32 M sucrose-10 mM calcium chloride, at pH 2.7, however, extraneous proteins were not removed in significant quantities by this procedure and it was discontinued. Histones were extracted from the nuclear pellet by three successive homogenizations in ice-cold 0.25 N HCl (hydrochloric acid) for 45 seconds on a Virtis "23" homogenizer (Gardiner, N.Y.) or by stirring for 30 minutes. The nuclear residue was separated by centrifugation at 12,000 X g and the combined supernatants were clarified by centrifugation at 17,300 X g for 20 minutes. The lack of TCA precipitable proteins in subsequent extracts and the absence of bands on electrophoresis in the fourth extracts suggested that the initial three extractions gave complete removal of histones. Goose and chicken nuclei were extracted similarly in 0.20 N hydrochloric acid (Purkayastha and Neelin 1966).

When required, H3 was oxidized to the dimer by a slight modification of the method described by Marzluff et al. (1972). Histone samples were adjusted to 6.0 M guanidinium chloride (Nutritional Biochemical Corp.)

buffered with 0.3 M tris at pH 8.3, then incubated at 38°C, overnight. The samples were then desalted by exclusion chromatography through Bio Gel P2 (BioRad, Richmond, Calif.) in 10% acetic acid (Vidali and Neelin 1968a) and lyophilized.

H3 dimers were easily and virtually quantitatively reduced to monomers by adjusting aqueous samples to 2.0% (v/v) 2-mercaptoethanol (Eastman Kodak, Rochester, N.Y.).

If the histone extracts were not immediately used, they were precipitated overnight by addition of ten volumes of ice-cold acetone containing 1.0 mM PMSF. The precipitate was recovered by centrifugation, redissolved in distilled water and then lyophilized.

D. Polyacrylamide Gel Electrophoresis

Ten to thirty micrograms of histone were electrophoresed through 0.6 x 15 cm gels containing 15% acrylamide and 6.25 M urea with 7% acetic acid as the running buffer, at 1.7 mA per gel. The gels were stained with 1% amido black in 40% ethanol-7% acetic acid for 15 minutes, then destained by successive washes in 40% ethanol-7% acetic acid. Electrophoresis samples contained 1.0-2.0 mg/ml histone protein in water or acid with 5% sucrose and 0.05% pyronin Y (Eastman Kodak). Polyacrylamide gels were prepared according to Panyim and Chalkley (1969)

except that only 1/7 of the amount of ammonium persulfate was required for polymerization. These gels were routinely pre-electrophoresed prior to use. Alternatively, gels were prepared with a similar recipe containing 25% more bis-acrylamide and polymerization was achieved by ultraviolet photooxidation of riboflavin (Brasch et al. 1974). These gels were not pre-electrophoresed prior to use. All the ingredients used in the preparation of polyacrylamide gels were supplied by Eastman Kodak except urea (Schwarz Mann, Orangeburg, N.Y.) and ammonium persulfate (British Drug Houses, Poole, England).

Optical density profiles were obtained at 600 nm on a Beckman Acta CIII spectrophotometer equipped with a gel scanner. The electrophoretic mobilities of H1, H5, H2A and H2B, relative to H3 and H4 were determined from these tracings by dividing the distance between the H3 dimer and H4 into the distance between the H3 dimer and the appropriate histone band. This method standardizes the conditions of separation. Relative peak areas of individual bands were calculated from their weights in excised paper tracings of the peaks.

E. Inhibition of Endogenous Proteases

The effectiveness of sodium bisulfite (Panyim and Chalkley 1971) and PMSF as inhibitors of proteolytic activity was tested in isolated carp erythrocyte nuclei by

incubations at 0°C for 0 and 7 hours, with these inhibitors present. In fact, inhibitors were included in all media prior to acid extraction of histones. Progressive histone proteolysis was monitored by polyacrylamide gel analysis.

Residual proteolysis was studied in unwashed whole blood, washed erythrocytes, the cell lysate and washed nuclei of carp by prolonged incubations at 0°C with sampling at 0, 4, 8 and 24 hours. All samples were processed as described above and 1.0 mM PMSF was present from the beginning. Residual proteolysis associated with White Sucker nuclei was assayed similarly at 0, 8 and 24 hours and that with trout nuclei at 0 and 24 hours.

To illustrate the importance of intact nuclei, samples of erythrocytes were processed in parallel: one sample was processed to nuclei, always in the presence of PMSF. The remainder was divided into two and both were lysed hypotonically in 10 mM Tris buffer, pH 8.0, with a Dounce-type homogenizer, and the chromatin fraction was collected and washed repeatedly by centrifugation at 12,100 X g. For one sample, 1.0 mM PMSF was included throughout. For the other, 50 mM sodium bisulfite was added rapidly, after lysis, because erythrocytes could not be hypotonically lysed in its presence. All chromatin and nuclear samples were extracted

into 0.25 N hydrochloric acid at the same time.

To test for protease activity in the carp erythrocyte cytoplasmic fraction following cell lysis, the carp nuclei in the cell lysate were removed by centrifugation and substituted with washed chicken erythrocyte nuclei. The mixture was incubated for 4 hours at 25°C. Then the nuclei were separated and washed free of carp cytoplasm by centrifugation. Histones were extracted in 0.25 N hydrochloric acid. Chicken nuclei without carp cytoplasm were incubated simultaneously in isotonic saline as a control for endogenous protease activity.

F. Cation-Exchange Chromatography

The initial separation of histone samples was performed by cation-exchange chromatography on Amberlite CG-50 (100-200 mesh; Rohm and Haas, Philadelphia, Pa.). Column sizes of 1.8 x 80 cm., 0.9 X 60 cm and 0.9 X 30 cm allowed the separation of total histone samples varying from 20 to 300 mg. Prior to loading, these samples were adjusted to 8.0% GuCl (guanidinium chloride), buffered at pH 6.8 by 0.1 M sodium phosphate and then eluted at room temperature by a discontinuous gradient of GuCl in 0.1 M sodium phosphate, pH 6.8. The flow rate varied between 20 to 40 ml/hr and fractions were assayed for protein by turbidity at 400 nm in five volumes of 18% (w/v) TCA (trichloroacetic acid) (Vidal;

and Neelin 1968b).

The gradient used with the largest column was generated by the continuous dilution of a mixing chamber initially containing 200 ml 8.0% GuCl from a reservoir containing successive additions of 120 ml 8.0% GuCl, 300 ml of 8.7% GuCl and 600 ml each of 9.4%, 9.7%, 10.1%, 11.8%, 12.7% GuCl, followed by 100 ml of 40.0% GuCl. All solutions were buffered to pH 6.8 with 0.1 M sodium phosphate. The volumes were scaled down four fold for columns of intermediate size and eight fold for the smaller columns. Usually, in the latter case, an additional gradient to 14% GuCl was included before the 40% GuCl wash.

Fractions constituting each peak were pooled and desalted by exclusion chromatography on Bio Gel P2 (Vidali and Neelin 1968a). They were concentrated in an ultrafiltration cell (Amicon Corp., Lexington, Mass.), with a PM-10 Diaflo membrane, at a constant nitrogen pressure of 55 psi, then lyophilized. Some very large samples were also concentrated prior to desalting, but this was avoided when possible since histones in the presence of GuCl will permeate the PM-10 membrane. The histone fractions contained within each peak were determined by polyacrylamide gel electrophoresis.

G. Exclusion Chromatography

Further separation and purification of the Amberlite CG-50 fractions were achieved by exclusion chromatography through Bio Gel P60 (BioRad, Richmond, Calif.), equilibrated with 0.01 N hydrochloric acid (Vidali and Neelin, 1968a). Samples were solubilized in 0.005 M sulfuric acid and eluted with 0.01 N hydrochloric acid, at a flow rate of 15-20 ml/hr. Five millilitre fractions were collected and assayed quantitatively for histone by turbidity in 18% TCA at 400 nm (Vidali and Neelin 1968b). Again, the fractions constituting each chromatographic peak were concentrated, lyophilized and analyzed by polyacrylamide gel electrophoresis.

H. Separation of Trout H3 and H4

Histones H3 and H4 of trout were mutually separated by a slight modification to Johns' method (Johns 1964). Samples were solubilized in 0.25 N hydrochloric acid in 80% ethanol, then dialyzed against eight volumes of ethanol for 18 hours at 4°C, then for an additional 4 hours against fresh ethanol. Histone 3 precipitated and was sedimented at 12,100 X g for 10 minutes. Histone 4 was precipitated from the supernatant by the addition of three volumes of cold acetone. Both precipitates were redissolved in distilled water, lyophilized and analyzed for purity by polyacrylamide gel electrophoresis.

I. Amino Acid Analyses

Histone fractions were hydrolyzed in 6 N hydrochloric acid for 24 hours, at 110°C, in evacuated, sealed tubes, then dried under vacuum. The separation and quantitation of the amino acids were performed on a Technicon TSM amino acid analyzer (Technicon, Tarrytown, N.Y.) equipped with a peak integrator. Reported values are not corrected for hydrolytic losses.

III. RESULTS AND DISCUSSION

A. Fish Erythrocytes

Differential cell counts were determined from at least a dozen microscope fields and a minimum of 500 cells. Generally, all the fish used in this thesis contained greater than 95% mature erythrocytes in their circulating blood, prior to removal of the buffy coat. Histone extracts were therefore representative of mature erythrocytes. All five fish species examined, contained nucleated erythrocytes (Fig. 1), however this was expected because only three antarctic species, certain eel larvae and a few deep-sea fishes do not (Satchell 1971). In appearance the erythrocytes closely resemble those of chicken. They vary in size but display similar ovoid shapes and contain ovoid nuclei, aligned with the long axis of the cells. The nuclear chromatin appears to be highly condensed. Polychromatic erythrocytes of white sucker, carp, trout and perch were seen often. From these, it was apparent that nuclear shrinkage and condensed chromatin aggregation was occurring as usual during the later stages of cell maturation. These events have been described for other fish species (Sekhon and Beams 1969, Catton 1951, Jakowska 1956). No obvious differences in the degree of nuclear condensation was observed among these species in the light microscope studies.

Figure 1. Photomicrographs of fish blood smears, each stained with May-Grunwald and Giemsa stains (Lucas and Jamroz 1961); (A) white sucker, (B) carp, (C) trout, (D) perch and (E) black crappie (magnification X 2000).

Photomicrographs were taken on a standard Zeiss microscope employing bright field optics and recorded on Kodak Panatomic-X 35 mm film.

C

D

E

B. Polyacrylamide Gel Electrophoretic Survey of Fish Erythrocyte Histones

The electrophoretic patterns of erythrocyte histones from five fish species and chicken are compared in figure 2. H3d and H4 are found to have invariant electrophoretic mobilities. This has been shown to be true for diverse organisms (Panyim et al. 1970), therefore H3d and H4 act as internal standards to which more variable histones can be compared (Fig. 2, Table 1).

Polyacrylamide gels, containing 15% acrylamide and 6.25 M urea resolve each of the fish erythrocyte histones into single bands (Fig. 2). With one exception, the order of migration of these histones are identical among the species tested. The chicken erythrocyte histones 2A and 2B migrated in a single band. In contrast, these proteins separate in polyacrylamide gels containing 2.5 M urea, but fish H2A and H2B do not (Panyim and Chalkley 1971). They also persist in relatively constant proportions among the various species examined but display small yet significant variations in electrophoretic mobility (Fig. 2, Table 1).

Among the fish erythrocyte histones, bands analogous to chicken H5 can be found. Their ultimate identification will be described later, but throughout the rest of this thesis they will be referred to as fish H5. That of black crappie stains less intensely than chicken H5 but has identical electrophoretic mobility (Fig. 2A,D, Table 1). Trout and perch H5 bands display weaker relative staining but differ from chicken and black crappie H5 in their greater electrophoretic mobilities (Fig. 2B,C). Carp H5 is a weakly staining band which might easily be overlooked as a contaminant or degradative artifact, since it migrates faster than H3, thus deviating from the other histones in its order of migration (Fig. 2E, Table 1). White sucker erythrocyte histones do not reveal an obvious H5 band, but two non-oxidizable bands with relative mobilities between H1 and H3m persist in replicate samples (Fig. 2F). Among different species of fish it is obvious that according to electrophoretic parameters, H5 is a highly variable protein, differing vastly in relative amounts even to the extent of its apparent absence in some fish species. Shifting H3 entirely to the dimer position by oxidation greatly facilitated the examination of these bands, migrating in the vicinity of the H3 monomer.

Figure 2. Densitometric scans of polyacrylamide-gel electropherograms of erythrocyte histones from: chicken (A), trout (B), perch (C), black crappie (D), carp (E) and white sucker (F). Only chicken and black crappie histones were not oxidized prior to electrophoresis. The conditions of electrophoresis, staining and recording have been described. The descending migration is from positive to negative poles, and the identity of the individual bands are indicated. All histone samples were prepared with 1.0 mM PMSF present.

Table 1
Relative Electrophoretic Mobilities of Individual
Erythrocyte Histone Bands in Polyacrylamide Gels.

	3d	1	5	3m ⁺	2A	2B	4
Chicken	0.00	0.40	0.45	0.63	0.67	0.73	1.00
Rainbow Trout	0.00	0.51	0.66	0.67	0.71	0.78	1.00
Perch	0.00	0.47	0.50	0.66	0.67	0.73	0.77 1.00
Black Crappie	0.00	0.46	0.52	0.63	0.67	0.72	0.76 1.00
Carp	0.00	0.53	-	0.69*	0.67	0.71	0.77 1.00
White Sucker	0.00	0.54	0.56	-	0.67	0.71	0.78 1.00

* The relative electrophoretic mobilities of H3m were determined from separate gels, simultaneously run with non-oxidized histone samples.

* The relative electrophoretic mobility of carp H5 was determined for isolated fractions co-electrophoresed with trout H3-H4. The reproducibility of the mobilities of isolated H5 fractions was verified for all fish H5s.

Among the fish erythrocytes, H5 levels in carp and white sucker erythrocytes are smallest or absent and the H1 levels are the highest (Figure 2). Chicken reveals the greatest H5 levels and the lowest H1 levels. Trout, perch and black crappie yield intermediate amounts of H1 and H5. It is unlikely that differences in molar staining ratios (Wright and Olins 1975, Mazen and Champagne 1972) among the H5 proteins can account for such extreme quantitative differences because staining intensities of the isolated proteins (Figs. 24, 25) are comparable when equivalent amounts are electrophoresed.

As well as varying greatly in relative amounts, H1 is heterogeneous and its subfractions vary most in electrophoretic mobilities (Fig. 1, Table 1). This is particularly evident on electrophoretic analyses of the isolated H1 components (Fig. 25).

It is doubtful that the heterogeneity of H1 and H5 variability can be explained by enzymatic degradation. All the histone samples described were prepared with the protease inhibitor, PMSF (phenylmethylsulfonyl fluoride) present in all media prior to acid extraction. The importance of this aspect cannot be overemphasized and will be dealt with in the next section.

C. Histone Proteolysis

Histones from a variety of sources are vulnerable to enzymatic degradation by endogenous protease. The pH optima, specificity, location and inhibition of these enzymes have been described thoroughly for calf thymus (Sarkar and Dounce 1961, Stellwagon et al. 1968, Furlan and Jericijo 1967a,b, Furlan et al. 1968, Bartley and Chalkley 1970, Kurecki et al. 1975) and rat liver (Dounce and Umana 1962, Brostrom and Jeffay 1972, Garrels et al. 1972, Chong et al. 1974, Nooden et al. 1973). Similar enzymes have also been studied in liver and erythrocytes of *Xenopus laevis* (Destrée et al. 1975), chicken erythroid cells (Harlow and Wells 1975b), fish erythrocytes (Panyim et al. 1971), trout spermatids (Marushige and Dixon 1971, Marushige et al. 1976), mammalian hepatomas (Chae and Smith 1975, Ballal et al. 1975) and cycad pollen (Brandt et al. 1975). Generally, a significant protease activity is present at neutral or weakly alkaline pH and displays several features common to serine-type proteases. It is inhibited to various extents by DFP (diisopropylfluorophosphate), PMSF, sodium bisulfite, Kunitz inhibitor or soybean trypsin inhibitor. Although isolated from calf thymus and rat liver chromatin (Kurecki et al. 1975, Garrels et al. 1972, Chong et al. 1974), the enzyme may be held originally in cytoplasmic organelles (Stellwagon

et al. 1968, Destrée et al. 1975, Heinrich et al. 1976).

The following experiments were performed to assess and to minimize proteolysis as a factor in the variable yields of individual fish erythrocyte histones. Carp erythrocytes reveal high levels of latent, endogenous protease activity when nuclei are isolated without protease inhibitors. During the 4.5 hours from bleeding to acid extraction, erythrocyte histones are consistently damaged by autolysis, despite the use of ice-cold media. Characteristically, histones 1 and 3 are preferentially attacked, while histones 2A, 2B and 4 are relatively unaffected (Fig. 3, gel 1). Three peptide fragments designated P1, P2 and P3 frequently appear (Fig. 3); P1 arises along with the H3 dimer under oxidative conditions, together with variable other bands of greater mobility. This is clearly shown when carp nuclei are incubated in it's homologous cell lysate with PMSE (Fig. 5). These bands clearly arise from H3 fragments, the only vertebrate histone containing cysteine. Whether the occurrence of this peptide is a common feature of histone autolysis is not certain because gel electrophoresis samples are frequently prepared with reducing agents. An electrophoretic band similar to P3 is observed from a variety of sources where proteolysis of histones has occurred (Panyim et al. 1971, Bartley and Chalkley 1970, Chong et al. 1974, Destree et al. 1975). Since

P3 appears in preparations commonly affected by proteolysis of H1 and since it is not subject to oxidative dimerization, peptides of H1 probably contribute significantly to this band. Although several bands seem to arise during histone proteolysis, P1, P2 and P3 are particularly consistent and can be used as a reliable index of proteolysis. A diffuse background of bound dye in the region between H1 and H4 may reflect a heterogeneous population of peptide fragments originating from less specific cleavages (Fig. 3).

This pattern of selective proteolysis is reminiscent of that described for calf thymus chromatin (Bartley and Chalkley 1970). The particular vulnerability of H1 has frequently been described (Panyim et al. 1971), Garrels et al. 1972, Chong et al. 1974, Destrée et al. 1975, Harlow and Wells 1975). Since exogenous proteases, such as trypsin, attack avian erythrocyte chromatin with the same selectivity as the carp protease (Weintraub et al. 1975), it is likely that this reflects the greater accessibility of vulnerable peptide bonds in particular histones on chromatin. This argument was strengthened by the finding that histones free in solution are preferentially attacked in reverse order to those bound in chromatin (Bartley and Chalkley 1970).

Figure 3. Polyacrylamide gel electropherograms of histones from nuclei prepared without inhibitors (gel 1), with 50 mM sodium bisulfite (gel 2), with 1.0 mM PMSF (gel 4). Histones from nuclei incubated for 7 additional hours at 0°C in the presence of 50 mM sodium bisulfite (gel 3) and 1.0 mM PMSF (gel 5) are also displayed. The intact histone bands are labelled and the major peptide fragments resulting from proteolysis are indicated P1, P2 and P3. The extra band in gels 2 and 3 is a presumed contaminant. Conditions of electrophoresis in acid-urea are described in Materials and Methods. The polyacrylamide gels were deliberately overloaded to reveal minor products of proteolysis.

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Because 50 mM sodium bisulfite was shown to be an effective protease inhibitor (Panyim et al. 1971), it was included in all media employed for the isolation of carp nuclei. Unfortunately, hemoglobin precipitated in the presence of this compound, yielding brownish nuclear pellets highly contaminated with cytoplasmic proteins. Subsequent acid extracts included a prominent protein band migrating in polyacrylamide gels between H3 dimer and H1 (Fig. 3, gels 2,3). Furthermore, prolonged incubation of carp nuclei in 50 mM sodium bisulfite yields a high proportion of proteolytic fragments (Fig. 3, gel 3). The limitations of this inhibitor in other tissues has been reported (Harlow and Wells 1975, Ballal et al. 1975, Destrée et al. 1972).

PMSF (Fahrney and Gold 1963) is now more commonly used as a protease inhibitor, since in some cases it apparently inactivates chromatin-associated proteases completely at much lower concentrations than usually employed with sodium bisulfite (Chong et al. 1974, Nooden et al. 1973, Ballal et al. 1975). Furthermore, PMSF is less toxic than DFP, another potent inhibitor of serine proteases. It was tested at 1.0 mM, the limit of its solubility. Nuclei isolated in its presence could be washed free of all traces of cytoplasmic hemoglobin, and ca histones extracted from them seemed to be free of

extraneous proteins and were less damaged than previously (Fig. 3, gels 4;5).

Nevertheless, residual proteolytic activity is detectable in the presence of PMSF, although it may not become obvious without prolonged incubation of nuclei after cell lysis. Washed erythrocytes of whole blood can be stored for up to 24 hours in PMSF, without evident degradation of histones (Fig. 4A). On cell lysis, however, proteolysis is initiated in the presence of PMSF. Again H1 and H3 are preferentially attacked and the activity cannot be washed free of the nuclei, even when all visible traces of cytoplasm have been removed (Fig. 4B, Fig. 5). The enzyme therefore appears to be strongly bound within the nuclear fraction. Increasing the pH of the nuclear wash media from pH 6.0 to 7.5 did not increase the inhibition of PMSF.

Sucker nuclei prepared identically and also incubated for prolonged periods in the presence of PMSF displayed no significant changes (Fig. 6), thus proteolysis seemed to be totally arrested. Similarly, trout nuclei incubated for 24 hours with PMSF displayed few changes, again suggesting the absence of proteolytic activity under these conditions (Fig. 7). This was not unexpected since proteolytic activity in trout erythrocytes is normally undetectable.

Figure 4. Progress of histone proteolysis in washed carp erythrocytes (A) and washed nuclei (B), at 0°C in the presence of 1.0 mM PMSF, during prolonged incubations. Polyacrylamide gels were quantitated as described under Materials and Methods. Values obtained for individual histone bands are indicated (H1 ●---●, H3 ○---○, H2A □---□, H2B ▲---▲, H4 Δ---Δ).

Figure 5. Polyacrylamide gel densitometric scans of carp erythrocyte histones undergoing progressive proteolysis, at 0°C, in nuclei not separated from the cell lysate, but in the presence of 1.0 mM PMSF. Samples were extracted at 0 hours (A), 8 hours (B) and 24 hours (C) and were oxidized prior to electrophoresis. The histone bands and the main proteolytic peptides are designated.

Figure 6. Polyacrylamide gel densitometric scans of white sucker erythrocyte histone isolated from washed nuclei that were incubated at 0°C in the presence of 1.0 mM PMSF for 0 hours (A), 8 hours (B) and 24 hours (C). The histone extracts were not oxidized prior to electrophoresis.

Figure 7. Polyacrylamide gel densitometric scans of trout erythrocyte histones from washed nuclei that were incubated at 0°C, in the presence of 1.0 mM PMSF, and extracted at 0 hours (A) and 24 hours (B). The samples were oxidized prior to electrophoresis.

The degree of proteolytic activity associated with the carp cytoplasmic fraction of the cell lysate was determined in the absence of inhibitors by removing the carp nuclei and replacing them with washed chicken nuclei. As illustrated in figure 8, proteolytic activity in the carp erythrocyte cytoplasm penetrated the chicken erythrocyte nuclei and produced a similar profile of histone digestion as did autolysis within carp nuclei. H1, H5 and H3 were preferentially degraded. This low level of activity presumably reflects enzyme associated with the cytoplasm rather than the chicken nuclear fraction. Homologous nuclei could not be used as substrate for cytoplasmic enzymes, since their endogenous proteases could not be totally inhibited (Fig. 4B). Although most of the activity appears to be firmly bound to the nuclear fraction, this does not exclude the possibility that the proteases were originally held in cytoplasmic organelles, from which they were released during cell lysis before bound by the nuclei (Destree et al. 1975, Heinrich et al. 1976).

The relatively good protection against histone degradation given by protease inhibitors was not obtained unless nuclei were kept intact. Washed carp erythrocytes were dispersed into 10 mM Tris, pH 8.0 in the presence of sodium bisulfite or PMSF. Histones from the chromatin dispersed in the lysates

Figure 8. Densitometric scans of stained polyacrylamide gels after electrophoresis of histones from chicken erythrocyte nuclei incubated with carp erythrocyte fractions of cell lysates for 4 hours at 25°C (A) and control chicken erythrocyte nuclei simultaneously incubated in isotonic saline (B). No inhibitors were present in either case.

and recovered by centrifugation were scarcely protected by either inhibitor (Fig. 9, gels 2,3) in contrast with histones simultaneously extracted from unswollen, isolated nuclei in the presence of PMSF (Fig. 9, gel 1). This evidence indicates that preservation of nuclear structure assists in the control of proteolysis. In cells where proteolysis is encountered, isolation of histones from clean nuclear preparations is clearly preferable to direct preparation of histone from cell homogenates (Hnilica 1972) or chromatin preparations.

D. Separation and Purification of Histones

In order to characterize the fish erythrocyte H5s and to compare them to other histones, an initial separation of the major histones were attained by cation-exchange chromatography on Amberlite CG-50, eluted with guanidinium chloride. Total histones from trout (Fig. 10), carp (Fig. 13), perch (Fig. 19) and black crappie (Fig. 20) separated into four predominant peaks containing H1, H2A-H2B, H5 and H3-H4. The eluting positions of these peaks were very similar among the fish histones, including white sucker (Fig. 21) which did not yield an H5 peak. The various H5s elute in distinctive regions of the gradient well spaced from the other histone peaks. Carp H5, however, elutes at a higher concentration of guanidinium chloride than trout, perch or black

Figure 9. Polyacrylamide gels of histones extracted from carp erythrocyte nuclei, prepared with 1.0 mM PMSF (gel 1); chromatin prepared from cell lysates with 50 mM sodium bisulfite present (gel 2); chromatin prepared from cell lysates with 1.0 mM PMSF present (gel 3).

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crappie H5 (Fig. 13). When goose erythrocyte histone was chromatographed under identical conditions (Fig. 18), it was found that goose H5 eluted even later than carp H5, emerging with the H3-H4 peak at the end of the guanidinium chloride gradient, as anticipated from previous studies (Vidali and Neelin 1968a, Adams and Neelin 1969). The goose H1 and H2A-H2B peaks also eluted at higher guanidinium chloride concentrations than the comparable fish histone peaks. It seems, from these elution profiles, that homologies among fish H1, H2A-H2B exist which distinguish them from goose H1 and H2A-H2B.

Freshly prepared whole histone extracts from trout erythrocyte nuclei usually contain most of the H3 in the form of reduced monomers (Fig. 12A). When chromatographed immediately, two peaks containing H3-H4 emerge at widely different positions in the gradient (Fig. 10B). As well as the typical peak eluting at the end of the gradient which corresponded to the H5-H3-H4 peaks in the goose chromatogram (Fig. 18) or H3-H4 of calf thymus (Rasmussen et al. 1962), a novel peak eluted closely behind that of H2A-H2B. Oxidation of unfractionated trout erythrocyte histone extracts prior to chromatography eliminated this early peak, while the final peak of histone H3-H4 increased in relative size (Fig. 10A). Such a distribution of avian

Figure 10. Cation-exchange chromatography of total trout erythrocyte histone, (A) oxidized prior to chromatography and (B) freshly prepared without oxidation prior to chromatography. Samples were eluted from 79 X 1.8 cm column of Amberlite CG-50 by a discontinuous gradient of GuCl in 0.1 M sodium phosphate, at pH 6.8. Protein was detected in the effluent by turbidity in 18% TCA at 400 nm and GuCl concentration was determined from its refractive index (Vidali and Neelin 1968b). Histone fractions are indicated at the top of each chromatogram (R0 = run-off peak, I = H1, IId = H2A-H2B, V = H5, III-IV = H3-H4).

(I = H1, Iib1 = H2A, Iib2 = H2B and
V = H5)..

Figure 12. Polyacrylamide gel analysis of the fractionation of trout erythrocyte histone. Photograph A illustrates the total histones of trout before (gel 1) and after (gel 2) oxidation, followed by the contents of the four successive CG-50 peaks after the run-off peak (Fig. 10A); H1 (gel 3), H2A-H2B (gel 4), H5 (gel 5) and H3-H4 (gel 6). Photograph B illustrates the final purification of BioGel P60 exclusion chromatography (Fig. 11); H1 (gel 2), H2A (gel 3), H2B (gel 4), H5 (gel 5) and by selective solubilities of H4 in ethanol; H3 (gel 6), H4 (gel 7). The sources of the samples for the second stage of purification (Fig. 12B) are indicated by the arrows. Individual bands were identified by their distance of migration relative to the bands of total histone samples, illustrated at the left of each photograph. (III dimer = H3d, I = H1, V = H5, III = H3m, IIb1 = H2A, IIb2 = H2B, and IV = H4). Polyacrylamide gels were prepared according to Brasch et al. (1974).

arginine-rich histone is obtained only after special treatment with 2-mercaptoethanol (Vidali and Neelin 1968a). This early peak is reminiscent of the IIa peak noticed among early calf thymus histone separations (Rasmussen et al. 1962). The reduced H3 may take a conformation which allows H3-H4 aggregation and possibly a reduction in apparent net charge. The elimination of this peak simplified the elution profile and facilitated a cleaner separation of histones, as well as improving the electrophoretic resolution of bands with mobilities similar to the H3 monomer.

The above trout histone samples were extracted in the absence of proteolytic enzyme inhibitors. However, since carp erythrocyte histones are susceptible to enzymatic digestion without inhibitors, similar chromatograms yield varieties of degraded histones. Amberlite CG-50 chromatographic profiles of carp histones isolated with PMSF (Fig. 13A) or sodium bisulfite (Fig. 13B) are very similar to those of trout and show no evidence of degradation. The samples loaded onto these columns also displayed trivial amounts of peptides P1, P2 and P3 which are diagnostic of proteolysis (Fig. 15,17). Despite this apparent minimization of endogenous proteolytic activity, the H5 peaks in the chromatographic profiles were very small, reinforcing the conclusions from the electrophoretic densitometric scans (Fig. 2) that carp H5 exists in very low relative quantities.

Figure 13. Cation-exchange chromatography of carp erythrocyte histones from nuclei treated with (A) 1.0 mM PMSF and (B) 50 mM sodium bisulfite. The total histone samples were oxidized prior to chromatography. The conditions of chromatography were stated in figure 10 except that a 1.6 X 87 cm column of Amberlite CG-50 was used.

Figure 14. Exclusion chromatography through Bio Gel P60 of PMSF-treated carp erythrocyte histone fractions from the cation-exchange chromatogram (Fig. 13A): (A) H1, (B) H1-H2A-H2B, (C) H5 and (D) H3-H4.

Figure 15. Polyacrylamide gel electrophoresis of individual PMSF-treated carp erythrocyte histone peaks obtained from exclusion chromatography through Bio Gel P60 (Fig. 14A-D); unfractionated histone (gel 1), H1 (gel 2), H2A (gel 3), H2B (gel 4), H5 (gel 5), H3 (gel 6) and H4 (gel 7). Polyacrylamide gels were prepared according to Panyim and Chalkley (1969).

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Figure 16. Exclusion chromatography through Bio Gel P60 of sodium bisulfite treated carp erythrocyte histone fractions from the cation-exchange chromatogram (Fig. 13B); (A) H1-H4, (B) H1-H2A-H2B, (C) H5 and (D) H3-H4. The column dimensions were 1.8 X 91 cm.

Figure 17. Polyacrylamide gel electrophoresis of sodium bisulfite-treated carp erythrocyte histone peaks obtained from exclusion chromatography through Bio Gel P60 (Fig. 16A-D); total histone (gel 1), H1 (gel 2), H2A (gel 3), H2B (gel 4), H5 (gel 5), H3 (gel 6) and H4 (gel 7). Polyacrylamide gels were polymerized according to Brasch et al. (1974).

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Cation-exchange chromatography alone yielded homogeneous fractions of H1 and H5 from perch and black crappie erythrocyte histones (Figs. 19,20) and homogeneous H1 fractions from white sucker histone (Fig. 21). Despite the apparent absence of H5 in white sucker, a broad region, normally occupied by other fish H5s, was collected and analyzed (Fig. 21). Since carp and trout H5 peaks contained extraneous protein bands (especially H3 and H4), further purification was necessary. Complete isolation of all the histone fractions was attempted with trout, carp and white sucker histones for comparative purposes.

By rechromatography of the CG-50 H5 peaks through Bio Gel P60, homogeneous H5 peaks were obtained for trout (Figs. 11C, 12B) and carp erythrocytes treated with PMSF (Figs. 14C,15) or sodium bisulfite (Figs. 16C, 17). In all cases, the H5 peak emerged just behind H1 in effluent volume (Figs. 11A,14A,16A), therefore the fish H5s may be slightly smaller than H1 in particle size but still much larger than the other histone fractions. This would be expected if fish H5s are analogous to avian H5 under similar chromatographic conditions (Vidali and Neelin 1968a, Seligy and Neelin 1971). Chicken H5 has been estimated to be 21,450 daltons from its amino acid sequence (Garel et al. 1975).

Figure 18. Cation-exchange chromatography of total goose erythrocyte histone of a 79 X 1.8 cm column of Amberlite CG-50. The histone sample was not oxidized prior to chromatography. The conditions of chromatography were reported in figure 10. (I = H1, IIb = H2A-H2B, V-III-IV = H5-H3-H4).

Figure 19. (A) Cation-exchange chromatography of perch erythrocyte histone on a 0.9 X 30 cm column of Amberlite CG-50. (B) Polyacrylamide gel electropherograms of the chromatographic fractions: The total histone starting sample (gel 1), H1 (gel 2); H2A-H2B (gel 3); H5 (gel 4), H3-H4 (gel 5). The individual histones can be identified by comparison of their migrations to the labelled fractions in the total histone gel. Polyacrylamide gels were prepared according to Panyim and Chalkley (1969).

2 2

OF/DE

Figure 20. (A) Cation-exchange chromatography of black crappie erythrocyte histone on a 0.9 X 30 cm column of Amberlite CG-50. (B) Polyacrylamide gel electropherograms of the chromatographic fraction: The total histone starting sample (gel 1); H1 (gel 2); H2A-H2B (gel 3); H5 (gel 4); H3-H4 (gel 5). Polyacrylamide gels were prepared according to Panyim and Chalkley (1969).

Figure 21. (A) Cation-exchange chromatography of total white sucker erythrocyte histone on a 0.9 X 60 cm column of Amberlite CG-50. (B) Polyacrylamide gel electropherograms of the chromatographic fractions: The total histone starting sample (gel 1); H1 (gel 2); H2A-H2B (gel 3); H3-H4 (gel 5). Proteins eluting in positions normally occupied by other fish histone H5s (11.0-12.5% GuCl) were collected and analyzed by polyacrylamide gel electrophoresis also (gel 4). Polyacrylamide gels were prepared according to Panyim and Chalkley (1969).

Figure 22. Exclusion chromatography through Bio Gel P60 of white sucker histones from carion-exchange chromatography (Fig. 21A); peak H2A-H2B (A) and peak H3-H4 (B). The column dimensions were 1.6 X 90 cm.

Figure 23. Polyacrylamide gel electrophoresis of white sucker erythrocyte histones; total histone (gel 1), H1 from ion-exchange chromatography (Fig. 21A), (gel 2), H1 from exclusion chromatography (Fig. 22A) (gel 3), H2A from exclusion chromatography (Fig. 22A) (gel 4), H2B from exclusion chromatography (Fig. 22A) (gel 5), H3-H4 from exclusion chromatography (Fig. 22B) (gel 6) and H3-H4 from the ethanol insoluble precipitate (gel 7): The polyacrylamide gels were prepared according to Panyim and Chalkley (1969).

H3d

H1

H2A

H2B

H4

Cation-exchange chromatography H1 peaks from trout (Figs. 11,12B) and carp erythrocytes treated with PMSF (Figs. 14A,15) or sodium bisulfite (Figs. 16A, 17) were purified to homogeneity by exclusion chromatography. The carp CG-50 H1 peak from sodium bisulfite treated erythrocytes eluted in association with prominent amounts of H4 (Fig. 15A). The large peaks in Bio Gel P60 chromatograms eluting at positions intermediate to H1 and H4 did not appear as dense bands on electrophoresis. This unusual feature was not produced in extracts from PMSF treated erythrocytes (Fig. 14A). Very pure H1 fractions were thus obtained from all fish species examined.

Exclusion chromatography also resolved individual fractions of H2A and H2B from the CG-50 mixed fractions for trout (Figs. 11,12B), carp treated with PMSF (Figs. 14B,15) or sodium bisulfite (Figs. 16B,17) and white sucker (Figs. 22A,23). Associated with the CG-50 H2A-H2B peak is late-eluting H1 which displays greater heterogeneity than is evident in polyacrylamide gel electrophoresis of total histone. These proteins elute at the same position on Bio Gel P60 as the main bands. Whether they are subfractions, modified histone or degradation products is not known. In white sucker, an unusually prominent band is evident (Figs. 22A,23, gel 3). This band may

represent an enrichment of the slower electrophoretic band intermediate to H1 and H3m.

Histones 3 and 4 of carp are readily separated by exclusion chromatography through Bio Gel P60 whether PMSF treated (Figs. 14D,15) or sodium bisulfite treated (Figs. 16D,17). The small peaks eluting at the void volumes, contain either highly aggregated or very large proteins, which barely enter the gels on electrophoresis. Neither trout H3-H4 nor white sucker H3-H4 can be fractionated by exclusion chromatography (Figs. 22B,23). In both cases they elute as a single peak, late in the column volume, Trout H3 and H4 however are separable by the selective solubility of H4 in ethanol (Fig. 12B). White sucker H3-H4 however is not separable by this alternative procedure, since both H3 and H4 precipitated in ethanol (Fig. 23). Exclusion chromatography of the white sucker H3-H4 mixed CG-50 fraction did not yield any protein peptides of molecular weight comparable to H5 from birds or other fish, nor did any other histone fraction from this fish.

Established techniques were successfully employed in the isolation of H1 from all the fish species (Fig. 25), H2 from all species except white sucker (Fig. 24), H2A and H2B from trout, carp and white sucker and H3 and H4 from trout and carp.

Figure 24. (A) Polyacrylamide gel electrophoresis of H5 isolated from erythrocytes of chicken (gel 1), goose (gel 2), carp (gel 3), trout (gel 4), perch (gel 5) and black crappie (gel 6). (B) The relative electrophoretic mobilities of H5 are compared to internal standards: trout H3d and trout H4 (gels 1-6) and goose H5 (gels 2-6); chicken H5 (gel 2), carp H5 (gel 3), trout H5 (gel 4), perch H5 (gel 5) and black crappie H5 (gel 6). The polyacrylamide gels were polymerized according to Panyim and Chalkley (1969).

A

B

Figure 25. Polyacrylamide gel electrophoresis of isolated H1 from erythrocytes of carp (gel 1), perch (gel 2), black crappie (gel 3) and white sucker (gel 4). The polyacrylamide gels were polymerized according to Panyim and Chalkley (1969).

E. Analysis of Individual Histones

Although no attempts were made to separate the individual H1 subfractions (Fig. 25), H1 and H5 are revealed as the most variable histone fractions by several criteria. These differences extend further to their amino acid compositions.

Almost all of the amino acids of fish H1 differ in relative proportions (percent of total recovered residues) but sufficiently high lysine and alanine values and low arginine values were obtained to classify these as typical H1 (Table 2). This confirms other observations reported on the heterogeneous and variable nature of H1 (Panyim et al. 1971). As a group, H1 from fish are not sufficiently unique in their compositions to imply any phylogenetic distinction from other H1 of a wide variety of sources (Hnilica 1972, Panyim et al. 1971, Sanders and McCarty 1972, Vidali and Neelin 1968a, Adams and Neelin 1976, Tsai and Hnilica 1975). Generally, the H1 compositions of carp and trout erythrocytes reported here (Table 2), are comparable to values reported for carp erythrocyte and liver H1 and trout testis H1 respectively (Panyim et al. 1971, Marushige and Dixon 1971).

All the fish H5 display high lysine, arginine, serine and alanine levels, unambiguously identifying these proteins as analogues of avian H5 (Table 3). Generally, the H5 proteins are not as variable as

Table 2

Amino acid composition of fish erythrocyte H1

Amino acid	Moles per 100 mol. of recovered amino acids					
	Perch	Black Crappie	Trout	Carp	White Sucker	White* Sucker
Aspartic acid	2.7	2.8	1.9	1.7	2.6	2.4
Threonine	5.2	4.8	2.7	3.9	5.3	5.0
Serine	4.2	5.0	5.4	6.5	6.5	7.2
Glutamic acid	4.6	4.3	2.6	3.1	4.3	3.3
Proline	7.4	7.5	10.1	10.3	8.2	9.3
Glycine	6.4	7.3	4.2	5.3	6.2	5.2
Alanine	21.0	20.3	27.4	25.9	20.5	22.9
Cysteine	0.0	0.0	0.0	0.0	0.0	0.0
Valine	6.6	7.4	7.4	4.7	6.6	6.0
Methionine	0.3	0.3	0.0	0.0	0.3	0.0
Isoleucine	2.5	2.6	0.6	1.5	2.1	1.3
Leucine	4.7	4.9	3.2	3.8	4.8	3.7
Tyrosine	1.2	1.2	0.3	0.4	1.2	0.5
Phenylalanine	0.9	0.8	0.9	0.4	0.8	0.5
Histidine	1.0	1.0	0.0	0.3	0.5	0.2
Lysine	27.6	25.4	32.1	31.1	26.9	31.1
Arginine	3.9	4.3	1.2	1.2	3.4	1.4
Lysine/Arginine	7.1	5.9	26.8	25.9	7.9	22.1

*H1 fraction retarded on Amberlite CG-50, but recovered from H2A-H2B by exclusion chromatography through BioGel P60.

Amino acid composition of fish erythrocyte H5

Amino acids	Moles per 100 mol of recovered amino acids					
	Perch	Black Crappie	Trout	Carp	Chicken	Goose*
Aspartic acid	1.8	3.2	4.5	2.1	1.6	2.5
Threonine	4.5	5.3	6.0	6.1	3.0	4.4
Serine	10.4	7.9	7.9	12.8	12.5	9.0
Glutamic acid	2.0	2.0	2.4	2.5	3.8	4.6
Proline	7.6	7.8	6.7	5.0	6.9	8.1
Glycine	5.8	5.5	6.5	5.9	5.0	5.6
Alanine	16.3	17.6	14.3	15.9	15.6	15.3
Valine	4.8	6.1	6.2	4.2	4.1	3.2
Methionine	0.5	0.1	0.6	0.0	0.5	0.7
Isoleucine	2.3	2.4	3.0	2.4	3.1	2.4
Leucine	5.9	5.6	6.0	5.8	4.3	4.7
Tyrosine	0.7	0.7	1.7	0.6	1.5	1.7
Phenylalanine	0.6	0.6	0.8	0.5	0.5	0.9
Histidine	0.2	0.2	0.3	0.1	1.6	1.6
Lysine	30.1	27.5	24.3	25.4	24.1	22.7
Arginine	6.8	7.6	8.9	10.7	12.0	11.8
Lysine/Arginine	4.4	3.6	2.7	2.4	2.0	1.9

*This fraction contained detectable amounts of H3 in polyacrylamide gels.

the H1. This is revealed by their comparable levels of glutamic acid, glycine, isoleucine and phenylalanine. When compared to chicken and goose H5 (Table 3) or values reported for several other avian H5 (Seligy et al. 1976), they yield more variable quantities of other amino acids and display group patterns in amino acid levels. Generally, threonine is somewhat elevated above avian levels but serine, arginine, glutamic acid and histidine are lower. The amino acid compositions reported for H5 of another fish species, *Caranx hippos*, (Hnilica 1972), also bears stronger similarities to these fish H5 than to avian H5. It too contains lower arginine and serine and higher threonine but glutamic acid is higher than in other fish and histidine is intermediate in content. Since amino acid composition of the chicken H5 (Table 3) is consistent with that determined from sequence data (Garel et al. 1975), the difference among the H5 are probably not artifactual. Certainly all H5 appear pure and undamaged (Fig. 24) before analysis.

No typical H5 is found among the white sucker erythrocyte histones, however, a prominent H1 band elutes late from the ion-exchange column. This protein has mobilities similar to the slower of the two bands found consistently among the sucker histones between H1 and H3m in polyacrylamide gels. This subfraction

tends to have lower arginine values than the main H1 and was clearly not similar to H5. From the CG-50 chromatogram of white sucker, it was shown that the volumes from 11.0-12.5% guanidinium chloride were collected because H5-like proteins of other fish eluted within this range. Amino acid analyses of the proteins in these volumes confirms polyacrylamide gel findings that no H5 was evident (Table 4).

H2A from trout, carp and white sucker erythrocytes, showed almost no significant differences (Table 5). They were also consistent with the compositions of a wide variety of animals including trout testes H2A (Bailey and Dixon 1973), buffalo fish erythrocyte H2A (Panyim et al. 1971), duck erythrocyte H2A (Sanders and McCarty 1972) and calf thymus H2A (Yeoman et al. 1972).

H2B from trout, carp and sucker erythrocytes are nearly identical in amino acid compositions (Table 5) and again are extremely similar to H2B from trout testis, buffalo fish erythrocytes, duck erythrocyte and calf thymus, to mention only a few species.

The similarities between trout and carp H3 and H4 are illustrated in Table 6. The compositions of H3 from carp and trout erythrocytes are consistent with those reported from carp testis (Hooper et al. 1973) and trout testis (Marushige and Dixon 1971). In fact the sequences are perfectly conserved among the H3 from

Table 4

Amino acid composition of White Sucker erythrocyte histone eluting from Amberlite CG-50 cation-exchange columns between 11.0-12.5% guanidinium chloride

Amino acid	Moles per 100 mol of recovered amino acids
Aspartic acid	4.1
Threonine	7.8
Serine	7.0
Glutamic acid	5.7
Proline	3.7
Glycine	9.8
Alanine	11.2
Cysteine	0.0
Valine	6.8
Methionine	0.3
Isoleucine	4.0
Leucine	6.6
Tyrosine	2.2
Phenylalanine	1.8
Histidine	1.4
Lysine	17.5
Arginine	10.0
Lysine/Arginine	1.8

Table 5

Amino acid composition of fish erythrocyte histones

Amino acids	Moles per 100 mol of recovered amino acids					
	H2A			H2B		
	Trout	Carp	White Sucker	Trout	Carp	White Sucker
Aspartic acid	6.8	5.3	6.1	4.7	3.6	4.2
Threonine	4.8	5.0	4.5	6.9	7.0	6.7
Serine	2.5	2.5	2.7	10.2	9.3	9.0
Glutamic acid	9.3	8.4	9.2	8.0	7.4	7.8
Proline	3.1	4.6	4.8	4.6	5.5	4.0
Glycine	11.6	10.4	11.9	8.1	6.8	7.8
Alanine	14.3	15.1	13.5	10.2	11.6	10.1
Cysteine	0.0	0.0	0.0	0.0	0.0	0.0
Valine	7.1	6.9	7.0	6.0	6.5	6.4
Methionine	0.0	0.0	0.0	1.3	1.6	1.5
Isoleucine	2.9	3.7	3.5	4.9	5.6	5.4
Leucine	12.3	12.6	12.3	5.5	5.3	5.6
Tyrosine	2.1	2.0	1.4	3.8	3.7	3.7
Phenylalanine	1.0	0.6	0.8	1.4	1.5	1.7
Histidine	1.4	1.6	1.5	2.2	2.4	2.3
Lysine	10.8	10.7	11.1	15.1	14.9	16.4
Arginine	10.3	10.5	9.8	7.3	7.2	7.4
Lysine/Arginine	1.0	1.0	1.1	2.1	2.1	2.2

Amino acid composition of fish erythrocyte histone

Moles per 100 mol of recovered

amino acids

Amino acids	H3		H3-H4	H4	
	Trout	Carp	White Sucker	Trout	Carp
Aspartic acid	4.5	4.2	5.4	4.7	4.7
Threonine	7.2	6.7	6.7	7.5	6.8
Serine	4.1	4.6	4.4	2.6	2.3
Glutamic acid	11.6	10.3	10.0	7.4	6.6
Proline	3.6	4.8	3.3	trace	1.6
Glycine	7.6	6.2	9.6	16.5	14.9
Alanine	10.6	13.7	9.9	7.8	8.7
Cysteine	0.0	0.0	0.0	0.0	0.0
Valine	5.9	5.1	6.0	7.0	7.6
Methionine	1.1	1.2	1.1	0.6	1.2
Isoleucine	4.9	5.2	5.2	4.7	5.8
Leucine	8.8	9.1	8.6	8.2	8.2
Tyrosine	3.0	2.2	2.9	4.2	2.7
Phenylalanine	3.0	3.0	2.9	2.0	2.1
Histidine	1.5	2.2	1.8	1.9	2.4
Lysine	8.7	9.2	10.4	9.5	9.9
Arginine	14.1	12.7	12.0	14.5	14.2
Lysine/Arginine	0.6	0.7	0.9	0.7	0.7

from carp (Hooper et al. 1973), chicken (Brandt and VonHolt 1971) and shark (Brandt et al. 1974), while species as diverse as pea and calf differ by only four of 135 total amino acids (Patthy et al. 1973). Since H4 from pea and calf differ by only 2 of 102 amino acids in primary sequence (DeLange et al. 1969), the similarity in trout and carp erythrocyte H4 (Table 6) was not unexpected.

IV. GENERAL DISCUSSION

Little is known about the erythrocytes from which the fish histones were isolated. The degree of metabolic and cytological maturation can only be assumed from limited information. The mature erythrocytes of trout, perch, black crappie, carp and white sucker are similar in shape but differ in size. Stained with May-Grünwald and Giemsa stains (Lucas and Jamroz 1961), they resemble chicken erythrocytes, showing eosinophilic cytoplasm and strongly basophilic nuclei. During the later stages of maturation, i.e. from orthochromatic to mature erythrocytes, the nuclear volume shrinks and chromatin becomes densely aggregated as in birds. In trout, these events, as well as the extrusion of cytoplasmic organelles, have been visualized by electron microscopy (Sekhon and Beams 1969). Whether replication and transcription are inhibited to the same degree as in chicken erythrocytes and whether the degree of chromatin condensation attained by fish erythrocytes compares with that of birds has not been reported. This lack of information limits functional interpretations based on the comparative biochemical measurements. Nonetheless the present studies provide new information on the species distribution of H5 and raise several questions concerning its function.

The central question concerning the universal presence of H5 among the nucleated erythrocytes of the

lower vertebrates has not been answered adequately. H5 has been isolated and unambiguously identified among the histones of trout, perch, black crappie and carp erythrocytes. Relatively high contents of lysine, arginine, serine and alanine are characteristic of all homologues of H5. If they were proteolytic fragments of H2A, H2B, H3 and H4, they could not display larger particle sizes than these four proteins on exclusion chromatography. Although slightly smaller than H1, all H5s contain arginine levels too high to be fragments of H1.

Among the white sucker erythrocyte histones, a clear H5 homologue was not found, however, two bands always appeared between H1 and H3m on electrophoresis. Cation-exchange chromatography failed to reveal an H5 peak in positions characteristic of other fish or bird H5s. The slower of the two unusual bands however eluted with H2A-H2B from Amberlite CG-50 columns and with H1 from BioGel P60 columns. Amino acid analysis revealed that it belonged to the H1 family. The faster band eluted with H3-H4 from cation-exchange columns, but did not subsequently display large particle size on exclusion chromatography, since it remained with H3-H4. Amino acid analyses of this mixture did not suggest a contribution by an H5-like protein.

Clearly an interpretation of the source of these white sucker histone bands is important since, among the fish erythrocytes examined, only they lack typical H5. The slower band may be a subfraction of H1, but it does not display the atypically high arginine values like the cell-specific Flb of sea turtle erythrocytes (Tsai and Hnilica 1975). Both bands may contain proteolytic fragments generated by proteases shown to have a marked preference for H1, H5 and H3. If early proteolysis has escaped detection, a low level of H5 may have been preferentially eliminated by the action of the proteases, despite the presence of inhibitors.

Whether or not H5 is actually present in sucker erythrocytes, this histone is the most quantitatively variable among the vertebrate histones, its presence ranging from an apparent absence in sucker erythrocytes through low levels in carp erythrocytes, intermediate levels in the erythrocytes of trout, perch and black crappie, to prominent levels in the mature erythrocytes of the definitive erythropoietic series in chickens and other birds.

This distribution of H5 levels among the erythrocytes of fish may reflect the more general pattern of H5 occurrence among the vertebrates. H5 is not found in the nucleated erythroid cells of phenylhydrazine-treated rabbit marrow cells (Adams and Neelin 1976), but since

mammalian erythroid cells eventually void their nuclei, it may not be required for terminal nuclear differentiation. The nucleated erythrocytes of the sea turtle, however, also lack H5 but may possess a cell-specific H1 sub-fraction with elevated arginine, (Flb), in its place (Tsai and Hnilica 1975). Histones were surveyed from a wide variety of species by polyacrylamide gel electrophoresis, and H5 was found only in avian erythrocytes (Panyim et al. 1971); however, this data must be considered with caution because of contradictions with other reports. H5 has been isolated and characterized from the erythrocytes of bull frog (*Rana catesbeiana*), and fish (*Caranx hippos*) (Edwards and Hnilica 1968, Miki and Neelin 1975) including carp, in which H5 has been overlooked by Panyim-et al. (1971). Furthermore, frog (*Xenopus laevis*) erythrocytes, free of proteolytic activity (Destrée et al. 1975), display low to intermediate levels of a protein resembling H5 in polyacrylamide gel electropherograms (Destrée et al. 1973).

These variable levels of H5 obviously do not correlate well with the state of maturation attainable by the various nucleated erythrocytes. For instance, the degree of morphological maturation reached by trout, sucker or carp erythrocytes do not appear to differ appreciably despite vastly differing levels of H5. Even the frog erythrocytes undergo nuclear inactivation

and marked chromatin condensation (Thomas and McLean 1975) in the presence of only low levels of H5 (Destrée et al. 1975). H5 does not appear to be essential for these maturational events.

It has been proposed that H5 may provide an alternative to H1 in mature chicken erythrocytes, thus accounting for the unusually low level of H1 in these cells relative to other tissues (Greenaway and Murray 1971). This survey provides evidence in support of this relationship. Carp and white sucker erythrocytes, which have prominent H1 levels, display the smallest H5 levels. On the other hand, chicken erythrocytes yield meager amounts of H1 but copious H5. Trout, perch and black crappie have intermediate amounts of both. In the erythrocytes of carp and white sucker, H1 may perform functions similar to the H5 in chicken erythrocytes, despite the differences in the primary structures of the proteins.

Consistent with this suggestion is the evidence that these two histones share certain characteristics in studies with avian chromatin. Neither participate in the assembly of chromatin monomers (Thomas and Kornberg 1975, Olins and Olins 1974, Oudet et al. 1975), but they bind to similar regions of DNA outside of the monomers (Ramsay Shaw et al. 1976). Both have dramatic effects on chromatin condensation (Billet and Barry 1974,

Lurquin and Seligy 1976), probably by cross-linking the monomers. Metabolically, H1 and H5 are phosphorylated but not acetylated (Seligy et al. 1973). Several structural similarities exist and will be discussed separately. It seems that the maturation of nucleated erythrocytes is a phylogenetically stable process in which the participation of H5 is secondary. The function of H5 may be substituted by H1 or by other less obvious adaptations of the mechanism determining erythroid cell maturation:

Among the several studies dealing with avian erythrocytes histones, it has been only occasionally noted that H1 subfractions change during maturation (Appels et al. 1972, Billett and Hindley 1972, Tobin and Seligy 1975). An apparent decrease in the most electrophoretically mobile H1 subfraction occurs late in maturation and this same subfraction is increased in chicken liver (Billet and Hindley 1972). In chick lens epithelium and lens fibre cells, which also undergo progressive genome inactivation, another subfraction becomes very prominent (Teng et al. 1974). Such tissue-specific expression of H1 subfractions may imply differing functions and separate regulation for these closely related proteins. Examples of such developmental changes extend even to the developing sea urchins, which appear to synthesize different H1 subfractions in morula and gastrula (Ruderman and Gross 1974).

That erythrocytes with small amounts of H5 selectively express a particular H1 subfraction is not yet certain, but preliminary observations paralleling those of Billett and Hindley have been made. Carp erythrocytes appear to express large amounts of a single H1 subfraction as revealed by polyacrylamide gel electrophoresis in acid-urea. Carp liver histones however possess smaller quantities of a faster migrating H1 subfraction (App. 1). Between these two tissues, there may be no overlap of the subfractions expressed. Since peptides P1, P2 and P3 are absent in the liver extracts, proteolysis is not a factor of elimination of the slower H1. White sucker erythrocyte and liver histones display the same pattern (App. 1). The erythrocytes again express the slower H1 band while liver possesses the faster H1. In this case, both tissues may have some overlap in the subfractions they possess. Trout on the other hand, yield the same H1 subfraction in both tissues (App. 1). Trout erythrocytes also possess intermediate H5 levels. Although the nature of the subfractions have not been investigated rigorously for authenticity or post-synthetic modifications, it is interesting that the functional complementarity between H5 and H1 might extend to a particular subfraction of H1. In trout erythrocytes, the presence of H5 may exclude the necessity for the particular H1 subfraction. Whether this H1 of white sucker and carp erythrocytes is cell-

specific cannot be determined without more extensive surveys of other tissues.

Qualitatively the H5s from diverse sources are more variable than all of the histones except H1, and as a group the fish H5 have characteristics which distinguish them from the bird H5. They tend to migrate faster in polyacrylamide gels than avian H5. Only black crappie H5, which is the slowest among the fish studied, has an identical mobility to the quickly migrating chicken H5 (Seligy et al. 1976). The fish H5 also elute more readily than avian H5 from Amberlite CG-50 cation-exchange columns. These differences reflect similar patterns in their amino acid compositions. Among the fish, threonine is elevated but serine, arginine, glutamic acid and histidine are generally lower than in avian H5.

Furthermore the amino acid compositions of the fish H5 are much more diverse than the avian H5, perhaps reflecting the great diversity of the group. This is particularly noticeable among the characteristic amino acids lysine, arginine and serine. By comparison, H1 does not seem to exhibit such group differences.

Diverse H5 from sources other than birds bear greater resemblances to the fish H5 than to the avian H5 (App. 2). As mentioned earlier, other fish (*Caranx hippos*) also contain lower arginine and serine and higher threonine levels but glutamic acid is higher

compared to the fish in this study and histidine is intermediate to fish and chicken (Edwards and Hnilica 1968). Frog (*Rana catesbiana*) erythrocyte H5 also have lowered serine, arginine, histidine, and glutamic acid. It also lacks methionine like carp and black crappie H5 and has aberrant tyrosine levels. Even the invertebrate worm, *Sipunculus nudus*, has an apparently homologous protein with lower arginine, serine, glutamic acid and histidine but with elevated threonine (Mazen and Champagne 1976). Viewing the spectrum of known H5, it seems that avian H5 are the most unique as a group.

Because of the similarities between H1 and H5, it is interesting to speculate on the possibility that these proteins have a common ancestor. They are similar in size and distribution of amino acids (Garel et al. 1975). Although antigenically distinct (Mura et al. 1974), long segments of sequence homologies are present (Sautière et al. 1975). Possibly, H5 evolved from an H1 subfraction with higher arginine content, such as the sea turtle Flb (Tsai and Hnilica 1975). The presence of multiple H1 subfractions possibly removed intense selective pressures from them. In contrast, H4 genes continue to mutate and evolve (Grunstein and Schedl 1976; Grunstein et al. 1976) but these mutations cannot be tolerated as changes in amino acid sequence due to the overwhelming selective pressures arising from the essential

topology of this protein in chromatin organization
(Camerini-Otero, 1976)

This phenomenon of H1 subfraction divergence may be more general than anticipated. The condensed sea urchin sperm chromatin has a highly specialized ϕ 1 histone with amino acid compositions very similar to H5 (Palau et al. 1969). It too, contains high arginine levels and extensive variability among its amino acids (Puigdomenech et al. 1976). H1-like HMG non-histone proteins have been found in small quantities (Johns et al. 1975) in H1 extracts and trout-specific HT which occurs in minute amounts has sequence homologies with H1 and H5 (Huntley and Dixon 1972, Wigle and Dixon 1971). These proteins may indicate the possibility of widespread evolution of H1 subfractions which go undetected among the mixture of non-histone proteins and degradative artifacts.

V. APPENDICES

Appendix 1. Oxidized Carp Histone (A) from erythrocytes (gel 1) and liver (gel 2) were compared by polyacrylamide gel electrophoresis, with oxidation. White Sucker histones (B) from erythrocytes (gel 1) and liver (gel 2) were compared without oxidation. Trout histones (C) from erythrocytes (gel 2) were compared to liver histones (gel 1) after oxidation. The polyacrylamide gels were polymerized according to Panyim and Chalkley (1969).

A

H3d

H1

H5

H2A

H2B

H4

B

H1

H3m

C

H1

H5

Appendix 2

Summary of amino acid compositions of various H5s

Moles per 100 mol or recovered amino acids

Amino acids	Common [†] Jack	Frog [†]	Sipunculid [†] worm	Chicken [†]	Quail [†]	Duck [†]	Goose [†]	Pigeon [†]
Aspartic acid	3.9	3.1	3.2	1.5	2.4	1.7	2.1	1.6
Threonine	4.9	4.2	2.3	3.0	3.6	2.0	4.0	3.5
Serine	8.8	7.8	6.5	11.9	12.2	9.8	10.1	10.8
Glutamic acid	3.9	1.7	3.1	3.8	3.4	3.6	3.3	3.2
Proline	6.2	7.0	6.1	7.8	6.5	9.3	9.1	10.9
Glycine	5.6	4.7	7.5	5.0	4.5	5.8	5.4	3.7
Alanine	16.2	17.0	20.7	15.9	16.0	19.0	17.3	18.2
Cysteine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Valine	6.2	5.8	3.2	4.2	4.5	3.0	2.8	3.6
Methionine	0.7	0.1	trace	0.4	0.2	0.2	0.3	0.4
Isoleucine	3.4	2.8	2.8	3.0	2.8	3.0	3.0	4.0
Leucine	5.8	5.3	3.2	4.3	4.5	3.5	3.6	3.1
Tyrosine	1.5	0.6	1.3	1.5	1.5	1.8	1.4	1.3
Phenylalanine	1.0	0.6	1.5	0.5	1.1	0.0	0.4	0.5
Histidine	0.9	0.5	0.8	1.5	1.7	1.5	1.5	1.5
Lysine	23.9	28.7	30.2	24.6	22.6	24.8	24.2	22.5
Arginine	7.1	9.9	7.8	11.0	12.7	10.9	11.5	11.3
Lysine/Arginine	3.4	2.9	3.9	2.2	1.8	2.3	2.1	2.0

[†] Edwards and Hnilica (1968)

^{*} Mazen and Champagne (1976)

[†] Garel et al. (1975)

^x Seligy et al. (1976b)

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