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**LA THÈSE A ÉTÉ
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IMMUNOLOGICAL CHARACTERIZATION OF LYSINE-
RICH HISTONES OF AVIAN ERYTHROCYTES

by

■ Casilda Victoria Mura

A thesis submitted to the Faculty of
Graduate Studies and Research in
partial fulfillment of the requirements
for the degree of Doctor of Philosophy.

Department of Biology
Carleton University
Ottawa, Ontario
May, 1979

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"Immunological Characterization of Lysine-
Rich Histones of Avian Erythrocytes"

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1979

A la memoria de mi tía Doña Rosa Silva Peña,
quien como tantas otras mujeres, hubiera
realizado tanto o mas, de haber tenido la
oportunidad.

ABSTRACT

The lysine-rich histones have long been implicated in maintaining and generating higher order chromatin structures. Other functions for these proteins have also been postulated. This study attempts to further investigate the relationship between the lysine-rich histones, H1 and H5, both of which occur in nucleated erythrocytes, the latter predominating in avian species.

Antibodies against histone 1 were raised in guinea pigs, and against histone 5, in guinea pigs and rabbits. Antibodies purified by affinity chromatography were used to localize histones H1 and H5 in isolated embryonic (3½, 6, 16 day-old) and adult chicken erythrocyte, liver and fibroblast nuclei by indirect immunofluorescence. Although virtually all nuclei were stained in isotonic conditions with anti-H5, variability in the staining was observed with both embryonic and adult erythrocyte nuclei staining in bright, medium and dim intensities or in rim patterns of fluorescence. Medium and dim categories were observed in liver and dim or no fluorescent staining in fibroblast nuclei. Staining with anti-H1 resulted in more uniform fluorescence, almost all fluorescent nuclei presenting bright and medium fluorescence. Localization of both histones in double-stained nuclei confirmed the results obtained in single-labelled nuclei. By scoring the distribution of different fluorescent categories in adult and embryonic

erythrocyte nuclei it was confirmed that H5 remained essentially constant in the primitive stages and appeared substantially higher in definitive line nuclei. The amount of H1 increased slightly during maturation of the primitive line but it was essentially constant in definitive cells.

This suggests that the mature erythrocytes do not comprise an homogeneous population of cells in terms of their content of H5, although H5 is present in virtually all cells from primitive erythroblasts through mature definitive erythrocytes. By staining mature erythrocyte nuclei in low ionic strength (0.03 M NaCl), it was shown that changes in chromatin condensation do not affect the availability of the antigenic determinants in either H5 or H1. The finding of anti-H5 binding antigens in liver and fibroblast nuclei would suggest that H1 and H5 share antigenic determinants.

Peptide fragments obtained from histone H5 were used as inhibitors in the formation of H5/anti-H5 complexes in the CF reaction. The strongest inhibitor was peptide 32-185 followed in decreasing order by 59-185 > 1-99 > 94-185 > 100-185 > 66-99 > 1-65 > 1-31. This reveals that most of the antigenic determinants are located in the middle of the H5 molecule in a region between residues 59 and 99 with little C-terminal to residue 99. The possibility of conformational determinants is also suggested. H1 histone also acts as inhibitor in

the reaction H5/anti-H5, and vice versa. This would stress the possibility that these histones possess common antigenic determinants. These results are consistent with the suggestion that H5 might represent an extreme variant of H1, added to erythroid cell chromatin already containing a reduced but constant level of common H1. This H5 might be involved in higher degrees of chromatin organization, leaving the globular region of the molecule, where the antigenicity of the molecule resides, accessible in mature as well as immature erythrocytes. The reason for the heterogeneity of the reaction of chromatin-bound H5 with its antibody, the general function of lysine-rich histones and the peculiarity of the erythrocyte-specific histone 5 await elucidation.

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LIST OF ABBREVIATIONS

anti-H5, H1 or IgG: antiserum against histone 5, histone 1 or immunoglobulin.

CF: complement fixation

CNBr: cyanogen bromide

DMSO: dimethyl sulfoxide

EDTA: ethylene diamine tetraacetic acid

FITC: fluorescein isothiocyanate

GuCl: guanidinium chloride

GuCl.PO₄: guanidinium chloride solution buffered with 0.1 M sodium phosphate

Histone nomenclature given at the Ciba Foundation Symposium in London 1975 (Bradbury, 1975) has been adopted as follows:

H1 = F1 (Johns, 1971); Ia & b (Rasmussen et al., 1962), (Macpherson and Murray, 1965); KAP (Huberman, 1973)

H2A = F2a2; I1b1; ALK

H2B = F2b; I1b2; KSA

H3 = F3; I1I; ARK

H4 = F2a1; IV; GRK

H5 = F2c (Hnilica, 1964); V (Neelin et al., 1964); KAS

ID = Immunodiffusion

IgG = Immunoglobulin

NBS = N-Bromo succinimide

PBS = phosphate-buffered saline (composition in Methods)

PFA = para-formaldehyde

PMSF = phenyl methyl sulfonyl fluoride

RITC = tetramethyl rhodamine isocyanate

TCA = trichloroacetic acid

TEMED = N,N,N',N'-tetramethylene ethylene diamine

TRIS = tris (hydroxymethyl) amino methane

VBS = veronal - saline buffer (composition in Methods)

VBS-G-M++ = veronal - saline buffer, gelatin, divalent
cations (composition in Methods)

A = angstrom

BIS = N-N' methylene bisacrylamide

INTRODUCTION

A. Histones and Chromosomal Organization

Chromatin is the name given to the chromosomal material when extracted. It consists mainly of DNA associated with an equal weight of small basic proteins called histones. Other proteins and RNA are also present, but in lesser amounts. Of all the components of chromatin, the histones have been the most extensively characterized so far.

According to their content in basic amino acids, histones are classified as very lysine-rich H1 and H5, moderately lysine-rich H2a and H2b and arginine-rich H3 and H4 (Elgin and Weintraub, 1975). These proteins are approximately equal in weight to the DNA within the cell nucleus and they are highly basic, since at least 25% of their amino acids consist of lysine, arginine and histidine compared with no more than 15% acidic amino acids. A consideration of the amino acid sequences of the four histones (H2a, H2b, H3 and H4) involved in the formation of the protein core of the nucleosome shows a predominance of basic residues towards the N-terminus, with a secondary cluster of basic residues in the C-terminal region. The central region contains most of the hydrophobic and acidic residues. In histone 1 the dominant basic region is at the C-terminal end. The apolar regions of the histones are thought to be part of the protein core of the nucleosome.

The very basic regions are thought not to be involved in histone complex formation but are major sites of interaction with DNA (Baldwin et al., 1975).

Another well known feature of histones is their unusual resistance to evolutionary change, histone H3 and H4 being the most conserved. The rates of protein evolution for histones have been estimated to be 400 million years to establish a 1% difference in amino acid sequence between two lineages of H4 (Wilson et al., 1977), 330 million for H3, 60 million for H2A and H2B and 8 million for H1. H1 is the most divergent protein, both in terms of the number of subfractions within any given tissue or species and in terms of its evolution. From one to eight subfractions of H1 including some different amino acid sequences have been observed in various species (Bustin and Cole, 1968a and b; Bustin and Stollar, 1972; Kinkade, 1969; Berdnikov et al., 1976). Variations in H1 have also been noted during development and cell differentiation.

During sea urchin development, lysine-rich histones exhibit the most striking changes. The pattern and developmental schedules of these changes show species specific differences (Seale and Aronson, 1973; Ruderman and Gross, 1974). Separation of rat testis lysine-rich histones showed that the main H1 in adult testis repre-

sents only a minor component in other tissues examined; its appearance could be correlated with the early phases of active spermatogenesis (Kistler and Geroch, 1975).

In cultured mouse mammary tissue the synthesis of certain H1 subfractions are elevated relative to others following hormonal induction (Hohman and Cole, 1971).

Several independent lines of work have shown that chromatin is composed of discrete subunits called nu bodies or nucleosomes. Studies with the electron microscope showed that the chromatin fiber appears like "beads on a string" (Olins and Olins, 1974; Woodcock, 1973; Woodcock et al., 1976), and nuclease digestion studies implied that the bead is a fundamental structural unit (Hewish and Burgogne, 1973; Axel et al., 1974; Noll, 1974a; Weintraub and van Lente, 1974). Studies in histone-histone interaction indicated that histone associations form the core of the bead structure (D'Anna and Isenberg, 1974a; Kornberg, 1974; Kornberg and Thomas, 1974).

The action of the micrococcal nuclease on chromatin is first as an endonuclease cleaving between nucleosomes, and then acting on the excised nucleosome shortening the DNA first to about 160 base-pairs, and then to about 140 base pairs (Noll and Kornberg, 1977). During this last stage the histone H1 is released leaving a metastable intermediate containing 140 base pairs of DNA complexed with the set of 8 histone molecules, called

the "core particle" (Shaw et al., 1976). Regardless of the tissue of origin, the nucleosome core contains approximately 140 base pairs of DNA wound around the outside of the histone complex (Noll, 1974b; Pardon et al., 1975; Baldwin et al., 1975). The remaining DNA connecting one core particle to the next varies in length from about 20 to 100 base pairs and is called "linker DNA".

The histone complex is composed of eight of the smaller histones, probably two each of H2a, H2b, H3, H4 (Kornberg, 1974; van Holde et al., 1974; Simpson and Bustin, 1976; Bustin et al., 1977). Histone H1 is not part of the nucleosome but is associated with linker-containing nucleosomes (Bradbury et al., 1975; Noll and Kornberg, 1976). It has been suggested that histone H1 determines the distance between nucleosomes; this could imply that the variation among H1 histone fractions could account for the observed variability of the chromatin DNA repeat length (Morris, 1976; Worcel, 1977).

This model is also compatible with data obtained using x-ray diffraction and neutron scattering techniques (Pardon et al., 1975; Baldwin et al., 1975; Finch et al., 1977). H1 has been found unnecessary either to generate the x-ray diffraction pattern of chromatin (Skidmore et al., 1973) or to observe nucleosomes in the electron microscope (Oudet et al., 1975). On the other hand, it has been shown that stabilization

of some of the larger limit-digest DNA fragments from chromatin after treatment with staphylococcal nuclease depends upon the presence of H1 or H5 (Axel et al., 1974; Camerini-Otero et al., 1976). Electron microscopic studies of histone H1-depleted "folded genomes" of Drosophila melanogaster (nuclear DNA in a complex with the intranucleosome histones and a fraction of the nascent nuclear RNA released by gentle lysis -- 0.4% Nonidet P40-0.9 M NaCl at 4°C -- of exponentially growing Drosophila melanogaster tissue culture cells) indicate that they are composed of long cables of 100 A diameter. Limited treatment with DNAase I relaxes the 100 A fiber to the "beads on string" structure (Worcel and Benyanati, 1977). Electron micrographs of oligonucleosome fractions containing H1 reveal the presence of spherical structures about 200 A in diameter which have been called "super-beads" and seem to contain 6 to 10 nucleosomes each (Finch et al., 1977). This evidence has implicated H1 in the modulation of the chromatin condensation, as have the changes in viscosity of H1-depleted chromatin (Gaubatz, et al., 1977), sedimentation coefficients of nuclease digests of products of bovine lymphocyte nuclei (Hozier et al., 1977; Renz et al., 1977a and b), and of H1-depleted SV40 mini-chromosomes (Griffith et al., 1977).

All this evidence suggests that the next order of chromatin structure is a coil of nucleosomes. Neutron diffraction and electron microscopic studies of the 300

A chromatin fiber indicates that the nucleosomes are arranged in a solenoid with a pitch of about 100 Å, containing about 6 nucleosomes per turn, a diameter of 300 Å and a 100 Å cavity down the central axis (Carpenter et al., 1976). Studies on micrococcal nuclease digestion of partially and totally H1-depleted nuclei of HeLa cell suggest that, where H1 is missing, the chromatin unfolds. Neutron diffraction of fibers from H1-depleted chromatin are also consistent with a coil implying that H1 is not essential to the coiling of a string of nucleosomes, at least at the very high concentration found in chromatin fibers. This led to the speculation that one of the functions of H1 may be to stabilize this coil, while other functions could involve the generation of higher order chromosome structure (Bradbury, 1978; Cole et al., 1977a).

The molecular properties of H1 are consistent with a multifunctional role. It is almost twice as large as the other histones. It has a very basic segment in the N-terminal region from residue 19 to 35, the middle region can be folded into a globular structure, and the segment 123-216 in the C-terminal region is very basic and consists mostly of lysine, proline and alanine (Hartman et al., 1977; Jones et al., 1974). The most conserved region is the globular middle (Cole et al., 1977b). The C-terminal peptide from residue 124 to 216 binds strongly to DNA and causes a behaviour resembling

the salt-induced contraction of chromatin, while the N-terminal half binds only weakly to DNA (Chapman et al., 1976).

Physical studies on H5 and its fragments suggest that it is structurally related to the H1 (Crane-Robinson et al., 1976 and 1977). These two histones have similar amino acid composition and sizes, H5 with 185 residues (Crane-Robinson et al., 1977) compared to H1 with 216 (Jones et al., 1974). The main distinction is that H5 contains substantially more arginine and serine than H1. Like H1, histone 5 in solution at high ionic strength consists of three domains: N-terminal disordered region 1-21, a compact globular central domain 22-100, and a disordered C-terminal chain 101-185 (Avilés et al., 1978). Tertiary folding of the two proteins might be less similar since the secondary structural predictions of H1 (Fasman et al., 1976) and H5 (Crane-Robinson et al., 1976) do not align well. One helical region is equivalent in the two proteins, 30-40 in H5 and 44-54 in H1, but no other distinctive secondary structural homologies are apparent. Both are soluble in 5% PCA or TCA and both are extracted relatively easily from chromatin (Murray et al., 1968). Since H1 and H5 do not aggregate at high ionic strength, they differ from the other histone fractions (Diggle et al., 1975; Champagne et al., 1970).

The gene coding for H5 is reiterated ten times in the chicken genome (Scott and Wells, 1976), like those for the other histone genes in mammals (Birnstiel

and Chiba (1977). Histone synthesis is tightly coupled to DNA synthesis in eukaryotes, but the stoichiometry of H1 or H5 differs from that of H4, H3, H2a and H2b. In addition H5 is the only histone synthesized after cessation of DNA synthesis (Appels and Wells, 1972).

Despite these recent advances suggesting that histones play a structural rather than regulatory role, the involvement of lysine-rich histones in higher levels of organization of chromatin does not exclude a regulatory role, since their position outside the nucleosome allows them to react with other proteins (e.g. non-histone proteins).

Up to now, two main approaches have been used in the study of chromatin, one involving the characterization of the whole macromolecular complex, which does not allow the study the individual components without a marker, and the other involving the study of isolated chromatin components and their assembly to form structures resembling the native structure; this does not reveal how the individual components are arranged in the native structure. Immunological techniques constitute a third option by allowing the study of the chromatin components "in situ" under conditions which do not alter the structure of chromatin and chromosomes. Furthermore, because

of their sensitivity and specificity these methods are particularly appealing. For these reasons, an immunological approach to study lysine-rich histones has been undertaken. This thesis describes the results of this investigation.

B. Development and Differentiation

All the genetic information required for the construction of an organism is present in the fertilized egg. The mechanism by which it develops into a multicellular organism has been a source of mystery to scientists for almost a century. Once the egg is fertilized, development proceeds by replication of the genome followed by cell divisions. The cleavage division does not lead to embryological development. The formation of specific cell types occurs rather by cell determination and cell differentiation. Determination occurs during embryogenesis when a cell becomes committed to eventually express a particular phenotype. Determined cells may divide for many generations before receiving a signal of differentiation; that is to express the predetermined phenotype (Hood et al., 1975). Differentiation is the process by which specialized cells, tissues and organs are formed during development of the individual from the fertilized egg. It implies thus the production from a single cell, of progeny that exhibit increasing heterogeneity as the individual develops, the fertilized egg

being by definition the undifferentiated cell (Harris, 1973). In most eukaryotes, the daughter cells from the first cleavage are identical in their developmental potential. These cells when separated from one another are capable of producing normal offspring, which are about half the normal size. The next cleavage from the 2- to 4-cell stage does not generally restrict developmental potential; in some animals each blastomere produces identical offspring if grown separately. After the second or third cleavage however many of the cells are no longer totipotent and are no longer capable of producing complete, normal animals. As development proceeds, quadrants or groups of cells become fated to differentiate and develop into cells of specific organs. In general normal cell division always produces daughter cells that have a level of determination identical to or more specific than the parental cell. As the development of the organism proceeds the ability to differentiate into a number of different phenotypes is severely restricted. This restriction seems to be irreversible, and normal cells, once committed to a specific pathway, are not able to turn back to a more undifferentiated point in their development.

The whole process of differentiation is carried out without any loss of genetic material; with few exceptions all differentiated somatic cells of an organism probably contain the same genetic information. This is

based on the finding that nuclei of differentiated somatic cells in amphibia can give rise to normal animals when transplanted into enucleated eggs (Gurdon and Vohlinger, 1966).

A model for control of development recently presented (Caplan and Ordahl, 1978), predicts that differential gene expression during development arises by restricting the transcriptional potential of cells. The genome of the fertilized egg should have all genes accessible to the transcriptional machinery. Differentiation and development will be accomplished by progressively repressing, in a selective and irreversible manner, genes that were previously active. This means that once a specific gene is repressed during a normal developmental event it cannot be transcribed at some time later. Thus some genes may be accessible to the transcription machinery but transcribed to only a minor extent in pre-differentiated and other differentiated states.

Increased transcription of such genes as a response to environmental stimuli would thus be an augmentation of an already established genomic state rather than a specific activation of a repressed gene. In this connection, it has been shown that the hemoglobin gene is transcribed at a low frequency in fibroblast cells and other nonhematogenic tissue (Humphries, 1976).

In the process of mammalian erythropoiesis, a precursor hematopoietic stem cell (CFU-S) is committed

to produce the three major types of blood cells: white blood cells (granulocytes), platelet-producing cells (megakaryocytes) and red blood cells (erythrocytes). The commitment of CFU-S cells to a particular hematopoietic lineage is influenced by the microenvironment in particular hematopoietic organs. After commitment to the erythroid lineage, erythroid stem cells seem to proliferate for a few generations before becoming sensitive to the hormone erythropoietin. This hormone increases the proliferation of committed erythroid stem cells and proerythroblasts which then differentiate into mature erythroid cells containing hemoglobin. During erythroid development, the globin genes become active between proerythroblast and basophilic erythroblast stages of development. Immature erythroid precursor cells (80-90% proerythroblast) from foetal liver cells contain negligible globin messenger RNAs (Harrison, 1976). Similar features of erythropoiesis in the embryos of mouse and chicken and in the metamorphosing larva of the bullfrog have been shown (Ingram, 1972).

Erythroblasts, the early dividing cells of the avian erythroid line found especially in bone marrow, are relatively active in DNA, RNA and protein synthesis. These cells divide every two hours and accumulate polyosomes with messenger RNA for hemoglobin (Cameron and Kastberg, 1969).

The polychromatic erythrocytes found in anemic blood synthesize hemoglobin, hnRNA and mRNA, but not ribosomal RNA or DNA (Attardi et al., 1966). Recent studies using erythroid cells of anemic chickens have shown that their polyA-containing mRNA contains fewer than 1000 species, about one quarter as many as the polyA nuclear RNA, while the number of mRNA species in the polysomes of the other cells varies from 2000 in sea urchin tube feet to 100,000 in mouse brain (Galau et al., 1974, 1976). Since the fraction of the genome transcribed in these cells is evidently lower than other vertebrate cells, and the sequence complexity of polyA hnRNA far exceeds that of functioning mRNA, Tobin et al., (1978) and Lasky et al., (1978) suggested that both selective transcription and post-transcriptional sequence selection contribute to the specialization of erythroid cells during terminal differentiation. The levels of RNA and protein synthesis gradually decrease in these cells essentially to zero in the mature nucleated erythrocyte (Cameron and Prescott, 1963; Williams, 1971).

The development of erythroid cells terminates in genetically inert, mature erythrocytes; while in the chick the nucleus is retained after maturation, in mammals it is lost. As they mature, erythrocytes become committed to the synthesis of one subset of polypeptides, the chains of hemoglobin, to the eventual exclusion of almost all other proteins.

In chicken there are at least two different erythroid cell lines; the primitive erythroid cells found in very early embryos and the definitive cell line found in later embryonic stages and in adult animals. Both erythroid cell lines mature through similar sequences. However, during the process of maturation of the definitive cell line, several cell maturation stages are in circulation at the same time (Lucas and Jamroz, 1961) while the maturation of the primitive cell line is better synchronized. Consequently the primitive erythroid population consists predominantly of particular cell types at certain stages in embryogenesis (Lucas and Jamroz, 1961; Burns and Ingram, 1973), and it is possible to correlate cytochemical and biochemical properties of the whole population with the dominant cell types at these stages.

The presence of a specific histone fraction in the terminally differentiated chicken erythrocyte led to the suggestion that this fraction plays a critical role in establishing and maintaining the highly repressed stage of chromatin in erythrocytes (Seligy and Neelin, 1970; Seligy et al., 1973; Brasch et al., 1974, 1976; Sung et al., 1978). However, histone 5 appears very early in maturation, being detected in erythroblasts at a significant but lower level than in mature erythrocytes (Appels et al., 1972; Ruiz Carrillo et al., 1976). Thus the presence of H5 seems to be necessary but not sufficient

to account for the inactivation of the avian genome. Metabolic studies have indicated that H5 is phosphorylated immediately after its synthesis and subsequently dephosphorylated (Tobin and Seligy, 1975; Sung et al., 1976). The timing of phosphate incorporation appears to correlate inversely with maturation of nuclei. Adams et al. (1970) showed that there is a significant increase of phosphate content in histone 5 from regenerating blood in comparison to H5 from normal blood. Subsequent studies further established the likelihood that phosphorylation and synthesis events of H5 were associated in cells which proliferate and synthesize protein, RNA and DNA (Seligy and Neelin, 1973; Seligy et al., 1973; Tobin and Seligy, 1975).

Recent studies have shown that the modified amino acid residues are located in highly basic regions of the histone 5 molecule. The absence of phosphates in the central region 8-94 is significant since this region is thought not to be involved in the DNA binding. Phosphorylation of both serine 3 and 7 produces an acidic region at the N-terminus, so that electrostatic repulsions between the phosphorylated amino end and DNA prevent "premature" condensation; removal of the negatively charged phosphates would relieve this repulsion and allow condensation (Sung et al., 1978). This is consistent with the observation that the more phosphorylated histone 5 from immature erythroid cells is more

weakly bound to chromatin than the less phosphorylated form of this histone from mature erythrocytes (Brasch et al., 1974; Tobin and Seligy, 1975).

Erythrocyte chromatin differs markedly from other types of chromatin, in that it retains only 1-2% of the transcription capacity of deproteinized erythrocyte DNA. Removal of H1 and H5 increases template capacity to levels very much like liver chromatin. H5 alone was found to be responsible for 50% of the template restriction of erythrocyte chromatin (Seligy and Neelin, 1970; Bolund and Johns, 1973; Seligy and Miyagi, 1974). By using bacterial RNA polymerase, the transcriptional capacity of chromatin from immature and mature pigeon erythroid cells was measured (Andreeva et al., 1978; Gasaryan et al., 1978). The erythroblast chromatin showed a template efficiency similar to that of liver chromatin and the selective extraction of H5 from this chromatin had little effect on its template efficiency, in contrast with the increase of transcription obtained when H5 is extracted from erythrocyte chromatin. It was suggested that in the early erythroblast the less active (phosphorylated) form of the histone accumulates, being subjected to dephosphorylation which would lead to an aggregation of chromatin and a progressive decrease of its transcriptional capacity.

During reactivation of erythrocyte nuclei in heterokaryons, which results in transcription and eventual

replication of chick DNA, one of the early events is the loss of H5 with apparent replacement by H1 and other proteins (Appels et al., 1974). It has been reported that the proportion of H5, relative to DNA or total histone content increases during embryonic development (Moss et al., 1973; Weintraub, 1978), maturation or regeneration (Appels et al., 1972; Billet and Hindley, 1972; Mazon and Champagne, 1972; Andreeva, et al., 1978). Although loss of H1 has been reported during regeneration of anemic duck blood (Dick and Johns, 1969), or pigeon blood (Andreeva et al., 1978), no change in the concentration of H1 was found in developing embryo erythroid cells (Weintraub, 1978) or anemic adult chicken erythrocytes (Billet and Hindley, 1972). Despite these contradictions, it has been suggested that histone H5 replaces H1 during maturation of chicken erythroid cells as it appears to do in mature erythrocytes of amphibian and fish species (Destree et al., 1975; Miki and Neelin, 1977). Complete absence of H1 has not been reported.

The exact cellular basis for the variation of H5 and H1 during erythropoiesis is still not known. Firstly it is not known if both H5 and H1 are present within the same cells, at least in all cells capable of hemoglobin synthesis. Secondly, it is not known if the increase of H5 during development is due to an accumulation of H5 within individual cells or to an increase in the proportion of cells having H5. A study of the

biological role of this unique histone should lead to a better understanding of the mechanisms regulating gene expression in higher organisms. Furthermore, because of the similarity H5 presents with the ubiquitous H1, the study of their relationship should shed light on the role of non-core nucleosomal histones during differentiation. Therefore the localization of histones H5 and H1 has been undertaken in isolated nuclei of chicken erythrocytes at different stages of maturation. Antibodies against H5 and H1 were prepared and the presence of these histones was analyzed by indirect immunofluorescence.

C. Localization of Antigenic Determinants

The lysine-rich histone of chicken erythrocytes include a major component, the specific histone H5. Variation in both N-terminal sequence and electrophoretic mobilities have been noted in H5 from five avian species (Seligy et al., 1976; Yaguchi et al., 1977). H5 has also been found in the erythrocyte nuclei of non-avian species, notably fish and amphibians, as well as the invertebrate sipunculid worm, but the proportions and properties vary at least as much as those of H1 (Vendrelly et al., 1968; Miki, Ph.D. Thesis, 1976; Mazen and Champagne, 1976). H5 from chicken erythrocytes has 185 residues (Crane-Robinson et al., 1977). The sequence has been determined up to residue 111 from the NH₂-terminus (Sautière et al., 1976).

However, the study of lysine-rich histones has been hampered by the lack of a sensitive and specific assay for these molecules. Within the last few years the antigenic structures for a variety of proteins have been proposed, among them, histone H1 from rat liver (Hekman and Sluyser, 1973), H1 from calf thymus (Bustin and Stollar, 1973), histone H3 from chicken erythrocyte (Sapeika et al., 1976), sperm whale myoglobin (Atassi, 1975), chicken lysozyme (Atassi and Lee, 1978) and bovine serum albumin (Benjamin and Teale, 1978).

The antigenic residues are arranged in groups called determinants, each of which binds to a specific class of antibodies. A typical determinant contains four to seven residues (White et al., 1978). Even though only a small fraction of the amino acid residues in the proteins participate directly in the antibody binding, the immunological techniques are able to detect differences among closely related proteins, including evolutionary substitutions. Although none of the 7 amino acid substitutions in turkey and chicken lysozyme occurs in the antigenic determinants, they are distinguishable from one another immunologically. Thus, substitutions elsewhere in the protein alter markedly the antigenic activity of the determinants (Arnhein and Wilson, 1967; White et al., 1978). Antibodies have been used to detect conformational changes in alkaline phosphatase of E. coli (Lazdunski et al., 1975), pancreatic trypsin inhibitor

(Creighton et al., 1978) and of bovine serum albumin (Teale and Benjamin, 1976; Chavez and Benjamin, 1978).

The identification of the immunologically reactive regions of histones is a prerequisite for studying the conformational changes of these proteins in interactions with DNA or other proteins in native chromatin in situ and for assessing the interspecific homologies among lysine-rich histones. This section describes experiments leading to the localization of the immunologically reactive regions of histone H5. This was accomplished by means of inhibition of the complement fixation reaction of H5 and its homologous antiserum by using peptides derived from purified H5.

METHODS

A. Preparation of Histones

1. Preparation of Chicken Erythrocyte Nuclei

Erythrocytes were obtained by severing the jugular veins of adult chickens. During the collection of blood 10% (w/v) trisodium citrate was added to a final concentration of 1% to minimize clotting. Alternatively 5-10 drops of a 1,000 USP unit/ml of heparin per 50 ml of blood was used for the same purpose. The blood was immediately chilled and all operations were carried out at 4°C.

The blood was centrifuged at 500 g for 10 minutes in a refrigerated centrifuge fitted with a swinging bucket rotor. Serum and the layer of white cells were discarded by aspiration and the erythrocytes were resuspended with sufficient cold 0.15 M NaCl to restore the original blood volume. Erythrocytes were recovered by centrifugation as before and resuspended in 0.15 M NaCl. Washing by centrifugation and suspension was repeated once more.

The erythrocytes were recovered by centrifugation as before and resuspended in half of the original volume in 0.15 M NaCl. An equal volume of 0.6% saponin in 0.15 M NaCl was then added to lyse the cells (Murray et al., 1968). The lysate was allowed to stand for about 15 minutes stirring in the cold, then centrifuged at 500 g

for 10 minutes. The pellet of unlysed erythrocytes, if any, was discarded and the supernatant was centrifuged at 8000 g in a refrigerated centrifuge.

The nuclear pellet was resuspended in 0.15 M NaCl, 2.5 mM PMSF to inhibit protease activity (Nooden et al., 1973), dispersed by 10 strokes of a loose-fitting hand homogenizer and centrifuged at 500 g for 10 minutes. This step was repeated until the nuclear pellet was free from evident heme pigment. The final clean nuclear pellet was resuspended either in 0.15 M NaCl, 2.5 mM PMSF or 0.15 M NaCl containing 10% DMSO, a cryo-protective agent, to the original blood volume, before sealing in glass ampules and storing at -90°C .

2. Extraction of Total Histones

For extracting total histones the pellet of washed erythrocyte nuclei was resuspended in 20-30 ml of 0.25 M HCl (Moss et al., 1973) for each 50 ml of original blood. The suspension was stirred in the cold for at least 4 hours before centrifugation at 8000 g for 10 minutes to remove any particulate material. The supernatant was saved and the pellet was extracted with 0.25 M HCl once more as before.

The pooled supernatants were extensively dialyzed against distilled water in dialysis tubing which had been previously boiled in 2.5×10^{-2} M EDTA and washed exhaustively with water and ethanol. The dialyzed his-

tones were then concentrated by ultrafiltration with a PM-10 Diaflo membrane at a constant nitrogen pressure of 55 psi ($1 \text{ psi} = 6.9 \times 10^3 \text{ N/m}^2$); the final concentrated histone solution was then lyophilized.

3. Extraction of Histones 1 and 5

For selectively extracting histone H1 and H5 the pellet of washed erythrocyte nuclei was resuspended in 5% TCA (Champagne et al., 1968), stirred in the cold for at least 4 hours and then centrifuged at 8000 g for 10 minutes in a refrigerated centrifuge. The supernatant was saved and the pellet was extracted once more with 5% TCA. The supernatants were pooled, extensively dialyzed against distilled water, concentrated by ultrafiltration and lyophilized as indicated in section II.

4. Extraction of Histones 2a, 2b, 3 and 4

The final pellet obtained after extracting H1 and H5 was resuspended in 0.25 M HCl to extract the remaining histones. The suspension was stirred in the cold as is 3 and centrifuged at 8000 g for 10 minutes. The supernatant was saved and the pellet was extracted again with 0.25 M HCl. A third extraction removed little or no additional protein. Supernatants were pooled, extensively dialyzed against distilled water, concentrated by ultrafiltration and lyophilized.

5. Fractionation of Histone Extracts by Column Chromatography

a) Cation-Exchange Chromatography

For the separation of the several classes of histones, solutions were applied to Amberlite CG-50, a carboxylic cation-exchange resin, equilibrated and eluted with guanidinium chloride solutions buffered at pH 6.8 with 0.1 M sodium phosphate (Adams and Neelin, 1969; Luck et al., 1958; Satake, 1960; Mura et al., 1974).

To prepare the resin, a thin slurry of Amberlite CG-50 was made in water. The heavier material was allowed to settle for several minutes before the suspension of finer particles was decanted. The resin was then successively suspended with stirring in each of the following solutions and collected under suction by filtration in a Buchner funnel containing two sheets of Whatman #4 filter paper: 2 N HCl; distilled water; 2 N NaOH; distilled water; 2 N HCl; distilled water; 2 N NaCl. Resin resuspended in 2 N NaCl was titrated to pH 7 with NaOH. Finally the resin was suspended and equilibrated in 8% GuCl-PO_4 (see below) and the slurry was used to pack the columns, or stored in this form.

Practical grade GuCl was purified by filtration of a solution through an activated charcoal column (Satake et al., 1960). To prepare the column, a thick slurry of celite and activated charcoal (2:1 w/w) was

rapidly stirred in distilled water and poured into a 30 x 8 cm column to a height of about 25 cm. The column was allowed to drain and used the same day. One or two liters of crude 60% w/v GuCl solution was prepared and the insoluble material was largely removed by filtration through Whatman #4 without suction. This solution was passed through the charcoal column and washed through with a small volume of distilled water. The removal of impurities was monitored by Absorbance at 400 nm which was less than 0.01 against a water blank.

The concentration of GuCl in the purified solution was determined from its refractive index using the relation

$$\text{weight \% of } \text{GuCl} \text{ in } \text{H}_2\text{O} = \frac{n^{25}_{\text{GuCl}} - n^{25}_{\text{H}_2\text{O}}}{0.00166}$$

Two column sizes were used: "large" (2.5 x 60 cm) and "small" (0.5 x 55 cm). For fractionation on "large" columns approximately 50-100 mg of histones, either H1 and H5 or H2a, H2b, H3, H4 mixtures, were dissolved in 2 ml of 8% GuCl [this and all subsequent solutions were buffered in 0.1 M sodium phosphate at pH 6.8]. All the procedures were done at room temperature. If the histone solutions were turbid the insoluble impurities were sedimented at 8000 g for 20 minutes. The clear supernatant was layered on top, allowed to flow into the column and then washed in with three 1 ml portions of 8% GuCl . Ten milliliters of 8% GuCl was then layered on the resin, and continuous

flow from the mixing reservoir was initiated. A linear gradient from 8-20% GuCl buffered in sodium phosphate at pH 6.8 was used to elute the lysine-rich and slightly lysine-rich histone fractions in a total 700 ml of solution. The column was treated with 100 ml of 40% GuCl to elute the arginine-rich histones followed by enough volume of 8% GuCl to re-equilibrate the column. The flow rate was 30-40 ml/hour and 4 ml fractions were collected.

For lesser amount of mixtures of histones (up to 10 mgs) a "small" column was used with a 100 ml gradient from 8-20% GuCl, followed by 20 ml of a 40% GuCl and then enough volume of 8% GuCl to re-equilibrate the column. The histone sample was dissolved in 0.2-0.4 ml of 8% GuCl and centrifuged as above to remove insoluble material. The clear histone solution was applied to the column and washed in with three 0.4 ml portions of 8% GuCl. Continuous inflow of GuCl was then initiated; the flow rate was 5 ml/hour and 0.3 ml fractions were collected.

Protein in the chromatographic effluent was assayed turbidimetrically in TCA (Luck, (1958), modified by Bonner et al. (1968)). Aliquots (0.2 ml) from every third effluent fraction were transferred into small tubes and diluted with 0.6 ml of distilled water. Then 0.4 ml of 3.3 M TCA per tube was added and the tubes were shaken vigorously for a few seconds. After 13 minutes measure-

ments of A400 were taken.

The protein from each peak was pooled and extensively dialyzed against water, then concentrated in an ultrafiltration cell with a PM-10 Diaflo membrane at a constant nitrogen pressure of 55 psi (losses were estimated to be approximately 20%). The concentrated solution was finally lyophilized and stored in airtight vials over Drierite.

b) Exclusion Chromatography

Fractionation of the Amberlite CG-50 fractions was achieved by exclusion chromatography through a 1.8 x 87 cm column of Bio-Gel P-60 equilibrated with 0.01 M HCl (Vidali and Neelin, 1968a, b). Samples were solubilized in 0.005 M sulfuric acid and eluted with 0.01 M HCl at a flow rate of 8.3 ml/hour. Three milliliter fractions were collected and assayed for histone at 230 nm. A standard curve of Absorption vs mg/ml was prepared using pure H5. The fractions constituting each protein peak were pooled, dialyzed against distilled water, concentrated by ultrafiltration and lyophilized as in Method A2.

6. Polyacrylamide Gel Electrophoresis

The acid-soluble nuclear proteins were separated and characterized analytically in the urea-polyacrylamide gel electrophoretic system of Panyim and Chalkley (1969). Gels contained 2.5 M urea, 15% (w/v) acrylamide, 0.125%

(w/v) BIS and 0.5% (v/v) TEMED. Ammonium persulfate 0.125% w/v was used as a catalyst of polymerization. Gels were made in glass tubes of 0.5 cm internal diameter and 10 cm in length. Polymerization was observed in 30 minutes but the gels were left for an additional 30-60 minutes.

All gels were pre-electrophoresed to remove residual reactants of polymerization. The process was done at room temperature in a standard vertical gel system. The electrode buffers were 0.9 N acetic acid and a Beckman Duostat power supply was used to provide constant current of 1 mA/gel under a voltage ranging from 80-100 V. Pre-electrophoresis was monitored by running Pyronin Y 0.01% in sucrose 15%, 7% acetic acid and it was judged complete when the dye was eluted.

Lyophilized histones were dissolved in distilled water to a concentration of 1 mg/ml. After mixing 25 μ l of total histone solution or 5 μ l purified histone solution with an equal volume of 15% sucrose in 7% acetic acid containing Pyronin 0.01% as the tracking dye, samples were layered onto the top of the gel column. Conditions for electrophoresis were as for pre-electrophoresis and the average run took 6 hours. The gels were stained for at least 15 minutes with 1% (w/v) Amido Black in 7% acetic acid, 40% ethanol and destained by changing the acetic acid-ethanol solution several times during two days. Gels were then placed in 7% acetic acid and

scanned one day later in a Beckman Acta C-III spectrophotometer with a slit width of 0.1 mm at 600 nm.

B. Preparation of Antibodies

1. Preparation of Guinea Pig Antisera Against H5 and H1

Purified lyophilized histone was dissolved in isotonic saline (0.15 M NaCl) to make a 1 mg/ml solution and emulsified with one part of complete Freund's adjuvant containing 10 mg of Mycobacterium tuberculosis H37.Ra per ml. The immunization schedule followed was that of Mura et al. (1974), using groups of 6 Hartley strain guinea pigs (250-300 g) for each experiment. Each guinea pig received 0.1 ml of the emulsion into each foot-pad for a total dose of 20 µg in case of H5 and 10 µg in case of H1. Booster injections consisting of 10 µg of histone in isotonic saline administered intradermally in five sites were given 8, 15 and 22 days later. Each animal was bled per cardium on the 30th and 41st day after primary immunization. An average of 8.5 ml of serum was obtained from each bleeding. Sodium azide was added as a preservative at a final concentration of 0.1% and the serum stored at -20°C.

2. Preparation of Rabbit Anti-H5 Serum

Purified, lyophilized histone was dissolved in isotonic saline and emulsified with two parts of complete

Freund's adjuvant. Two female rabbits received 0.2 ml of the emulsion into each foot-pad and 0.8 ml intradermally for a total dose of 1-5 mg of protein (Kabat, 1968).

Booster injections with the same amount of protein were administered 10-15 days later in the same way as the first injection except that the emulsion was made with incomplete Freund's adjuvant. Each animal was bled by ear vein 10-14 days after the booster injection. Each bleeding yielded an average of 20 ml of serum.

3. Preparation of Rabbit Antiserum Against Guinea Pig IgG

a) Preparation of Guinea Pig IgG

Blood from untreated guinea pig was obtained by heart puncture. The blood was allowed to clot for 2 hours at room temperature, and the clot was loosened from the test tube wall with a glass rod. After standing overnight in the refrigerator, the serum was removed carefully with a Pasteur pipette and centrifuged for 10 minutes at 1500 g. The clarified serum was removed by pipette without disturbing the sediment. Clarified serum was chilled to 0°C on ice and ice-cold saturated ammonium sulfate at pH 7.0 was added slowly to obtain 40% saturation, according to the formula:

$0.4 (\text{vol of serum} + X) = X$, where X is the required volume of saturated ammonium sulfate. The final solution was stirred slowly in the cold for 2 to

16 hours and the precipitate was collected at 8000 g for 10 minutes in a refrigerated centrifuge. The pellet was washed 3 times with 40% (w/v) ammonium sulfate at pH 7.0, collected by centrifugation and dissolved in a small volume of distilled water before extensive dialysis against 0.01 M phosphate buffer pH 7.5. The dialyzed protein solution was clarified by centrifugation at 8000 g for 10 minutes. The protein concentration of the sample was determined spectrophotometrically at 280 nm assuming $E \frac{1 \text{ cm}}{1\%} = 1.4$ for rabbit IgG (Handbook of Biochem. Selected Data for Mol. Biol., 1968).

The crude gamma-globulin solution was then fractionated in a DEAE-Sephadex A-50 column previously equilibrated with 0.01 M phosphate buffer pH 7.5 (Cebra and Goldstein, 1965); part of the gamma-globulin fraction passed directly through the column, but a second gamma-globulin was eluted from the column in a stepwise manner with a 0.05 M NaCl, 0.01 M phosphate buffer pH 7.5. Both gamma-globulin peaks were pooled separately, concentrated by ultrafiltration through PM-10 Diaflo membrane to a final concentration of 12 mg/ml and dialyzed against isotonic saline.

b) Raising of Rabbit Antiserum Against Guinea
Pig IgG

The concentrated globulin fractions were each emulsified with two parts of complete Freund's adjuvant.

Two female rabbits each received 0.2 ml of the emulsion into each foot-pad and 0.8 ml intradermally for a total dose of 4-5 mg of protein. Booster injections, with the same amount of protein were administered in the same manner 10-15 days later, except that the emulsion was made with the incomplete Freund's adjuvant. Each animal was bled by ear vein 10-14 days after the booster injections yielded 20-30 ml of blood. Serum was processed as in Method B1.

C. Assay of Antibodies

1. Immunodiffusion

Occasionally the detection and characterization of antibodies was tested by immunodiffusion by the method of Oudin and Williams (1971). Difco Special Agar Noble and agarose were used to prepare stock solutions of 2% (w/v) in distilled water in a boiling water bath. A volume of 1% Thimerosal solution was then added to make a final concentration of 0.01%. The hot solution was filtered through #4 Whatman filter paper on a Buchner funnel under moderate suction and 12 ml of the hot filtrate was stored at 4°C in screw-capped test-tubes.

Microscope slides (2.4 x 7.6 cm) were degreased successively in 90% (v/v) ethanol, ethanol:diethyl ether (1:1 v/v) and diethyl ether. They were dried, numbered and placed on a clean paper towel on a flat horizontal

surface. Two percent agar or agarose stock was melted on a boiling water bath. Meanwhile 10 ml of 0.01 M phosphate buffer, pH 7.5 (for agar) or 12.5 ml of buffer plus 2.5 ml of distilled water (for agarose) was preheated in the boiling water bath. After 5 minutes, 10 ml of the agar or agarose solution was mixed into the appropriate buffer to give a final concentration of 1% agar or 0.8% agarose. Two ml of the hot agar or agarose solution was evenly distributed by pipette onto each slide. While the gel set, the slides were protected from dust and in 5-10 minutes they were transferred into a moist chamber and stored at 4°C. For some preparations, 0.1 M VBS-G-M++, pH 7.35 was used.

The pattern for immunodiffusion in gels was cut with a metal punch. Wells of 2 mm internal diameter were spaced 0.7 cm from each other, and cleared with a Pasteur pipette before 0.3 μ l of antiserum was pipetted into the central well and 0.3 μ l of each antigen sample or a series of antigen dilutions was added clockwise to the peripheral wells. For immunodiffusion slides were left in a moist chamber at a constant temperature (4°C) from 16 hours to 4 days. The precipitation lines were then inspected directly or by using indirect illumination against a black matte-finish background. The unprecipitated proteins were washed off the slides by submerging them in 0.15 M NaCl solution for at least two days with frequent changes of the salt solution. Then the slides

were similarly washed in distilled water for one day before the remaining arcs were stained for 15 minutes at room temperature with 1% Amido Black in 7% acetic acid, 40% ethanol. The slides were destained by changing the acetic acid, ethanol solution several times and rinsed once with distilled water before drying by applying a filter paper to the surface of the gel and letting them stand in open air until next day. The stained immunoprecipitates were photographed.

2. Complement Fixation

Routine detection, characterization and quantitation of antibodies were made by complement fixation according to the method of Wasserman and Levine (1961; Levine and Van Vunakis, 1967; Howell and Perkins, 1968).

a) General

The complement fixation assay for an antigen antibody reaction makes use of the fact that complement is fixed by the antigen-antibody complex, making less complement available for reaction with the hemolysin-erythrocyte system. Since hemolysin contains antibody produced against certain elements in the surface of the erythrocytes, added complement leads to red cell lysis and the liberated hemoglobin can be determined by its absorbance at 550 nm and used as a measure of complement fixed by the unknown antigen-antibody system. The

standard test uses guinea pig complement, sheep erythrocytes and rabbit antibodies to sheep red cells (hemolysin). Other reagents include the following: stock VBS, consisting of 83 g of NaCl and 10.19 g of Na 5,5-diethyl barbiturate in 2000 ml of distilled water at pH 7.35 ± 0.05 . This stock solution is five times isotonic and is stable for at least 1 month at 4°C . Isotonic VBS-G-M++ is prepared by mixing 1 part of stock VBS with four parts of distilled water, adding and dissolving sufficient dry gelatin to yield a final concentration of 0.1% (w/v) and adding to each litre 1 ml of 1.0 M MgCl_2 in 0.15 M CaCl_2 . This buffer was prepared fresh every day.

b) Standardization of Components (Sheep Red Blood Cells, Guinea Pig Complement and Hemolysin)

The commercially available sheep blood (50% in Alsevers solution) was diluted with VBS-G-M++ and centrifuged at 600 g for 10 minutes. The supernatant was discarded and the pellet of red blood cells was washed several times with the same buffer until the supernatant was clear. Then 0.4-0.5 ml of packed red blood cells were diluted with VBS-G-M++ to 20 ml. From this cell suspension 0.30 ml was diluted to 2.0 ml with distilled water and the absorbance at 550 nm was measured. The volume of the cell suspension was adjusted with VBS-G-M++ to give a lysate with an absorbance at 550 nm = $0.50 \pm$.

for complete lysis were prepared with the initial arbitrary concentration of complement and its value was taken as 100 to determine % of hemolysis in each tube.

Commercially prepared hemolysin consists of a twofold dilution in glycerine of rabbit antiserum against sheep erythrocyte stroma. A stock solution was prepared by diluting 1/100 in isotonic saline, and from this different dilutions of hemolysin 1/2000, 1/3000, 1/4000, 1/6000, 1/8000, 1/12000 were prepared. For assay, 0.6 ml of hemolysin dilution was mixed with 0.6 ml of 2% sheep red blood cells (see above). After 10 minutes at room temperature, 0.6 ml of these sensitized cells were mixed with 0.3 ml of 1 unit of complement (1/5 dilution of 5 units of complement as above) and 0.6 ml of VBS-G-M++. Controls were prepared by mixing 0.3 ml of 5 units of complement, 0.6 ml of the 1/2000 dilution of hemolysin, and 0.6 ml of VBS-G-M++.

In the control for spontaneous lysis of red blood cells the 0.3 ml of complement was replaced by buffer. After incubation for 30 minutes at 37°C, 0.5 ml of buffer was added, the assay tubes were centrifuged at 600 g for 10 minutes and the absorbance of the supernatant was measured at 550 nm. The percentage of hemolysis was calculated as indicated in the case of the complement. The optimum dilution of hemolysin is the point where the curve of % of hemolysin versus dilution reaches a plateau.

c) Complement Fixation Test

All sera to be assayed were first treated at 56°C for 60 minutes in order to destroy their own complement systems.

The complement fixation was done in glass disposable culture tubes (10 x 75 mm). To each tube were added 0.1 ml of twofold serial dilutions of serum, 0.1 ml of twofold serial dilution of antigen (from 10 µg/ml to 0.03 µg/ml) and 0.1 ml of complement to its titered dilution. Controls were also prepared with no antibody, no antigen, and no complement respectively. Reactions were incubated 16-18 hours at 4°C. After incubation 0.1 ml of sensitized sheep erythrocytes were added to each tube, mixed, incubated at 37°C for 30 minutes, and then the tubes were centrifuged at 600 g for 10 minutes before decanting the supernatants to measure the absorbance at 550 nm.

Alternatively the complement fixation reaction was done in microtiter plates, to each well of which were added 25 µl of serially diluted antiserum, 25 µl of antigen and 25 µl of complement to its titered dilution. After incubation overnight at 4°C, 25 µl of sensitized sheep erythrocytes were added to each well and the plates were incubated for 30 minutes at 37°C. The plates were left in the cold until the sheep erythrocytes sedimented by gravity and the degree of hemolysis in each well was recorded semi-quantitatively. No hemolysis = 4+ CF;

complete hemolysis = 0 CF. The titer was recorded as the serum dilution producing 2+ (approximately 50% hemolysis).

d) Inhibition of Complement Fixation

Haptens that react with an antibody usually do so without fixing complement. If an antibody-antigen reaction is set up so that all antibody is saturated with antigen and all complement is fixed, then any agent that can bind antibody without fixing complement can be detected as a reduction in the amount of complement fixed. If a hapten is added along with an antigen with which it cross-reacts it will compete with the antigen for the antigen-binding site on the antibody and therefore will reduce the amount of complement fixed. This procedure is called complement fixation inhibition test.

For the inhibition test, peptides derived from H5 were provided by Dr. P. Sautière and G. Briard, of l'Institut National de la Santé et de la Recherche Medicale (INSERM), Université de Lille, France.

Fragments 1-31 and 32-185 were obtained by treating histone H5 with CNBr. Formic acid was added to a concentration of 70% by weight and the CNBr was added in 100-fold molar excess. After reaction at 24°C for 24 hours the sample was lyophilized, the fragments were applied to a CM-cellulose column and eluted with a linear gradient of 0-1 M NaCl (Garel et al., 1972). To obtain

fragment 94-185 the histone H5 was hydrolyzed at 37°C for 4 hours in 0.1 M ammonium bicarbonate pH 8 with trypsin at an enzyme/substrate ratio of 1:100. The hydrolysate was fractionated on a chromobeads P column (Technicon) with pyridine formate and pyridine acetate buffers (Sautière et al., 1976). The fragment 59-185 was obtained by NBS cleavage of the protein using 21 μ mole of NBS per μ mole of protein, the fragments were separated by chromatography on CM-cellulose equilibrated in 0.125 M sodium acetate buffer pH 4.3 and eluted with a linear gradient of 0-1 M NaCl in the same buffer (Garel et al., 1975).

Fragments 66-99, 1-66, 1-99, 100-185 were obtained by hydrolysis of histone H5 at 105°C for 6 hours with 0.25 M acetic acid (10 mg protein/ml acetic acid) and fractionation of the hydrolysate on Sephadex G-50 F equilibrated and eluted with 0.01 N HCl (Sautière et al., 1976). For the inhibition test, peptides derived from H5 were incubated for 15 minutes at 37°C with guinea pig anti-H5 antiserum at an appropriate dilution in VBS-G-M++ prior to complement fixation test (Sapeika et al., 1976).

D. Purification of Antibodies by Affinity Chromatography

The procedure followed was that of Cuatrecasas and Anfinsen (1971). Five ml of 50% suspension of Sepharose 4B was allowed to settle and washed several

times by resuspension in distilled water before final resuspension in 15 ml of 2 M Na_2CO_3 . While the suspension was stirred in the cold, 2 grams of CNBr were added all at once and the pH was maintained at 11 with 2 M NaOH until the CNBr was dissolved. The activated Sepharose was washed immediately in a sintered glass funnel at room temperature with 300 ml of cold 0.5 M NaCl buffered in 0.1 M NaHCO_3 pH 8.5, followed by 250 ml of distilled water and by 250 ml of bicarbonate buffer pH 8.5 before final resuspension in 15 ml of the same buffer. The antigen was then added. For each column 10-35 mgs of either H1 or H5 histone fractions were dissolved in bicarbonate buffer and the suspension was stirred for 16-20 hours in the cold before washing on a coarse Buchner funnel with large quantities of distilled water. The antigen-beads were then incubated with 30-40 ml of 1 M ethanolamine pH 8 for at least 2 hours at room temperature. They were then washed sequentially by 10 minutes exposure to and filtration from: 0.5 M NaCl in 0.1 M borate buffer pH 8.8; 0.5 M NaCl in 0.1 M acetate buffer pH 4.5. The final washed antigen-beads were resuspended in PBS.

Immune serum or purified immunoglobulin was added to the antigen-beads and allowed to react for 10 hours before the suspension was packed in a small column and washed extensively with PBS until $A_{280} \leq 0.001$. The

bound antibody was recovered from the column by eluting with a 0.1 M glycine-HCl buffer pH 2. The purified antibody was immediately neutralized to pH 7, extensively dialyzed against PBS pH 7.5, and concentrated to 1-2 mg/ml by ultrafiltration with a PM-10 Diaflo membrane at a constant nitrogen pressure of 35 psi.

E. Conjugation of Immuno Globulins with the Isothiocyanates of Fluorescein or Tetramethyl Rhodamine

Immune serum was obtained from rabbits immunized against purified guinea pig gamma globulin and the globulin fraction was precipitated from the rabbit antiserum with saturated ammonium sulfate at pH 7.0 to make serum 40% saturated. The crude globulin fraction was then fractionated on a DEAE Sephadex A-50 column as indicated for guinea pig IgG (Method B3a). The fraction of globulin which passed directly through the column was conjugated to prepare fluorescent antibody reagents.

The conjugation of FITC with the purified rabbit IgG fraction was done according to the procedure described by Cebra et al. (1965). Briefly, a solution of purified gamma globulin was concentrated by ultrafiltration to a final concentration of 10 mg/ml and dialyzed against 0.01 M phosphate buffer pH 7.5 and then against 0.01 M triethanolamine, 0.130 M NaCl pH 9.5. To the globulin solution stirred in the cold was added dropwise 0.15 µg

of FITC per mg of protein dissolved in a small volume of 95% ethanol, at constant pH 9.5. After stirring overnight at 4°C the supernatant was collected by centrifugation at 8000 g for 10 minutes, and extensively dialyzed against 0.01 M phosphate buffer pH 7.5 before loading on a DEAE-Sephadex A-50 column. The conjugate was fractionated by using a 0.05 - 1 M NaCl step gradient in 0.01 M phosphate buffer pH 7.5. Only effluent fractions showing ratios of $A_{280}/A_{495} \geq 2$ were used in immunofluorescence.

For preparing the rhodamine conjugate, goat antirabbit IgG serum was obtained commercially and the gamma globulin fraction was purified as described in Method B3a.

The conjugation procedure was the same as described in Method E except that 40 µg of RITC was used per mg of protein and once the conjugate was dialyzed against 0.01 M phosphate buffer, pH 7.5, it was then passed through a Sephadex G-25 column equilibrated with the same buffer to separate the conjugate from the free dye. The conjugated IgG was then fractionated in DEAE-Sephadex A-50 as described in Method E. In this case only fractions with $A_{280}/A_{515} = 4$ were used subsequently. The final conjugate solutions were concentrated by ultrafiltration to a protein concentration of 1 mg/ml, dialyzed against PBS and sterilized by filtration through a Millipore filter 0.45 µ.

F. Treatment of Adult Chicken Erythrocyte Nuclei With Detergent

For staining with the antibody in solution freshly prepared erythrocyte nuclei or nuclei stored frozen in DMSO (Method A1) were used. One ml of suspension containing about 1×10^9 of frozen nuclei at -90°C was thawed gradually by leaving at -20°C for at least 10 hours and at about 0°C for one hour. Nuclei were then washed twice with 0.15 M NaCl containing 0.1 mM PMSF to remove the DMSO and resuspended in 1 ml of the same solution before mixing with about 10 ml of 1% Tween 80 in 0.15 M NaCl (Barr, 1977). The nuclei were kept in suspension by agitation for 2 seconds on a Vortex mixer immediately and every 10 minutes for one hour thereafter at room temperature. Then the nuclei were pelleted at 600 g for 10 minutes and washed twice with 0.15 M NaCl, 0.1 mM PMSF before finally resuspension in the same medium.

G. Immunofluorescent Staining of the Nuclei in Suspension

1. Staining in Isotonic Saline

Histones H5 and H1 were detected by indirect immunofluorescent staining which required two sequential steps: untreated or detergent-treated nuclei were resuspended in 0.15 M NaCl, 0.1 mM PMSF to a concentration of $0.5 - 1 \times 10^7$ nuclei determined in a bright-line hemacyto-

meter with a phase-contrast microscope at 160X. Three or four drops of the appropriate antibody solution (purified by affinity chromatography), were added at concentrations in the range of 1 mg protein/ml ($A_{280} = 1.2 - 1.4$). The mixture was incubated at 4°C for 30 minutes with occasional stirring to keep the nuclei in suspension. After incubation the nuclei were sedimented at 600 g for 10 minutes. The supernatant was kept to measure Absorbance at 280 nm to ensure that an excess of antibody had been used. Meanwhile the nuclear pellet was washed twice by resuspension in 0.15 M NaCl, 0.1 mM PMSF and centrifugation.

To the antibody-treated nuclei resuspended in the same medium were added three or four drops of the appropriate conjugate solution: fluorescein-labelled, rabbit anti-guinea pig IgG immunoglobulin or rhodamine-labelled, goat anti-rabbit IgG immunoglobulin. The working solution of the conjugates was a 1:5 dilution of a 1 mg/ml protein stock. After these mixtures were incubated at 4°C for 30 minutes with occasional stirring the nuclei were sedimented at 600 g for 10 minutes. The supernatant was discarded and the pellet was washed by resuspension, centrifugation and a final resuspension in 0.15 M NaCl, 0.1 mM PMSF. Nuclei were observed in the fluorescence microscope and photographed before fixing in 3% glutaraldehyde for 1 hour or 2% paraformaldehyde for 15 minutes followed by washing, resuspension in 0.15 M NaCl, 0.1 mM

PMSF, and storage at 4°C.

2. Staining in Diluted Saline

Untreated or detergent-treated nuclei were resuspended in 0.15 M NaCl, 0.1 mM PMSF at a concentration of 1×10^7 nuclei as described above. The appropriate antibody solution was added and a dilution with distilled water was made to obtain a final NaCl concentration of 0.03 M. The suspension was incubated for 30 minutes at 4°C, and after incubation the nuclei were centrifuged at 300 g for 7 minutes. Resuspension and incubation with the conjugate was the same as described in Method G1 except that the ionic concentration was maintained at 0.03 M NaCl in all steps and centrifugations were at 300 g for 7 minutes. The final nuclear pellet was resuspended in 0.03 M NaCl, 0.1 mM PMSF, observed in the fluorescence microscope and photographed before nuclei were fixed as described above, washed, resuspended in 0.03 M NaCl and stored at 4°C.

3. Double Staining of Nuclei in Solution

The double staining of nuclei in suspension required three sequential steps: untreated or detergent-treated nuclei were resuspended in 0.15 M NaCl, 0.1 mM PMSF at a concentration of $1 - 5 \times 10^7$ nuclei and 0.4 ml of 1 mg/ml rabbit anti-H5 and 0.4 ml of 1 mg/ml guinea pig anti-H1 were added in rapid succession. When the double-staining

was made in 0.03 M NaCl, enough distilled water was added at this point to effect the fivefold dilution. After incubation as described in Method G1 the nuclear suspension was centrifuged, washed and resuspended as in Methods G1 or 3, before treatment with rhodamine-labelled, goat anti-rabbit IgG immunoglobulin in 0.15 M NaCl or 0.03 M NaCl, whichever was appropriate. After incubation, centrifugation and washing, the nuclear pellet was resuspended in 0.15 M NaCl or 0.03 M NaCl and treated with fluorescein-labelled, rabbit anti-guinea pig IgG immunoglobulin. After incubation and centrifugation the nuclear suspension in 0.15 M NaCl or 0.03 M NaCl was observed in the fluorescence microscope and photographed before fixation, washing and resuspension in 0.15 M NaCl or 0.03 M NaCl and storage as described above.

4. Light microscopy

Examination of the nuclei was done in a Reichert microscope equipped with an epi-objective and an Osram HBO 200/WL2 high pressure mercury lamp.

Rhodamine fluorescence (red light λ max = 515 nm) was observed with a green interference filter (λ max = 546 nm) for excitation and a red glass 30G 590 barrier filter.

Fluorescein fluorescence (green light λ max = 495 nm) was observed with a blue excitation filter 3BG 12 (λ max = 436 nm) and a combination of 1GG + 20G 515 barrier

filter. Photographs were taken using a Leitz camera on Kodak Ektachrome (ASA 200) colour film for slides, Kodacolor (ASA 400) colour film for prints, and Kodak Tri-X-pan (ASA 400) for black and white prints. Exposure time for fluorescent nuclei was 1 minute.

H. Preparation of Embryonic Chick Erythrocytes

This study needed embryos at selected morphogenetic stages as specified by Hamburger and Hamilton (1951). White leghorn (Hyline 934F) eggs were incubated at 37.5°C and 60% relative humidity. The number of embryos used at each stage for subsequent preparations are indicated in brackets, and the selected stages showed the following characteristics:

Stage 21 ($30 \leq n \leq 40$), after about 90 hours incubation, the allantois extends to the head, the extreme tip of the tail is unsegmented, eye pigmentation is faint, the wing- and leg-buds are slightly asymmetrical and the maxillary process is longer than the mandibular process.

Stage 29 ($7 \leq n \leq 15$), after about 154 hours incubation, the egg tooth is not yet visible on the beak, the mandibular process and second pharyngeal arch are fused, the second digit of the wing-bud is distinctly longer than the others and the rudiment of the fifth toe is visible.

Eggs were broken open into a deep bowl containing Hank's solution, in 0.1 mM PMSF at pH 7.5 at room temperature. The embryos with extra-embryonic membranes were explanted by trimming around the sinus terminalis. The vitelline membrane was removed and the explant was gently transferred with the aid of a scoop through three changes of Hank's solution in small petri dishes. In each change the explants were agitated for 1-2 minutes to remove yolk. Immediately following the last wash, the embryos were examined microscopically and staged. Explants were then transferred to 35 x 10 mm petri dishes containing 2 ml of Hank's balanced solution. The vitelline vessels of each embryo were cut and the blood cells allowed to drain into the solution. The blood suspension was drawn into a Pasteur pipette and transferred to a test tube at 0 - 4°C. The procedure was repeated until sufficient blood cells has been extracted to produce a pellet of 0.1 to 0.2 ml after low-speed centrifugation. The preparation of embryonic erythrocyte nuclei was the same as described in the case of adult (Method A1).

I. Preparation of Chicken Liver Nuclei

The method used was a modification of the procedure of Pogo et al. (1966). One liver was dissected from a freshly sacrificed chicken and minced with scissors

in 0.3 M sucrose, 3 mM $MgCl_2$, 0.1 mM PMSF, 0.05 M TRIS buffer pH 8, changing the buffer several times during the procedure; the buffer was discarded. When the pieces were small enough, they were blended with a Virtis homogenizer for 2 minutes at full speed. The homogenate was left in ice for about 3 minutes between each of three blendings. The final suspension was filtered through four layers of gauze and centrifuged at 800 g for 10 minutes in a refrigerated centrifuge.

The pellet was resuspended in the same buffer, centrifuged again, resuspended in 2 M sucrose, 1 mM $MgCl_2$ and homogenized by 10 strokes of a loose-fitting Dounce hand homogenizer. The final suspension was centrifuged at 57000 g for 90 minutes in a 30S rotor in a Spinco Model L ultracentrifuge. The final clean nuclear pellet was resuspended in 0.3 M sucrose, 3 mM $MgCl_2$, 0.1 mM PMSF 0.05 M TRIS, buffer pH 8.00.

For staining with the antibody the nuclei were previously treated with detergent as described in the case of chicken erythrocyte nuclei (Method F).

J. Preparation of Chicken Fibroblast Nuclei

Primary culture cells were provided by Dr. Victor Stollar, Rutgers Medical School, New Brunswick, N.J.. The method followed was a modification of that of Løvtrup and Ewen (1966). Cells were homogenized by hand in a Dounce homogenizer provided with a loose-fitting pestle

in 10 strokes, in a 0.3 M sucrose, 3 mM $MgCl_2$, 0.1 mM PMSF, 0.05 M TRIS buffer pH 8 containing 0.25% TRITON X-100. The homogenate was centrifuged at 800 g for 10 minutes in a refrigerated centrifuge. The final pellet was resuspended in the same buffer without detergent and centrifuged again as before. The crude nuclear pellet was resuspended in 2 M sucrose, 1 mM $MgCl_2$, and homogenized by 10 strokes of a loose-fitting Dounce hand homogenizer. The final suspension was centrifuged at 57000 g for 90 minutes in a 30S rotor in a Spinco Model L ultracentrifuge. The final clean nuclear pellet was resuspended in 0.3 M sucrose, 3 mM $MgCl_2$, 0.1 mM PMSF 0.05 M TRIS buffer pH 8. Nuclei were treated with detergent as described in the case of chicken erythrocyte nuclei (Method F). All operations were done at 0 - 4°C.

K. Development and Printing of Photomicrographs

During photography all phase micrographs were printed on Ilfospeed paper 3, with 5.5 seconds exposure at f11. The fluorescent micrographs of fluorescein-stained nuclei were printed on Ilfospeed paper 4, with 5 seconds exposure at f8, except where otherwise indicated. The fluorescent micrographs of rhodamine-stained nuclei were printed on Ilfospeed paper 4, with 2 seconds exposure at f11. Ilfospeed developer and fixer were used in all cases.

RESULTS

A. Preparation and Separation of Histone Fractions

To obtain total histones from adult chicken erythrocytes, nuclei were prepared according to Method A1. Once lack of cytoplasmic contamination was assured, nuclei were extracted with 0.25 M HCl (Method A2) and the extracted histones were analyzed by polyacrylamide gel electrophoresis (Method A6). A typical profile of total histones from chicken erythrocytes is shown in Graph 1A. 0.3% saponin or Nonidet P-40 was used to disrupt the cellular membrane without difference in the overall profile in polyacrylamide gel electrophoresis of total histone (not shown). Total histones extracted from fresh nuclei or from frozen nuclei stored in DMSO did not differ (not shown). Total histones (Method A2) were used as standards to compare mobility of purified histone fractions.

To obtain purified individual histones, nuclei were prepared and first treated at least twice with 5% TCA to extract lysine-rich histones H1 and H5 (Method A3). A densitometric profile of a stained polyacrylamide gel after electrophoresis of a typical TCA extract is shown in Graph 1B. Nuclei depleted of lysine-rich histones were then extracted with 0.25 M HCl to recover the remaining histones (Method A4). A densitometric profile of the stained gel after electrophoresis of the HCl extract is

Graph 1 - Densitometric profiles of polyacrylamide gels stained after electrophoresis of chicken erythrocyte histone extracts: A, total histones extracted in 0.25 M HCl; B, 5% TCA extract containing histone H1 and histone H5; C, 0.25 M HCl extract (after 5% TCA extraction) containing histones H2a, H2b, H3, H4.

Gels contained 15% acrylamide, 2.5 M urea and 0.9 N acetic acid. Electrophoresis was performed for 6 hr at 1 mA/gel in 0.5 x 10 mm tubes. The gels were stained with 0.1% Amido Black 10B in 20% ethanol, 7% acetic acid. The top of the gel (to the right) was taken as the origin of all scans.

shown in Graph 1C. No contamination by residual lysine-rich histones was detected in the latter extracts.

Separation of the TCA-soluble lysine-rich histones was attained by cation-exchange chromatography on Amberlite CG-50 eluted with guanidinium chloride gradient (Method A5a). Proteins eluted from the column were determined by turbidimetry (Graph 2). Fractions containing H1 eluted with 10% and H5 with 14% GuCl were pooled, dialyzed, concentrated by lyophilization and analyzed by polyacrylamide gel electrophoresis with which a contamination greater than 1 μ g (about 1%) would be detectable. Electropherograms of purified fractions H5 (Graph 3B) and H1 (Graph 3C) are compared to total histones (Graph 3A).

The HCl extract containing the remaining histones 2a, 2b, 3 and 4 was fractionated by exclusion chromatography through Bio-gel P-60 (Method A5b). Proteins eluted from the column were determined by Absorbance at 230 nm (Graph 4). Fractions containing proteins were pooled, dialyzed, concentrated by lyophilization and analyzed by polyacrylamide gel electrophoresis. Fraction I from the Bio-gel P-60 contains histone 2a or 2b or both (Graph 5B) while Fraction II and III correspond to a mixture of histones 3 and 4 (Graph 5C).

Graph 2 - Fractionation of lysine-rich histones on an Amberlite CG-50 column.

Histone solutions were placed on the column and eluted with increasing percentage of $\text{GuCl} \cdot \text{PO}_4$. The graph shows the separation of histones H1 and H5 selectively extracted from purified nuclei with 5% TCA (Figure 1B). Histone content was determined by turbidimetric Absorbance at 400 nm after 13 min at room temperature with TCA at a final concentration of 1.1 M. A sample of 25 mg of histone mixture was applied to a 1 x 52 cm column and 2.2 ml fractions were collected.

Graph 3 - Densitometric profiles of stained polyacrylamide gels after electrophoresis of chicken erythrocyte histone fractions: A, total histones as in Graph 1A; B, purified histone fraction H5 prepared chromatographically as in Graph 2; C, purified histone fraction H1 (Graph 2). The gels were made, electrophoresed and stained as in Graph 1.

Graph 4 - Fractionation of adult chicken erythrocyte histone fractions on Bio-gel P-60 column.

A sample of 55 mg of histone extracted with HCl after TCA extraction (see Graph 1C) was applied to a 1.8 x 87 column, equilibrated and eluted with 0.01 M HCl, and 2.6 ml fractions were collected. Histone content was determined by Absorbance at 230 nm.

Graph 5 - Densitometric profiles of stained polyacrylamide gels after electrophoresis of chicken erythrocyte histone fractions: A, total HCl extract after TCA extraction (see Graph 1C); B, unresolved histone fraction H2 (peak I from Graph 4); C, purified histone fractions H3 - H4 (peak II from Graph 4). The gels were made, electrophoresed and stained as in Graph 1.

B. Preparation and Detection of Antibodies

1. Anti-H5 and Anti-H1 Sera

Histone fractions H5 and H1, purified by Method A3 (Graphs 3B and 3C), were used as antigens to prepare antibodies. Two different groups of six guinea pigs each were used to obtain antibody I and antibody II against H5 (Method B1). Antibody complexes with H5 were detected by CF (Method C2), an initial block titration showing that the optimal reaction required a concentration of 5-10 $\mu\text{g/ml}$ of purified H5. The CF titers for each bleeding are shown in Table I. In the case of antibody I, the titer for the 1st, 2nd, 3rd and 4th bleeding was determined by using the semiquantitative CF method, while for the 5th bleeding the titer was determined by using the quantitative CF technique. In the case of antibody II the titer was determined by using the quantitative CF technique for all the bleedings. No cross-reactivity was detected with histone fractions 2, and 3-4 and either antibody I or II. However cross-reactivity was detected with histone 1 only at dilution 1/10 - 1/20 of antiserum in both antibody preparation. Cross-reactivity was eliminated by affinity chromatography (Method D).

Antibody prepared from six guinea pigs against H1 was detected by CF (Method C2). An initial block titration showed that the optimal reaction required a concentration of 5-10 $\mu\text{g/ml}$ of purified H1. The CF titers

TABLE I

Characteristics of antibodies raised in guinea pig against histones H1 and H5 and in rabbits against H5. Volume of serum obtained in the different bleedings and titers for each bleeding obtained by CF are also shown.

Anti-H5 Guinea Pig SeraAntibody No. I (5 Animals)

<u>Bleeding</u>	<u>Vol. (ml)</u>	<u>Titer CF</u>
1st	10.0	1 700
2nd	9.3	1 700
3rd	10.7	1 700
4th	9.0	1 700
5th	10.0	3 160

Anti-H5 Guinea Pig SeraAntibody No. II (6 Animals)

<u>Bleeding</u>	<u>Vol. (ml)</u>	<u>Titer CF</u>
1st	5.0	3 000
2nd	6.0	2 500
3rd	6.0	2 800
4th	32.0	3 000

Anti-H1 Guinea Pig Sera (5 Animals)

<u>Bleeding</u>	<u>Vol. (ml)</u>	<u>Titer CF</u>
1st	6.5	1 280
2nd	6.0	640
3rd	8.0	640
4th	8.5	2 820
5th	10.0	2 820

Based on 3 replicate tests.

for each bleeding are shown in Table I. Semi-quantitative CF technique was used to determine the titer of the 1st, 2nd and 3rd bleeding, while the quantitative CF technique was used to obtain the titer for the 4th and 5th bleeding. No cross-reactivity with histone fractions 2a, 2b, 3 and 4 at any dilution of the antiserum was detected by CF. However slight cross-reactivity was observed with H5 at 1/10 - 1/20 serum dilutions. Cross-reactivity was eliminated by affinity chromatography (Method D).

Antibody prepared from rabbit against H5 by Method B2 was detected by CF and ID (Methods C1 and C2). For obtaining an optimal CF reaction, the initial block titration showed that a concentration of 5-10 µg/ml of purified H5 was required. CF titers for each bleeding are shown in Table I. The titers for 1st, 2nd and 3rd bleeding were determined by semiquantitative CF technique; the titer for the 4th bleeding was determined by using the quantitative CF technique. For the 1st, 2nd and 3rd bleedings no cross-reactivity was detected against any other histone fraction by CF. Assay by ID (Plate I, d) shows precipitating lines of anti-H5 serum at 1/5 dilution with 2 mg/ml of H5 (wells 1 and 4) but not with H1 (wells 2 and 5), or H2 (wells 3 and 6). A parallel assay was done with H3 - H4 with negative results (not shown). However antiserum obtained from the 5th Bleeding showed substantial cross-reactivity with H1, although no cross-

Plate I - Characterization of antibodies by gel diffusion precipitation.

Assays were done in 0.8% Agarose with VBS-G-M++ buffer.

Part a shows precipitation of undiluted, goat anti-guinea pig IgG serum (central well) with normal guinea pig serum (well 3) and guinea pig IgG purified on a DEAE-Sephadex column (well 6), 1 mg/ml; no precipitation was observed with 2 M NaCl fractions from the DEAE-Sephadex column, 1 mg/ml (wells 1 and 4) or rabbit IgG, 1 mg/ml (wells 2 and 5).

Part b shows titration of rabbit anti-guinea pig IgG serum with purified guinea pig IgG at a concentration of 1 mg/ml in the central well and outer wells containing twofold dilutions of the antiserum starting in a clockwise direction from 1:10 dilution in well 1.

Part c shows titration of goat anti-rabbit IgG serum. The central well contains purified rabbit IgG at a concentration of 1 mg/ml and the outer wells contain twofold dilutions of the antiserum starting in a clockwise direction from 1:10 dilution in well 1.

Part d shows precipitation of 1/5 diluted rabbit anti-H5 with H5 at a concentration of 2 mg/ml (wells 1 and 4), but no precipitation with histone fractions H1 at a concentration of 2 mg/ml (wells 2 and 5) or H2 at a concentration of 2 mg/ml (wells 3 and 6).

reactivity was observed with the other histone fractions.

2. Preparation of Antisera Against IgG

For the preparation of rabbit anti-guinea pig IgG, a purified IgG fraction was obtained from normal guinea pig serum by ammonium sulfate precipitation, followed by DEAE Sephadex A-50 chromatography (Method B). The purified IgG fraction was identified by ID using a goat anti-guinea pig IgG serum (Plate I, a). Whereas undiluted goat anti-guinea pig IgG serum showed precipitating lines with undiluted guinea pig serum (well 3) and with 1 mg/ml purified guinea pig IgG (well 6) no precipitation was evident with 2 M NaCl fractions from DEAE-Sephadex A-50 (wells 1 and 4), or with rabbit IgG (wells 2 and 5). The titer of this antiserum could not be determined by CF, since the source of complement is guinea pig serum, which would increase the amount of antigenic IgG in the assay. The use of rabbit serum as a source of complement was attempted without success, because the low titer of rabbit complement demands a high concentration of serum proteins which allow non-specific interactions in the system. The titer of this antiserum was estimated by ID (Plate I, b). In this figure the central well contained purified normal guinea pig IgG at a concentration of 1 mg/ml while the outer wells contained twofold dilutions of anti-guinea pig IgG starting with a dilution 1:8 in well 1 and proceeding

clockwise. The last dilution at which a precipitating line is visible is 1/32.

Goat anti-rabbit IgG was obtained commercially and titrated by ID using purified rabbit IgG in the central well at a concentration of 1 mg/ml (Plate I, c).

Goat anti-guinea pig IgG serum was obtained commercially and was used without titration.

C. Purification of Antibodies by Affinity Chromatography

For purification of guinea pig anti-H5 and rabbit anti-H5, purified histone obtained as indicated in Method A3 and shown in Graph 3B was coupled to Sepharose 4B (Method D) and then packed in a column. Undiluted serum, crude IgG (fraction obtained by ammonium sulfate precipitation), or IgG purified by DEAE-Sephadex A-50, was passed through the column. After washing the column with PBS until the Absorbance at 280 nm of the effluent was less than 0.01, the antibody was eluted by using glycine-HCl buffer pH 2 (Graph 6A).

Rabbit anti-H5 was purified in the same way (Graph 6C). The fractions containing proteins were pooled, neutralized immediately to pH 7.0 and dialyzed against PBS. No cross-reactivity of the eluted antibody was detected by microquantitative CF with any other

Graph 6 - Purification of antibodies by Affinity
Chromatography on Sepharose 4B conjugated with histone
1 or 5.

Solutions of serum, crude IgG fraction obtained by
ammonium sulfate precipitation or IgG fraction purified
by DEAE-Sephadex chromatography were placed on the column
and non specific antibodies and other proteins from the
serum were washed off with PBS pH 7.5 until A280 was
less than 0.01; the specific antibody was then eluted
with glycine-HCl buffer pH 2.0. The samples and
adsorbents were as follows: Graph A - Guinea pig anti-
H5 serum on H5-Sepharose 4B conjugate; Graph B - Guinea
pig anti-H1 serum on H1-Sepharose 4B conjugate; Graph
C - Rabbit anti-H5 serum on H5-Sepharose 4B conjugate.
Proteins were detected by Absorbance at 280 nm and anti-
bodies by quantitative micro-CF.

histones even at IgG concentrations of 1 mg/ml (normal concentration of IgG in serum). In the case of rabbit anti-H5, cross-reactivity was also tested by ID (not shown).

For purification of guinea pig anti-H1, purified H1 obtained as indicated in Method A3 and shown in Graph 3C, was coupled to Sepharose 4B. The procedure was as described above. Purification of guinea pig anti-H1 is shown in Graph 6B.

No cross-reactivity was detected in the purified IgG by quantitative micro-complement fixation. The titers of antibodies purified by affinity chromatography were not determined.

D. Conjugation of Immune Globulins with the Isothiocyanate of Fluorescein or Tetramethyl Rhodamine

Purified IgG from goat anti-rabbit IgG serum characterized by ID (Plate I, c) was coupled to RITC (Method E). The conjugated IgG was separated from the free dye by Sephadex G-50 column. The fractions containing the conjugate were then concentrated and fractionated in a DEAE-Sephadex A-50 column (Graph 7A). Only those fractions with a ratio $A_{280}/A_{550} \geq 4$ were used in cytological experiments. The conjugate was further characterized by comparing the spectra of the RITC and of all conjugated fractions from the DEAE-Sephadex A-50 column.

(not shown). The conjugate presented one peak of maximum absorption in the UV region (280 nm) and another one in the visible (550 nm), which coincides with the maximum absorption of RITC.

For preparing the FITC conjugate, purified immunoglobulin from rabbit anti-guinea pig IgG was obtained as indicated in Method B3, characterized by ID (Plate I, a) and coupled by Method E. The conjugated IgG was then dialyzed against PBS and fractionated in a DEAE-Sephadex A-50 column (Graph 7B). Only fractions with A280/A495 greater than 2 were used in cytological experiments. Spectra of the FITC and all the conjugated fractions from the DEAE-Sephadex A-50 column were also obtained to characterize the conjugate further (not shown). The conjugate presented one peak of maximum absorption in the UV region (280 nm) and another one in the visible (495 nm), which coincides with the maximum absorption of FITC; therefore it is concluded the protein is linked covalently to the dye.

Graph 7 - Fractionation of fluorescent conjugate on a DEAE-Sephadex A50 column.

The conjugate was placed on the column and different fractions were eluted by using a stepwise NaCl gradient in 0.01 M phosphate buffer at pH 7.5. Absorbance was determined at 280 nm to detect protein, at 550 nm in the case of RITC to detect maximum absorbance of rhodamine and at 495 nm to detect maximum absorbance of fluorescein. Fractions were characterized by their A280/A550 or A280/A495 ratios for RITC and FITC conjugates respectively. Graph A shows the fractionation of rhodamine labelled, goat anti-rabbit IgG immunoglobulin and Graph B the fractionation of fluorescein labelled, rabbit anti-guinea pig IgG-immunoglobulin.

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E. Immunofluorescent Staining of the Erythrocyte Nuclei in Suspension

The presence of lysine-rich histones has been detected in this study by indirect immunofluorescence using a combination of purified antihistone immunoglobulin from guinea pig or rabbit serum and either anti-guinea pig or anti-rabbit IgG immunoglobulin, coupled to fluorescent dye.

The wavelengths of the exciting light used in the fluorescence microscope to visualize the presence of the fluorescent dyes FITC or RITC was close to the maximum absorption of hemoglobin, which is known to accumulate in nucleated erythrocytes as they mature (Coutelle et al., 1974; Small and Davies, 1972). To avoid the possibility of hemoglobin and other heme compounds preventing excitation of the fluorescent conjugate, the staining was done in isolated nuclei devoid of hemoglobin. None of the nuclei used in this study showed autofluorescence. Nor did they fluoresce when immunoglobulin from guinea pigs or rabbit not exposed to histones replaced the anti-histone immunoglobulin in the reaction (not shown).

1. Single-Staining of Histones in Adult Nuclei

When nuclei were stained for H5 and observed with the fluorescence microscope, almost all were fluorescent. The majority of the nuclei showed uniform staining.

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throughout with the intensity in different nuclei varying from bright (Plate II, Figure 1) through medium (Plate III, Figures 2 and 4) to dim (Plate II, Figure 1 and Plate III, Figure 2). A fraction of the nuclei stained heterogeneously, showing a bright rim surrounding a more weakly stained interior (Plate II, Figure 1 and Plate III, Figure 3). Frequencies of nuclei showing the different staining responses are summarized in Table II.

It was considered possible that the variability in the staining could arise from the presence of residual plasma or nuclear membranes which might prevent complete penetration of antibodies into the nucleus. To eliminate membrane lipids, nuclei were treated with the detergent Tween 80 (Method F), after which no membranes were observed by electron microscopy (Barr and Setterfield, personal communication). Detergent-treated nuclei stained for H5 showed the same general types of fluorescence as seen in the non-detergent preparations. Figures 2 and 4 show bright, medium and dim uniformly fluorescent nuclei. Figure 3 shows the intense peripheral rim surrounding a less intensely stained interior. Although all general types of staining were represented, the frequency of the different types changed in the detergent-treated samples (Table II). There was a substantial increase of the nuclei with light internal staining surrounded by an intense peripheral rim. At the same time the number of uniformly bright and medium nuclei

Plate II - Indirect immunofluorescence localization of histone H5 in adult chicken erythrocyte nuclei in suspension. Figure 1a, phase-contrast microscopy; Figure 1b, fluorescence microscopy of same field. Nuclei stained with fluorescein conjugate. All negatives of fluorescent nuclei taken with 1 minute exposure. X 1650

Plate III - Indirect immunofluorescence localization of histone H5 in detergent-treated adult chicken erythrocyte nuclei in suspension. Each pair of figures shows the same field with phase microscopy (a) and fluorescence microscopy (b). Nuclei stained with fluorescein conjugate. Figure 2 shows the homogeneous bright, medium and dim fluorescence. Figure 3 shows nuclei with bright peripheral ring surrounding an homogeneous stained nuclei. Figure 4 shows nuclei with bright and medium homogeneous patterns. X 1650

Table II. Frequency distribution of different types of fluorescent patterns in adult erythrocytes nuclei observed by indirect staining with anti-H1 and anti-H5.

Antibody	Salt Conc.	Tween Treatment	80 No. Nuclei Counted	No. Nuclei Positively Stained	Fluorescence Pattern %			
					Uniform			Peripheral Rim
					Bright	Medium	Weak	
anti-H5	0.15 M*	-	1 117	1 069	24.4 ± 1.2	38.3 ± 2.6	15.7 ± 2.3	17.3 ± 1.3
	0.15 M*	+	1 047	1 047	5.0 ± 1.0	12.0 ± 2.3	7.0 ± 1.3	76.0 ± 2.2
	0.03 M*	+	1 362	1 314	26.3 ± 5.2	44.0 ± 2.9	7.1 ± 1.7	19.1 ± 6.1
anti-H1	0.15 M*	+	400	400	-	3.0	-	97.0
	0.03 M*	+	1 317	1 277	8.5 ± 2.1	21.0 ± 5.8	8.0 ± 2.8	59.5 ± 6.6

* Mean ± SEM (standard error of the mean) of six separate experiments.

+ Mean of three separate experiments.

decreased (Table II).

Total histones were extracted from detergent-treated nuclei and analyzed by polyacrylamide gel electrophoresis. When the electrophoretic profiles were compared with those obtained from freshly prepared nuclei not treated with detergent, there was no change in the relative amounts of histones (not shown).

The appearance of a bright rim surrounding a more weakly stained interior is not understood at the present time. It was present in both non-treated and detergent-treated nuclei. In either case the fluorescence due to the presence of H5 showed variation in the intensity, which indicates that the "rim effect" does not seriously interfere with the availability of the antigen determinants. More crucial however was the consideration that the presence of membranes might interfere with the penetration of the antibodies inside the nucleus (Bustin, 1978). While the non-treated nuclei were examined qualitatively, detergent-treated nuclei were subsequently used for quantitative studies.

These observations were made on nuclei in isotonic saline (0.15 M NaCl) in which the erythrocyte chromatin is highly condensed (Brasch and Setterfield, 1971; Small and Davis, 1972; Ringertz, 1974). It is known, however, that chicken erythrocyte nuclei suspended in low ionic strength show uniformly dispersed chromatin and a decrease in fiber diameter from 200 to 75 Å.

(Brasch et al., 1971; Olins and Olins, 1972). It might be expected that the conformational change of chromatin in low ionic strength could affect the accessibility of histones to applied anti-histone immunoglobulins. To test this possibility, detergent-treated nuclei in 0.03 M NaCl were allowed to react with antibody. These nuclei showed the same variety of types of fluorescence as in isotonic conditions (Plate IV, Figure 5b). The proportion of nuclei presenting a peripheral rim decreased from 76% to 19%, while the bright and medium categories rose from 5% and 12% to 26% and 44% respectively (Table II). If the detergent-treated nuclei stained in low ionic strength were returned to saline conditions, the types of fluorescent nuclei were the same as observed in preparations stained directly in 0.15 M NaCl (Plate IV, Figure 6b). This was not established statistically.

Detergent-treated nuclei were also reacted with guinea pig anti-H1 in isotonic and hypotonic salt concentrations and stained indirectly. Typical results are illustrated in Plate V, Figures 7-9, and distributions of staining patterns are given in Table II. In contrast to the results with anti-H5, the nuclei which reacted with anti-H1 were relatively consistent in the staining reaction. In 0.15 M NaCl almost all nuclei showed uniform internal staining with a bright peripheral rim (Figure 7, Table II). Staining at low ionic strength gave some nuclei without the peripheral bright rim, but

Plate IV - Indirect immunofluorescence localization of histone H5 in detergent-treated adult chicken erythrocyte nuclei stained in different conditions of ionic strength. Each pair of figures shows the same field, with phase contrast microscopy (a) and fluorescence microscopy (b). Nuclei stained with fluorescein conjugate. Figure 5, nuclei stained in low ionic strength (0.03 M NaCl) and Figure 6, nuclei adjusted to isotonic saline after being stained in low ionic strength. X 1650

Plate V - Indirect immunofluorescent localization of histone H1 in detergent-treated adult chicken erythrocyte nuclei. Each horizontal series of micrographs shows the same microscopic field as either phase-contrast (a) or fluorescence (b). Nuclei stained with fluorescein conjugate. Figure 7, nuclei stained in isotonic saline; Figure 8, nuclei stained in low ionic strength (0.03 M NaCl), and Figure 9, nuclei in isotonic saline after being stained in low ionic strength.

During printing Figures 7c, 8b and 9b were exposed 5 seconds at f8, Figure 7b, 3 seconds at f11. X 1650

the majority were the same as in saline (Figure 8 and 9, Table II).

With biochemical methods histone H5 has been found exclusively in nucleated erythrocytes (Neelin et al., 1961, 1964; Hnilica, 1964; Purkayastha and Neelin, 1966), while H1 is apparently present in all the cells of multicellular organisms. To verify this cytochemically, liver nuclei were prepared by Method I, and, after being treated with detergent as in Method F, were allowed to react separately with guinea pig anti-H5 and anti-H1. With anti-H5, liver nuclei show chiefly medium and dim fluorescence (Plate VI, Figures 10 and 11), while with anti-H1 all nuclei gave strong, uniform fluorescence (Plate VI, Figures 12 and 13). Similar results were given by nuclei stained without prior detergent treatment.

The fluorescence shown in liver nuclei when stained with anti-H5 may indicate either that H5 is indeed present in trace amounts or that H5 shares enough common determinants with H1 to react with H1-containing nuclei. To help distinguish between these possibilities, nuclei were prepared by Method J from chicken fibroblast cells in culture which were presumably devoid of potential erythropoietic capacity, and thus of H5. Staining with anti-H5 revealed only weak fluorescence (Plate VII, Figure 14), but all fibroblast nuclei gave bright and uniform fluorescence with anti-H1 (Plate VII, Figure 15).

Plate VI - Indirect immunofluorescent localization of histones H1 and H5 in detergent-treated adult chicken liver nuclei. Each horizontal series of micrographs shows the same microscopic field, with phase-contrast microscopy (a) and fluorescence microscopy (b and c). Note presence of distinct nucleoli in phase-contrast micrographs. Nuclei stained with fluorescein conjugate. Figures 10 and 11 show localization of H5, Figures 12 and 13, localization of H1. During printing (b) figures were exposed for 3 seconds at f11, (c) figures, 5 seconds at f8. X 1650

Plate VII - Indirect immunofluorescent localization of histones H1 and H5 in detergent-treated chicken fibroblast nuclei. Each horizontal series of micrographs shows the same microscopic field, in phase microscopy (a) and fluorescence microscopy (b and c). Nuclei stained with fluorescein conjugate. Figure 14 shows localization of H5 and Figure 15, localization of H1. During printing, fluorescence micrographs were exposed as in Plate V. X 1650

2. Double-Staining of H1 and H5 in Adult Nuclei

To study more directly the relation between histone H1 and H5, nuclei were allowed to react simultaneously with antibodies against H5 and H1. Antibodies against H1 were raised in guinea pig and those against H5 in rabbits. A fluorescein-labelled, anti-guinea pig IgG immunoglobulin which gives green fluorescence (Plate VIII, Figures 16c, 17c and 18c) was used to detect H1; a rhodamine-labelled, anti-rabbit-IgG immunoglobulin which shows red fluorescence was used to detect H5 (Plate VIII, Figures 16b, 17b and 18b).

A test of the specificity of detection of the two fluorescent dyes, fluorescein and rhodamine, with the appropriate exciter and barrier filters was carried out as follows. Nuclei from erythrocytes of 16-day-old chick embryos were reacted separately with anti-H1 or anti-H5 and then singly stained with the appropriate anti-IgG immunoglobulin, fluorescein-labelled for H1 and rhodamine-labelled for H5 (see above). The fluorescence of the preparations was then observed with the microscope filter combinations used to detect each of the dyes (Plate IX, Figures 19 and 20). The fluorescein dye showed fluorescence only with the filter combination normally used for this dye; not with that used for rhodamine (Figures 19b and 19c). On the other hand rhodamine gave a weak fluorescence with the filters used for fluorescein. In double-stained preparations this small "leakage" of

Plate VIII - Indirect immunofluorescent localization of histones H1 and H5 in double-stained, adult erythrocyte nuclei in low ionic strength conditions. Each horizontal series of three micrographs shows the same microscopic field, phase contrast-microscopy (a) and fluorescence microscopy (b and c). (b), nuclei stained with rhodamine conjugate to detect anti-H5. (c), nuclei stained with fluorescein conjugate to detect anti-H1. During printing, Figure 16b was exposed for 2 seconds at f11, Figure 16c for 5 seconds at f8. X 1650

16a

b

c

17a

b

c

18a

b

c

Plate IX - Specificity of the indirect staining procedure for detection of histones H5 and H1 in isolated chicken erythroid nuclei. Each horizontal series of micrographs shows the same microscopic field, in phase-contrast microscopy (a) and fluorescence microscopy (b and c). Figure 19 shows 16 day old embryonic erythrocyte nuclei reacted with guinea pig anti-H1 and stained with fluorescein conjugate observed using excitation and barrier filters for visualization of rhodamine (b) and fluorescein (c). Figure 20 nuclei reacted with rabbit anti-H5 and stained with rhodamine conjugate observed using excitation and barrier filters for visualization of rhodamine (b) and fluorescein (c). Figure 21 nuclei treated with rhodamine conjugate in the absence of histone antibodies observed using excitation and barrier filters for visualization of rhodamine (b). Figure 22 nuclei treated with fluorescein conjugate in the absence of histone antibodies, observed using excitation and barrier filters for visualization of fluorescein (b). Figures 19b, 20b and 21b were printed as in Figure 16b, Figures 19c, 20c and 22b as in 16c.

X 1650

19a

b

c

20a

b

c

21a

b

22a

b

fluorescence could slightly exaggerate apparent H1 staining if substantial amounts of H5 were also present. H5 fluorescence however was due solely to staining by rhodamine-labelled anti-immunoglobulin. In control preparations with fluorescent-labelled anti-immunoglobulins, but with anti-histone omitted, no staining was observed (Figures 21 and 22).

Results from double-stained erythrocyte nuclei were consistent with the observations on single-stained nuclei. While the fluorescence for H1 had essentially the same intensity for all nuclei, the fluorescence for H5 showed variability. Thus in some cases, within the same nucleus, the two histones showed sharply different reactions, homogeneous bright fluorescence for H1, (Plate VIII, Figures 16, 17 and 18c) with medium or dim fluorescence for H5 (Figures 16, 17 and 18b). In other cases both histones stained strongly (Figures 16, 17 and 18). These results are illustrated with nuclei stained in low ionic strength, but the same results were obtained with nuclei stained in saline.

Double-stained liver nuclei gave only medium or dim fluorescence for H5 (Plate X, Figures 23b and 24b) but showed bright and homogeneous fluorescence for H1 (Figures 23 and 24c). Note that in Figure 24c one erythrocyte nucleus shows H1 fluorescence but almost no H5 fluorescence.

Plate X - Indirect immunofluorescent localization of histones H1 and H5 in double-stained chicken adult liver nuclei in saline. Each horizontal series of three micrographs shows the same microscopic field, in phase contrast microscopy (a) and fluorescence microscopy (b and c). (b), nuclei stained with rhodamine conjugate to detect anti-H5. (c), nuclei stained with fluorescein conjugate to detect anti-H1. Figure 23b was printed as in Figure 16b, Figure 23c, as in 16c.

X 1650

Double-stained fibroblast nuclei gave only faint H5 fluorescence (Plate XI, Figures 25b and 26b), in contrast to the strong homogeneous H1 fluorescence (Figures 25c and 26c). These results confirmed the observations with single-stained liver and fibroblast nuclei.

F. Immunofluorescent Staining of Embryonic Erythrocyte Nuclei

To compare the content of H1 and H5 in embryonic erythroid cells, chick embryos at stage 21 (3½ days), stage 29 (6 days) and 16 days were selected. The majority of the erythroid cells in these stages are as follows: stage 21 - polychromatophilic erythroblasts of the primitive line; stage 29 - reticulocytes or mature erythrocytes of the primitive line (Urban, Ph.D. Thesis, 1977); day 16 - mature erythrocytes of the definitive line (Lucas and Jamroz, 1961).

Nuclei were prepared by Method H, treated with detergent by Method F and allowed to react separately with guinea pig anti-H5 and anti-H1. Plate XII shows results of the staining of these nuclei for anti-H5. Figures 27, 28 and 29 show fluorescence of erythroid nuclei from 3½, 6 and 16 day embryos respectively, compared to nuclei from erythrocytes of adult chickens (Figure 30b). Although it is not apparent in the figures the preparations of embryonic erythroid nuclei all showed

Plate XI - Indirect immunofluorescent localization of histones H1 and H5 in double-stained chicken fibroblast nuclei in saline. Each horizontal series of three micrographs shows the same microscopic field, in phase-contrast microscopy (a) and fluorescence microscopy (b and c). (b), nuclei stained with rhodamine conjugate to detect anti-H5. (c), nuclei stained with fluorescein conjugate to detect anti-H1. Figures 25b and 26b were printed as in Figure 16b, Figures 25c and 26c, as in 16c.

X 1650

Plate XII - Indirect immunofluorescent localization of histone H5 in detergent-treated embryonic chicken erythrocyte nuclei. Each horizontal series of micrographs shows the same microscopic field, in phase-contrast microscopy (a) and fluorescence microscopy (b and c). Nuclei stained with fluorescein conjugate. Figure 27, 3½ day old embryo; Figure 28, 6 day old embryo; Figure 29, 16 day old embryo; Figure 30, adult chicken. Figures 27b, 28c, 29c and 30b were printed for 5 seconds at f8, Figures 28b and 29b for 3 seconds at f11. X 1650

some variability in staining for H5 (summarized in Graph 8, in which the bright category for nuclei from adults and 16-day embryos comprises both the bright homogeneous and the peripheral rim categories). The majority of the primitive-line nuclei of the 3½ and 6 day embryos showed medium, uniform (non-rimmed) fluorescence. In addition some bright homogeneous and dim homogeneous nuclei were also present. There appeared to be little difference in distribution of fluorescent types for the two early embryos. The distribution of fluorescent types was, however, significantly different for the definitive-line nuclei of both 16 day embryos and adults. Here bright fluorescent nuclei, both homogeneous and with rims, predominated. There may have been a small increase in the proportion of bright nuclei in the adult blood as against the embryos, although this was not established statistically.

Plate XIII illustrates staining of embryonic nuclei with anti-H1. Figures 31, 32 and 33 show fluorescence of erythroid nuclei from 3½, 6 and 16 day embryos respectively compared to adult chicken nuclei (Figures 34b and c). Quantitative distribution of the various fluorescent types are shown in Graph 9. With H1 staining of the embryonic preparations, brightly fluorescing nuclei predominated. The primitive-line nuclei of the early embryos showed greater staining variability than the definitive-line nuclei, with significant numbers of

Graph 8 - Distribution of fluorescent patterns in chicken erythrocyte nuclei from adult, 16, 6, and 3½ day embryos, after staining with anti-H5. Each bar shows the mean of determinations from six separate experiments in the case of adult, and three in the case of embryos. b = bright; m = medium; and d = dim. The ordinate is % of total fluorescent nuclei.

Plate XIII - Indirect immunofluorescent localization of histone H1 in detergent-treated embryonic chicken erythrocyte nuclei. Each horizontal series of micrographs shows the same microscopic field, in phase-contrast microscopy (a) and fluorescence microscopy (b and c). Nuclei stained with fluorescein conjugate. Figure 31, 3½ day old embryo; Figure 32, 6 day old embryo; Figure 33, 16 day old embryo; Figure 34, adult erythrocyte nuclei. During printing all (b) figures were exposed for 3 seconds at f11, (c) figures, 5 seconds at f8.

31a

b

c

32a

b

c

33a

b

c

34a

b

c

Graph 9 - Distribution of fluorescent patterns in chicken erythrocyte nuclei from adult, 16, 6 and 3½ day embryos, after staining with anti-H1. Each bar shows the mean of determinations of three separate experiments. b = bright; m = medium; and d = dim.

The ordinate is % of total fluorescent nuclei.

Plate XIV - Indirect immunofluorescent localization of histones H1 and H5 in double-stained embryonic erythrocyte nuclei in saline. Each horizontal series of three micrographs show the same microscopic field, in phase-contrast microscopy (a) and fluorescence microscope (b and c). (b), nuclei stained with rhodamine conjugate to detect anti-H5. (c), nuclei stained with fluorescein conjugate to detect anti-H1. Figure 35, 3½ day old embryo; Figure 36, 6 day old embryo; Figure 37, 16 day old embryo. X 1650

medium and dim types of nuclei appearing. The bright nuclei of the primitive-line were mostly homogeneously stained internally, while a large proportion of the definitive-line nuclei had bright-peripheral rims.

Results of the double-stained embryonic nuclei are presented in Plate XIV with Figures 35, 36 and 37 respectively showing fluorescence of erythroid nuclei from 3½, 6 and 16-day embryos respectively. The figures however do not illustrate well the variability for H5 in embryonic nuclei. Results obtained in single-labelled nuclei, summarized in Graphs 8 and 9, indicate that the amount of H5 in embryonic erythroid nuclei is variable and of H1 more uniform.

G. Inhibition of Complement Fixation Reaction of H5/
Anti-H5 by Peptides Derived from H5

To localize the antigenicity of H5 histone, peptide fragments were used to inhibit the formation of H5/anti-H5 complexes in the CF reaction. Inhibition was measured as the decrease in percent CF when a fragment was added at various concentrations to the CF reaction. Titers of the two separate lots of guinea pig anti-H5 antisera are shown in Table 1, and the homogeneity of histone H5, prepared and purified as described in Method A III, is shown in Graph 3b.

In initial experiments a 1/2000 dilution of the serum was divided into several aliquots and different

concentrations of H5 peptides were added to replicate samples containing serial concentrations of complete antigen H5. As shown in Graph 10, intact H5 histone at concentrations of 1.25-5.0 $\mu\text{g/ml}$ reacted with 1/2000 dilution of homologous antiserum to fix 95% of complement. Addition of peptide fragment 1-31 (MW 3400) produced no change in the CF reaction at optimal H5 concentration even when added at 25 or 50 $\mu\text{g/ml}$ concentrations (Graph 10a), which are greatly in excess of the H5 antigen.

In contrast, when fragment 32-185 (MW 16,900) was added under the same conditions, it inhibited the formation of H5/anti-H5 complexes. CF was partially reduced even at molar ratios of peptide/antigen less than 1.0, and it was almost completely inhibited with a slight excess of peptide/H5 at optimal antigen concentration (Graph 10b). This would indicate that all the antigenicity of histone H5 is located in this fragment and none in the first 31 most N-terminal residues.

The shorter fragment 59-185 (MW 13,900) added to the CF reaction between H5 and anti-H5 at 1/2000 serum dilution is scarcely less inhibitory at optimum H5 concentration than fragment 32-185 at comparable peptide/H5 molar ratios (Graph 11a). At 1/3000 serum dilution, fragment 59-185 is highly inhibitory at the optimum H5 concentration, even at a peptide/H5 molar ratio of 2.0 (Graph 11b). These results when compared with those obtained with fragment 32-185 would indicate that this

Graph 10 - Inhibitory effects of H5 peptides on CF by H5/anti-H5 at 1/2000 dilution of antiserum. Prior to the addition of the antigen the antiserum was incubated 15 min at 37°C with the inhibitor. In A, the inhibitor was peptide 1-31 at 0.4, 1.6, 6.25, 12.5 and 25 µg/ml. All curves coincided with the one obtained at 50 µg/ml □ - □ . In B, the inhibitor was fragment 32-185 at 0.8 and 1 µg/ml Δ - Δ; 1.5 µg/ml ▲ - ▲; 2.5 µg/ml ○ - ○; 3 µg/ml ∇ - ∇. No inhibitor, ■ - ■ , in both graphs.

Graph 11 - Inhibitory effects of H5 peptide 59-185 on CF by H5/anti-H5. In A, at 1/2000 dilution of antiserum, the concentrations of peptide were as follows: 2.5 $\mu\text{g/ml}$ Δ - Δ ; 5.0 $\mu\text{g/ml}$ $\blacktriangle$ - $\blacktriangle$; 10 $\mu\text{g/ml}$ $\circ$ - $\circ$; 20 $\mu\text{g/ml}$ $\bullet$ - $\bullet$; 40 $\mu\text{g/ml}$ $\square$ - $\square$. In B, at 1/3000 dilution of antiserum the concentrations of peptide were as follows: 1.7 $\mu\text{g/ml}$ ∇ - ∇ ; 17 and 53 $\mu\text{g/ml}$ coincided with 170 $\mu\text{g/ml}$ ∇ - ∇ . No inhibitor $\blacksquare$ - $\blacksquare$, in both graphs.

fragment also contains all the antigenic reactive regions of the molecule, perhaps in a slightly denaturated conformation. The 1/3000 dilution of antiserum was found to be more sensitive and this dilution was used subsequently.

When fragment 94-185 (MW 10,200) was added to the CF reaction of H5 and its homologous antiserum, it was scarcely inhibitory at the optimum antigen concentration and a peptide/H5 molar ratio of 2.0, only partially inhibitory at 20 fold excess, and almost completely inhibitory at a ratio of 60 (Graph 12a).

The C-terminal fragment 100-185 (MW 9,500) showed little inhibition of CF by the H5/anti-H5 complex at optimum antigen concentration and a 13-fold molar excess of peptides. However significant and complete inhibition was obtained at 38 and 100 fold excesses respectively (Graph 12b). This effect is about half that obtained using the slightly longer peptide 94-185.

The corresponding N-terminal fragment 1-99 (MW 10,800) inhibited CF by about half at a peptide/H5 molar ratio around 1, and virtually completely at a peptide/H5 molar ratio of 12 or more (Graph 13a); this is comparable to peptide 59-185 but slightly less than the 32-185 fragment or a complete H5 molecule.

In contrast, fragment 1-65 (MW 7,000) was only slightly inhibitory of CF by H5/anti-H5 even at a peptide/H5 molar ratio about 60 and moderately inhibitory

Graph 12 - Inhibitory effects of H5 peptides on CF by
H5/anti-H5 at 1/3000 dilution of antiserum. In A, the
inhibitor was fragment 94-185 at 1.2 $\mu\text{g/ml}$ ∇ - ∇ ; 12 $\mu\text{g/ml}$ $\bullet$ - $\bullet$; 36 $\mu\text{g/ml}$ coincided with 127 $\mu\text{g/ml}$ $\diamond$ - $\diamond$.
In B, the inhibitor was fragment 100-185, at 1.2 $\mu\text{g/ml}$,
 Δ - Δ ; 12 $\mu\text{g/ml}$, $\blacktriangle$ - $\blacktriangle$; 36 $\mu\text{g/ml}$ $\circ$ - $\circ$ and 120 $\mu\text{g/ml}$
 $\square$ - $\square$. No inhibitor $\blacksquare$ - $\blacksquare$, in both graphs.

Graph 13 - Inhibitory effects of H5 peptides on CF by H5/anti-H5 at 1/3000 dilution of antiserum. In A, the inhibitor fragment 1-99 at 1.3 $\mu\text{g/ml}$ $\square - \square$; 13 and 39 coincided with 130 $\mu\text{g/ml}$ $\bullet - \bullet$. In B, the inhibitor was fragment 1-65, at 0.9 and 8.7 $\mu\text{g/ml}$ coincided $\Delta - \Delta$; 26 $\mu\text{g/ml}$, $\blacktriangle - \blacktriangle$; 87 $\mu\text{g/ml}$ $\nabla - \nabla$. No inhibitor $\blacksquare - \blacksquare$, in both graphs.

at a ratio greater than 200 (Graph 13b). By deduction one would expect considerable antigenicity to lie between residues 65 and 100, but fragment 66-99 (MW 3,800) showed variation in its inhibitory effect on the CF reaction of H5 and its homologous antiserum. Graph 14a shows a weakly inhibitory effect at peptide/H5 molar ratio of about 20, but moderately strong inhibition required a more than 50 fold excess of peptide. In eight separate experiments done in parallel conditions the peptide/H5 molar ratio needed to obtain 50% of inhibition varied from 1.3-90. The variability of the inhibitory activity persisted when the antiserum was used at a dilution of 1/2000, but an increase in the optimum concentration of H5 was noted, in presence of inhibitor (Graph 14b). A moderately strong inhibitory effect was observed at peptide/H5 molar ratios of 1.1 and 1.4 and a strong inhibitory effect at peptide/H5 molar ratios of 6 and 12.

To compare the ability of each fragment to inhibit the CF reaction of H5 and anti-H5, the molar ratios of different peptides/H5 was plotted against the degree of inhibition at 1/3000 dilution of anti-H5 serum as illustrated in Graph 15. If half inhibition is taken for purposes of comparison, the strongest inhibitor was peptide fragment 32-185 followed in decreasing order by 59-185, 1-99, 94-185, 100-185, 66-99, 1-65, 1-31. Fragment 66-99 has a tentative position, due to its variability. Similar inhibition tests were done with anti-H5 serum

Graph 14 - Inhibitory effects of H5 peptide 66-99 on CF by H5/anti-H5. In A, at 1/3000 antiserum dilution, the concentrations of inhibitor were: 0.44 and 4.4 $\mu\text{g/ml}$ both $\square - \square$; 13 $\mu\text{g/ml}$ $\bullet - \bullet$; 44 $\mu\text{g/ml}$ $\circ - \circ$. In B, at 1/2000 antiserum dilution, the concentrations of inhibitor were: 0.3 $\mu\text{g/ml}$ $\Delta - \Delta$; 0.6 $\mu\text{g/ml}$ $\blacktriangle - \blacktriangle$; 2.5 and 5 $\mu\text{g/ml}$ both $\nabla - \nabla$. No inhibitor $\blacksquare - \blacksquare$, in both graphs.

Graph 15 - Distribution of H5 peptide fragments according to their ability to inhibit the CF of H5/anti-H5 at different molar ratios of peptide/H5. At 1/2000 dilution of antiserum I in case of peptide fragments 32-185, ▽ - ▽ and 59-185, Δ - Δ; and 1/3000 in case of peptide fragments 1-99, ● - ●; 94-185, ○ - ○; 100-185, ∇ - ∇; 66-99, ▲ - ▲; 1-65, □ - □; 1-31, ■ - ■. The strongest inhibitor (at half inhibition) was peptide fragment 32-185, followed in decreasing order by 59-185, 1-99, 94-185, 100-185, 66-99, 1-65, 1-31.

diluted to 1/2000 from a separate group of guinea pigs and the results are summarized in Graph 16. The order of inhibitory potency is almost the same; i.e., the strongest inhibitor was peptide 59-185 followed in decreasing order by 94-185, 1-99, 100-185, 1-65, 1-31. An exception is the position of fragments 94-185 and 1-99 in reverse order compared with antiserum I. (Two fragments 32-185 and 66-99 could not be tested with antiserum II.) The inhibitory activity of the peptide fragments appears to be amplified with antiserum II; for example, a lower ratio of peptide 59-185/H5 is needed to obtain 50% of inhibition with antiserum. Compare Graphs 15 and 16. In contrast, peptide fragment 1-65 appears less active with antiserum II, while fragment 100-185 presents the same inhibitory activity with both antisera.

These peptides do not fix complement by themselves and do not react with the antiserum to give complexes in a CF reaction (not shown). Since the inhibition by all the fragments involved a drop in the CF curve without a shift to higher or lower optimal antigen concentration, the effect was probably not due to an interaction directly between the peptides and protein molecule or to the peptides creating an effective excess of antigen in the formation of complex with antibody (Bustin and Stollar, 1973). That the inhibition is specific is indicated by the fact that the different

Graph 16 - Distribution of H5 peptide fragments according to their ability to inhibit the CF of H5/anti-H5 at different molar ratios of peptide/H5. At 1/2000 dilution of antiserum II, the strongest inhibitor was peptide fragment 59-185 Δ - Δ ; followed in decreasing order by 94-185, $\bigcirc$ - $\bigcirc$; 1-99, $\bullet$ - $\bullet$; 100-185 ∇ - ∇ ; 1-65, $\square$ - $\square$; 1-31, $\blacksquare$ - $\blacksquare$.

fragments had no effect on CF reaction of histone H3 and its homologous antiserum (Appendix A2).

H. Cross-Inhibition of Complement Fixation by Histones 1 and 5

Crucial to understand the biological role of H5 is to clarify its relationship with the most common lysine-rich histones. Histones H1 and H5 bear numerous similarities; for example, H5 has a MW = 21,450 (Garel et al., 1975) which is comparable to 22,000 for H1 (Jones et al., 1974); a segment of common sequence has been described between histone H5 and rabbit thymus H1 (Sautière et al., 1975). These and other similarities have led to the suggestion that these histones could have evolved from a common ancestral gene (Yaguchi et al., 1977).

When purified histone H1 was added to the CF reaction of H5/anti-H5, inhibition was observed. Similarly, the addition of purified histone H5 inhibited the CF reaction of H1/anti-H1. Histone H5 at a concentration of 1.25 µg/ml reacts with 1/2000 dilution of homologous antiserum to give 92% of CF. Addition of 0.1 µg/ml of H1 produces 17% inhibition of the CF reaction, 0.2 µg/ml of H1 produces 50% and addition of 10 or 20 µg/ml of H1 produces 90% inhibition (Graph 17a).

Conversely histone H1 at a concentration of 5 µg/ml reacts with 1/2000 dilution of the homologous anti-

Graph 17A - Inhibitory effect of chicken H1 on CF by
H5/anti-H5 complex at 1/2000 dilution of the antiserum.
H1: 0.1 $\mu\text{g/ml}$ $\square$ - $\square$; 0.2 $\mu\text{g/ml}$ Δ - Δ ; 10 and 20 $\mu\text{g/ml}$
coincided Δ - Δ . No inhibitor $\blacksquare$ - $\blacksquare$.

Graph 17B - Inhibitory effect of chicken H5 on CF by
H1/anti-H1 complex at 1/2000 dilution of antiserum. H5
concentration was 0.3 $\mu\text{g/ml}$ 0 - 0; 1.25 $\mu\text{g/ml}$ $\bullet$ - $\bullet$;
2.5 $\mu\text{g/ml}$ ∇ - ∇ , 5 $\mu\text{g/ml}$ ∇ - ∇ . No inhibitor $\blacksquare$ - $\blacksquare$.

serum to give 87% of CF. Addition of 0.3 $\mu\text{g/ml}$ of H5 produces 17% inhibition, 1.25 $\mu\text{g/ml}$ of H5, 25%, 2.5 $\mu\text{g/ml}$ of H5, 80% and 5 $\mu\text{g/ml}$ of H5, 84% inhibition (Graph 17b). No inhibition of the CF reaction of H5 and its homologous antiserum or H1 and anti-H1 was obtained when purified histone fraction H2, or H3-H4 was added.

These results would suggest that the histones compete with each other for antibody so that, even without sharing enough determinants to allow CF with heterologous serum, the sharing of one or two sites could allow competition. A second possibility is that the histones interact with each other in a way that masks or alters the determinants.

DISCUSSION

A. Immunofluorescent Studies of Nuclear Localization of Histones 1 and 5

In this study the lysine-rich histones H1 and H5 were detected in isolated erythroid nuclei by indirect immunofluorescence. Since the validity of the results depends ultimately on the specificity of the antibodies used, considerable attention was given to the preparation of these reagents. The purity of the antigens used for eliciting the antibodies was checked by electrophoresis of overloaded polyacrylamide gel which did not show impurities. Furthermore, prior to injection, the antigens were solubilized in 0.15 M NaCl to promote renaturation of acid-extracted histones (Smerdon and Isenberg, 1976; D'Anna and Isenberg, 1974; Sperling and Bustin, 1975).

In a previous study it was shown by complement fixation that guinea pig anti-H5 immunoglobulin reacts with H5 but not with H1, H2a, H2b, H3 or H4 (Mura et al., 1974). In the present study a slight cross-reactivity was observed between guinea-pig anti-H5 and H1 and between guinea pig anti-H1 and histone fraction H5. More pronounced cross-reactivity was detected between rabbit anti-H5 and histone fraction H1. However antibodies purified by affinity chromatography did not show cross-reactivity with any other histone fraction by quantitative CF. All fluorescence microscope studies were done with

antibodies purified by affinity chromatography.

The specificity of immunofluorescent localization of H1 and H5 in isolated nuclei is indicated by the following facts. First, nuclei do not show fluorescence when immunoglobulin from guinea pigs not exposed to histones replaces the anti-histone immunoglobulin in the indirect IF stain. Second, neither fluorescein nor rhodamine conjugates by themselves stain the nuclei. Third, the nuclei do not show autofluorescence.

In a previous study, only 80% of fixed intact adult chicken erythrocytes reacted with the anti-H5 as detected by IF (Mura et al., 1978). Possibly the presence of hemoglobin, which is known to accumulate in mature nuclei (Coutelle et al., 1974; Small and Davies, 1972), interfered with the IF technique. Since it has been established that isolated erythrocyte nuclei in saline, which lack hemoglobin, retain normal chromatin structure (Brasch et al., 1972; Dardick and Setterfield, 1976; Davies, 1968), all the immunofluorescence analysis were done on isolated nuclei.

When adult erythrocyte nuclei devoid of hemoglobin were stained for H5 there was considerable variability in fluorescence between different nuclei, and in some cases heterogeneity within the same nucleus, notably the bright-rim effect. Since one possible cause of this variable staining might have been the presence of plasma and nuclear membranes, which could interfere with

penetration of antibody molecules into the nucleus (Bustin, 1978), the isolated nuclei were treated with Tween 80, a non-ionic detergent which removes the lipids of all membranes without seriously disturbing chromatin organization as seen in the electron microscope (Parr and Setterfield, personal communication). Although such nuclei appeared devoid of membrane under the electron microscope, by phase contrast they looked more compact than untreated nuclei. These detergent-treated nuclei showed basically the same heterogeneity of fluorescence as the untreated nuclei when reacted with anti-H5, although the distribution of nuclei in different staining categories varied. In addition, when such nuclei were stained at low ionic strength, which markedly loosened and dispersed the chromatin, the same basic staining patterns were still observed.

The simplest interpretation of these results is that the nuclei which show bright fluorescence have more H5 than those in medium or dim categories. The few unstained nuclei may contain H5 in amounts not detectable by this method. It has been calculated that by indirect IF, 3,000 molecules of antigen per cell can be detected (Mura et al., 1978). An alternative explanation for the variability in staining is that post-transcriptional modifications affecting the interaction of H5 with DNA (e.g. different degrees of phosphorylation) will affect the availability of some antigenic determinants and thus

the intensity of fluorescence. Since phosphorylation is linked with maturity of erythrocytes, the more weakly staining nuclei would be less mature. Embryonic erythroid cells which progress synchronously through maturation should help in choosing between these interpretations.

In this regard four populations of cells were compared. Two were from the primitive erythroid cell line in early embryos. In the 3½-day embryo, the erythroid cells are largely composed of mid-polychromatophilic erythroblasts, while 6-day embryos are rich in immature reticulocytes (Urban, Ph.D. Thesis, 1977; Weintraub, 1978). The two populations from the definitive cell line were mature erythrocytes of 16-day old embryos and adult chickens.

A comparison of embryonic and adult cells was also of interest because of the observation that during erythropoiesis in the chick and chick embryo the DNA repeat-length in micrococcal nuclease digest increases (Wilhelm et al., 1977; Weintraub, 1978), while the proportion of H5 increases in chicken erythroid nuclei (Morris, 1977; Weintraub, 1978). At days 3 and 4 the repeat length is 190 base pairs, increasing at day 6 to 205, at day 16 to 207 and in adults to 212 base pairs in length, while the moles of H5 per nucleosome are 0.4, 0.6, 0.7 and 1.0 respectively (Weintraub, 1978). The same studies showed that the repeat-length of liver DNA coincided with that of 3½ day embryonic erythrocytes.

The same nucleosome core containing 140 base pairs of DNA was present in chicken liver, 3 day-old embryonic erythrocytes and adult erythrocytes.

In the preparations of embryonic nuclei the total proportion of nuclei reacting with anti-H5 remained fairly constant. Minor differences in the distribution of staining responses were observed. In 3½ day embryos 24% were brightly fluorescent and a slight increase to 33% was observed in 6 day embryos. The majority of the nuclei in both cases showed medium-bright fluorescence with homogeneous intranuclear distribution. The nuclei of the definitive line showed a much larger proportion of bright nuclei; 62% in nuclei from 16 day embryos and 81% in nuclei from adult chickens. These results might indicate either accumulation of H5 histone in erythroid nuclei during maturation in primitive cell line and in mature erythroid nuclei from the definitive cell line or that the different degrees of modifications which decrease as maturation progresses would produce a change in conformation in H5, leaving more antigenic determinants available to interact with the antibody. Circular dichroism studies indicate that phosphorylation of histone 5 modifies its conformation in relation to changes in ionic strength and temperature (Williams and Seligy, 1975). Previous biochemical studies, however (Mazen and Champagne, 1969, 1972; Appels et al., 1972; Moss et al., 1973; Ruiz-Carrillo et al., 1976; Weintraub, 1978;

Billet and Hindley, 1972) agree with the first alternative.

When detergent-treated adult nuclei were stained with anti-H1, all nuclei reacted and the fluorescence was more uniform than with nuclei stained with anti-H5. Embryonic nuclei stained with anti-H1 showed no change in the total number of reacting nuclei, but the ~~distribution~~ distribution of categories changed slightly. In 3½-day-old embryos the bright category constituted 61% of the reacting nuclei, increasing to 84% at 6 days. There was no difference in nuclei derived from the definitive line, 96-97% appearing bright. These results would indicate that the amount of H1 does not differ in nuclei from 16-day embryos and adult erythrocytes. The possible increase in H1 from immature to mature erythrocytes was however unexpected, since previous biochemical work has consistently showed that the amount of H1 is constant through erythroid development in chicks and chick embryos (Mazen and Champagne, 1972; Moss et al., 1973; Weintraub, 1978).

It should be noted that the larger nuclei in embryonic erythroid cells as compared with mature cells may appear less fluorescent because of chromatin "dilution". The average volume of 3½-day embryonic nuclei is $63.1 \mu\text{m}^3$, 6-day embryonic nuclei $23.8 \mu\text{m}^3$ and adult mature nuclei $13.8 \mu\text{m}^3$ (Dardick and Setterfield, 1976). The effect of nuclear volume on relative intensity of fluorescence is demonstrated by experiments in which adult erythrocyte

nuclei were stained with anti-H1 or anti-H5 in conditions of low ionic strength (0.03 M NaCl). In this condition an increase of nuclear volume was observed and the apparent number of nuclei in the bright category decreased about 30% in both cases. No removal of either H1 or H5 occurs. However even if one assumes that the amount of H1 remains constant from 3½ day-old onwards and the change in fluorescence represents "concentration" of chromatin by reduction of nuclear volume the relative amount of H5 still increases through the same stages.

The same basic results were obtained when H1 and H5 were located together through use of double-staining using guinea pig anti-H1 and rabbit anti-H5. The same number of nuclei reacted with both antibodies and the distribution of intensities was the same. Not only were H5 and H1 found in the same nucleus, but also while most nuclei showed similar fluorescence for H1 the heterogeneity of H5 was still present. These results would indicate that even though the adult erythrocyte population is considered homogeneous cytologically, and supposedly all cells are near the same stage of maturation, it is not homogeneous in the content of lysine-rich histones. The results do not support the idea that H5 replaces H1 during erythrocyte maturation (Dick and Johns, 1969; Sinclair and Brasch, 1975).

Reported patterns of fluorescent antibody staining of histones in nuclei within intact cells have not been

consistent. Fixed rat liver cells stained with anti-serum against lysine-rich rat liver histones showed homogeneous fluorescence in the nuclei (Hekman and Sluyser, 1973). Fixed HeLa-S₃ and HNG-100 cells stained with antiserum against calf thymus H1 displayed diffuse and coarse granular patterns. Some cells stained with anti-H4 showed intense immunofluorescence in the peripheral part of the nucleus. Similar results were obtained when the immunoperoxidase method was used (Tsutsui et al., 1975, 1976). Fixed mature chicken erythrocytes stained with anti-H5 also appeared as complete or partial halos by immunofluorescence (Mura et al., 1978).

In this study most detergent-treated nuclei from adult and 16-day embryo displayed an intense fluorescent rim surrounding an internal homogeneous region after staining with anti-H5 or anti-H1. This pattern was less evident in definitive-line erythrocytes and liver nuclei stained without pre-detergent treatment. This pattern mimics the patterns of peripheral heterochromatin of liver mouse nuclei stained with quinacrine fluorochrome dyes or Giemsa (Franke and Scheer, 1974), and the patterns of native DNA detected by indirect IF using antiserum specific to double-stranded DNA in fixed human lymphocytes in G₁ and S phase (Tan and Lerner, 1972).

The fact that this pattern persists in detergent-treated nuclei stained in low ionic strength, where chromatin appears homogeneous, indicates that it is not

due to simply a peripheral distribution of chromatin. Neither is it due to the detergent treatment alone, since it is found in untreated nuclei, although to a lesser extent. The fact that the rim is much sharper than the distribution of chromatin seen in electron microscope sections of erythrocyte nuclei suggests that it is some form of artifact, partially chemical and perhaps partially optical.

If the rim effect were produced by the detergent changing the conformation of nucleoprotein near the nuclear surface to expose more antigenic determinants one would expect this change to reverse by lowering salt concentration which does not happen.

It has been demonstrated that chicken erythrocyte and liver nuclei isolated and fixed in isotonic saline exhibit compact chromatin fibers at least 200 A in diameter. The exposure of these nuclei to reduced ionic strength caused reversible reduction in fibril diameter to 50-100 A. After selective removal of both H1 and H5, the liver and erythrocyte nuclei in saline contained no 200 A fiber, and it was concluded that these histones contribute directly to the organization of the 200 A fibers (Brasch et al., 1972; Brasch and Setterfield, 1974; Brasch, 1976). More recent studies in the electron microscope have shown a knobby 200-250 A fiber from isolated nuclei or whole cells, which unfold to reveal "the beads on string" configuration characteristic of nucleosomes

after lowering the monovalent ionic concentration (Renz, 1977; Rattner and Hamkalo, 1978).

When detergent-treated adult chicken erythrocyte nuclei were stained with anti-H5 or anti-H1 in conditions of low ionic strength (0.03 M NaCl), the number of reacting nuclei remain fairly constant, but bright nuclei decreased and medium nuclei increased. This result is expected simply as a result of nuclear expansion and chromatin dilution (see above). It is concluded therefore that the availability of antigen determinants in H1 and H5 do not change when nuclei are stained in conditions of low ionic strength,

Previous immunological work has shown that antigenic sites of lysine-rich histones are fully available in contrast with the determinants in nucleosomal core histones (Goldblatt and Bustin, 1975). By treating a given amount of chromatin with various anti-histone sera, the chromatin absorbs these antibodies to different degrees, so that the order of availability of the determinants in chromatin was determined to be $H1 > H2b > H3 > H4 > H2a$. Sonication of chromatin did not affect the availability of H1 but quadrupled the availability of determinants in H3 and H4 and doubled reaction with H2b. Inaccessibility of certain antigenic determinants has been demonstrated by the failure to stain nuclei of mature human spermatozoa with fluorescent antibodies. When the mature spermatozoa are artificially swollen after

treatment at pH 9.0 in presence of dithiothreitol and trypsin, determinants become exposed and accessible to antibodies against histones H2b, H3 and H4 (Samuel et al., 1976).

Relevant to the possible function of lysine-rich histones is the observation that chicken liver and chicken fibroblast nuclei show fluorescence when stained with anti-H5 in isotonic saline conditions. The reacting chicken liver nuclei were medium and dim in contrast to the bright fluorescence shown with anti-H1. Chicken fibroblast nuclei also fluoresced weakly when stained with anti-H5, but gave very bright uniform fluorescence when stained with anti-H1. One possibility is that H5 is indeed present in liver and fibroblast nuclei and that the faint band which appears in the position of H5 in all reported electrophoretic analyses of total histones from liver is not merely a contaminant from mature erythrocytes (Neelin and Butler, 1961; Purkayastha and Neelin, 1966). The sensitivity of the IF method is greater than that of zone electrophoresis. Another possibility is that histone H1 and H5 share common antigenic determinants, which are detected by IF but not by CF.

The inhibition observed of the CF reaction of H1 or H5 and its homologous antiserum by H5 or H1 respectively, would support the latter possibility (this study). Although the antibodies against the lysine-rich histones

were raised with H1 or H5 in solution, it is evident that antigenic determinants responsible for at least some of these antibodies are exposed to reaction in the native chromatin from primitive and definitive erythrocytes. It is of interest to localize these antigenic determinants within the erythrocyte H5 polypeptide chain in order to gain some insight into the gross conformation of this molecule in erythrocyte chromatin.

B. Localization of Antigenic Determinants Within Erythrocyte Specific Histones by Inhibition of Complement Fixation

Complement fixation is a sensitive reaction that depends on the presence of multivalent antigen in order to form a large cross-linked structure with a mixture of antibodies. Even two binding sites on each molecule of antigen may be insufficient to form a lattice-like complex, since such bivalent antigens would form linear polymers or closed structures such as triangles or quadrangles which may not fix complement. If a single antigenic determinant is present, the largest aggregate that can result consists of two molecules of antigen bound to one molecule of bivalent antibody Ag_2Ab , which would not fix complement but could inhibit CF of a multivalent form of the antigen (Reichlin et al., 1970, Arend and Mannik, 1974).

To study the antigenic structure of the H5 molecule, purified fragments derived from H5 were used to inhibit the reaction between the native antigen and its homologous antiserum. None of the fragments fixed complement, but the strongest inhibitory determinants are concentrated in the middle of the molecule, in a region from residue 59 to 99. Six of the eight fragments used in this study were found to be relatively efficient inhibitors, half-inhibiting at molar ratios of peptide to whole antigen ranging from 1.2 to 100.

Cleavage at the only methionine gave residues 1-31 and 32-185; although peptide 1-31 did not inhibit the reaction of H5 with its homologous antiserum, even at a peptide H5 molar ratio of 100, peptide 32-185 inhibited totally all its complement-fixing reactivity of H5 at a molar ratio of about 1. However since peptide fragment 32-185 did not fix complement when tested with anti-H5, this peptide had lost some antigenic quality found in the whole molecule. Peptide fragment 1-31 may have an effect either as a weak determinant or by contributing to the conformation of the whole molecule.

Peptide 59-185, obtained by cleavage of the tyrosine residue with NBS, inhibited reaction of H5 with its antiserum slightly less than did peptide 32-185 perhaps because larger fragments retain native conformation better and thus are more efficient at binding antibody. The remarks concerning the failure of peptide 32-185 to fix complement with anti-H5 despite its apparent richness to determinants, apply of course to this shorter less inhibitory peptide.

Cleavage of the H5 molecule with acetic acid at the aspartic acid residues gave the peptides 1-65, 66-99, 100-185 and 1-99, each of which showed different degrees of immunological activity. Fragment 1-65 inhibited marginally the reaction of H5 with either lot of antiserum used. This could indicate either a weak antigenicity in the region 32-65 or the restoration of the conformation

to the N-terminal and by the addition of 33 residues.

The fact that peptide 59-185 is nearly as good an inhibitor as peptide 32-185 suggests any inhibitory capacity N-terminal to residue 65 is located between the residues 50 and 65 inclusive, with the sequence Lys-Val-Gly-His-Asn-Ala-Asp (Sautière et al., 1976). There is also a possibility that these cleavages have split a reactive region so that neither fragment carries the whole of the inhibitory determinant. Since the single Tyr residue in H1 affects the structure of an antigenic determinant (Bustin and Stollar, 1973), by analogy residue 58, the homologous Tyr residue in H5 (Chapman et al., 1978a and b), might also be part of a determinant (although the preparation of the antibodies was different).

Peptide 1-99 highly inhibits the reaction of H5 with its antiserum. Since this fragment is as effective an inhibitor as fragment 59-185, it carries much of the inhibitory activity of the molecule and the loss of the C-terminal 86 residues has not disrupted its major determinant sites. However because this peptide lacks complement-fixing capacity, the loss of the C-terminal portion has more than a negligible effect of the overall antigenicity of H5.

Peptide 100-185, with 38 lysines (Sautière et al., 1976) inhibits the reaction of H5 with anti-H5 only moderately, indicating that this region is weakly antigenic or that the N-terminal 99 residues are necessary to stabilize

the conformation of any determinants in this portion of the protein. In contrast peptide 94-185, obtained by cleavage with pepsin (Alice Mazen, personal communication) and differing by only 6 amino acids from peptide 100-185, highly inhibits the reaction of H5 with anti-H5. This finding shows that residues 94-99 which have the inclusive sequence Arg-Leu-Ala-Lys-Ser-Asp (Sautière et al., 1976), play a very important role in the antigenic structure of the molecule either by serving as a binding site directly or being critical for its conformation.

The globular domain of histone 5, comprising the sequence 22-100 (Avilés et al., 1978), contains the bulk of the antigenicity or at least conserves its inhibitory capacity even without the N-terminal and C-terminal extremes.

While consistent results were obtained with both lots of antiserum with all other fragments, peptide 66-99 showed considerable variation in reactivity, even in replicate assays. This may be caused by conformational variations; antibodies against protein antigens are often directed against their three-dimensional structure predominating under the conditions of immunization (Atassi and Thomas, 1969). While short peptides may be in unfolded conformational states, their form is also less stable and they may be induced more readily by the antibody to fold into the shape intrinsic to the native antigen, forming an inhibitory complex. The probability of finding such an

ephemeral conformational state will improve with increase in peptide concentration (Atassi and Saplin, 1968). This is well illustrated by the consistency of the results obtained with the longer fragments, specially those in which the globular region of the molecule was kept intact, e.g. peptide 1-99 or 59-185, in contrast with the results obtained with the smaller peptide 66-99. The variable results of the latter may indicate that this region forms part of a conformational determinant in which contiguous surface residues are not in direct sequence, but form part of a determinant as a consequence of tertiary folding, stabilized by regions outside of residues 66-99 (Atassi and Lee, 1978). Other examples of conformational or split determinants have been reported among other histones. Fragments of chicken histone H3 (135 residues) containing 90, 30 and 15 residues, do not fix complement by themselves, but an equimolar mixture of the three restores 97% of the activity, indicating that the antigenic determinants could be reformed in this more globular histone (Sapeika et al., 1976). In contrast rabbit antibody against calf thymus H1 (complexed with RNA) forms complexes with determinants in either the amino-terminal third or carboxy-terminal two-thirds of H1, but gave complement fixing aggregates only with the latter. The extent of this complement fixation was less than with intact H1, and a mixture of the two fragments did not restore all the complement fixing activity of the whole molecule (Bustin and Stollar, 1973;

Bustin, 1978). In this case the antigenic determinants are concentrated in the lysine-rich C-terminus of H1.

In H5 most of the antigenic determinants seem to be located in the lysine-poor portion of the molecule (residues 1-99, with only eleven lysines).

A localization of regions inhibited by peptide fragments when used as inhibitors in CF of H5 and anti-H5 in the intact H5 molecule and peptide fragments is shown in Graph 18. This was deduced by comparing the inhibitory activities of fragments having overlapped regions; thus the inhibitory activity of fragment 1-65 was localized in the sequence 59-65, by comparison with fragment 59-185 which has an inhibitory activity similar to 32-185, and to the whole molecule. Similarly, the difference in inhibitory activity between fragments 94-185 and 100-185 implies the localization of antigenicity in another sequence 94-99.

The identification of the antigenic determinants of globular proteins has posed a difficult problem because of the dependence of the antigenic activity on integrity of the tertiary structure. Since conformational studies can profoundly affect antigenicity, many determinants must be studied in situ. That is, peptide inhibition of the reaction of the intact protein with the specific antibody will not be useful for the analysis of certain determinants in proteins because of this dependency (Reichlin and Noble, 1977). Therefore the complete delineation of the antigen structure can not be derived from a single approach since

Graph 18 - Localization of regions inhibited by peptide fragments when used as inhibitors in CF of H5 and anti-H5.

Regions which by themselves do not show inhibitory capacity (but the loss of which may affect complement-fixing qualities of histone 5), are shown as .

Regions which retained the capacity to inhibit CF by the whole molecule are shown as =====.

Regions; the loss of which markedly reduced inhibitory capacity of residual peptides, are shown as .

each has its limitations and shortcomings. When used to distinguish mutant hemoglobin from normal hemoglobin, the molar concentrations of reactants was 10^{-9} - 10^{-8} mole/liter for quantitative CF compared with 10^{-9} - 10^{-11} mole/liter for radioimmunoassay (Reichlin and Noble, 1977). This method is as sensitive as CF and has the advantage of detecting simple binding in contrast to CF, which requires a variety of antibodies and a multivalent antigen. Another advantage is that radioimmunoassay can be utilized with antisera made in any species, whereas CF can only be utilized with sera from species such as rabbits, whose immunoglobulins fix complement efficiently.

Other possibilities should include the use of antigenically active peptides as immunoadsorbants by attaching them to a suitable insoluble support. The antibody population which binds to the peptide can then be isolated by affinity chromatography.

Despite these limitations, valuable information has been obtained as a first step in elucidating the antigenic structure of histone 5.

C. Reflections and Conclusions

In this study lysine-rich H1 and H5 histones have been localized in isolated nuclei from erythroid cells at different stages of maturation by indirect immunofluorescence. It has been shown that the relative amounts of H5 remained essentially constant in the two primitive line stages studied, although the nuclei were quite variable in staining. H5 appeared substantially by higher in definitive line nuclei, but was still variable between nuclei. The amount of H1 appeared to increase slightly during maturation of the primitive cell line but was essentially constant in mature erythrocyte nuclei from the definitive line. Mature erythrocytes are therefore not a homogeneous population of cells in regard to the content of histone 5, but they show reasonably constant content of H1. The two histones did not show reciprocal proportions within single nuclei from erythroid cells at different stages of development.

Since the function of H5 in erythrocyte nuclei is unknown it is difficult to speculate on the meaning of the variability in amount of this histone. One possibility is that H5 is not strictly necessary for the function of erythrocytes. Certainly a substantial population of otherwise normal appearing nuclei showed a very low content or apparent absence of H5. H5 has been shown to be lacking in white sucker fish, which have erythrocytes structurally (and presumably functionally)

identical to those in fish species which do have H5 (Miki and Neelin, 1977). The variation in H5, at least in nuclei of mature cells, might also reflect variability in cell age. The immunofluorescence results would be compatible with a model in which H5 is progressively lost from nuclei of mature cells as they age. On this model H5 could have a necessary function during part of the cells' life cycle but become unnecessary as cell senescence approaches.

The variable staining of H5 might also be a reflection of modifications of the protein which reduce or eliminate its reactivity with antibodies, again this situation could be related to cell maturity and/or age. In fish erythrocytes, H5 varies from apparent absence in white sucker erythrocytes to 6% in carp and 11% in rainbow trout (Miki and Neelin, 1977). Furthermore the composition of H5 in different species shows as much variability as does H1. The reciprocal proportion of H1 and H5 in mature fish erythroid cells implies that H5 replaces H1 functionally and supports the idea that H5 is an extreme variant of H1.

The fact that, during maturation, chicken erythrocytes lose their capacity to synthesize RNA and there is an increase in the proportion of histone H5 with respect to total histones, led to the idea that H5 causes chromatin condensation and inactivates the avian genome. Further support for this notion was obtained from transcriptional

studies. Isolated chicken erythrocyte nuclei in saline have very restricted capacity for RNA synthesis while removal of H5 increases template activity of chromatin with exogenous RNA polymerase (Seligy and Neelin, 1970; Bolund and Johns, 1973; Seligy and Miyagi, 1974). The inactivity of intact chromatin is not due to a lack of enzymes, since an adequate level of active DNA-dependent RNA polymerase has been detected in mature chicken erythrocytes (Schechter, 1973; Scheintaub and Field, 1973; van der Westhuyzen et al., 1973). Furthermore, chromatin condensation itself seems to be unrelated to gene regulation, since in vitro transcription studies of ion-mediated condensed and dispersed chromatin showed that structural condensation of the mature erythrocyte does not have a major effect on restricted template properties of this chromatin (Brasch et al., 1971). The fact that the amount of condensed chromatin is fairly uniform through primitive maturation further supports this notion (Dardick and Setterfield, 1976).

Up to now, no specific relationship has been found between the appearance of histone H5 and the inactivation of the avian genome. Biochemical studies in regenerating goose blood showed DNA, RNA, and hemoglobin synthesis in early erythroid cells containing phosphorylated H5 (Seligy et al., 1973). By combining immunofluorescence and autoradiography, incorporation of (^3H) thymine has been

observed in cells containing H5 (Mura et al., 1978).

There is evidence that actively transcribed regions have an altered structure. Active genes have been shown to be preferentially digested by DNAase I (Weintraub and Groudine, 1976; Flint and Weintraub, 1977). More recently, work on 18-day embryonic erythrocytes showed that removal of H1 and H5 from erythrocyte nuclei appears to increase the rate of DNAase I digestion, but the preferential digestion of globin genes with DNAase I was not affected. Partial digestion of H1- and H5-depleted erythrocyte nuclei with DNAase I reduces the concentration of globin gene sequences in the remaining DNA by some 80%, as does DNAase I digestion of whole erythrocyte nuclei. The authors concluded that H1 and H5 are not required for the maintenance of the altered DNAase I sensitive conformation around the globin genes in chicken erythrocytes. Further evidence showed that the nucleosome cores themselves are altered in active genes (Villepontaux et al., 1978).

All these considerations support the notion that histones H1 and H5 are not directly responsible for fine gene regulation but may be involved in chromatin organization above the nucleosome level.

Other similarities between H1 and H5 include coincident homology of a stretch of 10 amino acids adjacent to the phenylalanine residue 106 in rabbit thymus, trout and sea urchin lysine-rich histone (Sautière

et al., 1975; Yaguchi et al., 1977). However using antibodies against H5 or H1, no cross-reactivity was observed by CF when heterologous antigen was used. In contrast, H5 added to the H1/anti-H1 system inhibits the CF reaction and vice versa.

These results can be explained as a competition between the histones for the antibody, indicating that they may share antigenic determinants. If this is true, the fluorescence observed in liver and fibroblast nuclei when stained with anti-H5 can be explained. There is also the alternative that H1 and H5 interact with each other so that some determinants become masked or altered, being unable to react with the antibody. At present it is not possible to distinguish between these possibilities, but the fact that H1 reduced the CF of anti-H2a, anti-H2b, anti-H4 and to a lesser extent, anti-H3 with their antigens would agree with the second alternative (Stollar, personal communication).

By CF studies it has been found that most of the antigenic reactive region of the H5 molecule is located in the middle of the molecule in a region from amino acid 59-99. This again would support the possibility of sharing determinants with H1, since the common sequence is located between amino acids 84-93 in H5 (see above). The globular domain of the molecule extends from residue 22-100 (Avilés et al., 1978), so the antigenic determinants are mostly in the globular region. The possibility of

conformational determinants are also suggested. Interestingly, the location of the phosphorylation sites of H5 have been localized in two regions of the molecule: between residues 1-28 of the N-terminal region and in the C-terminal region beyond residue 100 (Sung and Freedlender, 1978). None of the phosphorylation sites reside in the region where the presumptive antigenic determinants are, namely the region from amino acid 59-99. This indicates that the antibody against H5 may not distinguish between phosphorylated and non-phosphorylated H5 unless the conformational changes of the molecule were profound enough to distort the globular region containing the determinants.

The elucidation of the biological role of histone H5 should lead to a better understanding of the biological role of the lysine-rich histone family.

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APPENDIX A

Inhibitory Effects of Peptide Fragment 59-185 on CF by H5 Rabbit Anti-H5(RNA) Serum

Since fragment 59-185 (MW = 13,900) derived from histone fraction H5 inhibits strongly the CF by the complex between H5 and guinea pig anti-H5, this suggests that it contains all the antigenic activity of the molecule (this study). However when this fragment is tested as inhibitor of CF by the complex between H5 and antiserum raised in rabbit with H5 bound to RNA, it has no effect at a peptide/H5 molar ratio of 0.4, slight inhibitory effect (12%) at molar ratios of 1.6 and 6.5, and 57% inhibition at a ratio of 25 (Figure A1). This result suggests that rabbit anti-H5(RNA) has different specificity from guinea pig anti-H5.

Inhibitory Effects of Peptide Fragments Derived from Histone 5 on CF by H3 Rabbit anti-H3 Serum

The specificity of the inhibition of the CF reaction between H5 and its homologous antiserum by peptide fragments derived from the H5 histone fraction was demonstrated by testing the inhibitory capacity of these fragments in the CF reaction between H3 and anti-H3 serum from rabbit. Results of these experiments are shown in Figure A2. No inhibition was obtained when fragment 1-65 was added at a peptide/H3 molar ratio of

Figure A1 - Inhibitory effect of H5 peptide 59-185 on CF
by H5/anti-H5 serum raised in rabbit and diluted at
1/500. The fragment concentrations were 0.4 $\mu\text{g/ml}$ Δ -
 Δ ; 1.6 $\mu\text{g/ml}$ $\square$ - $\square$; 6.25 $\mu\text{g/ml}$ $\bullet$ - $\bullet$; and 26 $\mu\text{g/ml}$,
0 - 0. No inhibitor $\blacksquare$ - $\blacksquare$.

Figure A2 - Inhibitory effects of peptide fragments
from H5 on CF by H3/anti-H3 serum raised in rabbit at
1/300 dilution of the antiserum. Inhibitors were
peptides 1-65 at 50 $\mu\text{g/ml}$, coinciding with peptide 66-99
at 30 $\mu\text{g/ml}$ • - •; fragment 1-31 at 30 $\mu\text{g/ml}$ coinciding
with fragment 100-185 at 80 $\mu\text{g/ml}$ Δ - Δ ; and fragment
1-99 at 80 $\mu\text{g/ml}$ $\square$ - $\square$. No inhibitor ■ - ■.

70, when fragments 66-99 or 100-185 were added at a peptide/H3 molar ratio of 80 or when fragment 1-31 was added at a peptide/H3 molar ratio of 90. However if fragment 1-99 was added at a peptide/H3 molar ratio of 70, a slight inhibition was obtained (10%). It can be concluded that the inhibition by peptide fragments derived from H5 on the CF reaction by H5/anti-H5 was specific.

Note: This work was done in collaboration with Mme Alice Mazen. de l'Institut de Biologie Moléculaire et Cellulaire du CNRS. Laboratoire de Biophysique, Strasbourg, France.

APPENDIX B.

Cross-Reactivity of Guinea Pig Anti-H5 (Chicken) With H5 From Different Species of Fish and Birds

Serological studies have several applications in structure of proteins, because they can detect small alterations in structure allowing comparison of proteins isolated from various sources. It has been shown that H5 from erythrocytes of different avian and fish species can be distinguished qualitatively as well as quantitatively (Miki, 1976). The sequence of the 16 amino acid residues nearest the N-terminus of H5 from duck, goose, quail and pigeon also reveal differences among them (Seligy et al., 1976). In an attempt to further study such differences, cross-reactivity of H5 fractions from different species of fish and birds was determined by quantitative CF, using anti-H5 chicken serum.

The following histone H5 fractions from fish were provided by Dr. B.L.A. Miki (Division of Biological Sciences, National Research Council of Canada, Ottawa): carp (Cyprinus carpio), trout (Salmo gairdnerii), perch (Perca flavescens), black crappie (Pomoxis nigromaculatus). The following histone H5 fractions from birds were provided by Dr. V.L. Seligy (Division of Biological Sciences, National Research Council of Canada, Ottawa): goose (Anser anser), duck (Cairnia moscheta), pigeon

(Columbia livia), quail (Coturnix japonica).

When purified histone 5 from different species of fish was allowed to react with antiserum against H5 from chicken, no cross-reactivity was detected at 1/2000, 1/1500, 1/1000, 1/500 dilutions of antiserum, while in the same conditions between 80-95% of complement was fixed with the homologous H5 antigen (Figures B1 and B2). However inhibition of the CF reaction of anti-H5 chicken with the homologous antigen, was obtained when purified H5 histones from different species of fish were added to the reaction at a concentration of 20 $\mu\text{g/ml}$. Trout, perch and black crappie histone H5 gave 80% inhibition at the optimal antigen concentration of 2.5 $\mu\text{g/ml}$ and carp H5 gave 86% inhibition under the same conditions. (Figure B3). No inhibition was obtained when histone H5 from different species of fish were added at a concentration of 1 $\mu\text{g/ml}$ (not shown).

In contrast, purified histones H5 fractions from different species of birds cross-react with anti-H5 chicken at a dilution of 1/2500 (Figure B4). In conditions in which 85% of CF was obtained with the homologous antigen, 63% of CF was obtained with H5 from duck; 20% with H5 from quail, 14% with H5 from goose and 2% of CF with H5 from pigeon. These results would indicate that antigenically duck H5 is more similar to chicken H5 followed in decreasing order by quail H5, goose H5, pigeon H5. H5 from different fish species seem not to

Figure B1 and B2 - Cross-reactivity of guinea-pig anti-H5 (chicken) with H5 fractions from erythrocytes of different species of fish. The complement fixation of histone 5 from trout, perch, black crappie and carp all coincided at 1/2000 dilution of the antiserum $\square - \square$ (Figure B1), and at 1/500 dilution of the antiserum $0 - 0$ (Figure B2). Chicken erythrocyte H5 $\blacksquare - \blacksquare$, the homologous antigen.

H5 $\mu\text{g/ml}$

Figure B3 - Inhibitory effects of histone 5 from different fish erythrocytes on the CF by H5/anti-H5 (chicken) at 1/2000 dilution of the antiserum. CF in presence of H5 from trout, perch and black crappie at 20 µg/ml all coincided □ - □ ; carp at 20 µg/ml ▲ - ▲. Without inhibitor ■ - ■ .

Figure B4 - Cross-reactivity by CF with guinea pig anti-H5 (chicken) and H5 from erythrocytes of different species of birds, at 1/2500 dilution of the antiserum.

Duck erythrocyte H5 Δ - Δ ; quail erythrocyte H5 $\blacktriangle$ - $\blacktriangle$;

goose erythrocyte H5 0 - 0; pigeon erythrocyte H5 $\bullet$ - $\bullet$;

chicken erythrocyte H5 $\blacksquare$ - $\blacksquare$.

present any antigenic similarities with chicken H5.

The data presented demonstrate that H5 histone fraction from different species differ antigenically. No antigenic similarities were found when H5 from different species of fish were compared with chicken H5 using anti-H5 raised against chicken H5 as a probe in CF, but inhibition of the reaction chicken H5 with its homologous antiserum was observed when histone H5 from different species of fish were used as inhibitors, at high concentrations suggesting that they may have determinants in common. These results agree with recent studies in H5 histone fractions from different species of fish which showed that this histone fraction not only varies in the proportion in the different species of fish but also in relative electrophoretic mobility and amino acid composition (Miki and Neelin, 1977).

On the other hand, when chicken H5 was compared with H5 from different species of birds, using anti-H5 raised against chicken H5 as a probe, they do show cross-reactivity. Duck H5 presented the highest cross-activity followed in decreasing order by quail, goose and pigeon H5. The homology in amino acid sequence between goose and chicken H5 explain the cross-reactivity between these two fractions (Yaguchi et al., 1977).

Note: This work was done in collaboration with Margarita Rodriguez (Honours B.Sc. Research Project, Biology Dept. Carleton University, 1978).

This shows that histones H5 show as much variability as H1 histone fraction does.

APPENDIX C: MATERIALS

EQUIPMENT

Acta III Spectrophotometer, Model 3003 (Beckman)
B-20 refrigerated centrifuge (International)
Dounce-type glass hand-homogenizer with loose-fitting
pestle "A" (Kontes Glass Co.)
Duostat regulated DC power supply (Beckman)
Concentration and diafiltration system (AMICON)
Diaflo membrane, P-10
Giratory G 76, water bath shaker (New Brunswick Sc.)
J-21 Preparative centrifuge (Beckman)
L-5-50 Ultracentrifuge (Beckman Spinco)
Metrohm Herisace pH-meter Model E 300 B
Spectrophotometer Model 26 (Beckman)
Ultrorac fraction collector (LKB)
VIRTIS "23" homogenizer
VIRTIS lyophilizer

Note: All centrifugal forces in g are based on average rotor radius.

ANIMALS

Chickens, White Leghorns (Sterling McEwan, North Gower, Ont.)
Chicken embryos, White Leghorn strain Hyline 934F (same
supplier)
Guinea pigs, Hartley strain (Connaught Labs, Toronto)
Rabbits, New Zealand White (Double J. Ranch, Smiths Falls)

CHEMICALS

Chemicals Used For Chromatography:

Amberlite CG-50 H Type I 100-200 mesh (BDH Chemical Co.)

Bio-gel P-60 (Bio-Rad Richmond Calif.)

Carbon decolorizing NORIT A (J.T. Baker Chemical Co.)

Celite No. 545 lot 7648 44 (Fisher)

DEAE - Sephadex A-50 (Pharmacia)

Guanidinium chloride practical grade lot N 77C-00351
(Sigma Chemical Co.)

Sephadex G-25, G-100 (Pharmacia)

Sepharose 4-B (Pharmacia)

Chemicals Used For Electrophoresis:

Acrylamide (Eastman Kodak)

Amido Black 10B CI No. 20470 (J.T. Baker Chemical Co.)

N-N' methylene-bisacrylamide (Eastman Kodak)

Pyronin-Y CI No. 45005 lot No. 711B (Eastman Kodak)

TEMED (Eastman Kodak)

Ultrapure urea (Schwartz-Mann)

Chemicals Used For Immunology:

Agar Noble lot No. 621584 (Difco Lab.)

Agarose lot 024 CO 550 (Sigma Chemical Co.)

Bacto antisheep hemolysin (Canlab)

Complete adjuvant (Difco Labs)

FITC lot No. 95 C-5020 (Sigma Chemical Co.)

Gelatin (Difco)
Glycine (BDH Chemical Co.)
Goat antirabbit IgG (Gibco Canada)
Guinea pig complement (Gibco Canada)
RITC lot No. 0987440 (BDH Chem. Co.)
Sheep blood in Alsevers (Qualicum)
Thimerosal (Sigma Chemical Co.)

General Chemicals:

Ammonium sulfate (J.T. Baker Co.)
Barbital, Sodium (Fisher Sc. Co.)
Cyanogen Bromide (J.T. Baker Chemical Co.)
EDTA (BDH Chem. Co.)
Hank's Balanced salt solution (GIBCO)
Heparin, Sodium (BDH Chemical Co.)
PMSF (Sigma Chemical Co.)
Saponin lot No. 213313 (BDH Chem. Co.)
TRIS (Sigma Chemical Co.)
TRITON-X-100 (BDH Chem. Co.)

General Material:

Common glassware (Johns Sc. Co.)
Dialysis tubing (Fisher Co.)
Hemocytometer (Canlab)
Microscope slides and cover glasses (Corning)
Millipore filters 0.45 μ (Millipore Corp.)
Whatman filter #4 (Balston Limited)

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