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Histones in the Primitive Erythroid Line of
Embryonic Chickens

by

Michael K. Urban III

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

Department of Biology
Carleton University
Ottawa, Ontario
May 1977

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"The body of a worm and the face of a man alike have to be taken as chemical responses. The alchemists dreamed of old that it might be so. Their dream however supposed a magic chemistry. There they were wrong. The chemistry is plain everyday chemistry. But it is complex. Further, the chemical brew, in preparation for it, Time has been stirring unceasingly throughout some millions of years in the service of a final cause. The brew is a selected brew".

Sherrington, 1951

"The field of developmental biology is alive; the pot is boiling, but it must be stirred vigorously - and it needs more than a dash of flair and imagination and technical skill".

Ebert, 1965

The undersigned recommend to the Faculty of Graduate Studies acceptance of this thesis "Histones in the Primitive Erythroid Line of Embryonic Chickens" submitted by M.K. Urban, B.A., in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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May 1977

Abstract

The primitive red blood cell line of embryonic chickens is well suited for studying erythroid maturation, because it appears in one wave at about two days of incubation and matures synchronously until day six. Qualitative and quantitative changes in histone fractions during four primitive line maturation stages were examined by electropherograms of polyacrylamide gels of total histone extracts, in relation to changes in DNA and RNA synthesis. Relative proportions of the erythrocyte-specific histone 5 changed significantly, while the other histone fractions did not change appreciably during the maturation of the primitive cell line. The weight ratio of histone 5 to DNA increased from 0.06 at erythroblast and polychromatophilic erythrocyte stages to 0.11 at orthochromatophilic and mature erythrocyte stages. Incorporation of ^{14}C -thymidine into DNA and ^3H -uridine and ^{14}C -uridine-triphosphate into RNA decreased before histone 5 increased. Thus, the accumulation of histone 5 on chromatin during primitive red blood cell maturation may not be directly related to nuclear repression, but may involve chromatin condensation. During development from embryonic mature red blood cells to definitive mature red blood cells of adult the relative amount of histone 1 decreased while histone 5 increased and the other histones remained unchanged. It is suggested that the amount of both histone 1 and 5 bound to DNA affects the degree of condensation of chromatin.

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Abbreviations

Bis - N,N'-methylene-bisacrylamide

BSA - bovine serum albumin

CD - circular dichroism

cpm - counts per minute

DE - definitive line erythrocyte

DNA - deoxyribonucleic acid

DNase - deoxyribonuclease

dpm - disintegrations per minute

Eb - erythroblast

EP - early-polychromatophilic erythrocyte

H1,H2,H3,H4,H5 - respectively histones 1, 2 (a and b), 3, 4 and 5

Hb - hemoglobin

ME - mature primitive erythrocyte

MEM - minimum essential medium (modified Eagle-Spinner medium)

mRNA - messenger RNA

OE - orthochromatophilic erythrocyte

P - polychromatophilic erythrocyte

PCA - perchloric acid

PMSF - phenylmethyl sulfonylfluoride

PPO - 2,5-diphenyloxazole

RBC - red blood cell

RNA - ribonucleic acid

RP - residual protein

SDS - sodium dodecyl sulfate

S-period - that period of the cell cycle during which DNA is synthesized

Stage-17-18 RBC's (stage-21, stage-27, stage-29) - red
blood cells isolated from stage-17-18 embryos

TCA - trichloroacetic acid

TEMED - N,N,N',N'-tetramethylene-ethylenediamine

TPCK - L-1-tosylamine-2-phenylethylchloromethyl ketone

Tris-HCl - tris (hydroxymethyl) aminomethane hydrochloride

UV - ultraviolet

Introduction

The metazoan diploid zygote, through complex developmental processes, gives rise to an organism containing millions of cells of various types and performing a diversity of metabolic functions. Present evidence suggests that each of these cells contains the same genetic information (Gurdon and Uehlinger, 1966; McKinnell et al., 1969; King and Briggs, 1954). If this is the case then during differentiation the genetic material must be regulated such that specific cells are capable of performing specialized functions.

The assessments of gene regulation in a metazoan system is complex due to the multiple parameters involved in the differentiation of a cell which is part of a total organism and is subject to a host of regulatory factors. Furthermore, the intracellular complexity of eukaryotic cells makes them much less amenable to the types of studies which have been used to elucidate regulatory mechanisms in prokaryotic cells. The genetic material is sequestered from the site of translation (cytoplasm) and further regulatory events control the transport and processing of information prior to its translation (Comings, 1972; Darnell, 1968). Informational RNA (messenger RNA) may be transcribed as part of a much larger molecule (Imaizumi et al., 1973; Lindberg and Darnell, 1970), selectively cleaved and modified by addition of polyadenine at its 3' hydroxyl end (Adesnik et al., 1972; Scherrer and Marcaud, 1965), associated with protein for nuclease protection and/or transport (Monahan and Hall, 1975), and stored in the

cytoplasm for translation at a later stage of development or differentiation (Smith, 1975; Infante and Nemer, 1967; Wilt, 1965). During specific periods of development some genes may be selectively amplified (Brown and Dawid, 1968). Regulatory factors may be present in some cells which affect the rate and types of mRNA which are translated (Hunt et al., 1972; Lodish, 1971; Heywood, 1970). Enzyme activities may fluctuate due to post-synthetic regulatory mechanisms (Kielly, 1963; Duck-Chong and Pollak, 1973). The cellular environment may produce membrane alterations which affect the cells ability to enter a proliferative or differentiative state (Rutter et al., 1973).

Thus it would appear that the study of genetic regulation in a metazoan would be an overwhelming task. Operationally, however, it should be feasible to study gene regulation in a eukaryotic system in which the cells terminally differentiate into specific entities and produce a specialized predominant protein which can be detected. In the systems in which terminal differentiation has been studied, the evidence suggests that the initial regulatory event occurs at the transcriptional level. Upon entering the terminal stages of differentiation cells initiate the synthesis of highly specific proteins. In the differentiation of the lens cell (Papaconstinou, 1967), avian and mammalian blood cell (Wilt, 1965; Chan et al., 1974; Fantoni et al., 1975), chick oviduct tubular gland cell (Means et al., 1972; O'Malley and Means, 1974), and mouse mammary gland cell (Turkington, 1972) the synthesis of cell specific proteins is dependent upon de novo RNA

synthesis. In the estrogen-stimulated chick oviduct system and the avian red blood cell system the newly synthesized RNA has been shown to include the messenger RNA for the cell specific protein (Chan et al., 1974; Rosen et al., 1975).

How then is the eukaryotic cell transcriptionally regulated? Two series of experiments provide some insight into the mechanism of transcriptional regulation. When midblastula Xenopus laevis nuclei are injected into irradiated oocytes, they switch from DNA synthesis to RNA synthesis, and when Xenopus laevis brain nuclei are injected into irradiated eggs, they switch from RNA synthesis to DNA synthesis (Curdon and Woodland, 1968). Metabolic nuclear changes are preceded by nuclear swelling and an uptake of cytoplasmic proteins. Furthermore, injected differentiated nuclei are capable of directing the development of eggs to the tadpole stage (Curdon and Uehlinger, 1966). In fusion experiments between hen erythrocyte nuclei and HeLa cells, the initially dormant erythrocyte nuclei synthesize RNA and DNA (Harris, 1965). These nuclear metabolic changes are preceded by an increase in nuclear volume, dispersion of condensed chromatin, and nuclear uptake of both preformed and newly synthesized cytoplasmic proteins (Ringertz and Bolund, 1974; Appels et al., 1974). These experiments suggest: 1) Differentiation and/or maturation proceed via a process of general gene repression (negative control), which in some cases is reversible. The concept of differentiation as a process of general gene repression is not new. Much experimental

evidence indicates that the cells of the egg and early embryo are totipotent, with later cell populations gradually restricted in their developmental capacities (Ebert, 1965). The fact that this process is reversible, supports the original assumption that differentiation does not proceed via a process of gene deletion. 2) Gene activation is under positive control, since the active partner of the heterokaryon stimulates nucleic acid synthesis in the inactive partner, and the acceptor cytoplasm controls the metabolic pattern in the transplanted nucleus. The observation that chromatin structural changes and the rapid uptake of proteins by the nucleus were a prerequisite for nuclear metabolic changes, places chromatin changes as a cause rather than a result of renewed transcription and/or replication. Other cases of chromatin structure being related to genetic expression are the heterochromatization of the x-chromosome with concomitant loss of function of the genes carried by the chromosome and the "puffing" of a region of a polytene chromosome followed by transcription of that region (Berendes, 1968). Hence, this thesis is based on the hypothesis that the structure and/or composition of chromatin influences the specific pattern of gene transcription during cell differentiation and maturation.

Chromatin consists of DNA, RNA, and both basic proteins (histones) and acidic proteins. Cells of higher organisms contain large amounts of DNA (Thomas, 1971; Comings, 1972; Flamm, 1972; Rees and Jones, 1972). It was originally assumed that the quantity of DNA would increase

proportionally with the increased complexity of a cell. Recently, however, researchers have begun to question the necessity for such large amounts of DNA in a metazoan cell. A human cell contains approximately 800 times more DNA than a bacterial cell (Rees and Jones, 1972), in fact enough DNA to code for two million different proteins, each with a molecular weight of 50,000. A single band in a Drosophila salivary gland polytene chromosome, which can be genetically linked to a single structural gene, contains enough DNA to code for a protein which is approximately 50 times the molecular weight of the protein actually coded for by that band (Comings, 1972). Also some organisms, seemingly less complex than the human species, contain larger amounts of DNA (Rees and Jones, 1972). Furthermore, if all the DNA available did code for essential genes, the mutational load would be too great to allow survival of the species (Comings, 1972). In current theories then only a minor portion of this DNA is thought to code for proteins. Some of the remaining DNA is "highly" repetitive (Britten and Kohn, 1968; Thomas et al., 1970). "Highly" repetitive DNA has been localized in constitutive heterochromatin (Pardue and Call, 1970; Bastock and Prescott, 1972; Comings and Mattoccia, 1972) and is interspersed with nonrepetitive sequences throughout the genome (Davidson et al., 1973). The "highly" repetitive DNA clustered in heterochromatic regions may perform a structural role (Lee and Yunis, 1971) and interspersed repetitive sequences may serve a control function governing the transcription of contiguous nonrepetitive

structural genes (Britten and Davidson, 1969). Eukaryotic DNA also contains "moderately" repetitive sequences, some of which consist of repeated structural genes for ribosomal RNA, transfer RNA, and histones (Kedes and Birnstiel, 1971). Some of the excess DNA may be transcribed in the form of high molecular weight heterogeneous nuclear RNA (HnRNA), only a small portion of which is used for the translation of proteins (McCarthy and Janowski, 1974).

The acidic or nonhistone chromosomal proteins consist of those proteins in association with DNA in purified chromatin from which the basic proteins (histones) have been extracted (Comings, 1972; Prescott, 1971; Elgin and Weintraub, 1975; Huberman, 1973; Spelsberg et al., 1972). These proteins may be extracted from chromatin in salt at acid pH (Spelsberg and Hnilica, 1971), by sedimentation of chromatin through CsCl (Wang, 1967), in urea and salt (Sanders, 1974), in SDS (Marushige et al., 1968), and in buffered phenol (Shelton and Neelin, 1971). The extracted proteins are usually separated by polyacrylamide gel electrophoresis and/or column chromatography into twenty or more components ranging in molecular weight from 10,000 to several hundred thousand (Elgin and Bonner, 1972). The number of components identified not only depends upon the resolving power of the analytical technique but also upon the method used for the preparation of chromatin, which may also contain proteins from the cytoplasm, nuclear membrane, and nuclear sap (Comings and Tack, 1973). The nonhistone protein pattern in SDS-polyacrylamide gels appears to be similar in both different tissues and organisms, and these

proteins probably represent enzymes of chromosomal metabolism and structural proteins (Vidali et al., 1972; Cox, 1973). Nonhistone chromosomal proteins do, however, show limited tissue and species specificity (Wilson and Spelsberg, 1973; Yeoman et al., 1975). The pattern of nonhistone protein bands in SDS acrylamide gels has been reported to change both quantitatively and qualitatively with development, cell differentiation, and cell maturation (Shelton and Neelin, 1971; Vidali et al., 1973; Sanders, 1974; Elgin and Hood, 1973; Theriault and Landesman, 1974).

The fact that nonhistone proteins do exhibit quantitative and qualitative changes in response to changes in the genetic state of a cell has led to the suggestion that some of these proteins are gene regulators.

Nonhistone proteins have been reported to act as positive regulators of gene expression. An increased quantity of nonhistone proteins associated with chromatin results in a higher chromatin template capacity for RNA synthesis in vitro (Seligy and Miyagi, 1969; Elgin and Hood, 1973).

Euchromatin, which is considered to be active in transcription, contains a higher quantity of nonhistone proteins than heterochromatin, which is considered to be inactive in transcription (Warnecke et al., 1973). Addition of nonhistone proteins to a chromatin dependent RNA synthesizing system in vitro, results in a 2 to 3 fold increase in transcription (Wang, 1970). Nonhistone proteins accumulate in the puffs of polytene chromosomes which are active in RNA synthesis (Spelsberg et al., 1972). Chromatin reconstituted from constituents of normal and 6-hr-old

regenerating rat liver showed that nonhistone proteins, but not DNA and histones, are responsible for the derepressed state of regenerating liver (Kastraba and Wang, 1973). It has been suggested that nonhistone proteins act as positive regulators by competing with DNA for histones, and hence relieving histone repression (Wang, 1970). One would suspect, however, that if increases in nonhistone chromosomal proteins do result in derepression by altering histone-DNA interactions, then such gross changes would lead to general rather than specific derepression. Furthermore nonhistone proteins appear to be unable to remove histones from their complexes with DNA (Spelsberg and Hnilica, 1969) and nonhistone proteins in association with DNA (dehistonized chromatin) restrict the template capacity of that DNA in vitro (Seligy and Neelin, 1970; Spelsberg et al., 1971a; Seligy and Miyagi, 1972, 1974).

The interactions between histones, nonhistones, and DNA may determine the availability of chromatin from a given cell type for transcription and specific nonhistone proteins may then act as repressors or activators of gene transcription. Researchers have thus looked for specific products of transcription in RNA synthesizing systems in vitro containing chromatin reconstituted with nonhistones from different tissues. Gilmour and Paul (1970) and Spelsberg and Hnilica (1969) reconstituted chromatin by interchanging DNA, histones, and nonhistones from different organs of the rabbit and rat, and showed by DNA-RNA competitive hybridization that more effective competition

was observed when the competing RNA transcript and the nonhistone proteins used for reconstitution came from the same tissue. RNA-DNA hybridization, however, may not be an accurate index of the transcriptional fidelity of isolated chromatin (McCarthy and Church, 1968, 1970). More recently Chiu et al., (1975a) have used hybridization of transcribed RNA to a reverse transcript of globin mRNA to detect the fidelity of transcription. In their system a nonhistone protein fraction from reticulocyte chromatin but not from brain chromatin, promoted globin mRNA transcription in reconstituted chromatin. A similar nonhistone protein fraction has been isolated from rat liver chromatin (Chiu et al., 1975b). These two nonhistone protein fractions contain many of the characteristics of gene regulatory proteins: they are present in small quantities (2-4% of the total nonhistone proteins), are tissue specific, show high affinity for DNA, and transfer tissue specificity to other chromatin by mixed reconstitution.

Another approach to the study of the role of nonhistone proteins has been the action of hormones on the metabolism of chromosomal proteins and the binding of hormones to nonhistone proteins in target cells. This study has been conducted since hormones are thought to influence patterns of RNA and protein synthesis, and ultimately cell differentiation, in target cells through a primary action on the control of gene activity. Hormonal control of gene expression in the chick oviduct has been studied extensively by O'Malley and co-workers (Spelsberg et al., 1971b;

Steggles et al., 1971; O'Malley et al., 1972). In the chicken oviduct, when estrogen primed cells are administered progesterone, they begin synthesizing a specific product, avidin. Competitive hybridization between RNA's synthesized before and after progesterone administration have revealed an induction of a specific species of RNA. After ^3H -progesterone was incubated with oviduct cytosol, density gradient sedimentation analysis revealed that the labeled progesterone was bound to a cytoplasmic protein receptor. If the labeled progesterone-protein complex was then incubated with isolated chromatin of different tissues, significant binding occurred only to oviduct chromatin. Chromatin reconstitution experiments with histones and nonhistones of different chromatin demonstrated that the nonhistone protein fraction of oviduct chromatin was responsible for binding the cytosol receptor complex. Specific nonhistone protein synthesis has been reported in rat liver in response to cortisol and in the rat uterus in response to estradiol (Shelton and Allfrey, 1970; Teng and Hamilton, 1970). The specific rat liver nonhistone protein which binds glucocorticoids, appears to be similar to the cytoplasmic hormone receptor (Defer et al., 1974). These studies suggest that the nonhistone fraction of chromatin will interact with specific signals from the cytoplasm (i.e., hormone or hormone-receptor complex), and that this interaction may derepress specific genes. However, a suitable inductive system in vitro must be developed before this question can be fully answered.

The basic proteins (histones) found in chromatin can

be characterized as acid-soluble, low molecular weight proteins (11,000-21,000 d) found associated with DNA in most if not all eukaryotes (Comings, 1972; Elgin and Weintraub, 1975; Huberman, 1973; Georgiev, 1969; Phillips, 1971; Wilhelm et al., 1971; Hnilica, 1972). Histones are usually extracted from DNA in acid (HCl or H_2SO_4 at pH 1-2) or salt (1-2 M NaCl). Since there are other acid-soluble proteins present in chromatin and nuclei, these preparations are usually extensively washed in isotonic saline before histone extraction. The histones have been separated by polyacrylamide gel electrophoresis and cation-exchange and exclusion column chromatography into five major fractions: H1 (KAP; f1; Ia,b,c), H2a (LAK, f2a2, IIB1), H2b (KAS, f2b, IIB2), H3 (ARE, f3, III), and H4 (GRK, f2a1, IV) (Bradbury, 1975). All histone fractions contain a higher proportion of lysine and arginine compared to glutamic and aspartic acid, and hence carry a net positive charge. They all lack tryptophan.

The "very" lysine rich histone fraction, H1 (about 28% mole percentage lysine in calf thymus) is also rich in alanine and proline (Hnilica, 1972). Although H1 is the most basic of the histone fractions, it is the fraction most easily removed from chromatin. H1 can be selectively dissociated from chromatin at pH 1.76 to 2.1 (Seligy and Neelin, 1970), in 5% perchloric acid (Teng et al., 1974), and in 5% trichloroacetic acid (Kinkade and Cole, 1966). This histone fraction has the highest molecular weight, about 20,000 d (Rall and Cole, 1971). Histone fraction H1 contains two clusters of basic amino acids: amino acid

residues 15-38 and 107-212 (Rall and Cole, 1971; Jones et al., 1974). The COOH-portion consists of alanine, lysine, and proline arranged in groups spaced mostly by glycine. The region between amino acid residues 41 and 107 is mostly hydrophobic. It is this region that undergoes salt induced conformational changes, mostly in the form of loose folds with little β -or α -configuration (Bradbury et al., 1975a). The NH₂-terminal serine of the molecule is acetylated (Phillips, 1971). It has been suggested that histone fraction H1 resembles two joined histone molecules; the NH₂-terminal half resembling H2b, H3, and H4 and the COOH-terminal half resembling protamines, small basic proteins which become associated with chromatin during fish spermatogenesis (Unilica, 1972).

Histone fraction H1 is heterogeneous, the number and types of subfractions varying in both different tissues and species. There are four subfractions in calf-thymus chromatin (Kinkade and Cole, 1966), five subfractions in rat thymus chromatin (Zick et al., 1975), three subfractions in chicken erythrocyte chromatin (Vidali and Neelin, 1968b), four or five subfractions in rabbit thymus chromatin (Rall and Cole, 1971), four subfractions in rabbit mammary gland chromatin and three subfractions in rabbit liver chromatin (Bustin and Cole, 1969). Among the various subfractions of rabbit and calf thymus H1 the basic COOH-terminal half of the molecule and the first 13 NH₂-terminal residues are relatively constant in amino acid sequence (Bustin and Cole,

1969; Rall and Cole, 1971; Jones et al., 1974). The sequence differences between these subfractions are accumulated between residues 14 and 40. This would suggest that certain regions of H1, particularly the basic COOH-terminal half, may be more conserved than other aspects of the molecule. In Drosophila H1, however, the amino acid composition of the major CNBr peptide indicates that the COOH-terminal half of Drosophila H1 contains less than half as many proline residues as calf thymus H1 (Alfageme et al., 1974). Furthermore, Drosophila H1 contains three amino acids not present in calf, rabbit, rat, and chicken H1 histidine, methionine, and cysteine. The tissue and species specific heterogeneity of H1 has led to the proposal that this histone may act in gene regulation. An H1 subfraction with the lowest relative mobility in polyacrylamide gel appears to be present in tissues with a high rate of cell division and an H1 subfraction with the highest relative mobility appears to be present in tissues with no cell division (Panyim and Chalkley, 1969). During sea urchin and Xenopus laevis development H1 does not accumulate until late cleavage, and the first subfraction which is synthesized (lowest mobility on polyacrylamide gels) is different from the H1 subfraction which is synthesized at gastrulation (Crane and Villee, 1971; Cognetti et al., 1974; Adamson and Woodland, 1974; Ruderman and Gross, 1974). During rat spermatogenesis this pattern may be reversed, such that the H1 subfraction with the lowest relative mobility increases after a time when division ceases (spermatid stage) (Grimes et al., 1975a).

Some of the difference in the mobility of H1 subfractions in polyacrylamide gels may be due to histone post-synthetic modification rather than amino acid substitution (Balhorn et al., 1972; Lake et al., 1972). The incorporation of a phosphate group into H1 generates a molecule with reduced mobility. There appears to be a direct relationship between the rate of H1 phosphorylation and the rate of replication in cultured hepatoma cells (Oliver et al., 1972; Balhorn et al., 1972), normal and regenerating rat liver (Balhorn et al., 1971), and chinese hamster cells (line CHO) (Shepherd et al., 1971a). In chinese hamster cells H1 phosphorylation begins two hours before the initiation of replication and increases in rate throughout G_2 and mitosis, as measured by a shift in H1 to a slower electrophoretic mobility and the incorporation of ^{32}P (Curley et al., 1974; Lake and Salzman, 1972). The net phosphate content of H1 and the activity of a cAMP-independent, H1-specific phosphokinase appears to increase as the CHO cells traverse G_2 to mitosis (Curley et al., 1974; Lake et al., 1972; Lake and Salzman, 1972). In the slime mold Physarum polycephalum a net increase in the phosphate content of H1 in G_2 is preceded by an increase in the activity of an H1 specific phosphokinase (Bradbury, 1975; Bradbury et al., 1974). It has been suggested that the phosphorylation of H1 in cells traversing G_2 to mitosis may be an initial step in chromosome condensation. H1 may also be phosphorylated in response to hormone mediated cellular changes resulting in derepression of the genome as measured by the template activity of chromatin in vitro.

(Mallette et al., 1973; Watson and Langan, 1973).

The composition of the histone fractions, other than H1, from various tissues and species is remarkably uniform and the individual fractions are highly conserved (Hnilica et al., 1969; Hnilica, 1972; Fambrough et al., 1968; Phillips, 1971). The arginine-rich histone fractions H3 (MW 15, 324) and H4 (MW 11, 282) are the most highly conserved histones (DeLange et al., 1969, 1973; Brandt and Von Holt, 1972; Marzluff et al., 1972).

Calf thymus H4 differs from pea seedling H4 by only two amino acid residues (DeLange et al., 1969). Calf thymus H3 and chicken erythrocyte H3 differ by only a few amino acid changes and two cysteine residues in calf thymus H3 as compared to only one in chicken erythrocyte H3 (DeLange et al., 1973; Marzluff et al., 1972). H3 and H4 are structurally similar in that both have a strongly basic NH₂-terminal region, weakly basic COOH-terminal region, and a hydrophobic internal region. Both arginine rich histones will aggregate (self associate) to form dimers and tetramers and the degree of association increases with increasing ionic strength (Diggle et al., 1975). These histones can be methylated and acetylated. Histone fraction H3 in rat tissues, calf thymus, and rat testis contains E-N-mono, di, and tri-methyllysine (residues 9 and 27) and E-N-acetylysine (residues 14 and 23)

(Duerre and Chakrabarty, 1975; Honda et al. 1975b; Grimes et al. 1975b; Hnilica, 1972). Histone H4 contains predominantly E-N-dimethyllysine at residue 20 and E-N-acetyllysine at residue 16. In CHO cells H3 and H4 acetylation has been reported to occur early in S phase and methylation to occur in G₂ (Shepherd et al., 1971b). In Ehrlich ascites tumor cells H3 is methylated in S phase just after histone synthesis and H4 is methylated in G₂ (Thomas et al., 1975). It was suggested that since methyl group distribution does not show tissue microheterogeneity but does occur at specific times in the cell cycle, it may play a functional role in the final arrangement of newly replicated DNA in the nucleoprotein-complex (Thomas et al., 1975; Duerre and Chakrabarty, 1975). During trout spermatogenesis after H4 is synthesized, it is acetylated and deacetylated supposedly for proper binding to chromatin, and then phosphorylated and methylated (Louie and Dixon, 1973; Honda et al., 1975a). The phosphorylation of H4 may be important in regulating the gross physical structure of chromatin and hence allowing newly synthesized protamine molecules to bind to DNA. It has also been suggested that histone acetylation may be necessary for gene derepression (Pogo et al., 1966).

Histone — H2a (MW 14,500) and H2b (MW 13,800) show an intermediate rate of evolutionary divergence in amino acid substitution (Sautiere et al., 1974; Bailly and Dixon, 1973; Candido and Dixon, 1972; Iwai et al., 1972). As in the others, these two histones have most of their basic amino acid residues clustered in the NH₂- and COOH-terminal regions, with a mostly neutral and hydrophobic

central region. The conformation of H2a is more sensitive to salt and pH changes than H2b and H4 (D'Anna and Isenberg, 1974). In H2a an ionic strength dependent equilibrium exists between largely unstructured coil molecules, and fully structured (globular-like) and aggregated molecules (Garel et al., 1975a; Bradbury, 1975). Due to the salt-dependent conformational changes in the central region and lack of organization at the NH_2 - and COOH -terminal region in H2a, it has been suggested that H2a and probably H2b, H3, and H4 bind to DNA in the unstructured and charged NH_2 - and COOH -terminal regions (Garel et al., 1975a,b). Histone fractions H2a and H2b can be acetylated and phosphorylated, and the implications of these post-synthetic modifications are similar to those discussed for histone fractions H3 and H4 (Shepherd et al., 1971a,b; Sanders et al., 1973; Louie and Dixon, 1973; Seligy and Neelin, 1973).

The close association between histones and DNA in chromatin suggests that histone synthesis should parallel DNA replication. The bulk of the histone ~~synthesis~~ appears to be synchronized with DNA synthesis in hepatoma tissue culture cells (Balhorn et al., 1972), Chinese hamster ovary cells (Curley et al., 1972), and HeLa cells (Sadgopal and Bonner, 1969). Hybridization of DNA transcribed from histone mRNA to polyribosomal RNA from different phases of the HeLa cell cycle, indicates that histone mRNA sequences are present on polyribosomes only during the period of DNA replication (Stein et al., 1975a). A translational regulation for histone synthesis in its coupling to DNA

synthesis was suggested, since S phase HeLa cell chromatin, but not G₁ phase chromatin transcribed RNA in vitro which hybridized to -cDNA complementary to histone mRNA (Stein et al., 1975b). The availability of histone genes for transcription appears to depend on a group of nonhistone proteins associated with chromatin only during S phase (Stein et al., 1975c). Other researchers, however, have reported histone synthesis at other stages of the cell cycle (Hnilica, 1972). In particular H1 has been reported to be synthesized in rat liver cells, mouse macrophages, and kidney epithelial cells which show only low levels of DNA synthesis and of other histone fraction synthesis (Ohba et al., 1975; Appels and Ringertz, 1974). When DNA synthesis in rat spleen cells was inhibited by 95% with hydroxyurea, H2 but not H1 synthesis was completely inhibited (Ohba et al., 1975). Addition of excess thymidine to HeLa cells to block DNA synthesis had no effect on the synthesis of H3 and H4, and only slightly affected the synthesis of H1, and inhibited the synthesis of H2 (Sadgopal and Bonner, 1969). In unfertilized Xenopus laevis eggs histones H2, H3, and H4 but not H1 are synthesized in the absence of DNA synthesis (Adamson and Woodland, 1974). During cleavage there is a 20,000 fold increase in DNA synthesis, but only a threefold increase in histone synthesis, including H1. The high rate of histone synthesis in the egg may be necessary to stockpile histone for the extremely rapid period of nuclear proliferation during cleavage. Histone synthesis has also been reported to be uncoupled from DNA synthesis during sea

urchin (Crane and Villée, 1971; Cognetti et al., 1974; Shutt and Kedes, 1974; Poccia and Hinegardner, 1975), and Rana pipiens (Malacinski, 1974) early development.

In 1950 Stedman and Stedman proposed the hypothesis that histones function as genetic repressors. Experimental evidence for the hypothesis was first presented by Huang and Bonner (1962), who demonstrated that removal of histones from pea chromatin led to a large activation of RNA synthesis. Allfrey et al., (1963) reported a similar increase in RNA synthesis after nuclei were exposed to trypsin. They assumed that the histones were the major group of proteins in the nucleus digested by trypsin. Additional experiments involving the removal of histones from chromatin have led to similar conclusions (Spelsberg and Nillica, 1971; Kurashima et al., 1970). Addition of natural and synthetic polyanions to isolated nuclei resulted in chromatin dispersion, nuclear swelling, and an increase in the ability of chromatin to serve as a template for RNA and DNA synthesis (Miller et al., 1971; Arnold et al., 1972; Barry and Merriam, 1972; Ansevin et al., 1975). Since a variety of polyanions are able to form strong complexes with histones, it has been suggested that polyanions release chromatin from template inhibition via interaction with histones (Miller et al., 1972).

Do these experiments, however, have any biological significance; i.e., do the regions which transcribe RNA

have a lower quantity of histones associated with them?

When calf thymus chromatin is exposed to limited DNase II digestion, 18% of the DNA is rendered acid-soluble while the remainder is protected by bound proteins (Sugano and

Okado, 1975). Digestion of chromatin with DNase however, depends partially on the concentration of enzyme and time

of exposure to the enzyme, because 54% of the DNA is digested at higher concentrations of DNase and longer time periods (Mirsky and Silverman, 1972). This suggests that

a large percentage of DNA in chromatin is covered with proteins, but that the binding in many places is loose.

Renaturation kinetics and CD

measurements on chromatin also indicate that a

substantial portion of the DNA in chromatin (80% of the base pairs in calf thymus chromatin) is covered with

protein (Suzuki and Iwanami, 1975; Li et al., 1975). The

CD spectra of calf thymus "native" and dehistonized chromatin indicates that most of the DNA is covered with

histone. These results are consistent with the finding

that the template capacity of calf thymus chromatin is 22% of that of "naked" DNA (Paul and More, 1972). Transcriptionally

active chromatin (euchromatin) has been separated from

inactive chromatin (heterochromatin) by differential

solubilization of sheared chromatin and sedimentation in

sucrose gradients (DeBillis et al., 1969; Duerksen and

McCarthy, 1971; Anderson et al., 1975). The histone content

and distribution of individual fractions were reported to

be the same in both types of chromatin, but as already

discussed the nonhistone content was higher in

transcriptionally active chromatin. Bonner et al., (1973) have reported, however, that the template active segments of ascites tumor cell chromatin fractionated by limited DNase digestion and salt precipitation of about 80% of the chromatin have a histone to DNA content of 0.36 as compared to 1.43 for inactive chromatin. Similar results have been obtained for chromatin fractionated on ECTHAM columns (Simpson, 1974). These active chromatin fractions, however, also contain a higher content of nonhistone proteins. Therefore chromatin derepression may involve reduced histone-chromatin interaction through nonhistone interaction and/or post-synthetic modification.

The marked uniformity of histones in different tissues and organisms makes these proteins unacceptable candidates for a role as regulators of gene specific repression. As was already discussed, H1 does show some tissue and species specificity, but at present there is a lack of evidence that would link the presence of a specific H1 subfraction with a specific transcriptional pattern of a cell. Hence histones are presently considered to be general repressors to DNA transcription.

However, a tissue-specific histone, H5, (V, f2c, KSA), has been identified in avian erythrocytes (Mnilica, 1964; Neelin et al., 1964). Histone fraction H5 has been isolated by extraction in HCl at pH 1.8 followed by cation-ion exchange chromatography (Vidali and Neelin, 1968a) or by extraction in 0.2 M HCl (pH 1.0) followed by cation-ion exchange and exclusion chromatography (Adams and Neelin, 1969). Vidali and Neelin (1968b) demonstrated that H5 was

not a degradation product of other histones or a contamination of nuclei with ribosomal proteins (Neelin and Vidal, 1968). H5 was first identified as a histone fraction present in chicken erythrocytes, but absent in other chicken tissues by starch gel electrophoresis (Neelin and Butler, 1961). It has more recently been identified as a histone fraction which migrates with an intermediate mobility between H1 and H3 monomer in urea-polyacrylamide gels (Panyim and Chalkley, 1969).

H5 has been identified in the nucleated erythrocytes of chickens, geese, ducks, quail, pigeons, and turkeys (Purkayastha and Neelin, 1966; Panyim et al., 1971). This histone has a high lysine, arginine, serine, and alanine content (Vidal and Neelin, 1968a). The partial amino acid sequence of H5 (MW 20,000) shows some similarity to H1: slightly basic NH₂-terminal region, highly basic COOH-terminal region and hydrophobic central region (Greenaway, 1971; Greenaway and Murray, 1971; Garel et al., 1975c; Sautiere et al., 1975). H5 is also similar to H1 in showing sequence heterogeneity. Chicken erythrocyte histone fraction H5 contains a substitution at amino acid residue 15 which may be either glutamine or arginine (Greenaway and Murray, 1971). A comparison of the nineteen N-terminal amino acid residues of H5 from chicken, quail, duck, goose, and pigeon demonstrated some sequence heterogeneity; for example only ten amino acid residues were identical between chicken and goose H5 (Seligy et al., 1976). However, the next ninety residues were much more homologous (Seligy, personal communication).

Histone fractions H1 and H5 may interact with chromatin in a similar manner. Treatment of chromatin with urea appears to disrupt the compactness of chromatin, suggesting that protein-protein interactions are important to chromatin structure (D'Anna and Isenberg, 1974). H1 and H5 may not, however, be involved in these protein interactions. All histone fractions except H1 and H5 have been shown to self-associate; the degree of self-association increasing in solutions of higher ionic strength (Diggle et al., 1975). The conformation of histone fractions H2a, H2b, H3, and H4 are more sensitive to salt and pH changes than H1 and H5 (D'Anna and Isenberg, 1974; Lewis and Bradbury, 1974; Bradbury et al., 1973; Bradbury, 1975; Bradbury et al., 1975a,b; Burton et al., 1975; Garel et al., 1975a). The apolar regions of H2a, H2b, H3, and H4, which contain the most potential for helix formation, may take-up specific conformations which are the sites for histone-histone interaction (Bradbury, 1975). The basic segments of these histones would then interact with DNA. Other studies which are consistent with this hypothesis involve the treatment of chicken erythrocyte chromatin with formaldehyde (van Lente et al., 1975) and calf thymus chromatin with glutaraldehyde (Chalkley and Hunter, 1975) and dimethyl suberimidate (Thomas and Kornberg, 1975), resulting in the formation of crosslinked products containing H2a and H2b; H2a and H4; H2b and H4; and H3, H2a, and H2b. Furthermore H3 and H4 have been shown to dissociate and reassociate reversibly, with DNA in the form of a stoichiometric equimolar complex (Burton et al., 1975). Also, histones bind to DNA in a

cooperative manner; i.e., regions of bound histone tend to accumulate more histone possibly through histone-histone interactions (Rubin and Moudrianakis, 1972). These studies suggest that histone interaction with DNA involves a self-assembled, highly-ordered, regular arrangement of a H2a, H2b, H3, and H4 complex along DNA.

This complex may be arranged in globular particles each containing about 200 base pairs of DNA. Evidence for a globular subunit structure in chromatin came from the visualization of a linear array of spherical particles ($60 \text{ \AA}$ in diameter), nu-bodies or nucleosomes connected by thin filaments (width $15 \text{ \AA}$) in electron micrographs of formaldehyde fixed rat thymus, rat liver, chicken liver, chicken erythrocyte, calf thymus, and calf kidney chromatin (Olins and Olins, 1974; Olins et al., 1975; Oudet et al., 1975).

The structure of these globular subunits was elucidated by physicochemical studies of the products of "limited" (controlled) nuclease digestion of chromatin. Chromatin "sheared" by nucleases or high frequency sound could be seen as individual particles resembling beads in electron micrographs (Senoir et al., 1975; Oudet et al., 1975). Mild nuclease digestion of chromatin resulted in particles that sedimented at 10.5-12.0 S and contained between 185 to 200 base pairs of DNA (Noll, 1974; Sahasrabudhe and van Holde, 1974; Bakayev et al., 1975; Oosterhof et al., 1975; Oudet et al., 1975). About 50% of the DNA in isolated chromatin was resistant to digestion by micrococcal nuclease, (staphylococcal) nuclease, DNase I,

and DNase II. Each particle contained histone fractions H2a, H2b, H3, and H4, with H1 and/or H5 either absent or present in reduced amounts compared to undigested chromatin. No long special sequence of nucleotides appeared to be required for nu-body formation, since all types of DNA (highly repetitive, moderately repetitive, and unique) were present in the particles (Lacy and Axel, 1975). Sollner-Web and Felsenfield (1975) reported that a second digestion of duck reticulocyte chromatin produced particles containing 140 base pairs of DNA and that further digestion resulted in a series of ten discrete DNA lengths between 140 and 52 base pairs. The high susceptibility of DNA to extensive nuclease digestion was given as a strong argument for placing DNA on the outside of the chromatin subunit.

Extensive nuclease digestion of chicken erythrocyte chromatin followed by trypsin digestion produced eight subparticles visualized as discrete bands when electrophoresed on 6% polyacrylamide gels (Weintraub, 1975). Five of the bands contained protein-associated DNA and three of the bands contained protein-free DNA. When chicken erythrocyte chromatin was digested with trypsin alone, H1, H5, and 20 to 30 amino acid residues from the N-termini of histone fractions H2a, H2b, H3, and H4 were removed (Weintraub and van Lente, 1974). The authors suggested that the protein-free subparticles released by trypsin treatment of nuclease particles were originally bound to the NH₂-termini of these histones. From this data Weintraub (1975) has proposed a model in which a

nucleosome contains DNA in a series of loops held in place by the crosslinking action of H2a, H2b, H3 and H4, N-termini along one side and C-termini along the other.

Further insight into the structure of chromatin has come from a re-evaluation of x-ray diffraction data using data obtained from neutron scattering. X-ray diffraction analysis of 100 Å chromatin fibers revealed a major pitch at 120 Å and several low angle diffraction rings at 110, 55, 37, 27, and 22 Å (Pardon and Wilkins, 1972). The neutron scattering pattern of chromatin can be used to discern reflections specific for protein or DNA by altering the composition of the solvent (deuterium water and protium water), such that either DNA or protein gives the same neutron scatter as the solvent. The data for chromatin indicated that the 110 and 37 Å reflections came from histone and the 55 and 27 Å reflections came from DNA (Bradbury, 1975). From these data Bradbury proposed that the DNA in chromatin folded around a histone core containing fractions H2a, H2b, H3, and H4, but not H1, since the presence or absence of H1 did not affect the x-ray diffraction or neutron scattering pattern.

Since H1 and H5 were solubilized or digested during the nuclease or trypsin preparation of nucleosomes from chromatin, it is conceivable that they are located on the outside of the structure. H1 and H5 have been shown to associate with DNA (Hayashi, 1975; Garel *et al.*, 1975b), and this association appeared to be primarily electrostatic. If the lysine-rich histones are associated with nuclease-sensitive DNA located on the outside of the nucleosome,

then these histones would be lost during nuclease preparation of nucleosomes. Furthermore, in rat liver chromatin antigenic determinants of H1 were more available to interact with homologous antibody than histone fractions H2a, H2b, H3, and H4 (Goldblatt and Bustin, 1975). If the binding of anti-histone sera to histones in chromatin depends on the availability of antigenic sites within histones in chromatin, H1 may be located on the periphery of the chromosomal complex. In this position the lysine-rich histone fractions may cross-link chromatin particles to generate the higher-order structure of the eukaryotic chromosome, such as the 200 Å fibrils into which the DNA helix must be compacted 56-fold (DuPraw, 1970). The integrity of the 200 Å chromatin fibril may depend on the presence of lysine-rich histones, since the acid extraction of H1 and H5 from chicken erythrocyte nuclei resulted in the dispersion of the 200 Å fibril to 20-100 Å chromatin fibrils (Brasch et al., 1972). Also in electron micrographs of chicken liver nuclei lysed directly onto grids, the nucleosomes appeared to be more closely packed together in nuclear preparations containing H1 than in those in which H1 had been extracted (Oudet et al., 1975). Further support for the assumption that lysine-rich histones regulate the packing of chromatin has come from NMR, sedimentation, and viscosity measurements of chromatin (Bradbury et al., 1975b; Lewis et al., 1976). The ability of thymus chromatin to expand and contract in solutions of varying ionic strength was shown to be partially dependent on the presence of H1.

Hence about 50% of the DNA in eukaryotic chromatin may be associated with histone fractions H2a, H2b, H3, and H4 in structures resembling spheres each containing about 200 base pairs of DNA. These spherical particles connected by thin filaments of DNA may then be condensed into 200 Å chromatin fibrils via electrostatic interactions with the lysine-rich histones.

Despite the fact that H1 and H5 appear to interact with chromatin in a similar manner, H5 has been assigned a tissue-specific role due to its unique presence in avian nucleated erythrocytes (note: H5 has been reported to be also present in the nucleated erythrocytes of other, non-avian, vertebrates; see Miki and Neelin, 1976; Hnilica, 1972). In mammalian RBC's a complex process of denucleation takes place during maturation (Simpson and Kling, 1967). Avian RBC's, however, retain a nucleus even at the mature erythrocyte stage. Maturation of avian RBC's involves distinct morphological and metabolic changes: cessation of DNA synthesis (Attardi et al., 1970; Seligy et al., 1973), reduction in RNA and protein synthesis (Cameron and Prescott, 1963; Scherrer et al., 1966; Kabat and Attardi, 1967; Attardi et al., 1970; Seligy et al., 1973) and increased chromatin condensation (Brasch et al., 1971; Kernell et al., 1971). It has been suggested that the avian erythrocyte specific histone, H5, may be involved in genetic repression and chromatin condensation during erythroid maturation (Seligy and Neelin, 1970; Miller et al., 1972).

The depression of RNA synthesis in a eukaryotic cell may be associated with increased chromatin condensation (Comings, 1972). Most of the chromatin in mature erythrocyte nuclei of chicken is condensed (Brasch et al., 1971; Kernell et al., 1971; McCarthy and Jahowski, 1974). Erythrocyte chromatin is more condensed and has a significantly lower template capacity for RNA synthesis than liver chromatin (Seligy and Miyagi, 1969, 1974; McConaughy and McCarthy, 1972; Brasch et al., 1972; McCarthy and Jahowski, 1974. Removal of H5 from erythrocyte chromatin leads to a dispersion of condensed chromatin (Brasch et al., 1972; Miller et al., 1972) and an increase in the template capacity of chromatin for RNA synthesis (Seligy and Neelin, 1970; Seligy and Miyagi, 1974). However, the dispersion of chromatin produced when chicken erythrocyte nuclei are placed in a low ionic strength medium does not result in an increase in template capacity for RNA synthesis (Brasch et al., 1971; Leake et al., 1972). It is possible that factors other than the condensed state of chromatin may regulate the availability of chromatin for transcription. The nonhistone content of erythrocyte chromatin is lower than the nonhistone content of liver chromatin (Seligy and Miyagi, 1972; 1974). The suggested function of nonhistone chromosomal proteins as gene regulators has already been discussed. Furthermore, the chromatin decondensation and derepression produced after the removal of H5 is not necessarily related to the changes in chromatin structure and gene expression which occur during maturation. The

removal of either only H1 or only H2 from calf thymus chromatin results in similar changes in chromatin structure, yet the universality of H2 makes it an unlikely candidate for a cell specific role (Smart and Bonner, 1971).

It is possible that the quantity of a particular histone fraction on chromatin may reflect the degree of chromatin inactivation and/or condensation. Selective increases in H5 as maturation proceeds from basophilic erythroblast to mature erythrocyte have been reported in adult birds (Purkayastha and Neelin, 1966; Adams and Neelin, 1972; Appels et al., 1972; Billett and Hindley, 1972). In bone marrow cells of anemic birds fractionated on BSA or Ficoll gradients, H5 was present in the fraction containing the most immature stage cells (Adams and Neelin, 1972; Appels et al., 1972). Sotirov and Johns (1972) found H5 markedly reduced in erythroblasts from chickens suffering from erythroblastosis. Mazen and Champagne (1972) observed increased levels of H5 in adult hen compared to 5-day-old chicken erythrocytes. Only one report in the literature has suggested that H5 may be absent in the most immature erythroid stages. The lightest fraction of an isopycnic gradient-separation of Triton X-100 treated nuclei from anemic bone marrow cells, contained no detectable H5 (Neelin et al., 1976). The authors discounted non-erythroid cell contamination as the reason for the lack of H5 in this fraction, since the quantity of H1 associated with this fraction was characteristic of erythroid tissue. However, since H5 appears to be present in most, if not all, erythroid maturation stages, a simple relationship between

the presence of H5 and the cessation of macromolecular synthesis is not tenable.

H5 may interact with chromatin differently at immature and mature erythroid stages. Nonhistone chromosomal proteins, which are present in a higher quantity in erythroblast than in mature erythrocyte chromatin (Casaryan and Andreeva, 1972), may shield DNA from inactivation by H5. Alternatively the phosphorylation of H5 may modify its interaction with DNA (Kleinsmith et al., 1966). During avian erythroid maturation the levels of alkali-labile phosphate and the incorporation of radiophosphate into H5 decreases (Adams et al., 1970; Seligy and Neelin, 1973; Sung et al., 1974). The quantity of alkali-labile phosphate associated with H5 can be correlated with the relative ease with which H5 is extracted from chromatin (Brasch et al., 1974). Furthermore, CD studies indicate that phosphorylated H5 has more random structure and interacts with DNA in a different manner than nonphosphorylated H5 (Vandegrift et al., 1974; Williams and Seligy, 1975).

Appels and Wells (1972) have suggested that the turnover of H5 may be important to the potential of chromatin for transcription. It has been reported that H5 is the sole histone synthesized in normal goose blood (Seligy et al., 1973) or in chicken polychromatophilic erythrocytes (Medvedev and Kirpicheva, 1972) and that this synthesis may represent turnover of H5 rather than net accumulation of H5 on chromatin (Appels and Wells, 1972). This is also the erythroid maturation stage at

which RNA synthesis ceases (Cameron and Prescott, 1963; Seligy et al., 1973) and the incorporation of radiophosphate in H5 decreases (Seligy et al., 1973). At the polychromatic erythrocyte stage newly synthesized, non-phosphorylated H5 may replace the phosphorylated H5 associated with chromatin, and this could lead to the transcriptional repression of chromatin.

However, elucidation of the role of H5 has been hampered by incomplete separation of adult maturation stages (Adams and Neelin, 1972; Seligy et al., 1973). Use of the primitive line of embryonic chickens overcomes this problem, because immature RBC's appear in one wave about two days of incubation and mature synchronously (Romanoff, 1960; Wilt, 1967; Campbell et al., 1971; Weintraub et al., 1971; Bruns and Ingram, 1973).

The first erythroid cells of the embryonic chicken are formed in the area opaca surrounding the posterior and postero-lateral parts of the area pellucida (Romanoff, 1960). These erythroid elements are derived from premesodermal cells originally located in the epiblast, which pass through the streak and migrate laterally to form a mesodermal layer in the area opaca (Spratt and Haas, 1965; Wilt, 1967). At about the head process stage condensations of cells can be seen in the splanchnic mesoderm of the area opaca vasculosa. Localized thickenings become more apparent in the area opaca caudal and lateral to the area pellucida, between the second and fourth somite stage (Sabin, 1920). These cell aggregates are in close association with the underlying yolk-filled endoderm. Electron micrographs of sectioned area vasculosa

at the 4 somite stage reveal projections extending from the endodermal cells into the mesodermal cell aggregates, and accumulation of electron dense material between the two germ layers (Mato et al., 1961). By the 5 somite stage the peripheral cells of these aggregates flatten and coalesce into a plexus of cellular cords which become the vascular epithelium of the blood islands and which enclose cells breaking away from the luminal edge to proliferate and give rise to the first blood cells (Romanoff, 1960; Murray, 1932; Sabin, 1920; Sugiyama, 1926; Edmonds, 1966). Shortly after this period (7-9 somite stage) the blood island cells form detectable quantities of Hb (O'Brien, 1961; Wilt, 1962; Granick and Levere, 1965). As the embryo matures, the vascular network of the extra-embryonic membrane increases, with the additional lumina joining to form a network of blood vessels. This is accompanied by the proliferation of erythroid cells. By the 16 to 17 somite stage the extra-embryonic vessels have joined with the intra-embryonic vessels, and the embryonic circulation is established. The yolk sac remains the major erythropoietic site of the embryonic chicken until 18 to 20 days incubation (Romanoff, 1960).

The first cells in the blood islands at the 5-7 somite stage constitute the primitive erythroid line, which is the only erythroid line in the circulation until four to six days of incubation, at which time the definitive erythroid line appears (Edmonds, 1966; Lucas and Jamroz, 1961; Fraser, 1963; Ceresa-Castellani and Leone, 1969; Bruns and Ingram, 1973). Primitive erythroid cells appear to mature

homogeneously, that is most of the erythroid cells which can be isolated from the embryonic circulation at any particular time appear to be at the same maturation stage (Romanoff, 1960; Lucas and Jamroz, 1961; Table 1). The maturation stages of the primitive erythroid line (proerythroblast, basophilic erythroblast, polychromatophilic erythrocyte, orthochromatophilic erythrocyte, mature primitive erythrocyte) have been described on the basis of observations with light microscopy (Lucas and Jamroz, 1961; Fraser, 1963; Bruns and Ingram, 1973) and electron microscopy (Edmonds, 1966; Ceresa-Castellini and Leone, 1969; Small, 1969; Dardick, 1975). Maturation is characterized by decrease in nuclear to cytoplasmic volume, decrease in cell and nuclear size, decrease in cytoplasmic basophilia, a loss of prominent nucleoli, decrease in the size and number of mitochondria, decrease in the number of ribosomes, progressive chromatin clumping, and increase in cytoplasmic eosinophilia. The maturation process is complete by about 6-8 days of incubation.

From their inception at about 35 hr. incubation until day 5, there is a dramatic increase in the number of circulating primitive red blood cells. By measuring the total Hb content per embryo and the average Hb content per primitive erythroid cell, it is estimated that there are approximately 10^8 primitive erythroid cells present per embryo at day 5, an increase by a factor of 30-50 over the number of primitive cells at day 2 (Bruns and Ingram, 1973; Weintraub et al., 1971; Campbell et al., 1971). During this period the mitotic index falls from 2-3% to 0.1% (Romanoff,

Table 1

Percentage of various maturation stages of RBC's found in the Embryonic Circulation^a.

<u>Days</u>	<u>Eb</u> ^b	<u>P</u>	<u>ME</u>	<u>DE</u>
2½	100	-	-	-
3	14	86	-	-
4½	3	93	1	3
5	-	10	80	10
6	-	-	24	76

^aRomanoff (1960).

^bPrimitive line: Eb, erythroblast; P, polychromatophilic / erythrocytes; ME, mature erythrocytes; DE, definitive line at all stages.

1960; Weintraub et al., 1971; Bruns and Ingram, 1973).

This indicates that primitive cells, which are in the process of terminal differentiation and are already synthesizing a cell specific protein (Hb), are capable of cell division and that this division phase is restricted, since mitosis ceases at the mature primitive erythrocyte stage. Weintraub et al. (1971) attempted to delineate the relationship between cell division and maturation in the primitive line by introducing ^3H -thymidine into eggs between 35 hr and 5 days incubation, and at time intervals measuring uptake into blood cells by autoradiography. The cell cycle time was established by measuring the time necessary for all of the blood cells to be labelled with ^3H -thymidine. They assumed that the primitive erythroid line population was an asynchronous population of cells in exponential growth such that one cell in mitosis would give rise to two cells in G_1 . The length of the G_1 phase was estimated using both colcemid and ^3H -thymidine, and the mitotic phase was estimated using ^3H -uridine, assuming that cells in mitosis do not take up uridine. Primitive red blood cells actively incorporated labelled precursors into DNA from 35 hrs. to 5 days incubation. These cells pass through six division cycles with a cell cycle length of 9.6 hr. at inception, to 17.1 hr. at 3 and 4 days, and 29 hr. at 5 days. A bimodal distribution in grain counts, evident when late 4 day and early 5 day cells were pulsed in vitro indicated more than one population of cells. They suggested that the non-homogeneous labelling pattern at this period indicated that the primitive RBC

line is released into the circulation in one wave of entry over a period of approximately 30 hr. If one assumes a population of 8×10^5 precursor cells (erythroid stem cells) out of 2×10^6 embryonic cells at 35 hr. incubation and an entry period of 30 hr, six division cycles could account for the 10^8 primitive red blood cells found in 5 day embryos. This would suggest that all the primitive erythroid cells found at 5 days are derived from the original differentiation of erythroid stem cells into erythroid cells in the blood islands of 5-7 somite embryos. Bruns and Ingram (1973) suggest that this hypothesis is not compatible with the 4 fold increase in primitive cell number between day 3 and 4, when the cell cycle length is 17 hr. and the mitotic index is 2.7%. The hypothesis also predicts the existence of a large (30 hr.) variation in the maturation stage of primitive cells at any particular time. This has not been described in most cytological investigations (Edmonds, 1966; Lucas and Jamroz, 1961; Fraser, 1963; Ceresa-Castellani and Leone, 1969; Bruns and Ingram, 1973; Small, 1969). Dardick (1975) reported, however, that when sectioned blood vessels from a particular embryo (from day 2 to day 6 incubation) were observed in the electron microscope, often two or more erythroid stages were present.

When 6-9 somite, de-embryonated blastoderms were cultured with ^3H -adenosine, the cell cycle length and mitotic index of the cultured primitive cells were similar to the results obtained in ovo, yet only 3% or less of the number of red blood cells formed in ovo are in culture by

7.5 days (Hagopian and Ingram, 1971). The authors suggest that these results indicate that the production of primitive cells in the embryo is not merely due to the proliferation of precursor cells at 35 hr. but also due to massive recruitment from other mesodermal cells. It is also possible that the embryo has a stimulating effect on precursor cells, and these cells have remained in stationary phase in de-embryonated blastoderm cultures. Also the culture conditions may affect the survival and continued division of erythroid precursor cells. Both embryonated and de-embryonated stage blastoderms form less Hb in culture after 48 hrs. than same stage embryos in vivo (Betz et al., unpub). Bruns and Ingram (1973) suggest that in ovo the recruitment of additional mesenchymal elements into the primitive cell pool continues until 4.5 days. This would also lead, however, to a large variation in the maturation stages present at any particular time, unless these additional cells pass through a more rapid maturation process or the cells mature in sequestered compartments and enter the circulation at advanced stages. Cytological observations on primitive cells mechanically dissociated from blastoderms discount the second possibility (Bruns and Ingram, 1973). Dardick (1975) observed a considerable number of cells with morphological features of hemocytoblasts or proerythroblasts in sequestered mesodermal compartments at 3.5 to 5 days incubation. It was suggested that these sequestered populations were precursor cells for the definitive cell line, rather than the primitive cell line.

As already mentioned, the major assumption in this thesis is that chromosomal proteins regulate the pattern of gene expression during the differentiation and/or maturation of a particular cell line. Using the primitive erythroid line of embryonic chickens, I sought to ascertain whether it is possible to correlate a quantitative change in a histone fraction with the metabolic changes which occur during RBC maturation. In this context special emphasis was placed on the role of H5 in erythroid maturation.

During the period of time in which the research for this thesis was being conducted, Moss et al., (1973) and D'Amelio et al., (1974) independently published research which quantitatively examined the histone fractions of the primitive erythroid line. Using polyacrylamide gel electrophoresis Moss et al., (1973) detected only traces of H5 in primitive RBC's of 3 and 4 day chicken embryos, and indicated that the relative content of this histone increased during development. Using starch gel electrophoresis D'Amelio et al., (1974) reported that H4 and H2a were absent in primitive RBC's of 6 day chicken embryos and that the pattern of H1 subfractions changed during development. My observations are not in complete accord with either of these partly contradictory reports. In addition this study extends our knowledge of erythropoiesis in chicken embryos in that:

- 1) Histones are extracted from the blood of embryos at developmental stages corresponding to specific primitive line maturation stages in order to detect changes in histone fractions during the maturation of one cell line

- 2) The metabolic properties of these maturation stages are examined, and an attempt is made to correlate changes in the metabolic properties of maturing RBC's with alterations in histone fractions.
- 3) The possibility of proteolytic degradation affecting histone quantitation is examined.
- 4) Histone identities are corroborated by more than one method.
- 5) Since histone fractions are quantitated from electropherograms of Amido Black stained polyacrylamide gels, the affinity of each histone fraction for the Amido Black dye is examined and histone fraction quantitation is expressed in molar ratios.

Materials

General equipment: Beckman L3-50 ultracentrifuge; Beckman J-21 preparative centrifuge; Sorval GLC-1 centrifuge; Duostat, regulated D.C. power supply (Beckman); Packard Tri-Carb liquid scintillation spectrometer, Model 3003; Beckman Acta III spectrophotometer; Virtis "23" homogenizer; LKB Ultrarac fraction collector; Metrohm Herisacc pH-meter, Model E300B; gyrotory ,G76, water bath shaker (New Brunswick Sci.); Virtis lyophilizer; Sartorius '2600' analytical single pan balance; Cahn 'DTL' Electrobalance; Zeiss 'C40' photomicroscope; dual-chamber, tissue culture incubator (National); horizontal gel slicer (Hoefer); concentration and diafiltration system (Amicon); Dounce-type glass hand homogenizer with loose-fitting pestle 'A' (Kontes Glass Co.); PM-10 Diaflo membrane, exclusion limit 10,000 d (Amicon); 20 ml plastic and glass scintillation vials (Fisher); Pyrex '9880' tubes (Fisher); Pyrex tissue grinder, 13 mm in diameter, 100 mm in length (Fisher).

General chemicals: Tris (hydroxymethyl) aminomethane hydrochloride (Fisher); 30% (w/v) hydrogen peroxide (Baker); Triton X-100 (BDH); 151,000 units per gram laboratory grade sodium heparin (Fisher); indicator grade diphenylamine (Fisher); A.C.S. certified ethylenediamine-tetraacetic acid (Fisher); acetaldehyde (Eastman Kodak); phenol liquified (Fisher); phenol reagent, 2N Folin-Ciocalteu solution (Fisher); reagent grade orcinol (Fisher);

bovine serum albumin, Fraction V (Sigma); reagent grade yeast RNA (Nutritional Biochem); calf-thymus DNA, highly polymerized sodium salt (Sigma).

General materials: GN-6 Metrical filters, 25 mm in diameter, 0.45 μ m in pore-size (Gelman); 47 mm in diameter, 0.45 μ m in pore-size, membrane filters (Sartorius); 7 cm in diameter No. 1 filter paper; Whatman 3 MM filter-disks.

Chemicals used in chromatography: guanidinium chloride (Nutritional Biochem.); Bio-Gel P-2 (Bio-Rad); Amberlite CG-50, 100-200 mesh (Rohm and Haas).

Radiochemicals: 2-C¹⁴ Uridine-5'triphosphate tetrasodium salt, 53.3 mCi/m mole (New England Nuclear); 2-¹⁴C thymidine, 58.9 mCi/m mole (New England Nuclear); 5-H³ uridine, 28.5 Ci/m mole (New England Nuclear); 4,5-³H L-lysine (N), 38.9 Ci/m mole (New England Nuclear); ¹⁴C(U) L-lysine, 30.6 mCi/m mole (New England Nuclear); 2,5-diphenyloxazole (Fisher); Omnifluor (New England Nuclear).

Chemicals used for the incubation of RBC: 10,000 units each of penicillin-streptomycin mixture (Gibco); 200 mM sterile L-glutamine (Gibco); 100 x MEM Spinner salt-solution (B.B.L.); 10 x MEM amino acid mixture (B.B.L.); L-amino acids (Sigma).

Chemicals used for electrophoresis: N, N'-methylene-bisacrylamide (Eastman Kodak); acrylamide (Eastman Kodak); N,N,N',-tetramethylene-ethylenediamine (Eastman Kodak); 2-mercaptoethanol (Eastman Kodak); 'ultrapure' urea (Schwartz-Nann); riboflavin (Eastman Kodak); bromophenol blue (Fisher); pyronin-y (Eastman Kodak).

Stains: Amido Black 10B or naphthol blue, color index 20470 (Fisher); May and Grunwald, 65513 (BDH); Giemsa, 58395 (BDH); toluidine blue O, color index 52040 (Fisher); fuchsin-acid, color index 42685 (Hartman-Leddon).

Chemicals used for template capacity analysis: (Purchased from Sigma). adenosine-5'-triphosphate tris-salt from equine muscle; cytidine-5'-triphosphate tris-salt, Type VI; guanosine-5'-triphosphate tris-salt, Type VI, from equine muscle; E. coli K₁₂ RNA polymerase, 1,000 units/ml.

Proteolytic inhibitors: L-1-tosylamine-2-phenylethylchloromethyl ketone (Sigma); phenylmethyl sulfanylfuoride (Sigma); sodium bisulfite (BDH).

Methods

I Embryonic Stages

This study includes embryos at selected morphogenetic stages as specified by Hamburger and Hamilton (1951).

White Leghorn

(hyline 934 F) eggs were incubated at 37.5°C and 60% relative humidity. The following developmental stages showed the indicated distinguishing features:

Stage 17-18 ($60 \leq n \leq 70$) About 68 hr. incubation, General characteristics: the allantois forms a short, thick-walled pocket, not yet vesicular; the amnion is closed except for an oval hole over somites 28-36; the tail-bud mesoderm is unsegmented; the wing and leg buds are distinct swellings of approximately equal size.

Stage 21 ($30 \leq n \leq 40$) About 90 hr. incubation. General characteristics: 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; the maxillary process is longer than the mandibular process.

Stage 27 ($15 \leq n \leq 25$) About 130 hr. incubation. General characteristics: the beak is barely recognizable; distinct grooves are visible between three digits and toes; the tip of the third toe is not yet pointed.

Stage 29 ($7 \leq n \leq 15$) About 154 hr. incubation. General characteristics: 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; the rudiment of the fifth toe is visible.

II Preparation of Blood Cells

Eggs were broken open into a deep bowl containing chick Ringers solution (7.00 g NaCl, 0.42 g KCl, 0.24 g CaCl_2 in 1.0 l H_2O ; pH 7.4) at room temperature. The embryo and 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 chick Ringer's solution in small petri dishes. In each change the explants were agitated for 1-2 minutes to remove as much yolk as possible. 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 modified Seligman's balanced salt solution (Noble et al. 1968) SBSS, (7.65 g NaCl, 0.20 g KCl, 1.2 g Tris-HCl, 0.05 g NaH_2PO_4 , 0.10 g K_2HPO_4 , 0.70 g NaHCO_3 , 1.0 g glucose, 0.003 g ascorbic acid, 1.0 g EDTA in 1.0 l H_2O ; pH 7.3). The vitelline vessels of each embryo were cut and the blood cells allowed to drain into the solution. The blood cell suspension was aspirated using a Pasteur pipette and transferred to a test tube at 0-4°C. This procedure was repeated until sufficient blood cells had been extracted to produce a pellet of 0.1 to 0.2 ml on centrifugation.

Blood cells were pelleted in a Sorval GLC-1 centrifuge at 1200 x g for 10 minutes, passed through a single layer of muslin after resuspension in modified SBSS at 0-4°C and repelleted. Resuspension and centrifugation was carried out twice more with a further

passage of the cells through muslin between the second and third washings.

As a developmental end point, blood from an adult White Leghorn hen was collected without a topical anesthetic from the severed jugular vein. Thirty ml of whole blood was thoroughly mixed with 5.0 ml of 1% sodium heparin in modified SBSS at 0-4°C. Red blood cells (RBC's) were separated by centrifugation at 1600 xg for 10 minutes, the buffy coat was removed by aspiration, and then the RBC's were washed as for embryonic blood. The procedure was repeated three times, once for each of three hens.

As a procedural control to indicate erythrocyte specificity for H5, non-erythroid cells were obtained from two batches of stage 27 embryos (four and five each) which had been explanted, dissected free of extra-embryonic membranes, and washed in chick Ringers as for whole explants. Whole embryos or separate head and trunk regions, cut-off just caudal to the otic vesicle, were homogenized in 10 volumes modified SBSS at 0-4°C with three strokes of a loose-fitting pestle in a Dounce-type hand homogenizer. Cells were pelleted at 3000 xg for 10 minutes and then the cells were washed as for embryonic RBC's.

In an attempt to enrich the proportion of mature primitive RBC's in stage 29 blood samples, two preparations of washed RBC's from stage 29 embryos (15 and 10) were layered over a two-step barrier of 30% and 37% BSA, purified by deionization according to Lief (1970), in modified SBSS at 0-4°C. Density barrier separation was

accomplished by centrifugation at 2000 xg for 30 minutes. The cells from the supernatant, from the 30% and 37% BSA interface, and from the pellet were separately collected and washed twice by centrifugation and resuspension in modified SBSS.

III Identification of Erythroid Cells

The erythroid stages were identified by two methods:

- 1) Cells in modified SBSS were spread on slides with a Pasteur pipette, dried in a slide-warmer for 12 hr. at 25°C, and stained with May-Grunwald for three minutes and Giemsa for ten minutes (Lucas and Jamroz, 1961). The slides were then rinsed in distilled water, dried at room temperature for about 30 minutes, and mounted with a coverglass using Permount.
- 2) Alternatively, following the final washing in SBSS, pelleted cells were resuspended and fixed for 15 minutes in 5 ml. 1.5% glutaraldehyde in 0.02 M sodium cacodylate buffer (pH 7.0) with 0.9% (w/v) sodium chloride. Pelleted cells were then washed three times in buffer-saline alone, over one hour. The cells were resuspended in 50 volumes modified SBSS, spread on slides with a Pasteur pipette, dried in a slide-warmer for 12 hr at 25°C, and stained with 1% (w/v) aqueous acid fuchsin (Feder and O'Brien, 1968) and 0.05% (w/v) toluidine blue at pH 5.0 (Sidman et al., 1961) for 30 seconds each. The slides were then rinsed in distilled water, dipped in methanol, dried at room temperature for about 30 minutes and mounted with a coverglass using Permount.

Stained cells were compared (Zeiss photomicroscope, 100 x oil immersion objective) with typical stages illustrated by Lucas and Jamroz (1961) and differentially counted (~1000 cells per stage). Photographs were taken with Kodak panatomic-X film. Numbers of mitotic figures in RBC's were counted in five randomly selected fields (100 x) from two slides at each maturation stage. Nuclear and cellular volumes of individualized cells were estimated in photographs of fixed, toluidine blue and acid fuchsin stained cells; five slides per stage, 30 cells per slide. Nuclei were considered to be spheres and volumes estimated from an average of two diameters measured at right angles. Cells were considered to be prolate spheres and diameters measured along the major and minor axes.

IV Isolation of Nuclei

Embryos were explanted and blood pellets prepared at the stages and incubation times noted in Part I above. All procedures were carried out at 0-4°C, unless specifically noted.

Following the final washing in SBSS, pelleted cells were lysed for 30 minutes with intermittent stirring with a glass rod in 100 volumes of 0.05% (w/v) saponin, 0.1% (v/v) Triton X-100, 3.0 mM CaCl_2 in 0.9% (w/v) NaCl; sometimes containing either 5 mM PMSF, 5 mM TPCK, or 10 mM sodium bisulfite. Routinely the lysate was centrifuged at 10,000 xg for 10 minutes, the supernatant was decanted, and the crude nuclear pellet was homogenized in 0.1% Triton X-100, 3.0 mM CaCl_2 , 0.9% NaCl with three strokes of a

loose-fitting pestle in a Dounce hand homogenizer. Nuclear preparations which were not homogenized in Triton X-100 had protein to DNA ratios twice the ratios for homogenized nuclear preparations. Nuclei were pelleted and resuspended twice in 3.0 mM CaCl_2 0.9% NaCl. Except for blood cells lysed in the presence of sodium bisulfite, the nuclear preparations appeared free of visible heme pigment and extraneous membranes material (phase contrast microscopy, 600 x and electron microscopy, Dardick, 1975).

V Incubation of Nuclei

In order to follow the degradation of histones associated with chromatin, 0.1 ml of pelleted RBC's from stage 17-18 and stage 29 embryos was homogenized in 10 ml 0.05% saponin, 0.1% Triton X-100, 3.0 mM CaCl_2 in 0.9% NaCl and incubated in a 37°C water bath for 30 minutes or 3 hr. Pelleted RBC's (0.1 ml) or RBC nuclei (~500 µg DNA) were also incubated at 37°C in 10 ml of the post-nuclear lysis supernatant from RBC's of stage 17-18 embryos. Nuclei were prepared and washed as before, and extracted as for total histone extraction (Methods VIII). The degree of histone degradation was monitored by quantitative gel electrophoresis (Methods XI).

VI Preparation of Embryonic Total Cytosol Proteins

The post-nuclear lysis supernatant from RBC's of stage 27 embryos was precipitated in nine volumes acetone, washed once in ethanol:ether (1:1), and dissolved in an equal volume of 7% (v/v) acetic acid. This sample was used as a reference for globin contamination of histone samples electrophoresed on polyacrylamide gels (Results IVD).

VII Chromatin Preparation and Extracction of Total Chromosomal Proteins

Pelleted nuclei from primitive RBC's of embryos at stages 17-18, 21, and 27 and from adult hen erythrocytes were suspended in 100 volumes of 0.05 M Tris-HCl at pH 8.0, homogenized with three strokes of a loose-fitting pestle in a Dounce hand homogenizer, and centrifuged at $14,000 \times g$ for 10 minutes. Pelleted chromatin was suspended in 10 volumes of 0.01 M Tris-HCl at pH 7.4 and sheared in a Virtis "23" homogenizer; two minutes at medium speed, followed by one minute at high speed. Chromatin was tested for template capacity within 3 hr. after preparation (Methods XVII).

Chromatin (~400 ug DNA) in 10 ml 0.01 M Tris-HCl was pelleted at $30,000 \times g$ for 20 hr, suspended in 5 ml 1% (w/v) SDS in 0.05 M Tris-HCl pH 8.0, incubated at 37°C for 20 hr., dialyzed against 100 volumes 0.01% SDS in 0.01 M Tris-HCl, and centrifuged in polyallomer tubes at $40,000 \times g$ (25°C) for 24 hr. (Marushige et al., 1968). The top two-thirds of the supernatant was removed and adjusted to 5% TCA. The TCA precipitate was dissolved in an equal volume 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 50% (v/v) glycerol in 0.02 M sodium phosphate buffer, then incubated at 90°C for 10 minutes, and electrophoresed in 0.1% SDS, 10% polyacrylamide gels (Methods XII).

VIII Extraction and Fractionation of Histones

A. Histone Extraction

Following the final washing in 3.0 mM CaCl_2 , 0.9% NaCl, pelleted nuclei were acid extracted by various

methods: 1) Total histones were extracted from nuclear pellets in equal volumes of 0.25 N H_2SO_4 in 3.0 mM $CaCl_2$, 0.9% NaCl (intermittent stirring with a glass rod, 30 minutes). The supernatant was recovered at 10,000 x g for 10 minutes and the nuclei were washed three times in the above acid solution by resuspension and centrifugation; extracts were combined. In all cases a fourth control wash was checked separately for absence of histones. 2) Histone was removed from the nuclei by selective extractions in two steps (Vidali and Neelin, 1968b). Nuclei were stirred intermittently for 30 minutes in an equal volume of 3.0 mM $CaCl_2$, 0.9% NaCl with H_2SO_4 at pH 1.8 and the supernatant was recovered by centrifugation at 10,000 x g for 10 minutes. The residue was re-extracted at pH 1.8 and centrifuged again; the two extracts were combined. The extraction of the remaining histones was completed as for the total extraction of histones. 3) Histones were extracted sequentially from nuclei with equal volumes of 0.01 M citric acid, 0.1 M citric acid, H_2SO_4 at pH 1.8, and 0.25 N H_2SO_4 , all in 3.0 mM $CaCl_2$, 0.9% NaCl. Each extraction was for 30 minutes and was followed by a wash in the same solution, except for 0.25 N H_2SO_4 extraction which was conducted as for total histone extraction. The 0.1 M citric acid extract from RBC nuclei of an adult hen and eight stage 27 embryos, the 0.01 M citric acid extract from RBC nuclei of 63 stage 17-18 embryos, and the H_2SO_4 extract at pH 1.8 from RBC nuclei of eight stage 27 embryos were precipitated by storage for 24 hr at $-15^{\circ}C$ in nine volumes of 95% (v/v) ethanol and by

centrifugation at 1600 x g for 10 minutes. Each pellet was dissolved in one ml. of glass-distilled water and dialyzed against 100 volumes of the same at 5°C for 24 hr. The dialyzed histones were then frozen at -15°C, lyophilized, and hydrolyzed for amino acid analysis as described in Methods XIV.

B. Estimation of Histone Concentration

The total histone extract (~60 mg) from adult hen erythrocyte nuclei was precipitated for 12 hr at 0°C in 9 volumes of acetone. Precipitated histone was collected on Whatman No. 1 filter paper with a sintered glass funnel under slight suction and rinsed three times with 95% (v/v) ethanol. Histone dissolved in 10% (v/v) acetic acid was desalted by exclusion chromatography in Bio-Gel P-2, equilibrated and eluted with 10% acetic acid (Vidali and Neelin, 1968b). Sulfate ions in effluent fractions were located by formation of a precipitate after BaCl₂ addition. Sulfate-free protein fractions (50 ml) were combined, dialyzed for 12 hr at 0°C against 20 volumes glass distilled water and concentrated to 10 ml in an Amicon micro-ultrafiltration system at 35 p.s.i., with a PM-10 Diaflo membrane (exclusion limit: 10,000 d), frozen at -15°C, and lyophilized. This histone was used as a standard for the determination of histone concentration by turbidity in TCA according to Vidali and Neelin (1968a) and of protein concentration in 1 N NaOH according to Lowry et al. (1951); see appendix 1.

C. Isolation of Adult Histones H1 and H5

The sequential extracts in 0.01 M citric acid, 0.1 M citric acid, and H₂SO₄ at pH 1.8 from adult

hen erythrocyte nuclei (Methods VIII A) were separately precipitated, collected, and washed as for total histone. The 0.01 M citric acid extract (150 μ g) produced no apparent bands when electrophoresed on polyacrylamide gels (Results IVF). The 0.1 M citric acid extract was desalted, dialyzed, concentrated, and lyophilized as for total histone. This extract contains histone fraction H1 (Fig. 25). The pH 1.8 extract ($\sim$ 30 mg) was frozen, lyophilized, dissolved in 50 ml of 0.1 M sodium phosphate buffer at pH 6.8 and chromatographed at room temperature on Amberlite CG-50 (100-200 mesh) with a gradient from 9-20% guanidinium chloride buffered in 0.1 M sodium phosphate (Vidali and Neelin, 1968b). The effluent flow rate was maintained at 20 ml/hr. Fractions 52 (Fig 1,F) were pooled, concentrated, desalted, dialyzed, re-concentrated, frozen, and lyophilized as for total histone. Fraction F contains the erythrocyte-specific histone H5 (Fig. 26). H1 and fraction F (H5) were used for gel quantitation (Methods XI).

D. Isolation of Adult Histones H2a, H2b, H3, and H4

Total histone extract from the nuclei of adult hen erythrocytes was precipitated, collected, and washed as for total histone. The precipitated histone (wet weight 340 mg) was dissolved in 210 ml of 0.1 M sodium phosphate buffer at pH 6.8 and chromatographed as described (Methods VI (Fig. 2); Adams and Neelin, 1969. The effluent flow rate was maintained at 30 ml/hr. Fractions 30-65 (Fig. 2A), 85-10 (B), 165-175 (C), 176-190 (D), and 191-200 (E) were pooled

Figure 1. Cation-exchange chromatography of the erythrocyte-specific histone, H5. Histones were extracted sequentially from adult hen erythrocyte nuclei (Methods VIII, A). The pH 1.8 extract (~30 mg protein) was dissolved in 50 ml of 0.1 M sodium phosphate buffer at pH 1.8 and chromatographed at room temperature on Amberlite CG-50 with a gradient from 9-20% guanidinium chloride buffered in 0.1 M sodium phosphate. Fractions of ten ml of effluent were collected; flow rate was 20 ml/hr. Every fifth fraction was monitored for turbidity in TCA at 400 nm (●) and percentage guanidinium chloride (■) estimated by refractometry. Fractions 52-60, F, contain histone H5.

Figure 2. Cation-exchange chromatography of a total histone extraction from PEC nuclei of adult hen erythrocytes (Methods VIII, A). 340 mg of histone (wet weight) was dissolved in 210 ml of 0.1 M sodium phosphate buffer at pH 6.8 and chromatographed on Amberlite CG-50 as for Fig. 1. Fractions of five ml of effluent were collected; flow rate was 30 ml/hr. Every fifth fraction was monitored for turbidity in TCA at 400 nm (●) and percentage guanidinium chloride (■) estimated by refractometry. Collected fractions are labelled above (A).

separately, concentrated, desalted, dialyzed, re-concentrated, and lyophilized as described before. Histones were identified by electrophoresis on polyacrylamide gels (Figs. 25-27). Fractions B (H2a and H2b) and E (H3 and H4) were used for gel quantitation (Methods XI).

IX Estimation of the Nucleic Acid and Residual Protein Concentration of RBC Nuclei

Acid-extracted nuclei (Methods VIII, A) were suspended and stored overnight in ten ml 10% (w/v) TCA at 4°C and then washed in 95% ethanol by resuspension and centrifugation. RNA was next extracted from nuclei (~500 µg DNA) in either two ml cold 10% (v/v) PCA or two ml 0.3 N NaOH at 37°C for 20 hr; concentration was estimated by the orcinol reaction (appendix 2; Meijbaum, 1939). This extract contained 0.1% of the total DNA in the nuclear preparation. Nuclei were then incubated in two ml 5% PCA at 70°C for 20 minutes and washed once by resuspension and centrifugation in two ml 15% PCA; extract and wash were combined. DNA concentration in combined 10% PCA extract and wash was estimated by the diphenylamine test (Burton, 1966) using calf-thymus DNA as a standard (appendix 2; Meijbaum 1939). The remaining protein precipitate was solubilized in four ml 1N NaOH at 60-70°C for 24 hr. Protein content of this nuclear RP fraction was estimated according to Lowry et al. (1951) using BSA as a standard (appendix 1). The RP and DNA values did not change appreciably, if nuclei were first incubated in five ml chloroform:ethanol (1:3) at 70°C for 20 minutes to remove lipids. DNA concentration was also,

estimated by optical density at 260 nm on suspended, unextracted nuclei or chromatin (appendix 2).

X Preparation of Adult Chicken Erythrocyte DNA

Adult hen erythrocyte chromatin was suspended in 10 volumes of 5% (w/v) SDS in 0.01 M Tris-HCl, stirred continuously for 24 hr at 37°C, and centrifuged at 30,000 x g at 25°C for 20 hr. The pellet (protein/DNA:0.02) was dissolved in three volumes of 0.01 M Tris-HCl and mixed with one volume chloroform:isoamyl alcohol (24:1) by aspiration with a Pasteur pipette. The aqueous layer was removed and the DNA was gathered on the glass rod after the addition of five volumes of 95% ethanol at 0°C. The extraction was repeated once.

XI Urea-Polyacrylamide Gel Electrophoresis

Histones were separated and quantitated by the polyacrylamide gel electrophoresis method of Panyim et. al. (1971). Gels contained either 2.5 M or 6.25 M urea, 15% (w/v) acrylamide, 0.125% (w/v) Bis, and 0.5% (v/v) TEMED. Riboflavin (0.0002% w/v) was used to replace ammonium persulphate as a catalyst (V.L. Seligy, personal communication). Gels were made in "long" glass tubes, 6 mm in internal diameter and 25 cm in length or in "short" glass tubes, 5 mm in internal diameter and 15 cm in length. Polymerization occurred in 30 minutes between UV lamps. Acid extracts or lyophilized histones were mixed with 20% sucrose, 0.01% (w/v) pyronin-y in 7% acetic acid at a final concentration of 1-2 ug/ μ l histone. Samples (10-40 μ l) were layered directly onto gels under the "running buffer" of 7% acetic acid with a microliter syringe. Electrophoresis

in a standard vertical gel system (Chrambach and Rodbard, 1971), was at room temperature using a constant current of 1.6 mA/gel for 4½ hr in "short" gels and 12 hr on "long" gels. Gels were stained for 15 minutes in 15 ml of 1% (w/v) Amido Black in 7% acetic acid, 40% ethanol and destained twice a day for three days with the acetic acid-ethanol vehicle. Gels were then placed in 15 ml 7% acetic acid and scanned 12 hr later in a Beckman Acta C-III spectrophotometer with a slit width of 0.1 mm at 600 nm.

Acid extracts of nuclei were adjusted to 2% (v/v) 2-mercaptoethanol and incubated overnight at 0°C to obtain partial reduction of H3 to the monomer (Fig. 27 D).

Partial oxidation of H3 to the dimer was achieved by the following: 1) Nuclear acid extracts were precipitated in 20 volumes of 95% ethanol at -15°C for 12 hr. Precipitates were cleared by centrifugation at 1600 x g for 10 minutes, dissolved in an equal volume of 6 M urea in glass-distilled water, and incubated at 37°C for one to three hr, with intermittent mixing by a Pasteur pipette during the incubation. The samples were then frozen, lyophilized, dissolved in 7% acetic acid, and electrophoresed (Table 12). 2) Nuclear acid extracts were precipitated as described above, dissolved in ½ volume of 2.5 M urea, 5 mM potassium ferricyanide, and incubated at 0°C or 37°C for one to three hr. (Fig. 22).

Areas under gel scan peaks were estimated by tracing the peaks on paper of uniform weight (identical scans traced on three separate sheets of paper differed by 1% to 4% in weight), cutting out the peaks, and weighing.

Relative amounts of each histone was expressed as the weight each traced peak divided by the weight of all the traced peaks in the gel scan.

For this method to be valid, it is important that the staining affinity of each histone be similar. In order to assess for this variable, weighed quantities of histones H1, H5, H2, and H3, H4 were put on gels at various concentrations and the quantity of each histone fraction loaded on a gel was plotted against the weighed area of paper corresponding to each electrophoretic peak (Fig. 48). Weighed quantities of H1, H5, and H3, H4 were corrected for contamination by minor components, by subtracting the proportion in weight which these minor components occupied in gel scans. Areas under gel scan peaks for individual histone extracts were adjusted for differences in staining, in order to estimate the concentration (μg) of histone within an electrophoretic band (appendix 5). The relationship between histone concentration and staining was not examined above 15 μg . The quantity of each histone fraction is expressed per unit DNA, by equating the total weight of histone in a gel scan to the protein concentration (determined by the Lowry method) per unit DNA in acid extracts of nuclei.

XII Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Histones and nonhistones were separated by the SDS polyacrylamide gel electrophoresis method of Weber and Osborn (1969). Gels contained 10, 12, or 15% acrylamide, 0.4% Bis, 0.1% SDS, and 0.2% TEMED in 0.2 M sodium phosphate buffer at pH 7.2. Riboflavin (0.0002%) was used

as a catalyst for polymerization as described above. Gels were made in 15-cm glass tubes. Lyophilized histone samples (1 mg) were dissolved in one ml 2% SDS, 2% 2-mercaptoethanol in 0.02 M sodium phosphate buffer and were heated in glass tubes covered with aluminum foil in boiling water for five minutes. Some samples were then dialyzed against 100 volumes 0.1% SDS in phosphate buffer for 12 hr, but if 30 μ l or less were loaded on gels, this step (to prevent aggregation) did not appear to be necessary. However, histones recovered from urea-polyacrylamide gels in 1% SDS (Methods XIII), were always dialyzed before electrophoresis. Nonhistone samples were prepared as described in Methods VII. Samples (10-50 μ l) were layered directly onto gels under the running buffer of sodium phosphate (pH 7.2). Electrophoresis was at room temperature using a constant current of 8 mA/gel for 10 hr. Gels were stained and destained as described for urea-polyacrylamide gels. Pre-fixing for 15 minutes in 15 ml 12.5% TCA did not improve the staining.

XIII Histone Recovery from Polyacrylamide Gels

In each method co-migrating protein bands located by comparison with parallel, stained gels were sliced out of five gels with a razor blade, blotted on Whatman No. 1 filter paper, and treated as follows:

- 1) Gel slices were homogenized in a Pyrex tissue grinder in one ml 6N HCl. This suspension was transferred to a hydrolysate tube and treated as described below.
- 2) Gel slices were homogenized in a Pyrex tissue grinder in one ml 1N H₂SO₄, incubated at 37°C for one hr,

cooled to about 4°C , and then pelleted at $1600 \times g$ for 10 minutes. The supernatant was precipitated by addition of nine volumes acetone at -15°C for three hr and centrifugation at $1600 \times g$ for 10 minutes. The pellet after washing in 95% ethanol by suspension and re-centrifugation was dissolved in 100 volumes 6N HCl (see below).

- 3) Gel slices were homogenized in a Pyrex tissue grinder in 0.5 ml 30% H_2O_2 and incubated at 50°C for one hr. The suspension was mixed with 0.5 ml phenol by aspiration with a Pasteur pipette, and then the suspension was re-mixed with one ml 1N H_2SO_4 . The suspension was then cooled to about 4°C and centrifuged at $1600 \times g$ for 10 minutes, before removal of the aqueous layer. Protein from this solution was precipitated by addition of nine volumes acetone and centrifugation at $1600 \times g$ for 10 minutes, before washing by resuspension and centrifugation in 95% ethanol at 0°C . Pellet was treated as described below.
- 4) Gel slices were homogenized in a Pyrex tissue grinder in two ml 1% SDS in 0.2 M sodium phosphate buffer, incubated at 37°C for 12 hr, and centrifuged at $1600 \times g$ for 10 minutes. The supernatant was either dialyzed against 100 volumes glass-distilled water for 12 hr, lyophilized, and hydrolyzed for amino acid analysis as described below or electrophoresed on SDS-polyacrylamide gels as in Methods XII.

XIV Amino Acid Analysis

Histone samples from sequential nuclear extracts,

chromatographic separations of acid extracts, or from sections of polyacrylamide gels were hydrolyzed in 100 volumes of 6N HCl (Aristar) at 105°C for 24 hr in evacuated and sealed nitric acid rinsed hydrolysis tubes. The residue was collected from the hydrolyzates by evaporation in a dessicator under vacuum, containing fresh pellets of sodium hydroxide. Amino acids were separated in a Beckman automatic amino acid analyzer (Model 121). The chromatographic peaks (located by reaction with ninhydrin) were traced on paper of uniform weight, weighed, and converted to nanomoles by comparison with a standard amino acid separation (Baily, 1967).

XV Assay of Protease Activity in Primitive RBC Nuclear Preparations

Samples of 50 μ l each of a 1.0 mg/ml preparation of adult chicken erythrocyte histones H1, H5, and H2 (mixed 1:3:3 by weight) in distilled water were incubated at 37°C for 15 minutes to 3 hr with 50 or 75 μ l of the post-nuclear lysis supernatants, nuclear washes, and acid extracts of nuclei obtained from 0.1 ml of packed RBC's (1200 x g, 10 minutes) from stage-17-18 and stage 29 embryos. Certain samples contained 5 mM or 15 mM sodium bisulfite, 5 mM TPCK, or 5 mM PMSF as indicated (Results, V.C). After incubation the samples were frozen in dry-ice/acetone. Frozen samples were quickly thawed in tap water at 60°C and 35 μ l aliquots, made up of 25% sucrose, 0.01% pyronin-y, were electrophoresed on 2.5 M urea, 15% acrylamide gels as described. The degree of histone degradation was analyzed using polyacrylamide gel electrophoresis. Weight of traced peaks from each histone fraction obtained from

gel scans of the incubated samples were expressed as a percentage of the weight of the corresponding peaks in control incubations, consisting of a 50 μ l of H1, H5, H2 mixture incubated at 37°C for the same time with the solutions used for cell lysis and nuclear washes (Figs. 40-42). Since globin migrates with H1 in this gel system, the weight of H1 was obtained by subtracting the weight of the presumptive globin peak from incubated samples containing 75 μ l of the post-nuclear lysis supernatant and 50 μ l distilled water. The validity of this correction was shown with the following mixtures: 1) 50 μ l of H1, H5, H2 mixture and 75 μ l of lysis solution (Methods IV); 2) 50 μ l H1, H5, H2 mixture and 75 μ l post-nuclear lysis supernatant; 3) 50 μ l glass-distilled water and 75 μ l post-nuclear lysis supernatant. Immediately after mixing, the samples were frozen in dry-ice/acetone and promptly (within 30 minutes) thawed and layered on gels to minimize time for proteolytic action. From gel scans of these samples, the weight of H1 in sample #1 was found to be equal to the weight of H1 in sample #2 after subtracting the weight of globin from sample #3.

XVI. Embryonic RBC Metabolism

A. Incubation of RBC's for estimation of nucleic acid and protein synthesis.

RBC's from 62 stage 17-18, 38 stage 21, or 13 stage 27 embryos were prepared as usual except that solutions were sterilized by passage through Sartorius membrane-filters and glassware was sterilized by autoclaving for 30 minutes. Following the final washing in SBSS, pelleted cells ($\sim 10^8$) were suspended in 10 ml MEM (Appendix 3),

pelleted at 1600 x g for 10 minutes, resuspended in 1.0 ml MEM, and transferred to a 25 ml Erlenmeyer flask. For cell incubations for the measurement of protein synthesis the MEM medium was prepared without the addition of lysine (appendix 3). Cells were incubated with constant stirring for 30 minutes at 37.5°C in a presumably water-saturated atmosphere with a continuously flowing mixture of 5% CO₂ and 95% air. After 30 minutes the following were added to separate cell suspension cultures: 10 μ Ci (100 μ l) of thymidine 2-¹⁴C for the estimation of DNA synthesis, 100 μ Ci (100 μ l) of uridine-5-H³ for the estimation of RNA synthesis, and 100 μ Ci (100 μ l) of L-lysine-4,5-³H(N) or 10 μ Ci (100 μ l) L-lysine [¹⁴C (U)] for the estimation of protein synthesis. The cell incubations for the estimation of DNA and RNA synthesis were continued for 30 minutes and those for the estimation of protein synthesis were continued for one hour. After incubation, the cells were pelleted at 1200 x g for 10 minutes and the supernatant was removed for measurement of radioactivity to ensure that an excess of labelled precursor was still available for incorporation.

B. Measurement of Radioactivity by the Whatman Filter-Disk Method

After 10 minutes incubation for RNA labelling and 30 minutes incubation for RNA and DNA labelling, three 100 μ l samples were removed; two 100 μ l samples were used to spot two Whatman 3 MM filter-disks; one 100 μ l sample was diluted ten times in SBSS for cell counts as follows: Cell numbers were counted in a bright-line hemocytometer with a phase-contrast microscope at 40 x (50 fields per sample). 200

μl of 10% TCA at 0-4°C was added to the filter-disks containing blood cells and the filter-disks were left to dry overnight at room temperature. The filter disks were then placed on Whatman No. 1 filter paper in a Buchner funnel and washed under slight suction three times in 200 ml of 5% TCA, and three times in 200 ml of ether; ethanol (1:1) at 0-4°C. The disks were then dried at 50°C overnight.

After 15, 30, and 60 minutes incubation for protein labelling, two 100 μl samples were removed: one 100 μl sample was used to spot a Whatman 3 MM filter-disk; one 100 μl sample was diluted ten times in SBSS for cell counts as described above. The blood cells were precipitated on to the filter-disks and the filter-disks were dried as described above. The filter-disks were then suspended in 300 ml of 5% TCA, boiled for 15 minutes, and washed in TCA and ether:ethanol as described above.

The dried filter-disks were placed in 20 ml Fisher plastic scintillation vials (one per vial) and covered with 15 ml of 0.4% Omnifluor in toluene. Radioactivity in the vials was measured as described below; with an efficiency of about 10% for ^3H and 60% for ^{14}C .

C. Estimation of the Incorporation of ^{14}C -thymidine into DNA

Washed nuclei, prepared as previously described (Methods IV) from RBC's incubated in ^{14}C -thymidine, were extracted as described before (Methods IX) to yield DNA.

Three 100 μl aliquots per stage of DNA in 10% PCA were added to 20 ml Fisher plastic scintillation vials with

15 ml 0.3% (w/v) PPO, 25% (v/v) Triton X-114 in toluene. Radioactivity in the vials was measured as described below; with an efficiency of about 90% for ^{14}C . The concentration of DNA was assayed by the diphenylamine test (appendix 2).

D. Estimation of the Incorporation of ^3H -uridine into RNA

After their incubation in ^3H -uridine, RBC's were lysed and the lysate was centrifuged as previously described (Methods IV). The supernatant was divided into two; each half was precipitated with two volumes of 10% TCA at 0-4°C. for 10 minutes and by centrifugation at 1600 x g for 10 minutes. The pellets were suspended in two volumes 5% TCA at 0-4°C for 12 hr, centrifuged at 1600 x g for 10 minutes, and then washed in two volumes 95% ethanol by resuspension and centrifugation. The pellet from one half was then suspended in one ml 0.3 M KOH and incubated at 37°C for 24 hr. After incubation one ml of 10% PCA was added, and the suspension was centrifuged at 1600 x g for 10 minutes; the supernatant was used for an estimation of RNA concentration (appendix 2) and for radioactivity measured as described for DNA (with an efficiency of about 13% for ^3H). The pellet from the other half was incubated in one ml 5% PCA at 70°C for 20 minutes, centrifuged at 1600 x g for 10 minutes, and the pellet was washed in one ml 15% PCA by resuspension and centrifugation; supernatants were combined for estimation of RNA and measurement of radioactivity as described above. The pellets after "RNA" extraction from both halves were individually incubated in one ml 10% TCA at 90°C for 10

minutes and centrifuged at 1600 x g for 10 minutes; RNA concentration and radioactivity in the supernatants were measured as described above. The cpm per microgram RNA for both alkali and acid extractions were similar. The hot TCA extract yielded an additional 12% RNA reacting material.

E. Estimation of the Incorporation of ^3H - and ^{14}C -Lysine into Cytoplasmic Proteins

After their incubation in labelled lysine, RBC's were lysed and the lysate was centrifuged as previously described (Methods IV). The pellet was centrifuged at 30,000 x g for 3 hr. One fifth volume of the supernatant from this centrifugation was precipitated with five volumes of 10% TCA at 90°C for 20 minutes and by centrifugation at 1600 x g for 10 minutes; this supernatant, hot-TCA-soluble fraction, was measured for radioactivity as described before for DNA. The pellet from the 30,000 x g centrifugation (microsomal fraction) and the hot-TCA-insoluble-fraction from above were dissolved in two ml and four ml respectively of 1N NaOH at 60°C for 18 hr, three 500 μl aliquots of each were estimated for protein concentration (appendix 1) and three 100 μl aliquots of each were measured for radioactivity as described above.

F. Estimation of the Incorporation of ^3H - and ^{14}C -Lysine into Nuclear Proteins

Histones from washed nuclei, prepared as previously described (Methods IV) from RBC's incubated in labelled lysine, were either extracted totally in 0.25 N H_2SO_4 (^3H -lysine) or sequentially in two steps in H_2SO_4 at pH 1.8,

followed by 0.25 N H₂SO₄ (¹⁴C-Lysine) as described before in Methods VIII A. Histone extracts were either precipitated in 10 volumes of 95% ethanol at -15°C for 12 hr and dissolved in four ml NaOH for estimation of protein concentration (appendix 1) and measurement of radioactivity as described before for cytoplasmic proteins; or electrophoresed on polyacrylamide gels as described in Methods XI. These gels were cut into one mm slices with a Hoefer horizontal gel slicer and the slices were incubated at 50°C for 12 hr in 20 ml Kimble glass scintillation vials containing 100 µl 30% hydrogen peroxide. After the incubation, 15 ml of 0.3% PPO, 25% Triton X-114 in toluene was added to each vial and the radioactivity was measured as described below. The radioactivity in slices corresponding to histone bands was divided by the estimated weight of histone in the band. The values were then normalized for the lysine content of each histone fraction: H1, H5, H2 taken from Vidali and Neelin (1968b); H3 taken from Brandt and Holt (1972); and H4 taken from Delange et al. (1969). Residual proteins were extracted as described in Methods IX, analyzed for protein content (appendix 1), and measured for radioactivity as described for cytoplasmic proteins.

As a control, nuclei (~500 µg DNA) prepared from adult hen erythrocytes were incubated at 37.5°C for 30 minutes in 10 ml of the supernatant from a 30,000 x g for 3 hr centrifugation of a hen erythrocyte lysate containing 2.5 µCi/ml ¹⁴C-lysine. Nuclei were washed, histones were acid-extracted and then they were electrophoresed on

polyacrylamide gels as described before; gel slices measured for radioactivity as described above. Since the slices did not contain radioactivity above background, contamination of histones by labelled cytoplasmic proteins during nuclear preparation appeared negligible.

XVII In Vitro Assay of the Template Capacity of DNA and Chromatin

The reaction mixture (0.35 ml) contained: 3.5 μ moles Tris-HCl; ~~0.1~~ μ moles GTP, CTP, ATP; 0.25 μ moles MnCl_2 ; 1.0 μ moles MgCl_2 ; 0.01 μ moles ^{14}C -UTP (53.3 mCi/m mole); five units of E. coli RNA polymerase; and DNA or chromatin. The reaction mixture minus ^{14}C -UTP and RNA polymerase was added to nitric acid rinsed Pyrex 9880 tubes (Fisher), already held for 10 minutes in a 37°C water bath. RNA polymerase and ^{14}C -UTP were added at time-zero, and the mixture was incubated at 37°C for 10 minutes. The reaction was stopped by adding 10 volumes of cold ($0-4^\circ\text{C}$) 0.05 N HCl, 0.01 M pyrophosphate in 10% TCA and the tubes were left in crushed ice for two hr. The mixture was next poured onto a 25 mm Gelman GN-6 filter on a Millipore filter apparatus under a partial vacuum. Tubes were rinsed three times with 5 volumes each of cold 10% TCA and of 5% TCA. The rinses were poured again through the filter, and then the filters were rinsed three times with two ml each of 2% TCA, suction dried for two minutes, and oven dried at 50°C for 12 hr. As controls, certain samples were incubated for 1 hr at 37°C in 67 units RNase A. Others were mixed with one ml yeast RNA (1 mg/ml) and phenol-extracted (Kirby, 1964). The aqueous phase was washed with an equal volume

of ether and precipitated with ten volumes 95% ethanol at -15°C for two hr. The precipitate was incubated at 37°C for 20 hr in 0.5 ml of 0.5 N potassium hydroxide or 0.5 ml of 0.01 M Tris-HCl. After the incubation, the mixtures were neutralized with conc. HCl, precipitated with cold 10% TCA, and treated as described above for the reaction mixture.

Oven-dried filters were placed in 20 ml scintillation vials (one per vial) and covered with 15 ml 0.4% Omnifluor in toluene. Radioactivity in the vials was measured, as described below, with an efficiency of about 63% for ^{14}C .

XVIII General Liquid-Scintillation Counting Procedures

Radioactivity was measured in a Packard Tri-Carb Model 3003 liquid scintillation counter. Scintillation vials were exposed to radiation detection for two 10 minute periods separated by 12 hr at 5°C to guard against chemiluminescence. The background radioactivity was subtracted from all samples, averaged 25 cpm for ^{14}C and 15 cpm for ^3H . The measured radioactivity in scintillation vials containing samples from any one experiment, in which the incorporation of labelled precursors at different times or from different stages were compared, were normalized by the external gamma reference method (Beckman Technical Report No. 367). No more than 100 μl of liquid was added to 15 ml 0.3% PPO, 25% Triton X-110, in toluene, because at that volume of liquid the external standard channel ratios (Carbon-14 window/tritium window), for each vial containing samples prepared identically, were similar. When 200 μl of liquid were added to the above solution, the channel ratios changed and the internal standard

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OF/DE

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radioactivity was reduced, presumably due to quenching.

Radioactive counting efficiency was estimated by measuring the radioactivity in a standard of known specific activity, which had been treated in the same manner as the samples to which it was compared.

XIX Statistical Methods

The data in this thesis was statistically analyzed according to Games and Klave (1967) using the Student's t-test, outlined below.

The arithmetic mean ($\bar{x}$) was obtained by taking the sum of the values (x) from experiments which were to be compared and dividing by the total number of observations (N). The standard deviation (σ) was obtained by taking the square root of the average squared deviation. Then the standard error of the mean (S.E.) was calculated by the following formula:

$$\text{S.E.} = \frac{\sigma}{N}$$

The Student's t-test can then be used to compare groups of data by the following formula:

$$T = \frac{1 \bar{x}_1 - \bar{x}_2}{\sqrt{(S.E._1)^2 + (S.E._2)^2}}$$

$$\text{degrees of freedom} = (N_1 - 1) + (N_2 - 1)$$

Using a standard table, the T value in combination with the degrees of freedom will yield a "p" or percentage significance value. A $p \geq .05$ was considered not significant; e.g., the means from two sets of data are considered as significantly different, if the deviation between the means occurs by chance less than 5% of the time.

Results and Discussion

I Primitive RBC Types in Chicken Embryos

As discussed in the introduction, the primitive erythroid line of embryonic chickens was chosen as a system to study the pattern of histones during erythroid maturation, because the literature (Romanoff, 1960; Wilt, 1967; Campbell et al., 1971; Weintraub et al., 1971; Bruns and Ingram, 1973) indicates that at any particular stage of development one can isolate blood from embryos with the majority of the cells at a particular maturation stage. It was important to re-examine this maturation sequence process in order to directly correlate particular RBC types with the histones evident at the embryonic stage.

A. Stage 17-18 Embryos

Terminology used for RBC maturation stages is based on the tinctorial quality of the cytoplasm after May-Grunwald and Giemsa or acid fuchsin and toluidine blue staining. The cells at this stage were generally spherical (Figs. 3 and 4), with cytoplasmic staining ranging from intense basophilia (dark blue) to polychromatophilia (grey). Those cells with intensely basophilic cytoplasm are termed erythroblasts (Eb); with greyish-blue cytoplasm, early polychromatophilic erythrocytes (EP); and with grey cytoplasm, polychromatophilic erythrocytes (P). The majority of the RBC's in the circulation of stage 17-18 embryos were either Eb or EP (Table 2). They are counted as one group in Table 2, because it was often difficult to choose their precise stage.

Dardick (1975) was able to distinguish EP from Eb in electron micrographs on the basis of the presence of a peripheral band of microtubules and an increased concentration of ribosomes in polychromatophilic cells.

The cytoplasm of Eb cells was difficult to examine because the nucleus virtually fills the entire cell (Figs. 3 and 4). The cytoplasm of EP cells had a textured appearance, possibly due to the presence of mitochondria. Nucleoli (usually two), paler blue spheroid particles in the nucleus, were evident in all cells of this incubation stage. Nucleoli were prominent in unfixed smears in which the cells were swollen (Fig. 5). All of the cell types contained particulate chromatin in the nucleus, but the chromatin appeared to be aggregated into larger, more obvious clumps in EP and P cells. Mitotic figures were abundant (about 6%).

About 4% of the basophilic cells examined were larger and contained less clumped chromatin than erythroblasts. These cells could be proerythroblasts or thromboblats, but were counted as Eb-EP cells in Table 2. Thromboblats have been observed in the embryonic circulation as early as 50 to 60 hours incubation (Sugiyama, 1926; Lemez, 1964). Dardick (1975) reported that basophilic cells which can be distinguished as thromboblats in electron micrographs, often appear as swollen-looking cells or "ghosts" with intact nuclei.

B. Stage 21 Embryos

Most erythroid cells at this stage of development (Table 2) appeared to be polychromatophilic erythrocytes,

circular to elliptical cells with a grayish cytoplasm (Figs. 6 and 7). Nuclei contained clumped chromatin mostly arranged along the nuclear membrane and homogeneous areas in the spaces. Nucleoli were evident. Mitotic figures were present in 2.9% of the cells.

C. Stage 27 Embryos

The majority of the RBC's at this incubation stage were orthochromatic (OE) erythrocytes (Table 2). The cytoplasm in fixed toluidine blue and acid fuchsin-stained cells was pale pink (acidophilic) and had a uniform, finely granular quality (Fig. 9). The cytoplasm of unfixed, May-Grunwald and Giemsa stained cells was pink to light orange and contained many large nonrefractile vacuoles and smaller spherical bodies or refractile vacuoles (Fig. 8). Lucas and Jamroz (1961) have indicated that these vacuoles are artifacts commonly seen in unfixed slides of late polychromatic erythrocytes and are caused by the uneven drying of the cell surface.

The nucleus appeared more contracted at this stage compared to the previous stages. The blue stained chromatin now forms coarse masses, arranged mostly along the periphery of the nucleus. Nucleoli appeared to be still present but smaller in size over the last stage. Mitotic figures were infrequent in OE's (less than 1%).

Also present in the blood at this incubation stage were erythroblasts and polychromatophilic erythrocytes. These immature cells are probably of a later embryonic generation, the definitive series.

D. Stage 29 Embryos

The majority of the RBC's at this incubation stage were mature primitive erythrocytes (ME), but definitive line erythrocytes now constitute about a third of the circulating blood cells (Table 2). The cytoplasm of ME cells was more acidophilic than the previous stage after May-Grunwald and Giemsa or acid fuchsin and toluidine blue staining but there was a wide variation in the shades of red to orange in individual cells. Primitive mature erythrocytes also show a wider range in size and shape (Figs. 10, 11), but were generally larger and the acidophilic staining of the cytoplasm was more intense than in successive generations of erythrocytes. Mitotic figures were not evident in acidophilic cells, but were present in some definitive line cells.

The nucleus of ME cells was similar to the nucleus of OE cells. In some cases more of the chromatin appeared confined to large aggregates. Nucleoli were still present. It is probable that many of these cells are actually reticulocytes. Bruns and Ingram (1973) reported that mature primitive erythrocytes are not present in the circulation until 7 to 8 days of incubation.

About one out of fifty cells at stages 21, 27, and 29 examined had characteristics of embryonic thrombocytes. Granulocytic cells were not observed in any of the cell preparations. The granulocytic series of cells has been reported to enter the circulation by 5 days of incubation (Lucas and Jamroz, 1961; Sugiyama, 1926). However, in the electron microscopic investigation of embryonic primitive

RBC's by Dardick (1975), only one granulocyte was observed in a pooled blood sample from embryos at 6 days of incubation.

Plate 1. Light micrographs of primitive RBC pellets from chicken embryos at stages 17-18 and 21 (1520 X).

Figure 3. Unfixed, May-Grunwald-and Giemsa-stained stage 17-18 RBC's.

Figure 4. Glutaraldehyde-fixed, acid fuchsin-and toluidine blue-stained stage 17-18 RBC's. The cytoplasm of erythroblasts (Eb) is difficult to examine because the nucleus virtually fills the entire cell. The cytoplasm of early polychromatophilic erythrocytes (EP) has a textured appearance, possibly due to the presence of mitochondria.

Figure 5. Swollen, unfixed May-Grunwald-and Giemsa-stained stage 17-18 RBC's. Nucleoli (N) are prominent in these cells.

Figure 6. Unfixed, May-Grunwald-and Giemsa-stained stage 21 RBC's. Cells containing less clumped chromatin than erythrocytes may be thrombocytes (t).

Figure 7. Glutaraldehyde-fixed, acid fuchsin-and toluidine blue-stained stage 21 RBC's. Circular to elliptical shaped cells with a greyish cytoplasm are polychromatophilic erythrocytes (P). Cells with a darker cytoplasm and larger nucleus may be EP.

Plate 11. Light micrographs of primitive RBC pellets from chicken embryos at stages 27 and 29 (1520 X).

Figure 8. Unfixed, May-Grunwald-and Giemsa-stained stage 27 RBC's. Cytoplasm contains many large nonrefractile vacuoles and smaller spherical bodies or refractile vacuoles

Figure 9. Glutaraldehyde-fixed, acid fuchsin-and toluidine blue-stained stage 27 RBC's. Nuclei of orthochromatophilic erythrocytes (OE) appear more contracted at this stage compared to the previous stage. Also present in the circulation at this incubation stage are erythroblasts and polychromatophilic erythrocytes of the definitive series (DE).

Figure 10. Unfixed, May-Grunwald-and Giemsa-stained stage 29 RBC's.

Figure 11. Glutaraldehyde-fixed, acid fuchsin-and toluidine blue-stained stage 29 RBC's. Primitive mature erythrocytes (ME) show a wider range in size and shape, but they are larger and the acidophilic staining of the cytoplasm is more intense than in immature definitive erythrocytes (DE).

DOE

DE

Table 2

Percentage of Red Blood Cell Types in Chicken Embryos
from Differential Counts on Both Fixed and Unfixed
Preparations of Blood^a

stage (days)	Eb-EP ^b	P	OE	ME	DE
17-18 (2.5)	95	5	-	-	-
21 (3.5)	4	96	-	-	-
27 (5)	-	1	85	3	11
29 (6)	-	-	1	69	30

^aCell counts on nine preparations of stage 17-18 blood,
five preparations of stage 21, 27 and 29 blood; 200 cells
counted per preparation.

^bPrimitive line: Eb, erythroblast; EP, early
polychromatophilic erythrocyte; P, polychromatophilic
erythrocyte; OE, orthochromatophilic erythrocyte; ME,
mature erythrocyte; DE, definitive line red blood cells,
all maturation stages.

E. Nuclear to Cell Volume Ratios

The percentage groupings of RBC's by different ratios of nuclear to cellular volume (N/C) in Fig. 12 agreed with the proportion of RBC's of a particular maturation stage at each incubation age (Table 2). More than 90% of the cells at stage-17-18 had large N/C values distributed between groups 2 and 3 (Fig. 12A), largely representing erythroblasts and early polychromatophilic erythrocytes. The maturation stage of a cell, however, does not always match its N/C ratio. Some cells classified as erythroblasts had N/C values corresponding to either group 1 or 3, but not group 2 where the majority of the Eb's are placed. The Eb's of group 1 might be either proerythroblasts or thromboblats; and the Eb's of group 3 might be basophilic cells which have undergone the nuclear changes characteristic of a polychromatophilic stage. These results seem consistent with the notion that erythropoiesis is a continuous process (dynamic state), and hence often RBC's will not fit the static descriptions used to arrange them arbitrarily in maturation stages.

The cells of stage-21, almost all polychromatophilic erythrocytes (Table 2), had N/C values corresponding to groups 3 and 4. This incubation age showed the most homogeneity in RBC type by both cytological identification and N/C grouping.

The N/C groups 5 and 6 predominated in blood pellets from embryos at stages-27 and 29 (Fig. 12C,D). While there was a statistically significant difference (Methods XIX) between the other N/C groups at each incubation age,

there was no statistically significant difference between the H/C values for groups 5 and 6 at stages-27 and -29. This may indicate that stages-27 and -29 include a continuum of RBC's near the end of the maturation process; that is, cells classified as orthochromatophilic erythrocytes, reticulocytes, and mature primitive erythrocytes (Table 2).

II Nucleic Acid Synthesis in Primitive RBC's

A. DNA Synthesis

Primitive RBC's at stages-17-18, 21, and 27 incorporated ^{14}C -thymidine into DNA (Methods XVI, C). Incorporation per cell and per microgram DNA was highest at stage-17-18 and decreased with maturation (Table 3, Fig. 13). Thymidine incorporation per unit DNA decreased by a factor of 1.3 between stages-17-18 and 21 in two different incubations. The S periods of primitive erythroblasts and polychromatophilic erythrocytes have been reported to be respectively 3.6 hr and 7.2 hr (Campbell *et al.*, 1971; Weintraub *et al.*, 1971). Hence if stage-17-18 and stage-21 contained pure populations of RBC's one should see a 50% drop in the incorporation of label. The decrease of less than 50% is probably due to the lower level of incorporation of early polychromatophilic cells at stage-17-18. The S period of primitive late polychromatophilic erythrocytes (OE) has been reported to be about 11.9 hr (Campbell *et al.*, 1971; Weintraub *et al.*, 1971), which would suggest a 40% drop in incorporation between the P stage and the OE stage. The observed drop in incorporation is less than predicted in this experiment (Fig. 13), but more in agreement in a second experiment

Figure 12. Percentage groupings of RBC's (1,2,3,4,5,6) by different ratios of nuclear to cellular volume. RBC's were isolated from (A), 60 stage 17-18 embryos; (B), 30 stage 21 embryos; (C), 15 stage 27 embryos; and (D), 6 stage 29 embryos. Measurements made from photographs of glutaraldehyde-fixed, acid fuchsin-and toluidine blue-stained cells. Nuclei treated as spheres and volumes estimated from an average of two diameters measured at right angles. Cells considered prolate spheres and diameters measured along the major and minor axes. Thirty cells measured on each of five slides per embryonic stage. The width of each bar indicates $\pm$ one standard error of the center mean.

Table 3Incorporation of ^{14}C -thymidine into Primitive RBC's^a

Stage	CPM x 10^{-4} / 100 μg DNA ^b	μg DNA / 10^8 cells	CPM x 10^{-4} / 10^8 cells	
			Calculated ^c	Measured ^d
17-18	1.8	375	6.8	66.3
21	1.4	380	5.3	15.4
27	1.1	331	3.6	6.4

^aPrimitive RBC's from selected embryonic chicken stages were incubated with thymidine-2- ^{14}C as described in Fig. 13.

^bData for extracted DNA taken from Fig. 13.

^cCalculated from cpm/ μg DNA and the total micrograms DNA/cell.

^dAfter RBC incubation 100 μl samples were used to spot filter-disks and diluted ten times for cell counts.

Filter-disks washed in TCA (Methods, XVII B) and counted in 0.4% Omnifluor in toluene.

Figure 13. Incorporation of ^{14}C -thymidine into the DNA of primitive RBC's. Primitive RBC's from 62 stage 17-18, 38 stage 21, and 13 stage 27 embryos were incubated in MEM for 30 minutes in the presence of 10 μCi thymidine-2- ^{14}C . DNA was extracted from washed nuclei in PCA; counted in 0.3% PPO, 25% Triton X-114 in toluene and assayed for concentration by the diphenylamine test. Percentage of RBC types at each stage: 17-18) 98% Eb-EP, 2% P; 21) 6% Eb-EP, 94% P; 27) 80% OE, 8% ME, 12% DE. Experiment was repeated once, percentage of RBC types at each stage: 17-18) 95% Eb-EP, 5% P; 21) 4% Eb-EP, 96% P; 27) 91% OE, 4% ME, 5% DE. For the repeat experiment the CPM per 100 μg DNA was similar at stages 17-18 and 21, and lower (0.7×10^{-4} cmp/100 μg DNA) at stage 27 than the illustrated experiment.

Incorporation of thymidine 2-C¹⁴ (cpm x 10⁻⁴)
per 100ug DNA

(not shown) which included fewer immature definitive cells.

The differential uptake of labelled thymidine into DNA at the three stages examined is not due to a differential uptake of labelled thymidine into the cell since the label associated with the hot 10% TCA soluble fraction of the post-nuclear lysis supernatant is similar for all stages (slightly higher at stage-27). It is unlikely that the ^{14}C -thymidine label was converted into an RNA precursor and incorporated into RNA, since there was no indication of radioactivity labile to alkali (0.5 N KOH, 16 hr 37°C) in either the cell supernatant or the nuclear DNA. However, the incorporation of ^{14}C -thymidine into DNA would not reflect differences in the actual rate of DNA synthesis during maturation if the DNA-precursor pool sizes and thus the intracellular concentration of ^{14}C -thymidine varied with the stage.

Uptake of ^{14}C -thymidine was almost ten times higher at stage-17-18, three times higher at stage-21, and two times higher at stage-27 when assayed by precipitation of whole cells on filter disks (Methods XVI, B), rather than by calculation from ^{14}C -thymidine per microgram DNA in suspension (Table 3). Nonetheless, both sets of values for stages-21 and 27 agree on the same relative decrease in DNA synthesis from stages-21 to 27, but not from stages-17-18 to 21. Possibly immature cells are more efficient at trapping adventitious labelled thymidine on the filter disks.

B. RNA Synthesis

Blood cells from stage-17-18 to stage-27 embryos incorporate ^3H -uridine into RNA (Methods XVI, D) when incubated for 30 minutes in vitro. There was about a five fold drop in the incorporation per unit RNA from stages-17-18 to 21, and a two fold drop in specific activity between stages-21 and 27 (Fig. 14). Since the quantity of RNA per cell increased by about 50% from stages-17-18 to 21 and decreased at stage-27 by the same amount, the adjusted net synthetic rate fell about four fold from stages-17-18 to 21 and three fold from stages-21 to 27 (Table 4). When the synthetic rate is expressed per unit available template (100 μg DNA), the largest decline in synthetic rate still occurred from stages-17-18 to 21 (Table 5). However, the decrease in the RNA synthetic rate with maturation could also be due to an increase in unlabelled uridine in the RNA precursor pool at the orthochromatophilic stage.

Figure 14. Incorporation of ^3H -uridine into RNA in primitive RBC's. Primitive RBC's from 60 stage-17-18, 30 stage-21, and 10 stage-27 embryos were incubated in MEM for 30 minutes in the presence of 100 μCi uridine-5- ^3H . RNA was extracted in PCA from the post-nuclear supernatant and counted in 0.3% PPO, 25% Triton X-114, in toluene or assayed for concentration by the orcinol test. Percentage RBC types at each stage: 17-18) 95% Eb-EP, 5% P; 21) 6% Eb-EP, 94% P; 27) 90% OE, 10% DE.

Incorporation of uridine-5-H³ (cpm x 10⁻³)
per 100 ug RNA

Table 4

Incorporation of ^3H -uridine into Primitive RBC's^a

Stage	CPM $\times 10^{-3}$ / 100 μg RNA ^b	μg RNA/ 10 ⁸ cells	CPM $\times 10^{-4}/10^8$ cells	
			Calculated ^c	Measured ^d
17-18	3.23	485	1.57	21.1
21	0.65	690	0.45	4.8
27	0.40	390	0.16	1.2

^aPrimitive RBC's at the indicated embryonic stages were incubated with uridine-5- ^3H as described in Fig. 15.

^bData for extracted RNA taken from Fig. 15.

^cCalculated from cpm/ μg RNA and the total micrograms RNA/cell.

^dAfter RBC incubation 100 μl samples were used to spot filter-disks and diluted ten times for cell counts. Filter-disks washed in TCA (Methods XVI B) and counted in 0.4% Omnifluor in toluene.

Table 5Incorporation of ^3H -uridine per 100 micrograms DNA^a

stage	CMP x 10^{-3}
17-18	4.19
21	1.18
27	0.48

^aThe incorporation of ^3H -uridine per 100 micrograms DNA was calculated from an estimate of the quantity of RNA per cell (Table 4) and DNA per cell (Table 3) and the amount of ^3H -uridine incorporated per cell (Table 4).

Like the incorporation of ^{14}C -thymidine into primitive RBC's (Results, II A), the uptake of ^3H -uridine is higher when assayed by precipitation of whole cells on filter-disks than when calculated by incorporation of ^3H -uridine per microgram RNA (Table 4).

C. Template Capacity of Primitive RBC Chromatin

It is possible that the decrease in the RNA synthetic rate during maturation is caused by a decrease in the activity of RNA polymerase or a loss of factors needed for transcription, rather than a decrease in the availability of chromatin for transcription. Hence the template capacity of primitive RBC chromatin was estimated by using an exogenous RNA polymerase.

In Table 6 are shown some of the essentials for synthesis of polyribonucleotides by exogenous RNA polymerase. The reaction was dependent upon the addition of both RNA polymerase and a template, DNA. Some RNA was synthesized in the mixture lacking ATP, possibly due to contamination of the other nucleoside triphosphates with ATP (labelled triphosphates are synthesized with ATP as the donor). The rate of RNA synthesis was highest if the mixture contained both $1.0 \mu\text{moles MgCl}_2$ and $0.25 \mu\text{moles MnCl}_2$.

Washing the Gelman filters with 5% and 2% TCA appeared to be necessary to decrease the amount of background label adhering to filters, since filters washed with only 10% TCA trapped 20 times more radioactivity in control mixtures lacking RNA polymerase. The product of the reaction mixture was mostly RNA since the label trapped on the membrane filters was reduced to

Table 6

Requirements of the RNA Synthesis Reaction (In Vitro)

Reaction Mixture	pmoles UTP incorporated/ 0.35 ml/10 min.
Complete system ^a	612
- RNA polymerase	2
- template	2
- Mn ⁺⁺	197
- Mg ⁺⁺	372
- ATP	17
- RNA polymerase 10% TCA wash only ^c	39
+ RNase A ^b	5
+ 0.5 N KOH ^b	6

^aComplete system, incubated at 37°C for 10 minutes contained: 3.5 μ moles Tris-HCl; 0.1 μ moles GTP, CTP, ATP; 0.25 μ moles MnCl₂; 1.0 μ moles MgCl₂; 0.01 μ moles ¹⁴C-UTP; 5 units E. Coli. RNA polymerase; 9 μ g calf-thymus DNA (methods XVII).

^bAfter the 10 minute reaction period samples were incubated in 67 units RNase A for 1 hr or in 0.5 N KOH for 20 hr at 37°C.

^cFilters were usually also washed successively with 5% and 2% TCA.

background levels after alkali treatment or digestion with RNase. The addition of chromatin to the reaction mixture did not cause a significant increase in the quantity of ^{14}C -UTP trapped on filters, since in reaction mixtures lacking RNA polymerase, chromatin containing respectively 3.0, 9.0 and 17.0 μg of DNA trapped only 4.0, 3.0 and 4.0 pmoles of ^{14}C -UTP.

An ultraviolet absorption spectrum of the primitive RBC chromatin used for template assays was typical, with a peak at 260 nm, a shoulder at 280 nm, and a drop in absorbance at 320 nm (Fig. 15). The protein to DNA values estimated from the spectra of chromatin was similar to those estimated chemically for nuclei (Results, III), primitive RBC chromatin from stage-17-18 embryos had the highest ratio of protein to DNA. SDS polyacrylamide gels of proteins extracted from chromatin contained the usual pattern of histone bands (Results IV, E) plus some additional bands, the patterns of which differed in stage-17-18, stage-21 and adult RBC chromatin (not shown). The more condensed regions of chromatin can be separated from the more extended regions of chromatin by partial shearing and centrifugation (McCarthy and Duerksen, 1970). Applying this approach (Methods, VII) sheared chromatin from primitive and adult RBC's was respectively divided into a fraction which pelleted at 10,000 x g for 10 minutes (~75% of the DNA) and a fraction which remained in the supernatant, but could be pelleted at 30,000 x g for 20 hr. The latter fraction had a lower protein to DNA value than the 10,000 x g pellet. Since the assay for template

capacity assay was performed on the total chromatin preparation, it presumably reflects an average RNA synthetic activity of two different states of chromatin.

The template capacity of adult chicken RBC DNA, calf thymus DNA and primitive embryonic chicken chromatin at various concentrations was assayed in the presence of added E. coli. RNA polymerase (Fig. 16, 17). The template capacity of primitive RBC chromatin decreased from stage-17-18 to stage-21, but remained about the same from stage-21 to stage-27 (Fig. 17). Embryonic RBC chromatin had a lower template capacity than deproteinized adult chicken erythrocyte DNA; chromatin from stages 17-18, 21 and 27 respectively, 6%, 4% and 4% of the activity of naked DNA.

In my template assay system, the percentage template capacity was measured at saturating concentrations of chromatin; i.e., at a point where additional chromatin did not increase the incorporation of ^{14}C -UTP into (polymerized) RNA. If the different rates of transcription at the plateau level for RNA synthesis reflects the freedom of the polymerase to transcribe various regions of chromatin rather than different degrees of binding of RNA polymerase, then the concentration of chromatin necessary to produce a plateau level of RNA synthesis should be similar for all samples. This appears to be the case for primitive RBC chromatin, since the RNA synthetic plateau level is reached at the same concentration of chromatin (15 μg DNA) from RBC's of embryos at stages-17-18, 21, and 27. Chicken erythrocyte DNA and calf-thymus

Figure 15. Ultraviolet absorption spectrum of primitive RBC chromatin. A 235 to 395 nm spectrophotometric scan of stage 27 RBC sheared chromatin in 0.01 M Tris-HCl (Beckman Acta III spectrophotometer). Scans of stage 17-18, stage 21, and adult RBC chromatin are similar (not shown).

Figure 16. Template assay of calf-thymus DNA and adult chicken erythrocyte DNA. Calf-thymus DNA (five determinations per concentration point) ● and adult chicken erythrocyte DNA (one determination per concentration point) ▲ were assayed for template capacity in a complete reaction mixture (Table 6) containing ^{14}C -UTP for 10 minutes at 37°C . Polymerized RNA collected on Gelman filters and incorporation of ^{14}C -UMP measured by LS-counting of the filters in 15 ml 0.4% omnifluor in toluene (Methods, XVII).

pmoles C^{14} UMP Incorporated

Figure 17. Template assay of RBC chromatin from stage 17-18, stage 21 and stage 27 embryos. Reaction mixture as in Table 25, incubation for 10 minutes at 37°C. Polymerized RNA was collected on Gelman filters and radioactivity was measured as in Fig. 16.

Aliquots of chromatin were removed immediately before addition to the template reaction mixture for DNA estimation by the diphenylamine assay. For stage 17-18 ★ each point is the mean of three individual chromatin preparations (four determinations per concentration point); for stage 21 ● each represents two individual chromatin preparations (four determinations per concentration point); for stage 27 each □ represents three individual chromatin preparations (three determinations at each concentration point).

DNA may contain more polymerase binding sites, because the RNA synthetic plateau level is reached at 10 micrograms of DNA.

If one assumes that primitive RBC chromatin for all embryonic stages contain a similar number of RNA polymerase binding sites, the increased template capacity of chromatin from stage 17-18 RBC's compared to chromatin from stage 21 and stage 27 RBC's may reflect enhanced efficiency in making multiple transcripts from the same DNA sequences or transcription of additional DNA sequences. Schwartz et al. (1975) have attempted to resolve this dilemma for the increased template capacity of oviduct chromatin which follows estrogen stimulation. They found that the initial rate of elongation of E. coli RNA polymerase on estrogen-stimulated and control chromatin was similar, 360 nucleotides/min. However, stimulated chick oviduct chromatin initiated 2.4×10^{10} more chains per 100 micrograms DNA than unstimulated control chromatin. Estrogen-stimulated chromatin was also able to bind more RNA polymerase molecules than unstimulated control chromatin, but in neither case were all bound enzyme molecules utilized for chain elongation. However, their results are consistent with my original assumption, since the concentration of chromatin (DNA) needed to reach a plateau level of RNA synthesis was different for estrogen-stimulated and unstimulated chromatin.

In my study bacterial RNA polymerase rather than homologous avian RNA polymerase was used to estimate the template capacity of chromatin. At present there is no

evidence to suggest that homologous polymerases are more specific as template probes than heterologous polymerases. Competitive RNA-DNA hybridization studies have demonstrated that the labelled RNA synthesized from embryonic-mouse-liver chromatin, calf-thymus chromatin and pea chromatin by a homologous polymerase was similar to the RNA synthesized with heterologous polymerase (Paul and Gilmour, 1968; Bekhor et al., 1969; Smith et al., 1969). Heterologous RNA polymerase transcribes cell specific mRNA from the same cell's chromatin, but not from other cell chromatin in vitro (Axel et al., 1973; Wilson et al., 1975).

III. Composition of Primitive and Adult RBC Nuclei

Total histone content of RBC's was assayed by two methods, with somewhat different results. Analysis by turbidimetry in TCA (Methods VIII, B) revealed no significant change during primitive line maturation, but by the Lowry method showed a significant increase from the polychromatic (stage-21 embryos) to the orthochromatic (stage-27 embryos) erythrocyte stage (Table 7). Even when both measurements were conducted on the same samples, according to the former method, histone/DNA ratios remained low at about 0.7 from stage-17-18 through stage-29. In contrast adult blood samples handled similarly to the primitive blood samples yielded similar histone values for both methods of analysis (Table 7) and agreed with published results (Elgin and Bonner, 1973). The turbidimetric analysis shows greater variations between samples than analysis by the Lowry method, probably because it is more dependent on variables which affect the degree to which

Table 7

Mean Composition of Pooled Embryonic and Adult Red Blood Cell Nuclei

stage	Weight Ratio to DNA in Washed Nuclei				Residual Protein
	RNA	Histone, ^a TCA Method	Histone, ^b Lowry Method		
17-18(7) ^c	0.10	0.60 $\pm$.05 ^d	0.75 $\pm$.05		*1.19 $\pm$.10
21(8)	0.05	0.65 $\pm$.18	*0.74 $\pm$.07		*0.87 $\pm$.12
27(8)	0.05	0.73 $\pm$.10	*0.93 $\pm$.04		0.67 $\pm$.13
29(8)	0.03	*0.76 $\pm$.12	0.96 $\pm$.05		*0.43 $\pm$.10
Adult(8) ^c	0.05	*1.03 $\pm$.06	1.08 $\pm$.02		*0.20 $\pm$.03

^aTurbidimetric estimation of protein concentration in trichloroacetic acid (appendix 1).

^bColorimetric estimation of protein concentration by method of Lowry *et al.*, (1951) (appendix 1).

^cNumber of samples for protein determinations at each stage.

^d $\pm$ standard error.

^eBlood from 3 hens.

*Significant difference, Student's T-test, $p \leq .05$.

the protein aggregates and precipitates. Lowry method for histone quantitation will be preferred in the remainder of the thesis so that these results can be compared with the results of others.

Evidently most of the nuclear histone was extracted by the routine procedures, because the histone content of the third wash never exceeded 4.5% and fourth wash never exceeded 1% of the total extracted histone. It is possible that at the earlier stages histone was lost during nuclear washings, but unlikely since in the more extensive washing procedure used to prepare chromatin, the histone values did not differ significantly from the nuclear values shown. Acid extracted protein values were higher when nuclei were not homogenized in 0.1% Triton X-100, but this may be due to co-extraction of acid-soluble globin (Results IV, D). Also, it is unlikely that the lower values at stages-17-18 and 21 result from histone degradation during isolation, because when stage-17-18 RBC nuclei are incubated further in 3.0 mM CaCl_2 , 0.9% NaCl for 3 hr at 37°C , the total histone content is not lower than in unincubated nuclei (appendix 4).

The residual protein and RNA contents of nuclei (Table 7) decrease significantly as the cells pass from the Eb-EP (stage-17-18 embryos) stage to the P (stage-21 embryos) stage, and the residual protein content continues to decrease in mature primitive cells and in adult mature erythrocytes. This is consistent with the relative differences between embryonic and adult chicken RBC nuclei (Dingman and Sporn, 1964) and regenerating adult blood cells (Neelin et al., 1976).

IV. Acid-Soluble Proteins of Primitive Embryonic and Adult RBC Nuclei

A. Electrophoretic Separation of the Proteins in Nuclear Acid Extracts

The pattern of Amido Black stained bands after total acid extracts from primitive RBC nuclei were electrophoresed on 2.5 M urea 15% acrylamide gels does not change appreciably with primitive line maturation and was similar to the pattern obtained with acid extracts from adult chicken erythrocyte nuclei (Figs. 18 A-E, 20). This pattern of nine distinct peaks was consistent in 200 gels from 23 RBC preparations at stage 17-18; 130 gels from 14 RBC preparations at stage 21; 120 gels from 13 RBC preparations at stage 27; and 220 gels from 22 RBC preparations at stage 29. Protein peaks 6, 7 and 9 contained trailing edges in primitive RBC extracts and peaks 7 and 9 contained trailing edges in adult RBC extracts, suggesting heterogeneity. In some gels the trailing edge in peak 7 is resolved into two bands (Fig. 18 F, 20). Peak 4 consisted of two dense bands (a and b) partially separated by a lighter staining region. The most variable in intensity of staining was band 1, (Results IV,D; Fig. 22), which usually resolved into two components. In gels containing 6.25 M urea, relative mobilities of all protein bands were similar to those in 2.5 M urea, however peak 8 resolved into a single component and the trailing edge on peak 9 was more prominent in the former medium (Figs. 19, 21).

Figure 18. Densitometric tracings of 2.5 M urea 15% acrylamide gel electropherograms of primitive and adult RBC nuclear acid extracts. Acid (0.25 N H_2SO_4) extracts from embryonic RBC nuclei at: (A) stage 17-18; (B) stage 21; (C) stage 27; (D) stage 29; and (E) adult chicken erythrocyte nuclei. Electrophoresis at 1.6 ma/gel for 4½ hr in 7% acetic acid. The vertical dashed lines in A indicate where individual protein peak areas were cut for paper weight analysis.

B

OD 600 nm

OD 600 nm

c

Figure 19. Densitometric tracing of a 6.25 M urea 15% acrylamide gel electropherogram of a nuclear acid extract from stage 27 RBC's. Electrophoresis at 1.6 ma/gel for 4½ hr in 7% acetic acid.

Plate III. Pattern of Amido Black stained bands after embryonic and adult chicken histones were electrophoresed on 15% polyacrylamide gels. Electrophoresis at 1.6 ma/gel for 4½ hr in 7% acetic acid.

Figure 20. 2.5 M urea polyacrylamide gels of "freshly prepared" acid extracts from primitive RBC nuclei. RBC nuclei from (A) stage-17-18 embryos and (B) stage-21 embryos.

Figure 21. 6.25 M urea polyacrylamide gels of embryonic chicken histones and chromatographed adult chicken RBC histone fractions. Nuclear acid extracts from (A) stage-29 RBC's and (B) stage-27 embryonic cells; (C) adult histone fractions H1, H2 and H5, prepared according to Methods VIII, C and D and analysis reported in Results IV, D.

Figure 22. 2.5 M urea polyacrylamide gels of oxidized embryonic histones. Nuclear acid extracts from (A) stage-21 RBC's and (B) stage-27 RBC's were incubated in urea-potassium ferricyanide at 0°C for 1 hr before electrophoresis (Methods XI).

20

21

22

Percentages of peaks 1 + 7, 8, and 9 from successive embryonic stages did not differ significantly, but decreased in acid extracts from adult erythrocytes (Table 8). Peak 1 is combined with peak 7 for quantitation, because it is an oxidized dimer of the latter (Results IV.D). There was a significant increase in the percentage of peak 6 from stage 21 to stage 27 and again in the adult. Peak 4 increased and peak 2 decreased from stages 17-18 to 21, and both decreased in the adult. Acetylation and/or phosphorylation of a protein, would result in this protein migrating in this electrophoretic system with reduced mobility (Balhorn et al., 1972; Lake et al., 1972a; Sanders et al., 1973). It is possible that peaks 2 and 3 are post-synthetic modifications of the proteins in peak 4, because the total percentage of these species (peaks 2, 3, and 4) did not change with primitive line maturation, and there was a shift in the ratio of these peaks towards the faster migrating bands with maturation (Table 9). However, the ratio between subbands 4a and 4b, did not change with primitive line maturation (Table 9), but did decrease in the adult (not shown).

B. Storage of Nuclear Acid Extracts

The percentage of each protein band in polyacrylamide gels in Table 8 was obtained from "freshly prepared" nuclear extracts, electrophoresed within 30 minutes of preparation (about 8 hr after the first embryo was explanted). When nuclear extracts were stored in 0.25 N H_2SO_4 , 3.0 mM $CaCl_2$, 0.9% NaCl at $-15^\circ C$ for 5 days and then electrophoresed after quick-thawing in hot running

Table 8

Mean Percentage of Each Electrophoretic Band in Acid Extracts from Embryonic and Adult Chicken RBC Nuclei^a

stage	samples ^b	Gel Band Numbers ^c					1 + 7	8	9
		2	3	4	6				
17-18 (4) ^c	17	*1.4(.7) ^d	2.8(.7)	*10.8(1.0)	7.0(1.2)	21.4(1.7)	42.3(2.2)	14.3(1.2)	
21 (3)	9	*0.2(.2)	1.9(.6)	*12.9(.9)	*6.7(1.2)	21.0(1.0)	42.6(2.1)	14.7(1.8)	
27 (3)	16	tr	2.0(.7)	13.7(1.7)	*10.0(1.4)	20.7(2.0)	40.6(2.6)	13.0(1.8)	
29 (3)	9	tr	1.5(.5)	12.8(.8)	9.7(.5)	22.0(2.8)	41.0(2.5)	13.0(1.5)	
Adult (3)	10	-	0.2(.2)	*11.7(1.8)	*23.2(3.1)	18.8(1.9)	39.5(2.2)	11.6(3.1)*	

^aAcid extracts from embryonic and adult chicken RBC nuclei were resolved by polyacrylamide gel electrophoresis and the stained gels were scanned at 600 nm (Methods XI).

^bNumber of gels containing the indicated samples used for quantitation.

^cBracketed numbers indicate separate preparations of blood.

^dOne standard deviation.

^eSee Fig. 18 for Band Numbers.

*Significantly different, Student's T-Test, $p \leq .05$.

tr-trace (less than 0.1%).

Table 9

Ratio of Electrophoretic Band 3, 4a, 4b in Acid Extracts from
Embryonic RBC Nuclei^a

stage	3	4a	4b	4b/4a
17-18	1.0	1.4	2.4	1.7
21	1.0	2.6	4.1	1.6
27	1.0	2.6	4.2	1.5
29	1.0	3.3	5.2	1.6

^aSee Table 8; Fig. 18 A - D.

tap water, there was a change in percentages of protein bands in stage 17-18 samples, but not in stage 29 samples (Table 10). The initial protein degradation appeared to occur in peaks 2, 3, and 4 leading to an increase in peak 6. These peaks no longer correspond to sharply resolved bands, but instead the Amido Black dye was spread from band 3 to band 6 (Fig. 23, B). This spreading made it difficult to separate gel scan peaks for weighing, hence the standard deviations for the percentages of each peak were higher. In stage 17-18 samples after 30 days storage at -15°C the region from band 2 through 8 was poorly resolved, and an extra peak appeared in gel scans between peaks 8 and 9 (Fig. 23, C). The bands in the stage 29 sample, however, were still distinct after 30 days storage, except for some spreading of peak 7 into peak 6 (Fig. 23E) and for an increase in the percentage of peak 6 (Table 10). Adult erythrocyte nuclear acid extracts were also affected by prolonged storage (not shown), in agreement with previous findings (Vidali and Neelin, 1968a). Since the protein banding pattern of embryonic RBC nuclear extracts in polyacrylamide gels does appear to change after storage in acid at -15°C , all further analysis was performed on "freshly prepared" samples.

C. Acid Extraction of Primitive RBC Nuclei at Room Temperature

Moss et al. (1973) subjected embryonic RBC nuclei to a long (5 hr) acid extraction and reported that H5 (band 6) could not be detected in the total histone pattern from embryos at 4 days of incubation. Vidali and Neelin (1968a)

Figure 23. Densitometric tracings of polyacrylamide gel electropherograms of "freshly prepared" and frozen-stored nuclear acid extracts. 0.25 N H_2SO_4 extracts from embryonic RBC nuclei at: stage 17-18 (A), electrophoresed within 30 minutes of extraction; stage 17-18 (B), stored 5 days at -15°C ; stage 17-18 (C), stored 30 days at -15°C ; stage 29 (D), electrophoresed within 30 minutes of extraction; stage 29 (E), stored 30 days at -15°C . Samples were electrophoresed in 2.5 M urea, 15% acrylamide gels at 1.6 ma/gel for $4\frac{1}{2}$ hr.

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Table 10

Mean Percentage of Each Electrophoretic Band^a in "Freshly Prepared" and Frozen-Stored Acid Extracts from Stage-17-18 and Stage-29 RBC Nuclei

storage ^b	Gel samples ^c	Band Numbers ^a				1 + 7	8	9
		2	3	4	6			
Stage 17-18								
Fresh(4) ^d	17	1.4(.7) ^e	2.8(.7)	10.8(1.0)	7.0(1.2)	21.4(1.2)	42.3(2.2)	14.3(1.2)
5 days(2)	8	0.2(.2)	2.2(.9)	9.0(1.5)	8.9(2.5)	27.1(3.3)	37.8(3.1)	14.8(2.8)
30 days(2)	8	0.3(.3)	2.1(.4)	11.3(.9)	6.4(1.4)	24.4(4.9)	24.4(4.9)	15.8(1.2)
Stage 29								
Fresh(3)	9	tr	1.5(.5)	12.8(.8)	9.7(.5)	22.0(2.8)	41.0(2.5)	13.0(1.5)
5 days(2)	10	0.2(.2)	1.1(.3)	12.5(.8)	10.9(.5)	21.3(.9)	41.3(1.1)	12.6(1.0)
30 days(1)	5	0.3(.3)	1.4(.5)	12.3(1.1)	14.5(1.3)	19.2(2.7)	40.3(2.6)	12.0(.8)

^a As in Table 8.

^b Nuclear acid extracts either electrophoresed within 30 minutes of extraction ("freshly prepared"), or stored at -15°C for the times indicated.

^c Numbers in brackets indicate separate preparations of blood.

^e One standard deviation.

tr. trace; less than 0.1%.

reported that prolonged (overnight) extraction of avian erythrocyte nuclei at acid pH released additional non-histone basic protein without alteration of the histone fractions. These extractions were carried out in the cold ($0-4^{\circ}\text{C}$). In general

the rate of enzymatic degradation of proteins (Mihalyi, 1972) increases with increasing temperature. Hence, by raising the temperature during acid extraction of nuclei, one might be able to duplicate the effects of prolonged acid extraction, i.e., additional substances extracted and/or nuclear protein degradation.

Proteins extracted from nuclei at room temperature ($\sim 22^{\circ}\text{C}$) contained an additional slowly migrating band "X" on polyacrylamide gels in stage 17-18 samples, but not stage 29 samples (Fig. 24). The percentage loss in peak 8 in samples extracted at room temperature compared to samples extracted in the cold, was approximately equal to the percentage of the new component "X" (Table 11). Peak "X" may represent self-associated (aggregated) peak 8, H2, molecules. Both stage 17-18 and stage 29 extracts at room temperature revealed spreading of protein bands. In acid extracts from nuclei of stage 29 RBC's the elevation in temperature resulted in a decrease in the percentage of peak 4 with a comparable increase in peak 6. Otherwise, elevation of the extraction temperature to 22°C did not dramatically alter the pattern of histones from nuclear extract of stage 17-18 and stage 29 RBC's in polyacrylamide gels.

Figure 24. Densitometric tracings of polyacrylamide gel electropherograms of cold (0-4°C) and room temperature (22°C) acid extractions from embryonic RBC nuclei. 0.25 N H₂SO₄ extracts from embryonic RBC nuclei at: stage 17-18 (A), extraction at 0-4°C; stage 17-18 (B), extraction at 22°C; stage 29 (C), extraction at 0-4°C; stage 29 (D), extraction at 22°C.

Table 11

Mean Percentage of Each Electrophoretic Band^a in Acid Extracts at 0-4°C or 22°C from Stage-17, 18 and Stage-29.

Temp. ^b	Gel samples ^c X	2	3	4	6	1+7	8	9
Stage 17-18								
0-4°C(4) ^d	17	-	1.4(.7) ^e	2.8(.7)	10.8(1.0)	7.0(1.2)	21.4(1.7)	42.3(2.2)
22°C(1)	5	6.3(17)	.8(.6)	1.7(.2)	11.7(.9)	6.8(1.1)	20.2(2.9)	37.3(3.6)
								15.2(1.6)
Stage 29								
0-4°C(3)	9	-	tr	1.5(.5)	12.8(.8)	9.7(.5)	22.0(2.8)	41.0(2.5)
22°C(1)	5	-	0.2(.2)	1.4(.3)	11.0(.9)	12.2(1.1)	19.6(2.0)	41.6(2.5)
								14.0(1.0)

^a CAs in Table 8.

^b Acid extractions at either 0-4°C or room temperature (~22°C).

^d Bracketed numbers indicate separate preparations of blood.

^e One standard deviation.

D. Identification of Acid-Soluble Nuclear Proteins
by Acid-urea-polyacrylamide gel electrophoresis

Components of embryonic histone extracts were identified by parallel migration of co-electrophoresed, typical histone fractions from adult erythrocyte nuclei: band 4 a,b co-migrates with H1; band 6 co-migrates with H5; band 8 co-migrates with H2; and band 9 co-migrates with H4 (Figs. 21, 25-27). The major cytoplasmic protein (probably globin) had a similar electrophoretic mobility to H1 and band 4 (Fig. 25 C). Acid extracts from stage-27 nuclei prepared without a Triton X-100 homogenization had a higher percentage of H1 than nuclei prepared with such homogenization (18.5% vs. 13.5%), probably because of globin contamination; band 3 was not augmented significantly in such preparations. In "clean" preparations, the proportion of both band 3 and 4 decrease with maturation while the concentration of globin in the cell increases. Bands 1 and 7 respectively co-migrate with H3 in the oxidized and reduced stages (Figs. 22, 27). The percentage of band 1 increased in nuclear acid extracts which have been stored, incubated at 37°C in the presence of 6 M urea, or incubated in the cold in the presence of potassium ferricyanide (1 mg/ml). In each case the percentage of band 1 plus band 7 was similar to the percentage of band 7 in reduced samples (Table 12).

Figure 25. Densitometric tracings of polyacrylamide gel electropherograms of acid extracts from RBC nuclei and adult erythrocyte histone fractions H1 and H2. The samples include acid extracts of nuclei from (A) stage-29 RBC's and (B) adult erythrocytes, (C) Fraction A, containing H1 and H2, and (E) Fraction B, containing H2, both prepared from a 0.25 N H_2SO_4 extract of adult erythrocyte nuclei with fractionation by Amberlite CG-50 chromatography (Methods VIII, D); (D) 0.1 M citric acid extract, containing H1, from adult erythrocyte nuclei (Methods VIII, C). The main band from the post-nuclear supernatant (---) from lysed stage-27 RBC's is shown in (C). Electrophoresis at 1.6 ma/gel for 4½ hr in 2.5 M urea - 15% acrylamide gels.

Figure 26. Densitometric tracings of polyacrylamide gel electropherograms of embryonic RBC acid-soluble nuclear proteins and adult RBC histone fractions H5 and H3. The samples include acid extracts of nuclei from (A) RBC's of stage-27 embryos and (B) trunk cells of stage-27 embryos; (C) Fraction F, containing H5, extracted from adult erythrocyte nuclei in H_2SO_4 at pH 1.8, (Methods VIII, C); (D) and (E) Fractions C and D respectively containing H5 and H3, extracted from adult erythrocyte nuclei and chromatographed as in Figure 25 (Methods VIII, D). (E) Also shows fraction D containing mercaptoethanol (---). Electrophoresis as in Fig. 25.

A

B

C

D

E

Figure 27. Densitometric tracings of polyacrylamide gel electropherograms of embryonic RBC acid-soluble nuclear proteins and adult RBC histone fractions H3 and H4. Samples include acid extracts of nuclei from primitive RBC's of (A) stage-17-18 embryos and (B) stage-27 embryos. Fraction E, containing H3 and H4, was prepared from 0.25 N H_2SO_4 extraction of adult chicken erythrocytes by chromatography as in Fig. 25 (Methods **VIIID**), and electrophoresis as in Fig. 25, in the presence (C) and absence (D) of mercaptoethanol.

Table 12

Oxidation^a of Embryonic RBC Histones
(Percentage of Total Electrophoretic Components^b)

stage	Band Numbers ^a		
	1	7	1 + 7
17-18	-	21.4	21.4
	1.5	19.8	21.3
	3.6	18.0	21.6
	5.2	15.8	21.0
	9.1	11.6	20.7
29	0.2	21.4	21.6
	5.9	14.1	20.0

^aNuclear acid extracts were precipitated in 20 volumes of 95% ethanol (-15°C), dissolved in 6 M urea in HOH, incubated at 37°C (1 to 3 hr), aspirated intermittently during the incubation, lyophilized, dissolved in 7% acetic acid, and electrophoresed.

^bAs in Table 8.

Electropherograms of nuclear acid extracts from head, trunk (Fig. 26, B), and whole stage-27 embryos (Fig. 21, B) were typical of non-erythroid avian tissue in exhibiting all histone fractions except H5 (band 6). RBC's and non-erythroid tissue also differed in the electrophoretic properties of histone fraction H1 (band 4), since in 2.5 M urea gels this histone from erythroid tissue resolved into two bands of comparable staining intensity, whereas trunk H1 consisted largely of one primary band (Fig. 26, B).

When nuclear acid extracts were electrophoresed on 25 cm 15% acrylamide gels in 2.5 M urea the pattern of protein separation and the comparison of bands to adult histone fractions was similar to the 15 cm gels (Fig. 28). In this system peak 4 consisted of three bands, peak 2 consisted of two bands, and peak 9 resolved into two or three bands depending on the stage. Band 3 was not resolved from band 4a which consequently had an increased staining intensity.

E. Identification of Acid-Soluble Nuclear Proteins by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

With 0.1% SDS in 10, 12, or 15% polyacrylamide the following histone fractions could resolve in order of increasing mobility: oxidized H3 dimer, a mixture of H1 and H5, a mixture of H3 and H2b, and a mixture of H2a and H4 (Plate IV). When fractions H1 and H5 were electrophoresed separately, H5 showed a slightly higher relative mobility (.52) than H1 (.50) but when they were electrophoresed in the same gel the two bands bled (Figs. 29, 30). The

Figure 28. Densitometric tracings of polyacrylamide gel electropherograms of embryonic RBC acid-soluble nuclear proteins and adult RBC histone fractions H5 and H2. Amido Black stained bands 6 and 8 in nuclear acid extracts from (A) stage 21 RBC's and (B) stage 27 RBC's correspond in electrophoretic mobility respectively to (C) H5 and H2 prepared from adult erythrocyte nuclei according to the procedure in Figs. 25 and 26 (Methods VIII, fractions F and B, respectively). Electrophoresis at 1.6 ma/gel for 12 hr in 2.5 M urea - 15% acrylamide gels.

Plate IV. SDS-polyacrylamide gels of embryonic acid-soluble nuclear proteins and adult RBC histone fractions. Gel samples were dissolved in 2% SDS, 2% 2-mercaptoethanol in 0.02 M sodium phosphate buffer (pH 7.2) and heated in boiling water for five minutes. (Method XII, and electrophoresed at 8 ma/gel for 10 hr in 0.1% SDS, 15% acrylamide gels.

Figure 29. SDS-polyacrylamide gels of adult hen erythrocyte histone fractions: (A) H2 and (B) H1, H5, and H2. Histone was either successively extracted from nuclei in 0.1 N citric acid (to yield H1), and in H_2SO_4 at pH 1.8 or totally extracted from nuclei in 0.25 N H_2SO_4 ; then the extracts were respectively fractionated by cation-exchange chromatography on Amberlite CG-50 (Methods VIII) to give fraction F (H5) and fraction B (H2).

Figure 30. SDS-polyacrylamide gels of adult hen erythrocyte histone fractions (A) H1, H2a and (B) H5, H3. Histone was extracted from nuclei in 0.25 N H_2SO_4 and fractionated into the various histone components H1, H2a (Fraction A), H5, H3 (Fraction C) as in Fig. 29.

Figure 31. SDS-polyacrylamide gels of nuclear acid extracts from primitive RBC's of (A) stage-17-18 embryos and (B) stage-29 embryos.

Figure 32. SDS-polyacrylamide gels of nuclear acid extracts of (A) embryonic cells from stage-27 embryos and (B) RBC's from stage-29 embryos. (C) Re-electrophoresis in SDS-acrylamide of band 8 from five urea-polyacrylamide gels of acid extracts of nuclei from stage-17-18 RBC's (Methods XIII).

29

H1,5

H1

H2b

H2a

H5

H3

30

S1-

-γ

S2-
S3-

-z

A

B

31

S1-

S2-
S3-

A

B

C

32

spreading of band S-1 might be a result of the partial separation of H1 and H5 (Figs. 31, 32) or a subfraction of H1 (Fig. 31-2, band y). There appeared to be little nonhistone contamination in these samples, except possibly for the faint band Z (visible in the overloaded gel in Fig. 31-2). When the gels were not overloaded, these contaminating bands and subfractions of H1 were not visible and erythroid and non-erythroid tissue showed similar patterns (Fig. 32).

F. Identification of Acid-Soluble Nuclear Proteins by Selective Histone Extraction and Amino Acid Analysis

The concentration of acid influences the composition of extracted histone mixtures. The order of extraction of histones with decreasing pH has been reported to be H1, H5, H2b, H2a, H3, and H4, with the extractability of the very lysine-rich histone, H1, varying between pH 1.76 - 2; depending upon the source of nuclei (Murray *et al.*, 1968; Seligy and Neelin, 1970; Unilica, 1972; Brasch *et al.*, 1974). Histone fractions H1, H2b, and H5 have been extracted from adult erythrocyte nuclei with HCl at pH 1.8 (Vidali and Neelin, 1968b). Primitive RBC nuclei extracted with H_2SO_4 at pH 1.8 yield polyacrylamide gel bands 2, 3, 4a,b, 6, and 8 on (Fig. 33); corresponding in electrophoretic mobility to histone fractions H1, H5, and H2 respectively.

A more gradual series shows some distinctions among embryonic stages. Amido Black staining bands coincident with H1 are detectable in urea-polyacrylamide gels from

0.01 M citric acid extracts from RBC nuclei of stage-17-18 embryos (Fig. 34 A), but not from later stages including adult RBC's (not shown). Successive extracts from stage-17-18 RBC nuclei (Fig. 34) differed from stage-21, stage-27, and adult RBC nuclei in other aspects (Figs. 35 A-C, and 37 A-C) respectively.

The major histone extracted in 0.1 M citric acid from stage-21, stage-27, and adult RBC nuclei was H1 (band 4), while H1, H5, and H2 (bands 4, 6, 8) were extracted from stage-17-18 RBC nuclei. Histone fraction H5 (band 6) was the major histone fraction extracted in H_2SO_4 at pH 1.8 from stage-21, stage-27, and adult RBC nuclei, but histone fraction H2 (band 8) was the major histone fraction extracted at this pH from stage-17-18 RBC nuclei. The 0.25 N H_2SO_4 extract from all samples contained mostly H3, H2, and H4 (bands 7, 8, and 9) but the adult sample also contained a large proportion of H5. In all cases H5 (band 6) was more easily extracted from embryonic RBC nuclei than from adult RBC nuclei. H1, H2, and H5 may interact differently with chromatin of stage-17-18 RBC's than later stage primitive RBC's, perhaps because of post-synthetic modifications of the histones and/or interaction with non-histone chromosomal proteins. The non-histone protein content of stage-17-18 nuclei was higher than that of primitive RBC nuclei of later embryonic stages (Table 7). Brasch et al., (1974) separated the regenerating blood of anemic geese into mature and immature RBC populations, and found that H5 was fully extracted from immature RBC nuclei, but only partially extracted from mature RBC nuclei in 0.1 M

7

8

Figure 33. Densitometric tracings of polyacrylamide gel electropherograms of pH 1.8 and 0.25 N H_2SO_4 extracts of embryonic RBC nuclei. RBC nuclei from stage 21 embryos extracted in (A) H_2SO_4 at pH 1.8 and (B) 0.25 N H_2SO_4 , and from stage 27 embryos extracted in (C) H_2SO_4 at pH 1.8 and (D) 0.25 N H_2SO_4 . Similar patterns were obtained with pH 1.8 and 0.25 N H_2SO_4 extracts from stage 17-18 and stage 29 RBC nuclei (not shown).

Figure 34. Densitometric tracings of polyacrylamide gel electropherograms of histones selectively extracted from RBC nuclei of stage 17-18 embryos. Nuclei are successively extracted in the following solutions: (A) 0.01 M citric acid, (B) 0.1 M citric acid, (C) H_2SO_4 at pH 1.8, (D) 0.25 N H_2SO_4 .

Figure 35. Densitometric tracings of polyacrylamide gel electropherograms of histones selectively extracted from RBC nuclei of stage-21 embryos. Nuclei were successively extracted in 0.01 M citric acid followed by: (A) 0.1 M citric acid, (B) H_2SO_4 at pH 1.8, (C) 0.25 N H_2SO_4 .

Figure 36. Densitometric tracings of polyacrylamide gel electropherograms of histones selectively extracted from RBC nuclei of stage-27 embryos. Nuclei were successively extracted in 0.01 M citric acid followed by: (A) 0.1 M citric acid, (B) H_2SO_4 at pH 1.8, (C) 0.25 N H_2SO_4 .

2

Figure 37. Densitometric tracings of polyacrylamide gel electropherograms of histones selectively extracted from RBC nuclei of an adult hen. Nuclei were successively extracted in 0.01 M citric acid followed by: (A) 0.1 M citric acid, (B) H_2SO_4 at pH 1.8, (C) 0.25 N H_2SO_4 .

citric acid. The H5, more easily dissociated from immature RBC nuclei, had a higher content of alkali-labile phosphate, than the H5 extracted at a lower pH from mature RBC nuclei. However H1 was similarly extractable in 0.1 M citric acid in both cell populations, but initial extractions with 0.01 M citric acid, were not tried.

G. Identification of acid-soluble nuclear proteins by amino acid analysis of selectively extracted histones and of the contents of Amido Black staining bands in polyacrylamide gels

Unfractionated 0.1 M citric acid extract of the nuclei from adult hen erythrocytes and stage-27 RBC's and a 0.01 M citric acid extract of the nuclei of stage-17-18 RBC's were analyzed for amino acid composition (Methods XIV). The scanned polyacrylamide gels for each sample had indicated that the 0.1 M citric acid extract from adult RBC nuclei contained 89% H1, 9% H5, 2% H2; that from stage-27 RBC nuclei contained 93% H1, 5% H5, 2% H2; that from stage-17-18 RBC nuclei contained 83% H1, 10% H5, 7% H2. Since band 4 is the major species in each sample, it is no surprise that the amino acid analysis for each sample is similar (Table 13). The lysine:arginine ratios and the high lysine, proline, and alanine contents of these samples was indicative of H1, slightly contaminated with H5 (Vidali and Neelin, 1968). The serine content of each sample parallels the proportion of band 6, presumably reflecting the serine, contributed by H5; however, the 12% serine for stage-17-18 extracts is higher than can be accounted for by 10% H5. In all of the samples the glycine content is

higher than those reported for H1. Perhaps these discrepancies with published compositions arise from which co-extracted peptides, would not be revealed by the urea-polyacrylamide gel system used for histone separation.

The amino acid composition of a pH 1.8 extract of the nuclei from stage-27 RBC's (containing predominantly band 6; Fig. 36 B) was similar to that from adult RBC's (not shown) and approaches that of chromatographed adult histone fraction H5 (Table 14). The lower contents of basic amino acids in the pH 1.8 extracts reveal evident heterogeneity.

An attempt was made to extract proteins directly from gel bands of urea-polyacrylamide gels for amino acid analysis. Attempts to analyze bands extracted in 6 N HCl, 1 N H_2SO_4 , and 30% H_2O_2 were unsuccessful resulting in unidentifiable peaks and an ammonia peak, which obscured the lysine and arginine peaks. Analysis of gel bands extracted with SDS gave interpretable results (Table 15). The amino acid composition of SDS-extracted bands 6 and 8 were similar in both the adult and primitive RBC acid-soluble nuclear proteins. The lysine:arginine ratio for band 6 (2.7, 2.8) was similar to that for H5 (2.2), and the lysine:arginine ratio for band 8 (1.6, 1.7) was close to that for H2 (1.8, 1.7). However, the lysine content of gel band 6, from adults and embryos (stages-17-18 or 29), was only 60% of the lysine content of H5. This is probably due to co-extractants since the amino acid composition of the same adult sample (H5, fraction F), isolated conventionally, yielded an expected lysine content. Both SDS-extracted bands 6 and 8 contained a high serine content,

Table 13

Amino Acid Composition of Citric Acid Extracts of
Embryonic and Adult Chicken RBC Nuclei^a

stage	Adult	27	17-18	Adult
extract ^b	0.1 M	0.1 M	0.01 M	H1b ^c
amino acid				
serine	8	6	12	5
glutamic acid	4	4	3	4
proline	9	8	10	10
glycine	10	8	9	7
alanine	15	19	15	28
leucine	4	7	5	5
lysine	20	24	23	26
arginine	5	5	6	3
lysine/ arginine	4	4.8	3.8	8.7

^aAll values are expressed as a percentage of the total amino acids recovered.

^bCitric acid extracts from embryonic and adult RBC nuclei (Methods VIIIA). Molarity of citric acid is indicated.

^cVidali and Neelin (1968).

Table 14

Amino Acid Composition of pH 1.8 H_2SO_4 Extracts of Stage-27
and Adult Chicken RBC Nuclei^a

stage	27	Adult ^c	Adult ^b
amino acid		pH 1.8	
serine	11	11	14
glutamic acid	4	4	7
proline	7	7	7
glycine	7	7	4
alanine	16	15	10
leucine	5	6	4
lysine	18	19	26
arginine	6	8	12
lysine/arginine	3.0		2.2

^aAll values are expressed as a percentage of the total amino acids recovered.

^bHistone was extracted from adult RBC nuclei in H_2SO_4 at pH 1.8 and histone fraction H5 (fraction F) was separated from other components by cation-exchange chromatography on Amberlite CG-50 (Methods VIIIC).

Table 15

Amino Acid Composition of Proteins, SDS-extracted from Urea-Polyacrylamide Gel Band Slices or Purified by Chromatography^a

stage	29b	Adult ^c	Adult ^d	Adult ^e	17-18 ^f	Adult ^f	Adult ^e	17-18 ^h	Adult ⁱ
sample	Band 6	Band 6	H5	H5	Band 8	Band 8	H2	Band 1	H3
amino acids									
serine	17	14	14	10	12	12	9	6	4
glutamic acid	10	11	7	4	9	12	6	14	4
proline	7	7	7	9	7	9	3	4	4
glycine	10	9	4	5	11	11	9	15	5
alanine	14	12	10	17	12	12	10	11	13
leucine	6	6	4	4	9	7	14	8	9
lysine	14	16	26	24	14	13	16	11	10
arginine	5	8	12	11	8	8	8	9	13
lysine/arginine	2.8	2.7	2.2	2.2	1.7	1.6	1.8	1.2	0.8

^aAll values are expressed as a percentage of the total amino acids recovered. Amido Black staining bands, five in each sample, from urea-polyacrylamide gels extracted in SDS (Methods XIII).

^bBand 6 from a 0.25 N H_2SO_4 extract of nuclei from stage 29 RBC's after electrophoresis on urea-polyacrylamide gels.

^cBand 6 from a pH 1.8 extract of nuclei from adult RBC's after electrophoresis on urea-polyacrylamide gels.

^dChromatographic fraction F (histone fraction H5) from a pH 1.8 extract of nuclei from adult RBC's (Methods VIIIC).

^eVidal and Neelin, 1968.

^fBand 8 from a 0.25 N H_2SO_4 extract of nuclei from stage 17-18 or adult RBC's after electrophoresis on urea-polyacrylamide gels.

^gChromatographic fraction B (histone fraction H2) from a 0.25 N H_2SO_4 extract of nuclei from adult RBC's (Methods VIIID).

^hBand 1 from a 0.25 N H_2SO_4 extract of nuclei from stage 17-18 RBC's after electrophoresis on urea-polyacrylamide gels.

ⁱBrandt and Von Holt (1972).

but this appeared to be true of all of the histone fractions, including those isolated by conventional methods. The relative serine contents (band 6 > band 8 > band 1) were in the same order as H5, H2, and oxidized H3. The glutamic acid and glycine content was consistently high in all SDS-extracted bands, but not in purified histone fractions. It is possible that these represent gel contaminants. Also, some discrepancies in amino acid content probably are due to an unavoidable overlap in bands; e.g., band 6 slices contain some band 7 protein and band 7 slices contain some band 6 protein (Fig. 38).

In summary, the relative proportions of polyacrylamide gel bands 2, 3, 4, and 6 in nuclear acid extracts differed in successive primitive maturation stages (Table 8). Gel band 4 probably contains histone fraction H1 and gel band 6 probably contains histone fraction H5; based on their migration in urea-polyacrylamide and SDS-polyacrylamide gels, relative extractability from RBC nuclei and amino acid compositions. Also band 6, like H5 in adult chickens, was not detectable in non-erythroid tissue. Gel bands 2 and 3 may contain modified H1 molecules because: 1) the total percentage of bands 2, 3, and 4 does not change with maturation, but there is a shift with maturation in the ratio of these peaks toward the faster migrating bands; 2) they are extractable in H_2SO_4 at pH 1.8 with other lysine-rich histones; and 3) histone fractions from stage 17-18 and stage-21 RBC's differ only in the relative proportions of bands 2, 3, and 4; probably all derived from H1. Percentages of gel bands 1, 7, 8, and 9 containing histone fractions H3, H2, and H4 did not change appreciably.

Figure 38. SDS-polyacrylamide gels of bands extracted from urea-polyacrylamide gels. RBC's from stage-17-18 embryos were incubated for 1 hr in MEM containing 100 μ Ci 3 H-lysine; histones were extracted from nuclei in 0.25 N H_2SO_4 and electrophoresed on urea-polyacrylamide gels (see Fig. 18 for band numbers). Gel bands (A), 4a,b; (B), 6; and (C), 7 were cut out, protein extracted in SDS (Methods XIII), and electrophoresed on SDS-polyacrylamide gels.

Bands 6 and 7, respectively from electrophoresis of histone fraction F and fraction E were also sliced out of urea-polyacrylamide gels (Methods XIII), extracted in SDS, and electrophoresed in SDS-polyacrylamide gels in the same gel chamber as the above samples, to obtain the positions of H5 and H3 indicated by the arrows.

V. Histone Degradation During Nuclear Isolation and Histone Extraction

Since the results (section IV, A) suggest a smaller quantity of band 6 at earlier maturation stages and the presence of minor histone species (bands 2 and 3), it is important to consider the possibility that histones might be degraded during nuclear preparation and/or acid extraction of chromatin. The first step was to check for proteolysis in the procedure used for nuclear isolation and histone extraction. Mixed histones H1-H5-H2 in solution were degraded to a greater extent when incubated with the post-nuclear supernatant from stage 17-18 RBC's than when incubated with either the other nuclear washes from stage 17-18 RBC's or the post-nuclear supernatant from stage 29 RBC's (section A and B below). Histone degradation was not prevented by the addition of enzyme inhibitors (sodium bisulfite, PMSF, and TPCK) to the incubation media (section C). The next step was to examine the degradation of histones associated with chromatin (section D). When stage 17-18 RBC's and stage 29 RBC's were lysed in solution A and incubated at 37°C for 30 minutes or 3 hr, the histones of stage 17-18 RBC's were degraded but not those from stage 29 RBC's. Thus, in incubation of histones both in solution and bound to chromatin, the post-nuclear lysis supernatant from stage 17-18 RBC's appeared to have a higher activity of histone-proteolytic enzymes than a similar supernatant from stage 29 RBC's. However, histones in stage 29 chromatin were equally resistant to the degradative effects of the post-

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nuclear supernatant from stage-17-18 and stage-29 RBC's. It appeared, therefore, that not only do stage-17-18 RBC's appear to contain a higher activity of proteolytic enzymes, but that the histones bound to chromatin of stage-17-18 RBC's are more susceptible to degradation than histones bound to the chromatin of stage-29 RBC's; possibly because of their looser association with other chromatin components. Consideration of the evidence for these inferences follows.

A. Evidence for proteolytic enzymes in the post-
nuclear-supernatant of primitive RBC's

Samples of 50 μ g mixtures of adult chicken erythrocyte histones H1-H5-H2 (1:3:3) were incubated in each of the following media: distilled water, the solutions used for nuclear preparations and histone extraction, and the supernatants from nuclear preparations of stage-17-18 RBC's. The samples were then electrophoresed on polyacrylamide gels in order to detect products of histone proteolysis. These gels were not used for quantitating the degree of histone degradation, but as a preliminary test to decide where, during the nuclear isolation procedure, histone degradation (appearance of extra bands) would be the most prominent. Hence each gel does not contain the same quantity of histones. Solutions A through D and supernatants 1 through 4 (Fig. 39) were also run separately as controls. Incubation of the histone solution at 37°C in solutions A, B, C, and D did not affect the migration of bands corresponding to H1-H5-H2 in gels, except for some trailing of H5 in solutions B, C (not shown). Incubation at 37°C for 30 minutes with the post-nuclear supernatant 1 after cell lysis resulted in the appearance

Figure 39.

The procedure used for the extraction of histones from the nuclei of primitive RBC's. The following solutions were used in the procedure: (A) 0.05% saponin, 0.1% Triton X-100, 3.0 mM CaCl_2 , 0.9% NaCl; (B) 0.1% Triton X-100, 3.0 mM CaCl_2 , 0.9% NaCl; (C) 3.0 mM CaCl_2 , 0.9% NaCl; (D) 0.25 N H_2SO_4 , 3.0 mM CaCl_2 , 0.9% NaCl (Methods IV).

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Plate V. Polyacrylamide gels of adult histones H1-H5-H2 incubated with the various solutions used for nuclear preparation and histone extraction, and with the supernatants from nuclear preparations of stage-17-18 RBC's. (See figure 39). Samples of 50 μ l each of a 1 mg/ml preparation of adult chicken erythrocyte histones H1-H5-H2 in distilled water were incubated in the above media for 30 minutes at 37°C (Methods XV). Electrophoresis in 2.5 M urea, 15% polyacrylamide gels at 1.6 ma/gel for 4½ hr.

Figure 40. Histones H1-H5-H2 incubated with 50 μ l of the supernatants from a nuclear preparation of stage-17-18 RBC's. Supernatants: (A) 1, (B) 2, (C) 3, (D) 4.

Figure 41. Histones H1, H5, H2 incubated with various concentrations of the post-nuclear-supernatant 1 from lysis of stage-17-18 RBC's. Incubation in (A) distilled water and (B) 10 μ l, (C) 25 μ l, (D) 50 μ l, and (E) 75 μ l supernatant 1. (F) 75 μ l of supernatant 1 incubated in 50 μ l distilled water.

Figure 42. Histones H1-H5-H2 incubated with nuclear suspension 4 from stage-17-18 RBC's. Incubation of histones in (A) distilled water and (B) 50 μ l suspension 4. (C) 50 μ l suspension 4 incubated in 50 μ l distilled water.

A B C D

40

A B C D E F

41

A B C

42

of a trailing shoulder on H5, additional bands between H5 and H2, and an additional band ahead of H2 (Fig. 40, 1). The additional bands increased in number and intensity with an increase in volume of supernatant 1 (Fig. 41, 1-5) and were distinguishable from bands evident in the supernatant incubated alone (Fig. 41, 6). It is probable that the bands faster than H1 are proteolytic degradation products of histones. Incubation in supernatants 2, 3, and 4 produced an extra band between H1 and H5 (Fig. 40, 2-4). However, since both solutions B and C, and supernatants 2 and 3 similarly affected the migration of H5, it was not certain that the additional band between H1 and H5 (Fig. 40, 2 and 3) was the result of histone degradation. The spreading of H5 in incubations with suspension 4 (Fig. 42.2) was reproduced in only about 50% of the tests.

Histone degradation appears to be greater during incubations with the post-nuclear supernatant 1, than during incubations with the other nuclear washes. It is possible that cytoplasmic membrane-bound, proteolytic enzymes are released and adhere to chromatin during cell or nuclear lysis. Other researchers have reported the presence of histone-proteases associated with calf-thymus chromatin (Furlan and Jericijo, 1967b; Bartley and Chalkley, 1970), rat-liver chromatin (Chong et al., 1974; Carter and Chae, 1976), and cycad-pollen chromatin (Brandt et al., 1975); although it is not clear whether these proteases are chromatin proteins or represent cytoplasmic contamination (Stellwagen et al., 1968). However, Chong et al., (1974)

isolated a neutral protease from rat-liver chromatin which was not a cytoplasmic contaminant. It is possible that any endogenous proteolytic activity associated with the chromatin suspension of stage-17-18 RBC's went unnoticed, because of the acidity of the suspension. Primitive RBC's may also contain other proteolytic enzymes, which have optimal activities at different pH's. Calf-thymus chromatin has been reported to contain two histone-proteases, one active in an acid solution and the other active in a neutral solution (Furlan and Jericijo, 1967a). However, in the procedure used here for the preparation of nuclei and the extraction of histones, the post-nuclear supernatant appears to be the major source of proteolytic enzymes capable of degrading histones.

B. The extent of degradation of histone fractions

H1 - H5 - H2 when incubated in supernatant 1

Quantitative gel electrophoresis was used to assess the degree of degradation of histone (Methods XV) and to observe the capabilities of enzyme inhibitors to prevent this action (Results V, C).

The degree of degradation of histones H1 - H5 - H2 incubated with the post-nuclear supernatant 1 from stage-17-18 RBC's increased with time (Fig. 43). The three histone fractions were probably degraded proportionally, since the initial lag of H5 behind H1, and the final lag of H2 behind H1 and H5 in degradation, was probably due to electrophoretic overlap of the initial breakdown products of H1 with intact H5 and later breakdown products of H1 and H5 with intact H2. This may be consistent with the

Figure 43. The extent of H1-H5-H2 degradation during incubation with the post-nuclear-supernatant 1 from stage-17-18 RBC's. Samples of 50 μ l each of a 1 mg/ml preparation of Adult chicken erythrocyte histone fractions H1 $\bullet$ - $\bullet$, H5 $\blacktriangle$ - $\blacktriangle$, H2 $\square$ - $\square$, in distilled water, were incubated at 37°C with 75 μ l of the post-nuclear-supernatant 1 from a 0.1 ml RBC pellet from stage-17-18 RBC's. Degradation assessed by polyacrylamide gel electrophoresis of the samples. Weight of traced peaks from each histone fraction obtained from gel scans of the incubated samples expressed as a percentage of the weight of traced peaks in control incubations, which had been incubated with 75 μ l of solution A (Methods XV). Data were derived from two preparations of supernatant 1, incubations were repeated once per preparation, 3 gels per incubation. Brackets indicate one standard deviation.

Figure 44. Densitometric tracings of polyacrylamide gel electropherograms of histones H1 - H5 - H2 incubated in the post-nuclear supernatant 1 from stage-17-18 and stage-29 RBC's. Aliquots of 50 μ l from a mixture containing equal volumes of 1 mg/ml preparations of adult chicken erythrocyte histones H1 - H5 - H2, in distilled water were incubated for 1 hr at 37°C with 75 μ l of the following: (A) solution A; (B) supernatant 1 from 0.1 ml packed RBC's of stage 17-18 embryos; (C) supernatant 1 from 0.1 ml packed RBC's of stage 29 embryos. Supernatant 1 (75 μ l) from RBC's of stages-17-18 and 29 embryos incubated with only 50 μ l HOH (----) are also plotted on B and C respectively. 35 μ l of each incubation sample were electrophoresed at 1.6 ma/gel for 4½ hr on 2.5 M urea, 15% polyacrylamide gels.

eventual disappearance of the band evident between H1 and H5 at 30 minutes (Fig. 41, 5) and the concomitant prominence of a trailing edge on H5 at 1 hr. (Fig. 44 B). The supernatant protein (probably globin), which had a similar electrophoretic mobility to H1, was also degraded during the incubation period.

Incubation of H1-H5-H2 with the post-nuclear lysis supernatant 1 from stage-29 RBC's resulted in less degradation than incubations with stage-17-18 supernatant 1 (Fig. 44 c). After incubations in supernatant 1 from stage-29 RBC's for one hr at 37°C, only H2 was significantly reduced compared to the control (mean: 72% of the control).

C. Action of proteolytic enzyme inhibitors on histone degradation and the extraction of histones from
RBC nuclei

Protease inhibitors are used routinely to prevent the degradation of chromosomal proteins during chromatin preparation and protein extraction. Sodium bisulfite (10-50 mM) and PMSF (1 mM) have been used to inactivate the chromatin-associated, neutral proteases of rat liver and calf thymus (Panyim et al., 1968; Bartley and Chalkley, 1970; Chang et al., 1974; Carter and Chae, 1976). These proteases and the neutral protease of rabbit polymorphonuclear leucocytes have properties similar to chymotrypsin (Carter and Chae, 1976; Phillips and Johns, 1959; Davies et al., 1971). The chymotrypsin-specific inhibitor TPCK (Mihalyi, 1972) however, was effective in inhibiting the rabbit-polymorphonuclear leucocyte protease but not the calf-thymus protease.

Sodium bisulfite (15 mM), PMSF (1 and 5 mM), and TPCK (1 and 5 mM) did not prevent cell degradation of histones H1, H5, and H2 when incubated in supernatant 1 from stage-17-18 RBC's (Table 16), but TPCK, which forms a suspension upon addition to the supernatant, significantly decreased the extent of H1 and H5 degradation. Heating the supernatant for 5 minutes at 90°C eliminated evident histone degradation during the incubation period.

When primitive RBC's were lysed in the presence of 10 mM sodium bisulfite, the nuclei assume a reddish-brown coloration, which was not removed during the nuclear washes. Electrophoresed acid extracts of these nuclei revealed several bands not present in samples prepared without the inhibitor (Fig. 45 A, B). These additional bands are probably co-precipitated cytoplasmic proteins, the presence of which discourages the use of this proteolytic inhibitor for the study of chromosomal proteins in RBC's.

Polyacrylamide gels of nuclear acid extracts prepared from stage-17-18 and stage-29 RBC's lysed in the presence of 5 mM PMSF or 5 mM TPCK also contained additional bands (Fig. 45 C, D). Two slowly migrating bands (peak "X") were present in these gels. The percentage of peak "X" was approximately equal to the percentage loss of bands 8 and 9, especially occurring in band 8. This is similar to the aggregation of H2 in histones extracted at 22°C (Results IV, C). Possibly TPCK crosslinks histone molecules by alkylating the imidazole ring of histidine and guanidine groups of arginine, and then binding the phenylmethyl group

Table 16

Percentage of Histone Fractions H1, H5, H2 of the Control Remaining after Incubation with a Post-Nuclear Supernatant from Stage-17-18 RBC's^a

Inhibitor	H1	H5	H2
none	34(10.5) ^b	56(5.2)	47(5.1)
NaHSO ₃ 15 mM	33(3.2)	59(5.2)	47(2.0)
PMSF 1 mM	41(7.4)	48(6.5)	42(5.6)
PMSF 5 mM	40(1.0)	52(7.9)	54(3.9)
TPCK 1 mM	36(1.0)	56(1.0)	44(4.0)
TPCK 5 mM	47(1.0)	75(8.0)	59(5.4)
heated ^c	99(11.9)	94(9.0)	103(16.4)

^aSamples of 50 μ l each of a 1 mg/ml preparation of adult chicken erythrocyte histone fractions H1-H5-H2 in distilled water were incubated at 37°C for 1 hr with 75 μ l of the post-nuclear lysis supernatant 1 from a 0.1 ml RBC pellet of stage 17-18 embryos. Control samples were incubated with only 75 μ l solution A. Percentage of each histone fraction is expressed as in legend to Fig. 43. TPCK and PMSF were dissolved in isopropanol at 50 mM before addition to supernatant 1. (Isopropanol alone does not change the extent of degradation.)

^bOne standard deviation.

^cSupernatant 1 heated at 90°C for 5 minutes prior to incubation.

Figure 45. Densitometric tracings of polyacrylamide gel electropherograms of RBC histones from stage 17-18 embryos lysed in the presence of enzyme inhibitors. RBCs lysed in solution A containing no additions (A); (B) 10 mM sodium bisulfite; (C) 5 mM PMSF; (D) 5 mM TPCK.

to the hydroxyl group of serine (Mihalyi, 1972). Incubation of the samples in guanidinium chloride did not reverse the production of peak "X" as one might expect with TPCK complexes. TPCK and PMSF also promoted an increase in band 1 and an equivalent decrease in band 7. The percentage of band 6 in acid extracts from stage-17-18 RBC's lysed in the presence of PMSF and TPCK was 8.3% and 10.0% respectively, compared to 7.0% in samples without the proteolytic inhibitors. Stage-29 and adult RBC's lysed in the presence of TPCK contained 12.3% and 21.7% band 6 respectively, compared to 10.0% and 23.2% for control samples. Although stage-17-18 samples show a significant increase in band 6 when prepared in the presence of protease inhibitors, this is matched by increases in stage-29 samples and may merely reflect artifacts caused by protein aggregation in such samples.

D. Degradation of chromatin-bound histones

Incubations of histone fractions in solution suggested that the post-nuclear supernatant from the lysis of stage-17-18 RBC's contained proteolytic enzymes which degraded histones.

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If these enzymes associate with chromatin, they may degrade chromosomal proteins during the nuclear washes or acid extraction procedures. Hence the degradation of histones associated with chromatin had to be assessed.

When stage-29 RBC's were lysed in solution A and then incubated at 37°C for 30 minutes, there was no change in the histone pattern in polyacrylamide gels (Fig. 46 A) or in the percentage of each protein band (Table 17). In this assay band 6 is sub-divided into band 6 and band 5 (the trailing edge of band 6) in order to reveal any overlap with degraded band 4. When the incubation was extended to 3 hr, there was a significant increase in the percentage of gel bands 2 and 6, with a commensurate decrease in band 4 (Table 17). Some spreading was evident between gel bands 4 and 6 in 3 hr incubations, but otherwise the protein bands were sharply resolved and band 5 was unchanged (Fig. 46 B). Since in stage-29 samples even after 3 hr incubation at 37°C there was a small (2%) change in the proportions of two gel bands and little spreading of the gel bands due to protein degradation, it is probable that "freshly" prepared histone extracts after lysis for 30 minutes at 0-4°C, revealed the true percentage of each histone fraction associated with undegraded chromatin from RBC's of stage-29 embryos. This was supported by the absence of evident degradation of histones in stage-29 RBC nuclei incubated in the post-nuclear supernatant 1 from stage-29 RBC's (Fig. 47 A). Also, the total histone:DNA ratio for these incubation samples are not significantly different from "freshly" prepared nuclei (appendix 4). Hence, it should be feasible

to use the histone-polyacrylamide gel pattern of acid-soluble nuclear proteins from stage-29 RBC's as a reference to assess the protein degradative effects of the post-nuclear lysis supernatant from stage-17-18 RBC's.

When stage-17-18 RBC's were lysed in solution A and then incubated at 37°C for 30 minutes, there were alterations in the relative proportions of most gel bands (Table 17) and spreading of all bands (Fig. 46 C). This trend continued during a 3 hr incubation (Fig. 46 D).

These results and the results from the incubation of histones H1-H5-H2 in supernatant 1 of stage-17-18 and stage-29 RBC's (Results, V B) suggest that stage-17-18 RBC supernatant 1 contains a higher activity of histone proteolytic enzymes than the stage-29 RBC supernatant 1. However, histones in stage-29 RBC nuclei appear to be equally resistant to the degradative effects of stage-17-18 and stage-29 RBC supernatant 1 at 37°C (Fig. 47 A and B). Compared to "freshly" prepared samples, incubated samples showed a decrease in band 4 and an increase in bands 3 and 6 (Table 18). When the incubation of stage-29 RBC nuclei in stage-17-18 RBC supernatant 1 was extended to 3 hr, the percentage of bands 3 and 6 continued to increase, and band 7 decreased. A concomitant decrease in band 4 was not apparent, but a decrease in this band may be masked by co-extracted and electrophoretically coincident globin, since in this preparation the contamination in nuclei incubated in supernatant 1 for 3 hr. was more resistant to washing than other preparations. These results contrast with the incubation of stage-17-18 RBC nuclei in stage-17-18 RBC supernatant 1, where all of the gel scan peaks appeared to show some degradation (Fig. 46 D).

Figure 46. Densitometric tracings of polyacrylamide gel electropherograms of histones from stage-17-18 and stage-29 RBC nuclei incubated in solution A. RBC's from stage-29 embryos were lysed in solution A and incubated at 37°C for (A) 30 minutes, and (B) 3 hr; RBC's from stage-17-18 embryos lysed in solution A and incubated at 37°C for (C) 30 minutes, and (D) 3 hr.

Table 17

Mean Percentage of Each Electrophoretic Component in Acid Extracts from "Freshly" Prepared^a and Incubated^b Stage-17-18 and Stage-29 RBC Nuclei

Incubation	Gel Samples	Band Numbers ^a							
		2	3	4	5	6	1+7	8	
Stage-17-18									
Fresh(4) ^c	17	1.4(.7) ^d	2.8(.7)	10.8(1.0)	1.7(.4)	5.3(1.0)	21.4(1.7)	42.3(2.2)	14.3(1.2)
30 min.(1)	5	1.9(.7)	4.0(.8)	10.3(.9)	1.8(.6)	5.2(1.2)	27.3(2.8)	37.2(2.5)	12.3(2.2)
3 hr.(1)	5	3.8(.5)	4.6(.1)	13.2(1.0)	1.4(.5)	4.4(.5)	25.3(2.1)	35.1(3.0)	11.8(.4)
Stage-29									
Fresh(3)	9	tr	1.5(.5)	12.8(.8)	3.3(.3)	6.4(.5)	22.0(2.8)	41.0(2.5)	13.0(1.5)
30 min.(2)	7	0.4(.4)	2.1(.9)	12.0(.9)	3.8(.6)	6.0(.9)	20.7(1.5)	41.0(2.0)	14.0(.6)
3 hr.(1)	5	1.0(.5)	2.3(.3)	10.5(.6)	4.0(.7)	8.3(1.1)	20.0(2.7)	41.1(.6)	12.8(1.4)

^aSame as in Table 8.

^bStage 17-18 and 29 RBC's lysed in solution A and incubated at 37°C (Methods V).

^cBracketed numbers indicate separate preparations of blood.

^dOne standard deviation.

Figure 47. Densitometric tracings of polyacrylamide gel electropherograms of histones from the nuclei of stage-29 RBC's incubated in the post-nuclear supernatant 1 from stage-17-18 and stage-29 RBC's. Nuclei from stage-29 RBC's incubated at 37°C in the following: (A) stage-29 supernatant 1 for 30 minutes; stage 17-18 supernatant 1 for (B) 30 minutes and (C) 3 hr.

Table 18

Mean Percentage of Each Electrophoretic Component in Acid Extracts from "Freshly" Prepared^a and Incubated Stage-29 RBC Nuclei^b

Incubation	Gel Samples	Band numbers ^d						
		2	3	4	5	6	1+7	8
Fresh(3) ^c	9	tr	1.5(.5) ^d	12.8(.8)	3.3(.3)	6.4(.5)	22.0(2.8)	41.0(2.5)
Sol.A 30	5	tr	0.9(1.)	11.4(.7)	3.1(.6)	7.7(.7)	21.5(1.2)	40.9(2.5)
29 Sup.1 30	9	0.4(.5)	2.0(.8)	11.0(.5)	4.1(.6)	7.8(.7)	21.1(2.1)	40.1(2.0)
17-18 Sup.1 30	9	0.4(.3)	1.6(.5)	11.5(.9)	3.0(.7)	7.3(.8)	20.4(1.4)	41.2(1.0)
17-18 Sup.1 3hr.(1)	6	0.8(.5)	2.6(.6)	11.6(1.0)	3.9(.5)	9.2(.8)	18.7(.8)	39.7(1.7)

^aAs in Table 8.

^bNuclei from stage 29 RBC's incubated at 37°C for 30 minutes or 3 hr in the following: solution A, stage 29 supernatant 1, and stage 17-18 supernatant (Fig. 39).

^cBracketed numbers indicate separate preparations of blood.

^dOne standard deviation.

It would appear, therefore, that the susceptibility of histones in chromatin to proteolytic degradation differs at stage 17-18 and stage 29. This could be due to a difference in the intimacy of binding of histones to DNA perhaps as a consequence of modifications to the histone molecules themselves or because of their interactions with other chromosomal proteins or RNA's.

When stage-29 RBC's were lysed in stage-17-18 RBC supernatant 1 at 37°C for 30 minutes, and the nuclei were washed and incubated for 12 hr., at 37°C in 3.0 mM CaCl₂, 0.9% NaCl, all histone fractions were degraded and the histone:DNA ratio was significantly reduced compared to "freshly" prepared nuclei (not shown). In contrast, when stage-29 RBC's were lysed in stage-29 RBC supernatant 1 at 37°C for 30 minutes, and the nuclei are washed and incubated for 12 hr. at 37°C in 3.0 mM CaCl₂, 0.9% NaCl, only H1 and H5 appeared degraded (not shown). These results suggest the following: 1) cytoplasmic proteolytic enzymes can become associated with chromatin during cell lysis, and 2) stage 29 RBC histones probably are not modified directly to make them resistant to degradation by the stage 17-18 RBC post-nuclear supernatant, rather it would appear that stage 17-18 RBC histones are more susceptible to degradation than stage 29 RBC histones, because of their altered association with DNA (chromatin).

To what extent does the susceptibility of stage 17-18 RBC histones to degradation affect the results for the percentages of each histone fraction in "freshly" prepared samples? The sharpness of Amido-Black staining bands in

polyacrylamide gels of extracts from "freshly" prepared samples suggest that histone molecules are not appreciably degraded. The percentage of gel bands 4, 5 and 6 did not change after nuclei are incubated in supernatant 1 at 37°C for 30 minutes, which could indicate that any degradation which might occur in these fractions was not different enough to affect their quantitation. Even after 3 hr incubation at 37°C H5 (band 6) was not eliminated from stage-17-18 RBC's. One would expect that the degradative process would be slower at the reduced temperatures (0-4°C) used for nuclear preparations. Hence the remainder of this study will be based on the assumption that "freshly" prepared histone extracts from stage-17-18 RBC nuclei closely reflect the true proportion of each histone fraction in chromatin.

VI. Histone Stoichiometry in Primitive and Adult RBC Nuclei

The percentage area of stained gel scan peaks reflects the staining affinity of each histone fraction for Amido Black. In order to express these proportions in terms of protein weights, the relation between microgram quantities of each histone in electrophoretic bands and the intensity of Amido Black staining was determined (Methods XI). The staining with Amido Black was a linear function of the quantity of histone applied to the gel up to 15 μ g histone, (Fig. 48). Histone H1 had the least and H2 the most affinity for the dye. Areas under

Figure 48. Affinity of chicken histone for the Amido Black dye. Weighed quantities of adult chicken RBC H1 (0.1 M citric acid extract) ● , H5 (pH 1.8 extract, fraction F) ○ , H2 (0.25 N H_2SO_4 extract, fraction B) Δ , and H3 - H4 (0.25 N H_2SO_4 extract, fraction E) ■ were loaded onto polyacrylamide gels at various concentrations and subjected to electrophoresis (Methods XI). The quantity of each histone fraction loaded is plotted against the mean weighed area of paper corresponding to each electrophoretic peak (6 gels per concentration; one standard deviation for the weighed peaks is about 6% of the weight).

Table 19Mean Weight Ratio of Each Histone Fraction to DNA^a

stage	H1	H5	H3	H2	H4
17-18	.16	.06	.16	.28	.11
21	.16	.05	.16	.27	.11
27	.20	.11	.19	.32	.12
29	.19	.11	.20	.33	.13
Adult	.10	.24	.21	.37	.16

^aAreas under gel scan peaks (Table 8) were adjusted for differences in the staining of individual histone fractions in order to estimate the concentration in micrograms of histone fractions within an electrophoretic band (Methods XI). Quantity of each histone fraction per unit DNA was obtained by extrapolating from the total weight of histone in a gel scan to the histone concentration per unit DNA in nuclear extracts (Table 7). Gel bands 2, 3, and 4 were considered as histone fraction H1 (see appendix 6).

gel-scan peaks for individual histone extracts were adjusted for these differences in staining affinity in order to estimate the microgram concentration of histone within an electrophoretic band (appendix 5). The histone to DNA values (Table 7), the total quantity of histone in a gel scan, and the quantity of histone in an individual gel scan peak were used to obtain the quantity of each histone fraction per unit DNA (Table 19). The combined standard deviation for this procedure would include: 1) 6% for the relative staining affinities of the histone fractions; 2) about 15% for the weight of gel scan peaks for different samples at the same stage; and 3) a maximum of 10% for the histone concentration per unit DNA. The ratios of histone fractions to DNA was not different in stages 17-18 and 21, and in stages 27 and 29 (Table 19). The increase in H1, H3, H2, and H4 from stage 21 to stage 27 reflects a 28% increase in the total quantity of histone per unit DNA during that period. The twofold increase in H5, however, was significantly higher than the increase in other histone fractions. From mature primitive embryonic RBC's to definitive adult RBC's H1 decreased, H5 increased, and H2, H3, and H4 remained unchanged, when the increase in the total quantity of histone was considered.

In order to develop a model for chicken RBC chromatin structure, the relative molar ratios of the five major histone components in primitive embryonic and definitive adult RBC nuclei were measured.

The molar ratios of histone fractions H3, H2, and H4 did not change significantly during the maturation of the

Table 20

Mean Molar Ratios of Histone Fractions in Acid Extracts
from Primitive Embryonic and Definitive Adult RBC Nuclei^a

stage	H1	H5	H3	H2	H4
17-18	0.76	0.28	1.03	1.83	1.00
21					
27	0.82	0.48	1.14	2.06	1.00
29					
Adult	0.33	0.83	0.97	1.82	1.00

^aMean weight ratio of each histone fraction to DNA in Table 19 divided by its molecular weight and presented relative to the molar quantity of H4 (appendix 6). Molar ratios for stages 17-18 and 21, and stages 27 and 29 are similar.

primitive embryonic RBC line nor in the definitive adult RBC line (Table 20). If one considers the total standard deviation inherent in these values ($\sim 30\%$) and assumes that the molar ratios are integral values, the data would be consistent with the following molar ratios: H3, 1.0; H2, 2.0; and H4, 1.0. Histone H2, which is computed as a single component in this thesis, actually contains histone fractions H2a and H2b. The molar ratios of histone fractions H1 and H5 in nuclear extracts do not exhibit a simple integral relationship. H5 increased significantly from stages 21 to 27, and again in the adult; and H1 decreased significantly from the primitive RBC line to the adult RBC line. Consequently the molar ratios of H1 and H5 in primitive RBC's are almost a reversal of the ratios in adult RBC's.

VII. Partial Fractionation of Stage 29 RBC's

Since the circulating blood of stage 29 embryos contains some immature definitive RBC's, it is possible that the quantitative analysis of histone fractions at this stage does not fully represent the distribution of histone fractions in mature primitive RBC's. In an attempt to enrich the proportion of mature primitive cells used for histone analysis, blood from stage 29 embryos was centrifuged through a two-step barrier of 30% and 37% BSA (Methods II). The majority of the cells which pelleted through the 37% BSA were mature primitive erythrocytes (Table 21). The ratios for histone fractions to DNA in this population enriched in primitive ME's were not significantly different from the values obtained from whole (unfractionated) stage-29 blood (Table 22).

Table 21

Percentage of RBC Types from the Blood of Stage-29 Embryos
Fractionated in BSA^a

source	PME ^b	DE ,
UF Original ^c	66.4	33.6
Top	17.8	82.2
Med	20.0	80.0
Pellet	85.1	14.9

^aWashed stage 29 RBC's were layered over a two-step barrier of 30% and 37% BSA in mod. SBSS and centrifuged at 2,000 x g for 30 minutes. Cells were collected above the 30% barrier (Top), at the 30% and 37% BSA interface (Med.), and from the pellet. Collected cells were washed twice in SBSS, smeared on slides, and stained; cell types counted as for whole blood (Methods II). Repeated once.

^bPME, primitive mature erythrocytes; DE, definitive erythrocytes.

^cUnfractionated stage 29 blood.

Table 22

Mean Weight Ratio of Each Histone Fraction to DNA^a for
Whole and 37% BSA-Pelleted Stage-29 Blood^b

Source	gels	H1	H5	H3	H2	H4
Original ^c (3) ^d	9	.19	.11	.20	.33	.13
Pellet (2)	13	.19	.11	.18	.32	.12

^aAs in Table 19.

^bAs in Table 21.

^cUnfractionated stage 29 blood.

^dBracketed numbers indicate number of blood preparations.

VIII. Nuclear Protein Synthesis

The close association between histones and DNA in chromatin suggests that histone synthesis may parallel DNA replication. In many tissues the bulk of the histone synthesis appears to be synchronized with DNA synthesis (Sadgopal and Bonner, 1969; Balhorn et al., 1972; Gurley et al., 1972). Non-histone chromosomal protein show a more complex pattern of turnover, which is consistent with their tissue and species specificity and hypothesized role in gene regulation (Shelton and Allfrey, 1970; Teng and Hamilton, 1970; Spelsberg et al., 1972). However, several researchers have reported that H5 synthesis in RBC's of adult birds may not be closely coupled to DNA replication (Appels and Wells, 1972; Medvedev and Kirpicheva, 1972; Seligy et al., 1973). This point was investigated in the primitive RBC line.

Figure 49 shows the kinetics of L-³H-lysine incorporation into hot 10% TCA-precipitates by RBC preparations from stages 17-18, 21 and 27 embryos in vitro. In blood cell populations from stages 17-18 and 21 embryos amino acid incorporation was approximately linear for one hr, while for stage 27 incorporation is linear for the first 30 minutes and then continues at a decreasing rate up to one hr. The initial rates of incorporation were similar for the stage-17-18 and stage-21 blood cells and about 45% lower for stage-27 blood cells.

This pattern was reflected in the specific activity of the hot TCA-precipitates from the post-nuclear-supernatant after lysis of RBC's from each stage (Table 23, HS

Figure 49. Uptake of labelled lysine by hot, TCA-insoluble proteins of embryonic RBC's. RBC's (10^8) from 62 stage 17-18 embryos ●, 38 stage 21 embryos ○, and 13 stage 27 embryos □ were incubated for 1 hr in MEM in the presence of 3H -lysine ($10 \mu Ci/ml$) at $37.5^\circ C$ (Methods XVI A). At 15 minutes, 30 minutes, and 1 hr after the addition of label two 100 μl samples were removed: one 100 μl aliquot used to spot Whatman filter-disks and the other 100 μl aliquot diluted 10 times for cell counts (Methods XVI B).

From stages 21 to 27 there was a 35% increase in the quantity of protein per cell in this fraction and about a 54% decrease in specific activity; indicating about a 20 to 30% drop in synthetic rate. This appeared not to be due to changes in the uptake of free labelled lysine by the cells, since the cpm in the TCA-soluble fraction were similar for all stages (not shown), but could be due to dilution of the labeled precursor in the intracellular amino acid pool.

The specific activity of the microsomal fraction increases by 50% from stages 17-18 to 21, and decreases only slightly (16%) at stage 27 (Table 23). The increase from stage 17-18 to 21 could be due to a 75% increase in the amount of labeled lysine associated with a unit of RNA in the monosome peak of microsomal pellets centrifuged through a 15% to 30% (w/v) sucrose gradient (not shown). Possibly stage 21 microsomal pellets contain more newly synthesized ribosomes.

The nuclear residual proteins increased in specific activity from stage 17-18 to 21, and decreased by about the same amount at stage 27 (Table 23). Since the residual protein content of nuclei decreased significantly from stages 17-18 to 21 (Table 7), the net rate of residual protein synthesis at stage 21 was unchanged from stage 17-18 but it was halved by stage 27.

The specific activity of the histones in the nuclear acid extract was similar for stages 17-18 and 21, and decreased markedly by stage 27 (Table 23). The changes in histone content (Table 7) were not sufficient to appreciably

Table 23

Incorporation of ^3H -lysine into Protein Fractions of Primitive RBC's^a

Fraction	CPM X 10^{-4} / 100 μg protein ^b		
	17-18 ^c	21	27
microsomal ^d	4.20	6.49	5.44
HSSP ^e	18.36	19.34	9.02
histone ^f	11.82	12.27	3.37
RP ^f	5.72	8.63	4.39

^aRBC's from 62 stage 17-18, 38 stage 21, and 13 stage 27 embryos were incubated for 1 hr in MEM in the presence of ^3H -lysine (10 $\mu\text{Ci/ml}$) at 37.5°C (Methods XVI A).

Repeated once with ^{14}C -lysine (2.5 $\mu\text{Ci/ml}$).

^bProtein estimation by Lowry et al., 1955 (appendix 1).

^cStages of embryos.

^dPellet from the centrifugation of the post-nuclear cell lysis supernatant at 30,000 x g for 3 hr (Methods XVI E).

^eThe supernatant from the above centrifugation was mixed with five volumes of 10% TCA and heated at 90°C for 20 minutes. The resultant hot TCA-insoluble fraction was dissolved in 1 N NaOH, for protein radioactivity estimation (Methods XVI E).

^fHistone and residual protein of nuclei, see Table 7.

affect the net drop in histone synthesis. This pattern was similar for individual histone fractions, after radioactivity in each polyacrylamide-gel band (Fig. 50) was expressed per microgram histone and then normalized for differences in lysine content (Table 24). The rates of incorporation of labelled lysine into all histone fractions at each stage was generally similar, except for a higher synthetic rate for H3 and H4 at stage 17-18, a higher synthetic rate for H2, H3, and H4 at stage 21, and a higher synthetic rate for H5 at stage 27. The rate of incorporation of lysine into cytoplasmic proteins (HSSP) was several-fold higher than the rate of incorporation of lysine into histones, especially at stage 27. Late polychromatophilic erythrocytes and reticulocytes of adult chickens synthesize hemoglobin and other proteins after the cessation of RNA synthesis (Cameron and Prescott, 1963; Kabat and Attardi, 1967). This synthesis is accounted for by the translation of stable (long-lived) mRNA's (Scherrer and Marcaud, 1968). Whereas, histone synthesis at least in HeLa cells appears to be more dependent upon the continued synthesis of RNA (Stein et al. 1975a). Hence in stage-27 RBC's where the rate of RNA synthesis is low compared to other stages (Results II, E), one would expect to see a reduction in histone but not cytoplasmic protein synthesis. Nuclear proteins may also be synthesized at a lower rate than cytoplasmic proteins, because of a lag in the transport of newly synthesized proteins to the nucleus (Wu and Warner, 1971). This situation may not be as significant for histones as for nonhistones, because the elapsed time

Table 24

Incorporation of Radioactive Lysine into Polyacrylamide-Gel-Separated Histone Fractions and Cytoplasmic Protein of Primitive RBC's^a

stage	gels	CPM X 10 ⁻⁵ /μmole lysine ^b					HSSP ^c
		H1	H5	H3	H2	H4	
17-18 ^d	3	8.0	8.8	11.0	8.4	10.9	22.1
21 ^d	3	5.3	5.0	8.3	8.7	9.3	23.3
27 ^d	5	0.7	1.1	0.6	0.6	0.5	10.9
27 ^e	3	0.2	0.6		0.3		1.5

^aPrimitive RBC's labelled as in Table 23.

^bLysine labelled histone fractions separated on polyacrylamide gels (Methods XVI F). Radioactivity in each peak expressed per μg of protein included in that band. These values were then adjusted for the moles of lysine in each molecule of a histone fraction.

^cTCA-insoluble fraction of the post-nuclear cell lysis high speed supernatant (Table 23).

^d³H-lysine labelling, Fig. 50.

^e¹⁴C-lysine labelling, Fig. 51.

between histone synthesis and nuclear deposition has been reported to be less than 4 minutes for hepatoma tissue culture cells (Oliver et al., 1974).

The radioactive peaks correspond approximately with the protein bands in urea-polyacrylamide gels (Fig. 50). H1 was separated into several radioactive peaks, which could correspond to modified H1 molecules (Balhorn et al., 1972). Fraction H4 was divided into three radioactive peaks, the slowest peak predominates at stage-17-18 and the middle peak predominates at stage-21. The three peaks could represent H4 molecules containing different quantities of phosphate (Seligy and Neelin, 1973) and acetate modifying groups (Sanders et al., 1973). A radioactive peak appears between H1 and H5 at stage-27, this was considered a modified H5 for the values in Table 24, but even without it the specific activity of H5 would be higher than those of the other histones. There was some overlap between the radioactive peaks for H5 and H3 at stages-17-18 and 21, due probably to slower mobilities of modified H3 molecules. Hence the radioactive pattern for histone fractions H1, H5, and H2 extracted in H_2SO_4 at pH 1.8 was also examined (Fig. 51). The pattern of incorporation of labelled lysine into polyacrylamide bands containing histone fractions H1 and H5 was similar for both the total histone extract and the selective (pH 1.8) extract. Again the rate of incorporation of lysine into H5 was more than double that of the other fractions at stage-27 (Table 24).

Figure 50. Distribution of ^3H -lysine in 0.25 N H_2SO_4 nuclear extracts electrophoresed on polyacrylamide gels. Primitive RBC's from (A) stage 17-18, (B) stage 21, and (C) stage 27 embryos were incubated for 1 hr at 37.5°C in MEM in the presence of ^3H -lysine; 10 $\mu\text{Ci/ml}$ (Methods XVI A). Histones were extracted from nuclei in 0.25 N H_2SO_4 and separated on 2.5 M urea -15% polyacrylamide gels. (electrophoresis at 1.6 ma/gel for 4½ hr). Radioactive peaks were identified by co-electrophoresis of adult histone fractions (indicated above the peaks). The gels were cut into 1 mm slices. Each slice was incubated for 12 hr. in 100 μl 30% H_2O_2 at 50°C and analyzed for radioactivity as described under Methods XVI F.

Figure 51. Distribution of ^{14}C -lysine in pH 1.8 nuclear extracts electrophoresed on polyacrylamide gels. Primitive RBC's from (A) stage 17-18, (B) stage 21, and (C) stage 27 embryos were incubated for 1 hr at 37.5°C in MEM in the presence of ^{14}C -lysine; $1.0 \mu\text{Ci/ml}$ (Methods XVI A). Histones were extracted from nuclei in H_2SO_4 at pH 1.8. Histone fractions were separated and analyzed for radioactivity as in Fig. 50. Radioactive peaks were identified by co-electrophoresis of adult histone fractions (indicated above the peaks).

General Discussion

This study complements reports that primitive RBC's of the embryonic chicken pass through successive maturation stages characteristic of adult avian erythropoiesis (Romanoff, 1960; Lucas and Jamroz, 1961; Fraser, 1963; Edmonds, 1966; Ceresa-Castellani and Leone, 1969; Bruns and Ingram, 1973). Light microscopic identification of RBC maturation stages indicated that primitive cells appear to mature as a uniform population. This contrasts with the definitive erythroid line, where several cell maturation stages are in the circulation at the same time (Romanoff, 1960; Lucas and Jamroz, 1961). Thus one may isolate a particular primitive erythropoietic stage at a specific time in development and examine the nuclear changes, which accompany terminal differentiation.

Weintraub et al. (1971) have proposed that the primitive RBC line is released into the circulation in one wave of entry over a period of approximately 30 hours. This hypothesis predicts the existence of a rather large (30 hr) variation in the maturation stage of primitive cells at any particular time. In the electron microscopic investigation of primitive RBC maturation, Dardick (1975) often observed two or more RBC maturation stages in the same embryo; basophilic erythroblasts, early polychromatophilic erythrocytes, and polychromatophilic erythrocytes were all noted within the same embryo of 2.5 days incubation. In my study EB and EP cell types were counted as one group, because it was difficult to choose their precise stage. Perhaps subtle ultrastructural differences

among the various primitive-RBC-maturation stages are not readily discernible in air dried smears (Lucas and Jamroz, 1961 vs. Dardick, 1975). This is consistent with the finding that when primitive RBC's were grouped by different ratios of nuclear to cellular volume (N/C), the majority of the RBC's in stage-17-18 embryos had N/C values distributed between two groups; probably representing erythroblasts and early polychromatophilic erythrocytes.

However, blood populations from both stage-21 and stage-27 embryos were more homogeneous, as classified by both cytological identification and N/C grouping. The majority of the RBC's in stage-21 embryos were polychromatophilic erythrocytes and the majority of RBC's in stage-27 embryos were orthochromatophilic erythrocytes. The majority of the RBC's in stage-29 embryos were reticulocytes or mature primitive erythrocytes, but blood pellets from this stage embryos also contained RBC's from the definitive erythroid line.

The study of sequential differentiative events allows one to distinguish three main stages in the maturation of primitive erythroid cells (Fig. 52). The first stage is characterized by the presence of immature (Eb-EP) RBC's which are actively engaged in DNA, RNA, and protein synthesis. During the second stage, maturing (P) RBC's continue to synthesize protein at the same high level as immature cells, but the replicative and transcriptional activity of the cells has decreased. During the final stage mature (OE and ME) RBC's maintain the capacity to synthesize cell-specific proteins, but abolish or continue

Figure 52. A diagram representing relative changes in the
chromosomal protein content and metabolic
properties of primitive RBC's during
maturation. Symbols, , ,
-----, indicate the relative decrease in the
listed parameters.

Predominant Cell Type

Eb-EP

P

OE

Embryonic Stage

17-18

21

27

at a low level other cellular activities, among them cell division.

One of the common features of terminal differentiation in several different cell lines, is that once the capacity to synthesize cell-specific proteins is established, the cells divide a specific number of times (Papaconstantinou, 1967; Holtzer et al., 1973; Weintraub et al., 1973; Fantoni et al., 1975). Weintraub et al., (1971) have reported that the primitive erythroid line precursor cell gives rise to six generations of RBC's, the last division cycle occurring in RBC's from embryos of 5 days of incubation. However, some blood cells in late 5-day chicken embryos are apparently still capable of synthesizing DNA (Campbell et al., 1971). The successive decrease with maturation in the incorporation of ^{14}C -thymidine into DNA by primitive RBC's (observed in this report) corresponds to the increase in the primitive RBC generation time with maturation reported by others (Campbell et al., 1971; Weintraub et al., 1971). In my study stage-27 (5-day) RBC's incorporated ^{14}C -thymidine into DNA. Comparison of the DNA synthesis in RBC's from two preparations of stage-27 embryos, revealed a 40% lower incorporation of ^{14}C -thymidine into DNA from the RBC population which contained 60% fewer definitive erythroid cells. If the number of definitive erythroid cells in the RBC population from stage-27 embryos is extrapolated to zero and the incorporation of ^{14}C -thymidine adjusted for this extrapolation, then a pure population of primitive orthochromatophilic erythrocytes would still synthesize some DNA. If one considers the infrequency of mitotic

figures observed in orthochromatophilic erythrocytes, then it may be inferred that the majority of the primitive RBC's at this stage do not synthesize DNA, but they are presumably finishing the last division cycle.

Another common feature of terminal differentiation in several different cell lines is that the transcription of the genes for both cell-specific products and normal cell metabolism is discontinued early in maturation, and the messenger RNA's (mRNA) for the specialized proteins are stabilized (Papaconstantinou, 1967; Wilt, 1967; Fantoni et al., 1975). The transcription of the mRNA for globin appears to be an early event in primitive erythroid differentiation. RNA extracted from chicken blastodiscs at the head-fold stage was able to hybridize with DNA complementary to globin mRNA isolated from RBC's of a 5-day chicken embryo (Chan et al., 1974). The mRNA for globin is apparently stabilized, because when one-somite chicken embryos were cultured on a medium containing actinomycin D, detectable quantities of hemoglobin formed in 6-8-somite embryos (Hell, 1964; Wilt, 1965). In adult avian RBC's, RNA synthesis is highest in the early erythroid maturation stages and falls rapidly by the polychromatophilic erythrocyte stage (Cameron and Prescott, 1963; Scherrer et al., 1966; Attardi et al., 1970; Seligy et al., 1973). The erythroblast-early polychromatophilic erythrocyte stage (stage-17-18 embryos) may be considered the transcriptional phase of primitive RBC maturation, since both the incorporation of ^3H -uridine into RNA of RBC's pulsed in vivo and the incorporation of ^{14}C -UMP into RNA transcribed from chromatin by E. coli RNA polymerase

were highest at this stage. The decrease in the template capacity of RBC chromatin from stages-17-18 to-21, may reflect a loss in the transcription of ribosomal RNA rather than the loss of a cell-specific product (globin mRNA). The template capacity of stage-17-18 RBC chromatin for RNA synthesis was 6% of deproteinized DNA as compared to 14% of deproteinized DNA for the least mature population of fractionated anemic goose blood (Seligy et al., 1973) and 20% of deproteinized DNA for the least mature population of fractionated anemic pigeon blood (Casaryan and Andreeva, 1972). I feel that the template capacity observed, in this study for the chromatin of immature (Eb-EP) cells is more compatible with the differentiative-stage of the cells than that reported by others (Casaryan and Andreeva, 1972; Seligy et al., 1973) for the following reasons: 1) The proportion of chromatin in a condensed state does not vary much during primitive RBC maturation (Dardick, 1975). Thus only a relatively small amount of the genome should be available for transcription at any time during the maturation process. 2) When the capacity of chromatin to bind the basic dye acridine-orange was used as an assessment of the degree of chromatin condensation during chicken RBC maturation, erythroblast chromatin bound 1.7 times more dye than polychromatophilic erythrocyte chromatin (Kernell et al., 1971). This is consistent with the 1.5 fold similar in template capacity reported here for the same primitive RBC maturation stages, but contrasts with the 6-fold difference between the same adult RBC maturation stages reported by Seligy et al., (1973). It is unlikely that the unexpectedly low template capacity in stage-17-18 RBC chromatin was due to a mixture of high and low RNA synthetic capacities,

since in a preliminary autoradiographic analysis from stage-17-18 RBC's incubated in ^3H -uridine, all cell types were labelled (Urban, work in progress).

The similar template capacity for RNA synthesis of chromatin from polychromatophilic erythrocytes and orthochromatophilic erythrocytes reported in this thesis agrees with the results of others (Seligy et al., 1973), but contrast with a concomitant decrease in the incorporation of ^3H -uridine into the RNA of RBC's labelled in vivo. Possibly this is due to a decrease in the activity of RNA polymerase in orthochromatophilic erythrocytes. The RNA synthetic activity of orthochromatophilic erythrocytes is probably lower than that reported for stage-27 RBC's, because the immature definitive blood cells were probably synthesizing RNA at a higher rate than the mature primitive blood cells.

The introduction to this thesis considered the hypothesis that the structure and/or composition of chromatin influences the specific pattern of gene transcription during cell differentiation and maturation. Since much experimental evidence indicates that histones can assume a repressive association with DNA (Allfrey et al., 1963; Georgiev, 1969; Hnilica, 1972), they may act as genetic repressors during the process of terminal cell differentiation. The histone content of transcriptionally active chromatin has been reported to be the same as (DeBillis et al., 1969; Duerksen and McCarthy, 1971; Anderson et al., 1975) or lower than (Bonner et al.)

1973; Warnecke et al., 1973; Simpson, 1974) transcriptionally inactive chromatin. During development the histone to DNA ratios of chromatin have been reported to increase in proportion to a decrease in the template capacity of chromatin for RNA synthesis (Fambrough et al., 1968; Elgin and Hood, 1973). However, the histone to DNA ratios have been reported to be similar for chromatin from tissues with large differences in transcriptional activity (Elgin and Bonner, 1973). I found that the nuclear histone to DNA ratios of primitive RBC's increased from 0.74 in polychromatophilic erythrocytes to 0.93 in orthochromatophilic erythrocytes. Similar histone to DNA ratios have been reported by Dingman and Sporn (1964) for RBC's from 3½-day chicken embryos (0.80), but my values are lower than the ratios reported by Moss et al. (1973) for RBC's of 4-day chicken embryos (1.03). My results also contrast with the reported constancy in the nuclear histone to DNA ratios during adult RBC maturation (Appels et al. 1972), despite the fact that both cell lines undergo similar metabolic changes with maturation. Possibly factors other than an increase in the total nuclear histone to DNA ratios during RBC maturation may reflect the degree of chromatin inactivation.

The quantity of a particular histone fraction in chromatin may reflect the degree of chromatin inactivation. Histone H5 has been reported to increase during adult avian erythropoiesis (Purkayastha and Neelin, 1966; Adams and Neelin, 1972; Appels et al., 1972; Billet and Hindley, 1972; Mazen and Champagne, 1972; Sotirov and Johns, 1972).

Moss et al. (1973) detected only traces of H5 in primitive RBC's of 3 and 4 day chicken embryos and indicated that the relative content of this histone increased during development. Vidali and Allfrey (1974) reported that H5 increased from 6% of the total histone in erythroblasts of 7-day duck embryos to 28% in adult duck erythrocytes. It has also been suggested (Appels et al. 1972; Sotirov and Johns, 1972) but contested (Billett and Hindley, 1972; Mazen and Champagne, 1969), that H5 may replace H1 during definitive erythropoiesis. I have detected H5 as early as the erythroblast-early polychromatophilic erythrocyte stage in primitive RBC's of stage-17-18 chicken embryos. It is unlikely that all of the H5 (7% of the total histone) detected in stage-17-18 RBC's was localized in early polychromatophilic erythrocytes, because the level of H5 did not increase in stage-21 RBC's, where 96% of the RBC's were polychromatophilic erythrocytes. The level of H5 approximately doubled from the polychromatophilic erythrocyte stage to the orthochromatophilic erythrocyte stage, remained constant in mature primitive erythrocytes, and more than doubled again in adult erythrocytes. Our results for primitive RBC's do not support the contention that H5 replaces H1 during RBC maturation, because the increase in H5 was not accompanied by a decrease in H1. However, our results for definitive erythropoiesis were consistent with this contention, because the histone fraction to DNA ratios of H1 and H5 in primitive RBC's were almost a reversal of the ratios in adult RBC's.

H5 may be involved in genetic repression. Removal of H5 from chromatin increases template activity (Seligy and Neelin, 1970; Bolund and Johns, 1973; Seligy and Miyagi, 1974). Removal of H5 from pigeon erythrocyte chromatin brings the template capacity for RNA synthesis to the level of erythroblast chromatin (Casaryan and Andreeva, 1972). Reactivation of dormant erythrocyte nuclei in heterokaryons is associated with a loss of H5 from the erythrocyte nuclei (Appels et al., 1974). However, since H5 appears to be present at all stages of primitive and definitive erythroid maturation, a simple relationship between the presence of H5 and the cessation of nucleic acid synthesis is not tenable. Furthermore, H5 accumulation on chromatin during primitive RBC maturation does not appear to be directly related to nuclear repression, because the incorporation of ^3H -uridine into RNA, ^{14}C -thymidine into DNA, and ^{14}C -UTP into RNA (template activity) decreased before H5 increased.

Unilica (1972) suggested that if histones function in genetic repression, it would be reasonable to expect the biosynthesis of individual fractions to reflect the changing transcriptional activity of each cell type. However, if histones function at the permanent repression of genes not required by a cell after differentiation, then their biosynthesis should be invariable and parallel DNA replication. In this study, histones H3, H2, and H4 were synthesized in equivalent rates and their synthetic rate decreased during RBC maturation, from the polychromatophilic erythrocyte stage to the orthochromatophilic erythrocyte stage. H1 and H5 were not synthesized at a rate

equivalent to the other histones in PE cells, which may 223.
reflect separate lysine pools for H1 and H5, and H2, H3,
and H4. If the equivalent rate/^{of}H1, H3, H2, and H4 synthesis
in stage-27 RBC's resulted primarily from the incorporation
of labelled lysine into histones of immature and maturing
definitive RBC's, then the synthesis of these histone
fractions is probably coupled to the replication of DNA
(both low in orthochromatophilic erythrocytes). H5 may be
uniquely synthesized by orthochromatophilic erythrocytes,
because the synthetic rate of H5 was approximately twice the
synthetic rate of the other histone fractions in stage-27
RBC's. Others have reported that H5 is uniquely synthesized
late in RBC maturation (Appels and Wells, 1972; Medvedev
and Kirpicheva, 1972; Seligy et al., 1973). Perhaps H5
synthesis in mature RBC's represents turnover rather than
net accumulation on chromatin, because orthochromatophilic
erythrocytes already contain an increased quantity of H5
compared to maturing cells, and the level of H5 did not
increase in primitive mature erythrocytes. Appels and
Wells (1972) have suggested that in maturing and mature
RBC's, newly synthesized, non-phosphorylated H5 may replace
the phosphorylated H5 associated with chromatin, leading
to the transcriptional repression of chromatin. Alternatively
it is also possible that the higher rate of H5 synthesis in
stage-27 RBC's results from the inception of H5 accumulation
by maturing definitive line cells.

The apparent presence of H5 in erythroblasts may indicate
that its availability for interaction with DNA rather than
its presence in chromatin is important for gene repression
(Adams and Neelin, 1972). Perhaps the interaction of H5
with DNA at immature stages is modified by

acidic macromolecules (Casaryan and Andreeva, 1972) Seligy and Miyagi, 1969, 1974) and by direct reduction of H5 basicity (Vandegrift et al. 1974). In primitive RBC's the decrease in the proportion of nonhistone "residual" protein to DNA corresponds to the decrease in nucleic acid synthesis as cells mature from the erythroblast-early polychromatophilic to the polychromatophilic-erythrocyte stage. Brasch et al. (1974) reported that H5 was fully extracted from immature but only partially extracted from mature RBC's of anemic geese in 0.1 M citric acid. Extractability was found to be directly correlated with the phosphate content of H5. The authors suggested that the decrease in phosphate content of H5 during RBC maturation may be required to maintain the repressed state of the mature erythrocyte genome. I found that H5 was more easily extracted from erythroblast-early polychromatophilic-erythrocyte nuclei than from RBC nuclei of later maturation stages. In particular, the major histone extracted in 0.1 M citric acid from stage-21 and stage-27 RBC nuclei was H1, while H1, H5, and H2 were extracted from stage-17-18 RBC nuclei in 0.1 M citric acid. Furthermore, H1 was extracted in 0.01 M citric acid from stage-17-18 RBC nuclei, while apparently no histones were extracted in 0.01 M citric acid from RBC nuclei of later maturation stages. Perhaps the more easily extracted histones have been modified by phosphorylation. This is supported by the finding that polyacrylamide gel electrophoresis of histones extracted from stage-17-18 RBC nuclei revealed two Amido Black staining bands (bands 2

and 3) with a slightly lower relative mobility than H1 and similar in mobility to the phosphorylated H1's reported to be present in tissues with a high rate of cell division (Panyim and Chalkley, 1969; Balhorn et al., 1972). There was a decrease in the proportion of these two bands and a commensurate increase in the proportion of the faster migrating H1 band with primitive RBC maturation.

Perhaps H5 participates in erythrocyte chromatin condensation. Removal of H5 from erythrocyte nuclei results in some chromatin dispersion (Brasch et al., 1972; Miller et al., 1972; Seligy and Lurquin, 1973). During primitive RBC maturation, the increase in H5 at the orthochromatophilic erythrocyte stage observed in this study, corresponds to the primitive maturation stage at which Dardick (1975) observed the largest decrease in nuclear volume and the largest increase in the proportion of condensed chromatin. Although Dardick and Setterfield (1976) reported that the volume of condensed chromatin did not increase with primitive RBC maturation, they did observe a decrease in the volume occupied by dispersed fibrils with maturation. Possibly the increased quantity of H5 in mature RBC's places chromatin in an irreversibly repressed state by condensing the remaining diffuse chromatin and increasing the aggregation of the already condensed chromatin.

Adult hen erythrocyte nuclei contain more than twice as much H5 as do mature primitive erythrocyte nuclei, yet both RBC nuclei contain about the same volume of condensed chromatin (Dardick, 1975). Perhaps the amount of both H1 and H5 bound to DNA affects the degree of condensation

of chromatin, since there is a reciprocal relationship between H1 and H5 in mature primitive and mature definitive RBC nuclei. This is supported by the finding that an exchange of H5 and H1 between chicken-erythrocyte and calf-thymus chromatin had no significant effect on the template activity of either chromatin for RNA synthesis (Bolund and Johns, 1973).

Eukaryotic chromatin may be arranged in repeating globular subunits, each containing equal molar quantities of H3, H2a, H2b, and H4 and 200 base pairs of DNA (Kornberg, 1974). The molar ratios of histone fractions H3, H2, and H4 (1:2:1) remained constant during primitive RBC maturation and were similar in adult erythrocytes. Perhaps the nucleosome structure is retained during RBC maturation, but alterations in the molar ratios of H1 and H5 affect the degree of coalescence of the chromatin particles and thus generate the higher-order structure of the eukaryotic chromosome (Bradbury et al. 1975b; Oudet et al. 1975; Lewis et al. 1976). My ratios for adult chicken erythrocyte nuclei differ from those of Wright and Olins (1975), who found no simple relationship among these histone fractions. However, if one considers the error inherent in these calculations, both interpretations for histone stoichiometry in chicken erythrocyte chromatin are feasible.

In conclusion, concerning the role of H5 in avian erythropoiesis, I propose the following:

- 1) The acquisition of H5 by avian RBC's is an early erythropoietic event, which may function in the differentiation of the hemopoietic stem cell to the erythroid cell line.

- 2) In immature RBC's H5 may repress a large portion of the genome no longer required by a differentiated cell. However, in immature RBC's H5 may be less repressive than in maturing RBC's, because of its interaction with non-histone proteins and by a direct reduction of histone basicity through phosphorylation.
- 3) The increased quantity of H5 in mature RBC's may be necessary in order to place chromatin in an irreversibly repressed (condensed) state. In this context, the total quantity of H1 and H5 associated with DNA may be imported for chromatin condensation, rather than the individual levels.

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Appendix 1

Trichloroacetic acid turbidimetric estimation of histone concentration

Histones, prepared as described in Methods VIII B were dissolved in either 7.0% acetic acid or 0.25 N H_2SO_4 in 3.0 mM $CaCl_2$, 0.9% NaCl at the following concentrations: 25 $\mu g/ml$, 50 $\mu g/ml$, 100 $\mu g/ml$, 150 $\mu g/ml$, 250 $\mu g/ml$, 500 $\mu g/ml$ and 1,000 $\mu g/ml$. Then three aliquots of 0.6 ml from each histone solution were mixed with 3.0 ml of 1.1 M TCA and allowed to stand at room temperature before the optical density was measured at 400 nm against a blank, containing the solutions used for histone dilution and the same final concentration of TCA. Lambert-Beer's Law was followed at concentrations ranging between 50 and 500 $\mu g/ml$. Turbidity of histone in TCA was not affected by the presence of 0.25 N H_2SO_4 , the media in which histones were extracted from nuclei and the media in which histone concentrations were routinely estimated.

Lowry estimation of protein concentration

Ten mg of BSA or ten mg of histone, prepared as described in Methods VIII B were dissolved in 10 ml 2N NaOH for 10 minutes and then diluted to 1N NaOH with 10 ml glass-distilled water, and incubated at 60°C for 12 hr. Subsequent dilutions were made with 1N NaOH; concentration range 25 $\mu g/ml$ to 500 $\mu g/ml$. One ml samples (three per concentration) from the above dilutions and one ml samples of nuclear acid extracts precipitated in 10 volumes 95% ethanol at -15°C and then dissolved in 1N NaOH as described above, were treated sequentially as follows: 1) added 5 ml of Reagent C, 2)

agitated for one minute in a Vortex mixer, 3) allowed to stand at room temperature for 10 minutes, 4) added 0.5 ml of Folin Reagent, 5) agitated within 30 seconds for one minute in a Vortex mixer; 6) allowed to stand at room temperature for 30 minutes. Optical density was measured at 750 nm against a 1N NaOH blank, treated as described above. Lambert-Beer's Law was followed for both histone and BSA at concentrations ranging between 50 and 250 μ g protein.

Reagent A: 20 g Na_2CO_3 , 0.2 g NaK Tartrate in 1 L glass-distilled water.

Reagent B: 5.0 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1 L glass-distilled water.

Reagent C: Reagents A:B, 50:1.

Folin Reagent: 5 ml 2N phenol per 7 ml glass-distilled water.

Appendix 2

Diphenylamine estimation of DNA concentration

15 mg of calf-thymus DNA was dissolved in 30 ml of 10% PCA at 90°C for 10 minutes. PCA nuclear acids (Methods IX) and the above DNA solution were diluted in 10% PCA. Then two ml samples were mixed with 4 ml of a 1.5% diphenylamine solution, incubated for 16 hr at 30°C and optical density was recorded at 595 nm against a 10% PCA blank treated as described above. Lambert-Beer's Law was followed at concentrations ranging from 50 and 500 µg DNA per ml.

Acetaldehyde solution: 0.2 ml acetaldehyde per 100 ml glass-distilled water.

Diphenylamine stock solution: 1.5 g diphenylamine, 1.5 ml concentrated H_2SO_4 made up to 100 ml with glacial acetic acid. Within 30 minutes of utilization, 0.1 ml of the acetaldehyde solution was mixed into 20 ml of the diphenylamine stock solution.

UV estimation of DNA concentration

One mg of calf-thymus DNA per ml of 3.0 mM $CaCl_2$, 0.9% NaCl and subsequent dilutions of DNA in the same medium were added in 100 µl aliquots to 3 ml 10% SDS in glass-distilled water. The DNA was solubilized by forcing the suspension five times through a 20 gauge glass-syringe needle and the optical density was recorded at 260 nm against a SDS-saline blank treated as described above. Lambert-Beer's Law was followed at concentrations ranging from 250 to 1,000 µg DNA per ml. RBC nuclei and chromatin, prepared as previously described (Methods IV

and VII) were analyzed for DNA concentration as described above.

Orcinol estimation of RNA concentration

Ten mg of yeast RNA was solubilized in 10% PCA at 90°C for 10 minutes and serial dilutions were made in 10% PCA. Two ml of the above dilutions or two ml of RNA extracted from RBC's (Methods IV and XVI, D) were mixed with two ml orcinol solution, heated in boiling water for 20 minutes, cooled at 5°C for 10 minutes, and the optical density was recorded at 660 nm against a 10% PCA blank treated as described above. Lambert-Beer's Law was followed at concentrations ranging between 50 and 200 μ g RNA per ml.

Appendix 3RBC incubation medium for the estimation of nucleic acid synthesis

The incubation medium at pH 7.3 contained the following:

10 x MEM Spinner salt solution	50 ml
10 x amino acid mixture	50 ml
100 x MEM vitamin solution	5 ml
100 x antibiotic mixture	5 ml
L-glutamine solution	5 ml
7.5% (w/v) sodium carbonate	13.5 ml
glass-distilled water	371.5 ml

The above medium was filtered through a Sartorius membrane filter (0.45 μ m pore size).

RBC incubation medium for the estimation of protein synthesis

The incubation medium contained the same ingredients as described above, except the 10 x amino acid mixture was replaced by 50 ml of the following:

arginine	105 mg	phenylalanine	32 mg
cysteine	24 mg	threonine	48 mg
histidine	31 mg	tryptophan	10 mg
isoleucine	52 mg	tyrosine	35 mg
methionine	15 mg	valine	45 mg

per liter of glass-distilled water

Appendix 4Composition of pooled embryonic RBC nuclei after
incubation at 37°C in various media

Weight ratio of histone^a to DNA of RBC nuclei treated as follows: (A) "freshly prepared"; (B) incubated at 37°C for 3 hr in 3.0 mM CaCl₂, 0.9% NaCl; (C) stage-29 RBC's lysed in stage-17-18 RBC supernatant 1^b at 37°C for 30 minutes, and then the stage-29 nuclei were washed and incubated for 12 hr. at 37°C in 3.0 mM CaCl₂, 0.9% NaCl; (D) stage-29 RBC's lysed in stage-29 RBC supernatant 1^b at 37°C for 30 minutes, and then the stage-29 nuclei were washed and incubated for 12 hr at 37°C in 3.0 mM CaCl₂, 0.9% NaCl.

RBC source

of nuclei	Treatment	Histone/DNA
stage 17-18	A	0.70
stage 17-18	B	0.71
stage 29	A	0.92
stage 29	B	0.94
stage 29	C	0.81
stage 29	D	0.91

^aLowry et al. (1951) colorimetric estimation of protein concentration

^bSee Fig. 39

Appendix 5

Mean weight ratio of each histone fraction in polyacrylamide gels

Histones were quantitated by analysis of Amido Black stained bands in polyacrylamide gels of electrophoresed acid extracts from RBC nuclei (Methods IX). Areas under gel-scan peaks were estimated by tracing the peaks on paper, cutting out the peaks, and weighing. These weights were adjusted for differences in the staining of individual histone fractions in Fig. 48 in order to estimate the concentration in micrograms of histone fractions within an electrophoretic band. The quantity of each histone fraction per unit DNA in Table 19 was obtained by extrapolating from the total weight of histone in a gel scan to the histone concentration per unit DNA in nuclear extracts (Table 7). A sample calculation for a nuclear acid extract from stage 17-18 RBC's treated as described above follows:

The cut-out traced paper peaks, corresponding to histone fraction gel-scan peaks, weighed: 12.3 mg for H1, 10.8 mg for H5, 31.7 mg for H3, 60.1 mg for H2, and 22.5 mg for H4. These weights were divided by the slope of the Amido Black affinity curve (Fig. 48) for the corresponding adult histone fraction, to yield the following microgram histone concentration within an electrophoretic band: 5.5 μ g H1, 2.0 μ g H5; 5.6 μ g H3; 9.4 μ g H2 and 4.0 μ g H4. The total weight of histone in the gel scan, 26.5 μ g, corresponds to 35.3 μ g of DNA in nuclei (Table 7). Hence the weight ratio of each histone fraction to DNA was obtained by

dividing the histone microgram values above by 35.3 to
yield the following: 0.16 H1/DNA, 0.06 H5/DNA, 0.16 H3/
DNA, 0.27 H2/DNA, and 0.11 H4/DNA.

END

04 [REDACTED] 78

FIN