Metabolism of Histones in Avian Erythroid Cells†

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ABSTRACT: The synthesis and enzymatic modifications of histones by phosphorylation, acetylation, and methylation during erythroid cell maturation have been studied. All newly synthesized histones, H1, H5, H2a, H2b, H3, and H4 undergo phosphorylation; histones H2a, H2b, H3, and H4, are acetylated and histones H3 and H4 are methylated. This type of histone metabolism is common to all dividing cells and therefore may be related to the assembly of histones into chromatin subunits. In the nondividing reticulocytes, the synthesis of histone H5 continues, while all the other histones show negligible incorporation of [^{3}H]amino acids. Furthermore, the reticulocytes show a unique pattern of enzymatic modification: phosphorylation of histone H2b, acetylation of histones H2a, H2b, H3, and H4, and methylation of histones H3 and H4. These "differentiation-linked" modifications are not dependent on histone synthesis, nor related to RNA synthesis, but may be related to the reorganization of chromatin in preparation for genomic inactivation.

Enzymatic modification of histones following polypeptide chain synthesis has been documented in a number of systems (for review, see Hnilica, 1972). Four types of histone modification can occur, namely, phosphorylation (Kleinsmith et al., 1966), acetylation (Allfrey, 1964), methylation (DeLange and Smith, 1971), and ribosylation (for review, see Sugimura, 1973). The exact biological role of these reactions is unknown. However, chemical studies on the positions of acetylation, phosphorylation, and methylation in histones have demonstrated a small number of modified residues which are all clustered in the basic domain of the histone molecule (Sung and Dixon, 1970; Dixon et al., 1975). This region has been implicated in DNA binding, while the more hydrophobic portion of the molecule may interact with other histones or with acidic proteins. Clearly, enzymatic modification at these sites may alter its association with DNA, changing the structure and function of the chromatin.

Histones of the avian erythroid system have been studied extensively by others (for review see Hnilica, 1972). In addition to the five major somatic histones, there is a sixth avian erythrocyte-specific histone H5 (Neelin, 1964). In the present paper, we describe the phosphorylation, acetylation, and methylation of these histones. The aim of this work is to assess the generality, as well as the biological role of these enzymatic modifications, in cellular differentiation. The methodology employed in this paper also provides the basis for additional work presented in an accompanying paper.

Materials and Methods

Induction of Anemia. White leghorn hens were obtained from Southern Illinois University Poultry Farm. Anemia was induced by daily intramuscular injections of a 2.5% solution of 1-acetyl-2-phenylhydrazine-HCl in 47.5% ethanol (Williams, 1972). Following four to six injections with 0.4 ml/kg of body weight, the chickens usually developed severe anemia. The hematocrit decreased from 30 to about 15 and the proportion of immature reticulocytes in the peripheral blood rose from near zero to 95%, as demonstrated by brilliant cresyl blue staining (Lucas and Jamroz, 1961). The birds were used 24 h after the last injection.

Blood and Bone Marrow. "Nonanemic" and "anemic" chicken blood was obtained from chickens by cardiac puncture and/or decapitation. In both procedures, blood coagulation was prevented by the addition of 1/5 v/v of buffered saline–EDTA[1] (solution A containing 0.15 M NaCl, 0.01 M Hepes, 0.05 M EDTA, pH 7.4).

For preparation of bone-marrow cells from the chickens, the tibiae and femora were excised quickly from exsanguinated birds. A bone cutter was used to remove the two ends of the leg and thigh bones and the red marrow was flushed from the open ends with anticoagulant solution B (0.15 M NaCl, 0.01 M Hepes, 0.01 M EDTA, pH 7.4). To remove the residual marrow, the bones were further cracked open and washed several times with buffered saline solution C (0.15 M NaCl, 0.01 M Hepes, pH 7.4).

Cell Incubation. The blood and bone-marrow cell suspensions were filtered through four layers of cheesecloth and centrifuged at 2000*g* for 10 min at 4 °C. The sedimented cell pellets were suspended and centrifuged as before in incubation medium (modified Eagles spinner medium supplemented with 10 mM Hepes and 5% bovine foetal calf serum). The incorporation of ^{32}P (300–500 μCi/ml) was carried out at 40 °C in the incubation medium at a cell density of 1×10^9 cells/ml (roughly, 1 volume of packed cells/2 volumes of incubation medium). For acetylation and methylation experiments, the cells were first preincubated in a shaker water bath with 2×10^{-4} M cyclohexamide (Sigma) to inhibit protein synthesis. After the preincubation period, [^{14}C]acetate (20 μCi/ml) or [*methyl*-^{14}C]methionine (5 μCi/ml) was added to the cell suspension and incubation was continued for an additional 90 min in an atmosphere of 95% air and 5% CO_2.

Nuclei and Chromatin Preparations. Following incubation, an equal volume of ice-cold incubation medium was added to the cell suspensions and the mixture was chilled on ice to terminate the incorporation of the radioactive isotope. The cell suspensions were centrifuged at 2000*g* for 10 min and the cell pellet was washed once in buffered saline solution C. Nuclei were isolated from blood and bone marrow cells by the method of Panyim et al. (1971) with all steps performed at 4 °C. The washed cell pellet was stirred vigorously in washing medium containing 5% Triton X-100, 0.25 M sucrose, 0.01 M $MgCl_2$,

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[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

TABLE I: Amino Acid Composition of Histone H5.

	n mol	mol/100 mol	Residues
Asp	21.8	1.59	3
Thr	39.4	2.89	6
Ser	150.3	11.02	22
Glu	52.4	3.84	7
Pro	93.1	6.82	13
Gly	63.7	4.67	9
Ala	207.0	15.17	30
Val	53.4	3.91	8
Met	7.0	0.51	1
Ile	38.2	2.80	5
Leu	57.6	4.22	8
Tyr	16.0	1.17	3
Phe	6.8	0.50	1
Lys	360.4	26.4	52
His	28.4	2.08	4
Arg	168.6	12.36	24

0.05 M $NaHSO_3$, 0.01 M Tris at pH 7.4, and the lysed erythroid cells were centrifuged at 2000*g* for 10 min. The sedimented pellet was stirred and centrifuged again in washing medium. The pellet obtained was usually white and free from hemoglobin contamination. To remove the residual detergent and $MgCl_2$, the nuclear pellet was washed twice and centrifuged in a solution containing 0.02 M EDTA, 0.05 M $NaHSO_3$, and 0.01 M Tris-HCl at pH 8.0. The final pellet was either used immediately for chromatin preparation or stored at −20 °C.

Chromatin was prepared from isolated erythroid nuclei by the method of Bonner et al. (1968). The nuclei were lysed by homogenizing in 0.01 M Tris buffer (pH 8.0) in a Waring blender at low speed for 30 s. Chromatin was sedimented at 6000*g* for 20 min and washed once in the same buffer. The chromatin was stored at −20 °C.

Histone Isolation. Perchloric acid extraction was performed using a modification of the procedure of Johns and Diggle (1969). The chromatin or nuclear pellet was stirred vigorously in a beaker on ice, while an equal volume of 10% perchloric acid was added slowly. Stirring was continued for 1 h and the extract was centrifuged at 10 000*g* for 30 min. The supernatant containing histones H1 and H5 was desalted on a 5 × 100 cm Sephadex G-25 column, eluted with 0.01 M HCl, and lyophilized. The pellet was resolubilized in 6 M urea and 0.05 M HCl, and centrifuged. The supernatant containing histones H2a, H2b, H3, and H4 was desalted on a Sephadex G-25 column as described above. The desalted histone fraction was further purified by adsorption to and elution from a CM-cellulose column, as described by Sung and Dixon (1970).

For more quantitative recovery of histone H1 and H5, the chromatin or nuclei was first extracted with an equal volume (as in chromatin) or 10 volumes (as in nuclei) of 0.2 M HCl for 1 h by stirring the mixture on ice. The extract was centrifuged and the supernatant was desalted on a Sephadex G-25-column. Histones H2a, H2b, H3, H4, and other contaminating nucleoproteins were precipitated from the desalted supernatant by adding 70% perchloric acid dropwise to a final concentration of 5%. The suspension was stirred on ice for 1 h and then centrifuged at 10 000*g* for 10 min. The lysine-rich histones H1 and H5 in the supernatant were desalted on a Sephadex G-25 column in 0.01 M HCl and lyophilized. The protein pellet containing histone fractions H2, H3, and H4 was solubilized in 5 volumes of 6 M urea containing 0.05 N HCl and any insoluble clumps were removed by centrifugation. The urea supernatant was desalted on a Sephadex G-25 column eluted with 0.01 M HCl. The protein fractions were neutralized by the addition of 0.1 volume of 0.5 M lithium acetate buffer (pH 6.0) and the histones were purified by a cation-exchange CM-cellulose column as before.

Chromatography of Histones on Bio-Gel Columns. To prepare the histones for Bio-Gel chromatography, it is necessary to reduce and alkylate the disulfide bonds in H3. The CM-cellulose-purified histone fraction was dissolved in 5 ml of 0.1 M sodium borate buffer, pH 8.9, containing 8 M urea and 0.02 M dithiothreitol and incubated at room temperature for 20 min. Iodoacetamide (recrystallized from acetone–chloroform, 1:1, v/v) was then added to a final concentration of 0.04 M and the incubation was continued for an additional 30 min. The reaction was stopped by the addition of 6 M HCl to pH 2.0. The reduced and alkylated histone sample containing H2a, H2b, H3, and H4 was recombined with the perchloric acid solubilized H1 and H5 and applied directly to the Bio-Gel column. Alternatively, the sample was desalted on a Sephadex G-25 column, lyophilized, and then chromatographed on the Bio-Gel column. These conditions are appropriately indicated in the legend to the figures.

In early experiments, we used two Bio-Gel P-10 columns (3 × 200 cm) connected in series and eluted with 0.01 M HCl with 0.01% sodium azide (as a preservative) according to Sung and Dixon (1970). In this procedure, the total chicken histones were eluted from the column in the following order: H1, H5, H2a, H2b, H3, and then H4. All the histones were well resolved, except H2a and H2b which were eluted on the ascending and descending portion of a single peak (Figure 2). Later, H2a and H2b were resolved from each other on a single Bio-Gel P-60 column (3 × 200 cm), which was also eluted with 0.01 M HCl (Figure 4 and 6). The Bio-Gel column purified histones have been shown to be suitable for sequence work as well as for the chemical characterization of the sites of modifications in individual histones (for collective references, see Dixon et al., 1975).

Since histone H5 has not been previously isolated on a Bio-Gel column from total chicken histones, we attempted to establish its purity. A sample of the Bio-Gel P-10 fractionated H5 (Figure 2) was hydrolyzed in 6 N constant boiling HCl and its amino acid composition analyzed. The amino acid composition (Table I) is similar to that reported by other workers. The homogeneity of histone H5 was further examined in a Panyim and Chalkley (1969) 10% polyacrylamide gel (Figure 1b). The single-stained zone seen in polyacrylamide gel electrophoresis corroborates the purity indicated by amino acid composition data.

Starch and Polyacrylamide Gel Electrophoresis. Histones were analyzed by electrophoresis in the urea–aluminum lactate starch gel system (Sung and Smithies, 1969), as modified by Sung and Dixon (1970). The electrophoresis was carried out for 16 h at 8–12 V/cm in a water-cooled gel tray using methyl green as a tracking dye (the dye migrates approximately twice as fast as the histones). Following electrophoresis, the gel was sliced and stained by the guanidinium-specific staining method (Sung and Smithies, 1969) and/or by the conventional amido black 10B stain. Alternatively, the stained zones may be conveniently viewed by rapid surface staining for 1 min with Coomassie brilliant blue and washed with tap water.

The advantage of the urea–aluminum lactate starch gel system is that it is highly resolving for phosphorylated histones. A significant difference is noted when a highly phosphorylated H5 was compared by electrophoresis in urea–aluminum lactate starch gel and a Panyim and Chalkley (1969) urea–acetic acid,

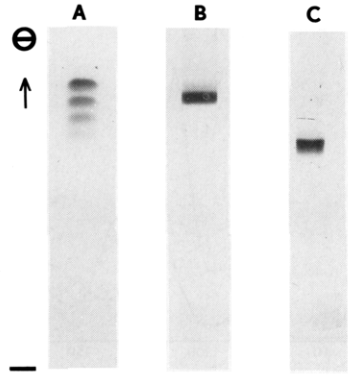

FIGURE 1: Comparative gel electrophoresis of highly phosphorylated histone H5 in (A) urea–aluminum lactate starch gel, (B) 10% acetic acid–urea–polyacrylamide gel (Panyim and Chalkley, 1969), and (C) 10% urea–aluminum lactate–polyacrylamide gel.

10% polyacrylamide gel. The phosphorylated components are resolved in the starch gel (Figure 1A) but not in the polyacrylamide gel (Figure 1B). The same result was obtained when the urea–aluminum lactate buffer was substituted for the urea–acetic acid in polyacrylamide gel (Figure 1C).

Amino Acid Analysis. Quantitative amino acid analysis was performed with the Beckman 120C amino acid analyzer. Semiquantitative amino acid analysis of column fractions was performed by one-dimensional high-voltage paper electrophoresis in 6.7% formic acid using a Gilson, Model DW, Electrophorator (10 000 V and 500 mA), as described by Dreyer and Bynum (1967).

Results and Discussion

One extremely convenient aspect of the erythropoietic system is the physiological and anatomical separation of blood and bone marrow tissues. In the adult chicken, 95% of the circulating cells are mature red blood cells. During phenylhydrazine-induced anemia, the proportion of immature cells may be increased dramatically such that 90% of the blood cells are late- or mid-polychromatic (the majority being reticulocytes). In the anemic bone marrow tissue, nearly 75% of the cell population may be very early and immature, i.e., erythroblast, early, and mid-polychromatic. These three classes of tissues form a developmental sequence in which the dividing and metabolically active cells in the anemic bone marrow progress to the metabolically active, nondividing cells in the anemic blood and then become inert mature erythrocytes. Since large quantities of tissues which show enrichment in certain cell types are readily available, the system lends itself to many biochemical investigations. We studied the synthesis, phosphorylation, acetylation, and methylation of histones in these three metabolically distinct cell types.

Histone Synthesis. Histone synthesis occurs during S phase of the cell cycle (Robbins and Borun, 1967), and is coupled to DNA replication. Accordingly, inhibition of DNA synthesis brings about a concomitant decrease in histone synthesis. During avian erythropoiesis, DNA synthesis occurs exclusively in the dividing erythroblasts of the anemic bone marrow tissue, while the reticulocytes of the anemic blood and the mature erythrocytes are nondividing cells and do not synthesize DNA. This implies that the bulk of histone synthesis occurs in the very immature cells of the anemic bone marrow. When anemic bone marrow cells are incubated in the presence of [^{3}H]lysine and [^{3}H]arginine, all six histone species contain label (figure 2A). By contrast, in nondividing reticulocytes, the five ubiquitous

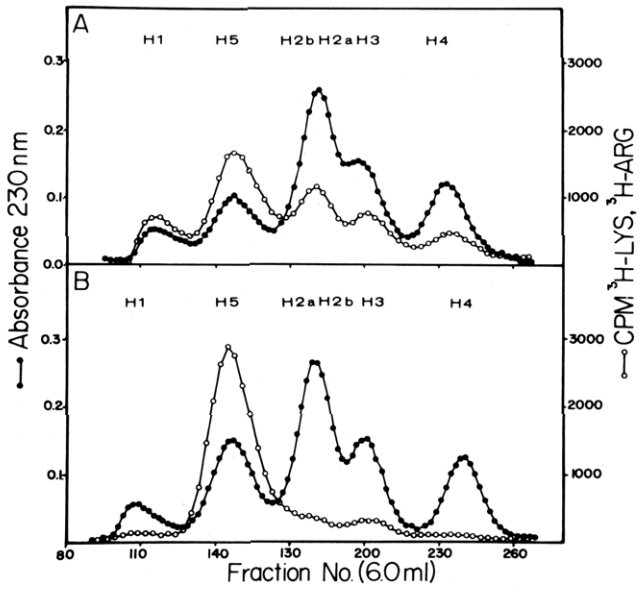

FIGURE 2: Pattern of histone synthesis in anemic bone marrow (A) and anemic blood (B) as analyzed by Bio-Gel P-10 chromatography. Erythroid cell suspensions were centrifuged and resuspended in modified Eagle's medium and incubated for 1.5 h as described under Methods. Histones were extracted, purified, reduced and alkylated to convert histone III dimer to the monomer form, and the protein was applied to a 3.0 × 200 cm Bio-Gel P-10 column. The first 500 ml was discarded and 6-ml fractions were collected. Radioactivity (0.2-ml aliquots) and absorbance at 230 nm in the column eluant fractions were monitored.

histones (H1, H2a, H2b, H3, and H4) show only background level of labeling; only the avian-specific histone H5 continues to incorporate radioactive amino acids (Figure 2B). Our observation on the selective synthesis of histone H5 in nondividing cells is in good agreement with the work of Appels and Wells (1972) and indicates that the synthesis of this unique histone is not dependent on DNA synthesis.

The specific radioactivity of [^{3}H]lysine and [^{3}H]arginine in the H5 from anemic bone marrow is 1.87×10^6 cpm/mg, while that in anemic blood is 2.09×10^6 cpm/mg. Since reticulocytes contain 1.33 times the amount of histone H5 in less matured cells, the higher specific activity in anemic blood indicates a higher rate of synthesis. This conclusion assumes that the lysine and arginine pool sizes in the various cell types are identical.

Histone Phosphorylation. In earlier work, we have shown that urea–aluminum lactate starch gel electrophoresis can resolve modified histones, either phosphorylated or acetylated from the unmodified species. Figure 1A shows the resolution of the anemic bone marrow histone H5 in this system. The four evenly spaced bands suggest that this histone may also be modified. To determine the nature of this modification, the H5 fraction obtained by Bio-Gel P-10 chromatography was treated with *E. coli* alkaline phosphatase. As seen in Figure 3, this treatment quantitatively converts the slower migrating components to the main component, indicating that phosphorylation is responsible for the observed electrophoretc heterogeneity.

In starch gel electrophoresis, we have also noted a significant difference in phosphorylation of H5 and the other histone species. The proportion of phosphorylated H5 in actively dividing anemic bone marrow is very high, roughly 70–80% of the total H5. This contrasts sharply with the low level of phosphorylation of the other histones (data not shown).

In order to compare rates of histone synthesis and phos-

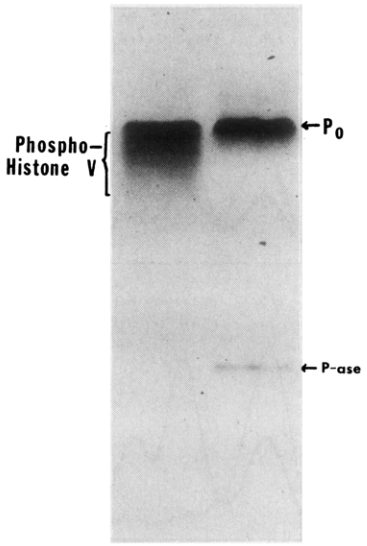

FIGURE 3: Dephosphorylation of phospho-H5 by alkaline phosphatase. A sample (125 μg) of histone H5 from anemic blood was incubated with 25 μg of *E. coli* alkaline phosphatase (Worthington) for 3 h at 37 °C in 0.2 M Tris-HCl, pH 8.0. Control vs. alkaline phosphatase-treated samples were compared in an urea–aluminum lactate starch gel. Note that following phosphatase treatment all the protein-specific staining disappeared from the phospho-H5 region of the gel.

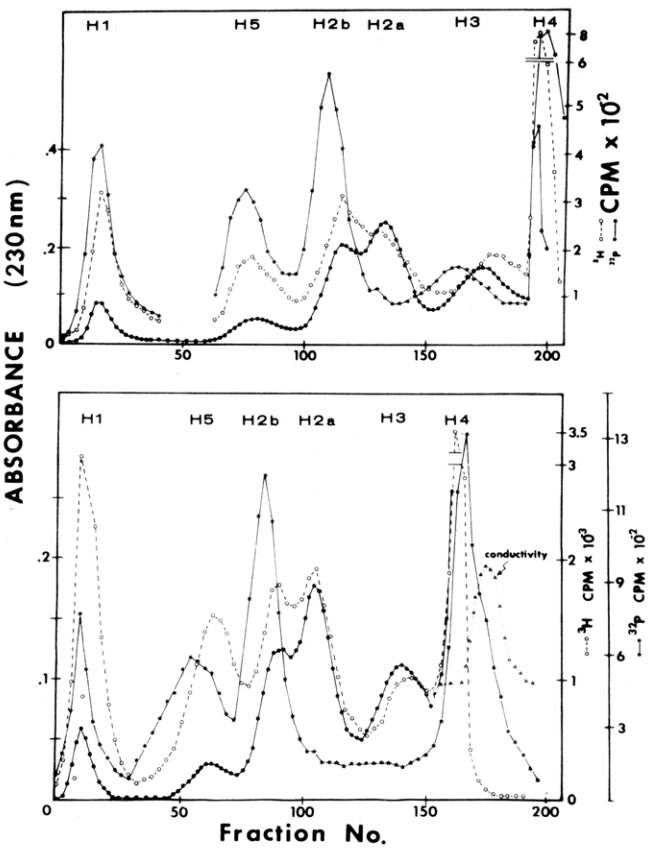

FIGURE 4: The time dependence of ^{32}P incorporation into histones. Anemic bone marrow cells were incubated in the presence of ^{32}P (1 mCi/ml), [^{3}H]lysine (50 μCi/ml), and [^{3}H]arginine (50 μCi/ml). At 10, 30, 90, 180, and 300 min, aliquots of cells were removed and histones were extracted. The reduced and alkylated total histones were chromatographed on a single Bio-Gel P-60 column (3 × 200 cm) as described under Materials and Methods. The synthesis of all histones and phosphorylation of histones H1, H5, H2b, and H4 are linear with time. However, the phosphorylation of histones H2a and H3 are time dependent as illustrated at two periods of labeling, 30 min (upper panel) and 180 min (lower panel). Note: H4 peaks are contaminated with salt and urea.

phorylation, anemic bone marrow cells were pulse labeled with both isotopes. The incorporation of [^{3}H]lysine and [^{3}H]arginine into all six histones were linear for at least 3 h. By contrast, the distribution of ^{32}P in histone varies: H1, H5, H2b, and H4 are highly phosphorylated, while H3 and H2a have incorporated little label. Furthermore, in Figure 4, the levels of ^{32}P incorporation into histones H2a and H3 are high at 10 min (data not shown), relatively low at 30 min, and decreased to background by 3 h. This time-dependent histone phosphorylation is similar to the observation of Louie et al. (1973) in their study on the phosphorylation of histones in the dividing spermatogonia cells of trout testis. Histone H5 is consistently phosphorylated in nondividing cells of the anemic blood and, frequently, we also find a high level of ^{32}P in histone H2b. In trout testis histones, newly synthesized H2b becomes rapidly phosphorylated and then dephosphorylated. The synthesis of this histone is only minimal in chick reticulocytes (Figure 2B). Therefore, the ^{32}P label cannot be solely on nascent H2b molecules. This phosphorylation may involve "old" histones.

The phosphorylation of all the major histones has been reported to occur in goose and duck erythroid cells (Seligy and Neelin, 1971, 1973a). The authors also noted a differential incorporation of ^{32}P into the histone fractions after 1 h of in vitro cell incubation. However, they did not consider the time dependence of phosphorylation as presented above. Seligy et al. (1973) have also studied histone phosphorylation during avian erythropoiesis and are in agreement with our result. They observed a slow decline in ^{32}P incorporation into the histones with increasing cell maturity. However, as pointed out in the following paper of this issue, the decline in ^{32}P incorporation in H5 simply reflects differences in the intrinsic phosphate pools.

Histone Acetylation. The pattern of acetylation, as measured by the incorporation of [^{14}C]acetate into the histones chromatographed on a Bio-Gel P-10 column, is shown in Figure 5. When histone synthesis is inhibited by cyclohexamide, extensive incorporation of [^{14}C]acetate is found only in H2a, H2b, H3, and H4; histone H1 shows no labeling. Most significantly, histone H5 is not acetylated. This is in accord with the gel electrophoresis experiment shown above, which indicated that all the modified H5 disappears upon treatment with *E. coli* alkaline phosphatase. In the nondividing cells of the anemic blood, again, significant levels of radioactivity are found in histones H2a, H2b, H3, and H4. Comparative values for the degree of acetylation for two tissues are shown in Table II. Evidence that this radioactivity truly reflects acetylation in the histones can be substantiated by two independent experiments: (1) all the ^{14}C counts are quantitatively volatilized upon acid hydrolysis of the histones, and (2) upon exhaustive protease digestion (trypsin plus proteinase K) ^{14}C counts are detected in the ϵ-acetyllysine (data not shown). The acetylation pattern for anemic blood histones is near identical to the anemic bone marrow. The specific radioactivity (cpm/O.D.$_{230}$) in the histones from the two tissues are not significantly different (see Table II) and, therefore, cannot be solely due to contaminating nonerythroid cells. Furthermore, Sanders et al. (1973) have shown high levels of acetylation in mature erythrocytes (see also Table II). This histone acetylation in nondividing cells is interesting and is clearly not related to the general phenomena of gene activation, as seen during rat liver regeneration or following stimulation by hormones or phytohemagglutinin (Pogo et al., 1966; Pogo et al., 1968). During

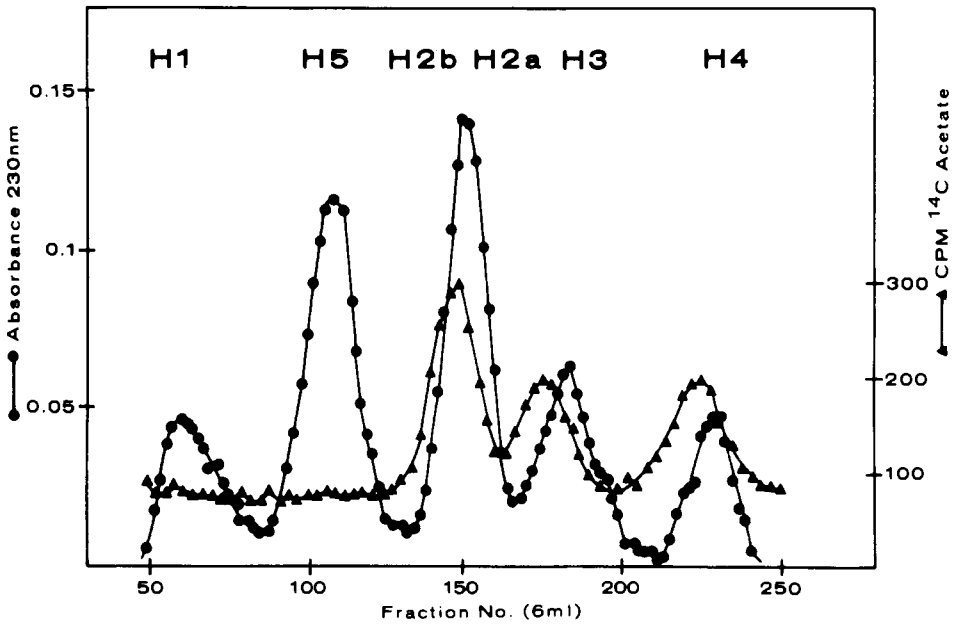

FIGURE 5: Bio-Gel P-10 chromatography of acetylated histones. Anemic bone marrow cell suspensions were preincubated for 15 min in the presence of cyclohexamide (2×10^{-4} M) and [^{14}C]acetate (20 μCi/ml) was added. Incubation was continued for an additional 90 min. Histones were extracted and chromatographed on a Bio-Gel P-10 column, as described in the legend to Figure 2.

TABLE II: Levels of Acetlyation and Methylation.

	Acetylation cpm/OD_{230nm}			Methylation cpm/OD_{230nm}	
	H2a and b	H3	H4	H3	H4
Anemic bone marrow	16 600	16 370	22 600	14 500	7500
Anemic blood	16 000	35 600	20 500	5 000	2200
Normal blood	47 000	44 000	57 000		

spermatogenesis in trout testis, there is extensive histone acetylation in the nondividing spermatid cells which may be related to the replacement process (Candido and Dixon, 1972). In the avian erythroid cells, only histone H1 is partially replaced, and, clearly, the observed acetylation cannot be correlated with this type of function. Ruiz-Carrillo et al. (1974) using high-resolution gel electrophoresis reported the progressive decrease in the proportion of acetylated H3 and H4 with the increasing maturity of the avian erythroid cells. This result together with our observation of high levels of [^{14}C]acetate incorporation and no apparent accumulation of acetylated species would suggest a rapid turnover of acetyl groups in the histones during the genomic inactivation, an observation well documented by Sanders et al. (1973).

Histone Methylation. The pattern of histone methylation in the dividing cells of the anemic bone marrow (not shown) and the nondividing cells of the anemic blood (Figure 6, upper panel) appears to be similar to that observed for other tissues. Methylation is observed in only the arginine-rich histones H3 and H4, and, more importantly, there is no methylation in histone H5. Since the labeling was performed in the presence of cyclohexamide to inhibit protein synthesis, the internal [^{14}C]methionine incorporation was not observed. The nature of ^{14}C-label incorporated into histones H3 and H4 was examined by acid hydrolysis of the histones. The methylated amino acids were identified as dimethyllysine by high-voltage paper electrophoresis (Dreyer and Bynum 1967).

The extent of methylation in the anemic blood and bone marrow was also compared by measuring the specific activity of the *methyl*-^{14}C label in H3 and H4. As expected, the methylation of histones H3 and H4 is approximately three times higher in the anemic bone marrow than in the anemic blood (Table II). In the metabolically inert erythrocytes, as can be seen in Figure 6 (lower panel), there is no observable incorporation of [*methyl*-^{14}C]methionine in histone H3 or H4. Since the incorporation of the [*methyl*-^{14}C]methionine in the histone H3 from reticulocytes is relatively high, we were interested in knowing if the bulk of methylated derivatives in the arginine-rich histones is retained in the mature erythrocytes. The histone peaks were hydrolyzed and the methylated amino acids were identified by amino acid analysis using the procedure of Morse et al. (1975) to resolve all the methylated derivatives of lysine and histidine. The histone H3 from anemic blood and normal blood showed nearly equal contents of ϵ-1-methyl- and ϵ-1,1-dimethyllysine with the latter being the predominant species. The result of this amino acid analysis indicates that the histone H3, and possibly H4 (not analyzed), in the highly condensed erythrocyte chromatin is methylated.

In synchronous HeLa cell cultures, histone methylation occurs during the late S and G_2 phase of the cell cycle (Borun et al., 1973). Similarly, in regenerating rat liver, Tidwell et al. (1968) showed that the peak of histone methylation occurs after the peak of DNA synthesis. During spermatogenesis in trout testis, histone methylation is confined to the dividing spermatocytes and stem cells which are actively synthesizing DNA and histones (Honda, 1975). In the nondividing spermatids, no histone methylation was observed. This suggests

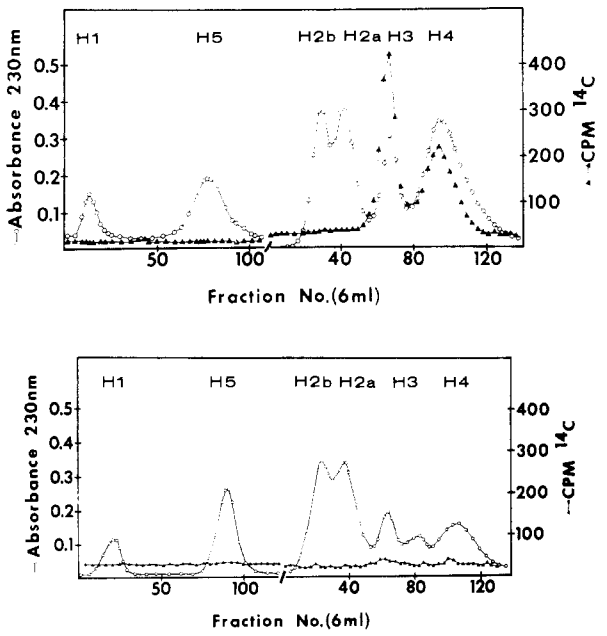

FIGURE 6: Bio-Gel P-60 column chromatography of methylated histones. Histones were extracted from anemic bone marrow cells after a 90-min period of labeling with [*methyl*-^{14}C]methionine (5 μCi/ml), as described under Material and Methods. The lysine-rich histones H1 and H5 were differentially solubilized from histones H2a, H2b, H3, and H4 by 5% perchloric acid treatment. The arginine-rich histone fraction was reduced and alkylated, and the protein was desalted and lyophilized. The lysine-and arginine-rich histone fractions were chromatographed separately in two different runs on a Bio-Gel P-60 column (3.0 × 200 cm). The above chromatogram represents recombined data for the lysine- and arginine-rich histones from anemic (upper) and normal blood (lower).

that histone methylation is a late event in the cell division cycle and may be limited to the newly synthesized histone molecules. Our observation on the methylation of histones H3 and H4 in the nondividing cells of the anemic blood is again unique to the differentiating avian erythroid cells.

Conclusion

The metabolism of histones in avian erythroid cells can be classified into two distictive types: first, the dynamic process which is occurring in the immature erythroblasts of the anemic bone marrow and appears to be common to all dividing cells, and, second, the limited range of activities seen in the highly differentiated, nondividing erythroid cells. All the histones in the immature cells are first synthesized and then undergo postsynthetic modifications: phosphorylation of all histones, acetylation of histones H2a, H2b, H3, and H4, and methylation of histones H3 and H4. The phosphorylation and acetylation of newly synthesized histones follow a series of complex metabolic pathways (Louie and Dixon, 1972; Ruiz-Carrillo et al., 1975). Since these transient modifications are occurring at a time when new DNA is being synthesized, the functional role which has been proposed is the facilitation of the proper binding of histones to DNA or the organization of histones into chromatin subunits. The biological role of histone methylation is more vague. However, methylation of the basi amino acids does increase the basicity and hydrophobicity of the protein. In the dividing cells, methylation occurs during the G_2 phase of the cell cycle and this histone methylation may be of importance in subsequent chromosome condensation or mitosis. The main conclusion that can be drawn from the above study is that the dividing avian erythroblasts appear to exhibit no great metabolic difference in the five commonly occurring histones from other tissues studied. In addition, we have demonstrated that histone H5 in these early cells is highly phosphorylated. In a separate paper, we have postulated that the phosphorylation is of great importance to the differentiating erythrocytes (Sung, 1976).

Histone metabolism in the nondividing reticulocytes is primarily restricted to the synthesis and phosphorylation of histone H5, phosphorylation of histone H2b, acetylation of histones H2a, H2b, H3, and H4, and methylation of histones H3 and H4. In the mature erthrocytes, both phosphorylation and methylation have ceased to occur, while acetylation of histones H2a, H2b, H3, and H4 continues. In the absence of histone synthesis in these nondividing cells, the observed enzymatic modifications cannot be correlated with the chromatin assembly function, since only preexisting histones are involved. It has been shown that the RNA content in these cells is significantly reduced. Therefore, these reactions cannot be correlated with a template modulation function in RNA synthesis. It appears that this class of histone structural modification in the avian reticulocyte chromatin is unique to the terminal differentiation process. A plausible function for the "differentiation-linked" phosphorylation of histone H2b, acetylation of histones H2a, H2b, H3, and H4, and methylation of histones H3 and H4 is the reorganization of chromatin structure in preparation for the final genomic inactivation. This suggestion is in accord with the current knowledge of chromatin subunit structure in that all the histones present in the subunit are extensively modified. Further studies are necessary to establish the spacial correlation of these events involving detailed kinetic and biochemical investigations. In addition, knowledge of "differentiation-linked" histone modifications in other biological systems may assist in evaluating the significance of the present observation. In this context, the acetylation of trout testis histones in the spermatid chromatin may represent another prototype "differentiation-linked" modification reaction.

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