Biochemistry

Volume 12, Number 5 February 27, 1973

A Comparative Study of Histone Acetylation, Histone Deacetylation, and Ribonucleic Acid Synthesis in Avian Reticulocytes and Erythrocytes†

Lee A. Sanders,*‡ Norman M. Schechter,§ and Kenneth S. McCarty

ABSTRACT: The processes of histone acetylation and deacetylation and their possible relationships to RNA synthesis have been studied in both avian reticulocytes and erythrocytes. In contrast to the erythrocytes, reticulocytes actively synthesize RNA. Investigation of the relative amounts of the six histones and the histone:DNA ratios of these cells showed no significant differences. During pulse-labeling experiments [^{14}C]-acetate was incorporated as N^{ϵ}-acetyllysine in four of the histones present in each cell type. The magnitudes of acetate incorporation into the histones were in the order F2a1,F3> F2b>F2a2 in both cell types during the entire time course studied. These patterns of acetylation are similar to those observed in other species, except for the significant acetylation of histone F2b. The lysine-rich histones F1 and F2c did not incorporate [^{14}C]acetate in either cell type. Pulse-chase experiments revealed that histone deacetylase activities for each of the four acetylated histones were significantly higher in the mature erythrocytes than in immature reticulocytes suggesting a possible role of histone deacetylation in the differentiation of the avian erythroid cell line.

Several chemical modifications of preexisting chromosomal proteins are known to occur within the nuclei of eukaryotic cells. Recent studies have examined the processes of acetylation of lysine residues (Allfrey, 1970; Marzluff and McCarty, 1970; Candido and Dixon, 1972a), methylation of arginine, lysine, and histidine residues (Paik and Kim, 1970; Benjamin, 1971; Gershey *et al.*, 1969), and phosphorylation of serine residues (Ord and Stocken, 1966; Langan, 1969). Only one of these modifications, the phosphorylation of histone F1, has been associated with a specific cellular function, namely DNA replication (Balhorn *et al.*, 1972; Oliver *et al.*, 1972). In contrast, very little information is available on the exact metabolic role of the acetylation of histones, although this process has been implicated in the control of transcription (DeLange and Smith, 1971).

The developing nucleated avian erythroid cell line provides a useful system for the investigation of histones and their biochemical modifications in relation to RNA synthesis. In the experiments reported here, immature avian reticulocytes have been compared to mature erythrocytes in terms of their histone contents as well as their capacities to acetylate and deacetylate histones.

Materials and Methods

Chemicals. Phenylhydrazine hydrochloride (recrystallized) and N^{ϵ}-acetyllysine were obtained from Sigma. Sodium [1-^{14}C]acetate (58.8 Ci/mol), [5,6-^{3}H]uridine (42.4 Ci/mmol), and Omnifluor were purchased from New England Nuclear Corp. Actinomycin D was a gift from Dr. Sidney Pestka. Trypsin (Tos-PheCH$_2$Cl treated) was obtained from Worthington Biochemicals. Pronase (45,000 PUK/g) was purchased from Calbiochem. Cyanogen bromide was obtained from Aldrich.

Preparation of Avian Reticulocytes and Erythrocytes. In order to obtain avian reticulocytes, anemia was induced in adult white Peking ducks (2.5–3 kg) by three daily injections of phenylhydrazine hydrochloride (18 mg/kg, intraperitoneally, 2% solution in sterile saline). The course of the induced anemia was monitored by following the hematocrit and reticulocyte count. Cells were stained with new methylene blue by the method of Brecher (1949) except that the blood was diluted with an equal volume of saline prior to staining for 15–20 min. Reticulocytes were readily distinguished from mature erythrocytes by the presence of numerous blue granules in the cytoplasm as noted by Lucas and Jamroz (1961). The anemic

† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received May 18, 1972.* This work was supported in part by Grant 5 R01-GM-12805 and NCI-G-72-3857 from the National Institutes of Health.

‡ M.D.–Ph.D. candidate, trainee of the National Institutes of Health, National Institute of General Medical Sciences (T5-GM-01678).

§ Supported in part by Predoctoral Traineeship GM-00233-12 from the National Institutes of Health.

ducks were routinely exsanguinated within 48–52 hr after the last injection. Blood from both anemic ducks and normal controls was obtained as described previously (Sanders and McCarty, 1972), and clotting was prevented by the presence of citrate (10 ml of 10% trisodium citrate/100 ml of blood). Cells were sedimented by centrifugation at 600*g* for 10 min at room temperature, and both the plasma and buffy coat were removed by aspiration.

Measurement of Uridine Incorporation. Packed cells were washed by suspension in 4 volumes of modified Eagle's medium, pH 7.4 (Levintow and Darnell, 1960; Attardi *et al.*, 1970), followed by centrifugation at 600*g* for 10 min to sediment the cells. In order to measure RNA synthesis, 2.0 ml of washed, packed reticulocytes or erythrocytes was suspended in 23 ml of modified Eagle's medium (pH 7.4) containing 5% (v/v) autologous duck plasma. Cell concentrations in the incubation mixtures were 5.0×10^8 and 5.3×10^8 cells per ml for reticulocytes and erythrocytes, respectively, as determined with a Model A Coulter counter. Aliquots (8 ml) of each incubation mixture were placed in separate flasks, brought to 39° in a shaking water bath, and incubated either with or without actinomycin D (56 μg/ml). After 15 min, 300 μCi of [^{3}H]-uridine (0.3 ml, 42.4 Ci/mmol) was added to each mixture. At various times after addition of the [^{3}H]uridine, 1.0-ml aliquots were removed and added immediately to 4.0 ml of SSC[1] at 4° in conical glass centrifuge tubes. After gentle agitation, the cells were centrifuged, the supernatants were discarded, 1.0 ml of SSC was added with stirring, and the suspensions were quick-frozen at −76° and stored at −20° for 2–5 hr. The samples were thawed (leading to hemolysis of the cells) and after rapid vortexing to ensure homogeneity, 0.1-ml aliquots of each sample were pipetted onto filter disks (Whatman No. 3, 2.3 cm). The disks were dried and washed by the method of Bollum (1966). Radioactivity was determined in Omnifluor–toluene solution (4.0 g/l. of toluene) using a Packard liquid scintillation spectrometer (Model 3003).

Acetylation and Deacetylation of Histones. Packed reticulocytes or erythrocytes were washed twice by suspension in 2.0 volumes of modified Krebs–Henseleit solution, pH 7.4 (Marks *et al.*, 1963), followed by centrifugation at 600*g* for 10 min at 20°. Aliquots (29.0 ml) of the washed, packed cells were suspended in 76.0 ml of an incubation medium of the following composition: a mixture of L-amino acids (Lingrel and Borsook, 1963) diluted 1:4 in modified Krebs–Henseleit solution, 116 mM NaCl, 4.56 mM KCl, 1.13 mM KH_2PO_4, 1.13 mM $MgSO_4$, 24 mM $NaHCO_3$, 3.46 mM glucose, 0.58 mM inosine, 0.41 mM $Fe(NH_4)_2(SO_4)_2$, 2.0 mM glutamine, and 4% (v/v) autologous duck plasma at pH 7.4.

Cell suspensions were incubated for up to 90 min with 5 μCi/ml (0.085 mM) of sodium [1-^{14}C]acetate at 39° in a shaking water bath. For pulse experiments, 15-ml aliquots of the cell suspensions were rapidly chilled to 0° after various times and the cells were centrifuged at 600*g* at 0°. The packed cells were washed by suspension in 5 volumes of radioisotope-free SSC followed by sedimentation as above. This washing procedure was repeated twice more using fresh SSC prior to preparation of the labeled histones as described below. For pulse-chase experiments, 30-ml aliquots of the cell suspensions were withdrawn, the cells were centrifuged for 4 min at 600*g* at 20°, and the supernatants were discarded. The cells were then resuspended in 38 ml of fresh incubation medium containing 6.25×10^{-4} M sodium acetate and incubated for 20 and 60 min at 39° with gentle shaking. At the end of the chase periods, 15-ml aliquots of the cell suspensions were sedimented and washed as described above.

As a control, the amounts of acetate taken up by reticulocytes and erythrocytes were compared by determining the radioactivity remaining in 0.01-ml aliquots of cell incubation supernatants at various times after addition of the sodium [1-^{14}C]acetate.

Preparation of Histones.[2] Histones were selectively extracted from both isolated reticulocyte and erythrocyte chromatin and fractionated as described previously (Sanders and McCarty, 1972). Where present, the cysteine-containing F3 histone was usually oxidized to the dimer form (F3-S-S-F3) in 6 M Gdn·HCl at pH 8.3 to facilitate its separation from other histones during gel electrophoresis (Sanders and McCarty, 1972; Marzluff *et al.*, 1972a).

Gel Filtration. Oxidized reticulocyte histones F1 + F2c + F2b were separated by gel filtration on Sephadex G-100 as described previously (Sanders and McCarty, 1972). Gel filtrations of histone F2b cyanogen bromide digests were performed on a Sephadex G-50 column (2.2 × 170 cm) equilibrated with 0.1 N acetic acid (Marzluff *et al.*, 1972b) at a flow rate of 13.5 ml/hr.

Electrophoresis. Disc gel electrophoresis was performed by a modification of the methods of Panyim and Chalkley (1969a,b) as described previously (Sanders and McCarty, 1972). The gels (10 × 0.4 cm) contained 15% acrylamide and either 2.5 M urea at pH 2.8 or 6.25 M urea at pH 3.2. Ethylene diacrylate was utilized as the cross-linking agent allow the gels to be solubilized (Choules and Zimm, 1965). Electrophoresis of cyanogen bromide digested histones was performed for 5 hr at 0.89 mA/gel; all undigested histone samples were electrophoresed for 6.5 hr at the same amperage. Gels were stained for 5 hr in 1% Amido Black in 7% acetic acid and destained in 7% acetic acid. The gels were scanned with a Model 2410 Gilford linear transport scanner. After staining and destaining, the gels had swollen reproducibly to 13.5 cm; migration distances refer to the final position of various bands in the swollen gels. Staining was quantitated by integration of the gel scans as described by Marzluff and McCarty (1970).

In order to determine specific activities of various labeled histones, the gels were frozen on Dry Ice and sliced into 1-mm disks with a manual device (Marzluff *et al.*, 1969). Gel slices were hydrolyzed in 0.2 N NaOH for 40 hr at 37°, neutralized with acetic acid, and counted in Triton–toluene at a ^{14}C efficiency of 79% (Patterson and Green, 1965). Alternatively, specific activities were determined by direct scintillation counting of samples of known protein concentration.

Cyanogen Bromide Digestion. Reticulocyte ^{14}C-labeled acetylated histone F2b (12 mg) and unlabeled histone F2b (23 mg; added as carrier) were dissolved in 5.0 ml of 70% formic acid. Crystalline cyanogen bromide (~40 mg) was added, and the solution was incubated for 16 hr at room temperature. Six volumes of water were added and protein was recovered by lyophilization.

Determination of Histone and DNA in Isolated Nuclei. Reticulocyte and erythrocyte nuclei were isolated by a modification of the method of Sanders and McCarty (1972); all washes contained 3 mM $MgCl_2$, 60 mM KCl, and 50 mM Tris buffer at pH 7.9. Histone was determined by quantitative gel

[1] Abbreviations used are: SSC, 0.14 M NaCl–0.01 M trisodium citrate; Gdn·HCl, guanidine hydrochloride.

[2] The histone nomenclature used is that of Johns and Butler (1962). The corresponding designations of Rasmussen *et al.* (1962) are given in parentheses as follows: F1 (I), F2a1 (IV), F2a2 (IIb1), F2b (II2b), F2c (V), and F3 (III).

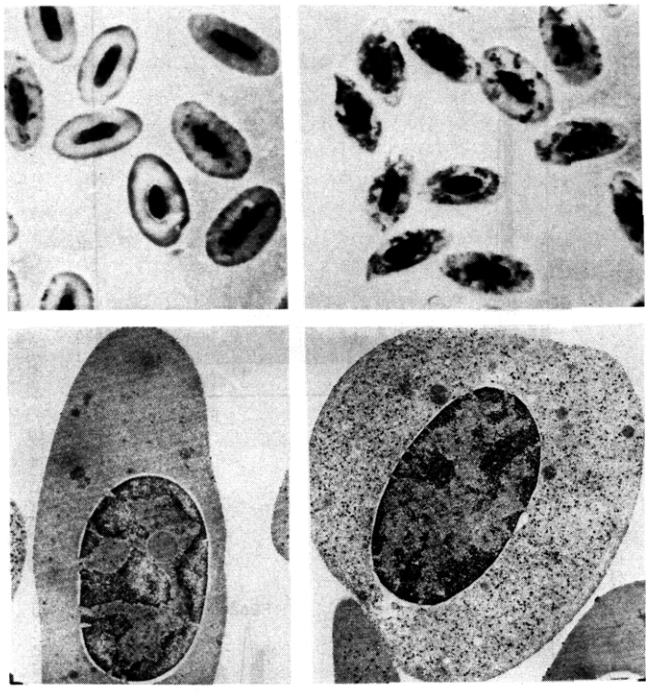

FIGURE 1: Phase-contrast and electron microscopy of avian erythrocyte and reticulocyte preparations. Top, left and right: phase-contrast micrographs of erythrocytes and reticulocytes, respectively (total magnification 925× for each preparation). Bottom, left and right: electron micrographs of erythrocyte (total magnification 3620×) and reticulocyte (total magnification 4170×), respectively.

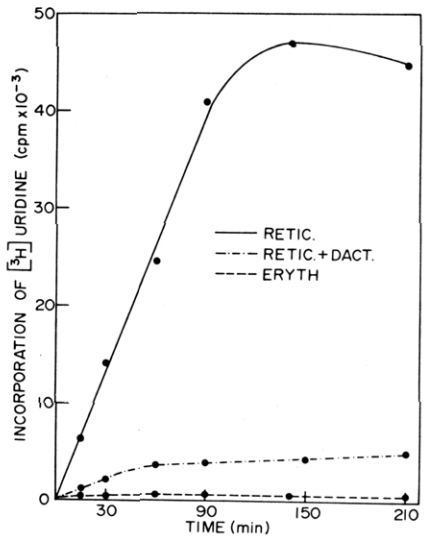

FIGURE 2: Measurement of RNA synthesis in reticulocyte and erythrocyte populations. Each value represents the mean of two independent determinations; DACT. = actinomycin D.

electrophoresis and/or by the method of Lowry *et al.* (1951) using bovine serum albumin as standard (assuming $A_{280\ nm}^{1\ cm} = 0.66$ for a 1-mg/ml solution; Cohn *et al.*, 1947). Both methods gave identical results. DNA was extracted from the nuclei by the method of Ogur and Rosen (1950) and assayed by reaction with indole (Ceriotti, 1955; Short *et al.*, 1968).

Analysis of Labeled Amino Acids. Labeled amino acids were analyzed after digestion of the histones with trypsin and pronase using a single long column Technicon amino acid analyzer equipped with a Model 320E Packard Tri-Carb flow monitor (Marzluff and McCarty, 1970).

Electron Microscopy. Erythrocyte and reticulocyte samples were prepared for electron microscopic examination by suspending the SSC-washed cells for 1 hr at room temperature in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The fixed cells were centrifuged, postfixed in 1% OsO_4 in cacodylate buffer for 1 hr, and stained with lead citrate and uranyl acetate. The samples were dehydrated in ethanol, embedded in epon, and examined in an RCA EMU-3 electron microscope.

Results

In response to phenylhydrazine, large numbers of immature erythroid cells were released from the marrow into the peripheral circulation of the treated ducks. Within 48–52 hr after the final injection of phenylhydrazine, the red blood cell population of the treated ducks reproducibly consisted of 90 ± 5% immature cells, predominantly reticulocytes, with only a small number of mature erythrocytes (10 ± 5%). In contrast, normal duck blood was found to consist of 90 ± 2% mature erythrocytes and only 10 ± 2% reticulocytes.

Each population of cells was examined by both phase-contrast microscopy and electron microscopy. As shown in Figure 1, numerous polyribosomes were present in the cytoplasm of reticulocytes, but not erythrocytes. This indication that active protein synthesis was occurring only in the immature cell type is in agreement with the findings of earlier autoradiographic studies (Cameron and Prescott, 1963).

The synthesis of RNA in populations of intact reticulocytes or erythrocytes is shown in Figure 2. The reticulocyte population rapidly incorporated uridine into acid-insoluble material, in contrast to the mature erythrocyte population in which negligible synthesis of RNA occurred. The incorporation was almost completely inhibited by actinomycin D. These findings are in agreement with autoradiographic studies of Sherrer *et al.* (1966), but are in contrast to the findings of Attardi *et al.* (1966, 1970), which suggested that significant RNA synthesis was occurring in normal duck erythrocytes. These discrepancies might reflect different treatments of the cells prior to precipitation of incorporated [^{3}H]uridine with CCl_3COOH. In our experiments we found it necessary to freeze and thaw the cells prior to acid precipitation in order to minimize trapping of unincorporated radioactivity, since 5% CCl_3COOH did not satisfactorily lyse the cells.

Because the histones may play a role in the control of gene expression in higher organisms (Allfrey *et al.*, 1963; Bonner *et al.*, 1963; Pogo *et al.*, 1966–1968), both the histone:DNA ratios and the relative amounts of the individual histones in each cell population were compared to see whether an increase in the amount of one or more histones could play a role in the terminal stages of differentiation of the erythroid cell line. There were no significant differences in the absolute amounts of total histones as evidenced by histone:DNA ratios of 1.3 and 1.2 for erythrocytes and reticulocytes, respectively. Furthermore, when analyzed by gel electrophoresis at pH 2.8, the total histones from each cell population gave almost identical patterns as shown in Figure 3.

Table I presents the relative proportions of each histone fraction in duck erythrocytes, duck reticulocytes, and calf thymus. Although only minor differences were detected between the histones of circulating avian erythrocytes and reticulocytes, the histones of both of these cell types differ markedly from those of calf thymus (Table I). Unlike calf

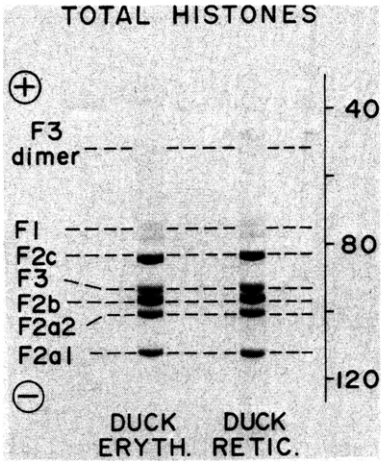

FIGURE 3: Gel electrophoresis of erythrocyte and reticulocyte total histones. Electrophoresis was performed at pH 2.8 as described under Materials and Methods. Scale at right denotes migration distances in millimeters.

thymus, the avian red blood cells contain the "erythrocyte-specific" histone F2c (Purkayastha and Neelin, 1966; Greenaway and Murray, 1971), and a smaller percentage of histone F1.

In view of the possible importance of bichemical modifications of histones in the control of processes such as DNA replication and/or transcription, the processes of histone acetylation and deacetylation in the avian erythroid cells were studied. A sample of the erythrocyte population of normal blood was incubated in the presence of sodium [1-^{14}C]acetate for 90 min. The total histones were isolated, and digested with trypsin and pronase; the digest was chromatographed as described under Materials and Methods. Radioactive material eluted from the column as a single major peak corresponding to N^{ϵ}-acetyllysine with no evidence of *N*-acetylserine, suggesting that there was negligible synthesis of new histones during the 90-min pulse period (Marzluff and McCarty, 1970).

TABLE I: Percentage of Individual Histone Fractions.[a]

Cell Type	Histone Fraction					
	F1	F2c	F2b	F2a2	F2a1	F3
Duck erythrocyte	3.5	17.1	21.4	18.2	15.2	24.6
Duck reticulocyte	3.9	16.5	22.4	19.1	14.9	23.1
Calf thymus	13.1	0	32.4[b]	20.5	15.5	18.5

[a] Total histones were extracted from each cell type indicated, and the relative amounts of each histone were determined by quantitative gel electrophoresis (pH 2.8). [b] It is possible that this value might be higher than the actual value for histone F2b due to the presence of some oxidized calf thymus F3 histone

which would coelectrophorese with histone F2b (Marzluff *et al.*, 1972).

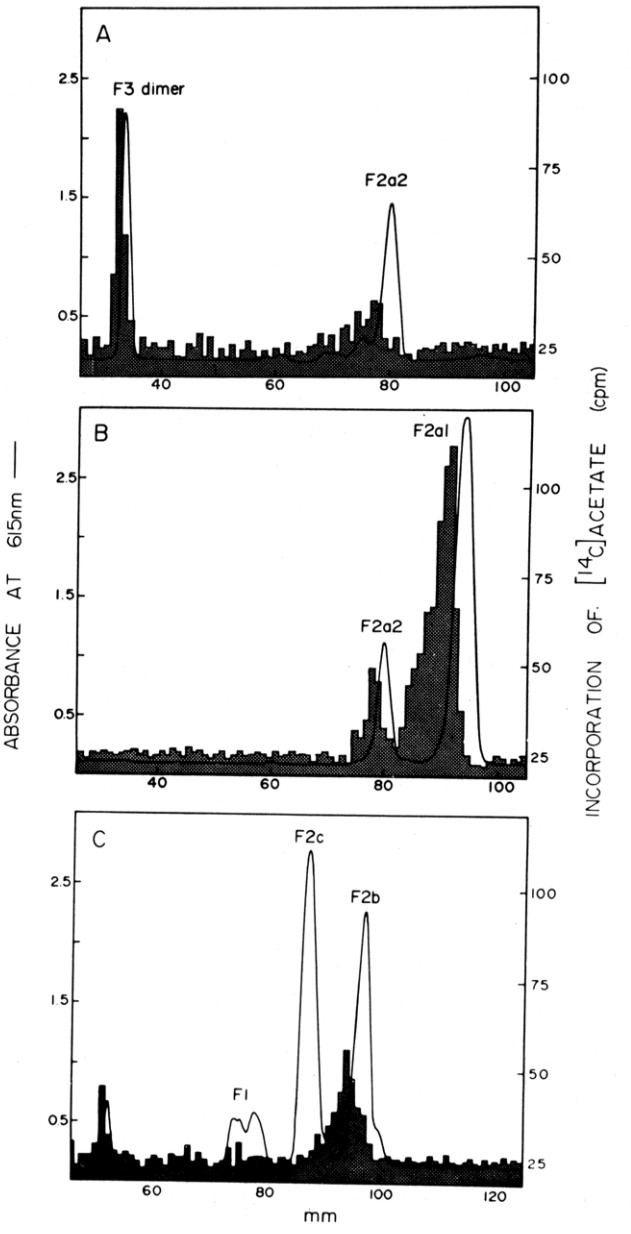

FIGURE 4: Distribution of [1-^{14}C]acetate in erythrocyte histone fractions. Erythrocytes were incubated for 90 min in the presence of [1-^{14}C]acetate, and the labeled histones were fractionated as described under Materials and Methods. After electrophoresis, the gels were scanned, frozen, and sliced into 1-mm disks from which radioactivity was determined. Shaded areas represent radioactivity. (A) F3 + F2a2 histones, electrophoresed at pH 3.2; (B) F2a2 + F2a1 histones, electrophoresed at pH 3.2; (C) F1 + F2c + F2b histones, electrophoresed at pH 2.8. The distribution of [^{14}C]acetate in reticulocyte histones (not shown) was very similar to that for erythrocytes (see Figures 5 and 6).

To investigate the processes of histone acetylation and deacetylation in greater detail, intact erythrocytes or reticulocytes were pulse labeled with [1-^{14}C]acetate for up to 90 min or pulse labeled for 20 min and then subjected to either 20- or 60-min "chase" periods in the presence of unlabeled acetate. Histones were selectively extracted from the isolated chromatin as described earlier (Sanders and McCarty, 1972). The distribution of [^{14}C]acetate in the various histone fractions was examined by gel electrophoresis as shown in Figure 4. Significant amounts of [^{14}C]acetate were associated with all of the histone fractions except the lysine-rich histones, F1 and

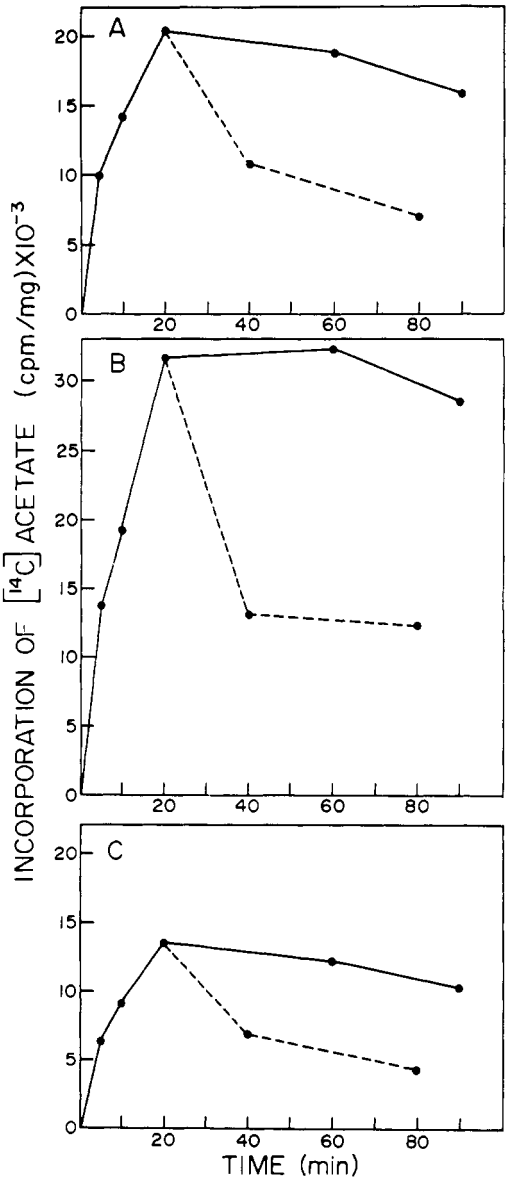

FIGURE 5: Incorporation of [^{14}C]acetate into histones of the avian erythrocyte population. Erythrocytes were either pulse labeled for various times (solid lines) or pulse labeled and then submitted to chase periods in the presence of unlabeled acetate (dashed lines). The labeled histones were fractionated as described under Materials and Methods, and their specific activities were determined by scintillation counting of aliquots of known protein concentration. (A) F3 + F2a2 histones; (B) F2a1 histone; (C) F1 + F2c + F2b histones.

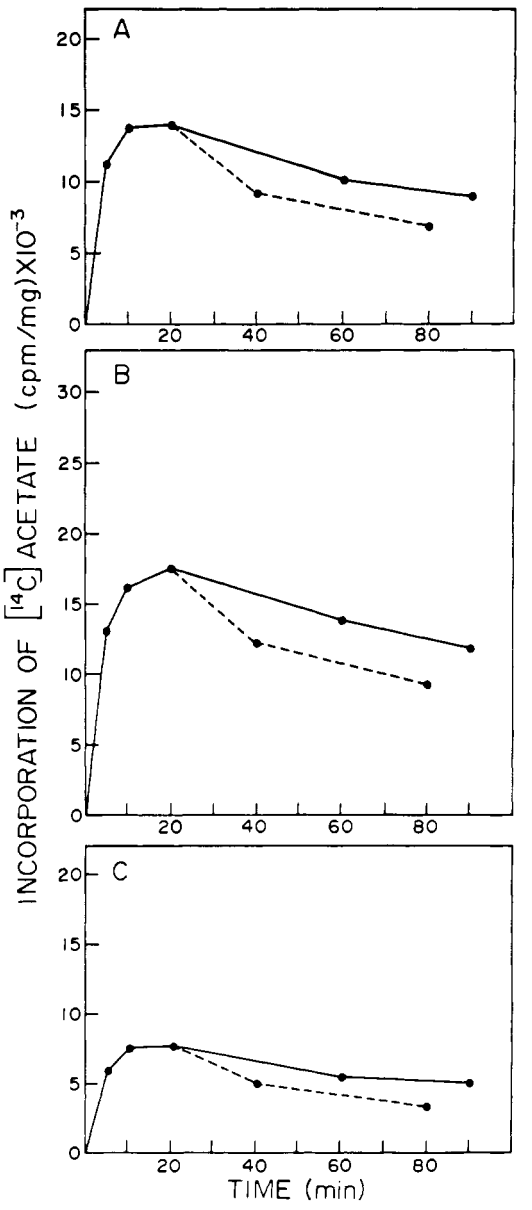

FIGURE 6: Incorporation of [^{14}C]acetate into histones of the avian reticulocyte population. Reticulocytes were incubated and their histones isolated exactly as described in the legend to Figure 5. Pulse labeling studies (solid lines); pulse-chase studies (dashed lines). (A) F3 + F2a2 histones; (B) F2a1 histone; (C) F1 + F2c + F2b histones.

F2c. The acetylated histone molecules of each fraction had slightly lower electrophoretic mobilities than the unacetylated parent molecules in agreement with the findings of Wangh *et al.* (1972) [as expected since the acetylation of lysine ϵ-amino groups reduces the net positive charge]. It was also apparent from the distinctive electrophoretic migration of the labeled histones that only a relatively small proportion of the molecules of each histone fraction were acetylated. In histones containing multiple sites of acetylation, *e.g.*, histone F2a1 (DeLange *et al.*, 1968, 1969), those molecules containing two or three acetate groups appeared to lose label more rapidly during chase periods than did monoacetylated molecules.[3]

[3] Our unpublished observations.

Figures 5 and 6 present the time course of histone acetylation and deacetylation for the various histone fractions of erythrocytes and reticulocytes. It is apparent that the erythrocyte histones exhibit a greater magnitude of acetate incorporation than reticulocyte histones. Further examination using either disc gel electrophoresis followed by gel slicing and scintillation counting as described under Materials and Methods (or slab gel electrophoresis and subsequent autoradiography[3]) revealed that in both cell types the magnitudes of acetate incorporation into the histones were in the order F2a1, F3 > F2b > F2a2. The lysine-rich histones F1 and F2c of either erythrocytes or reticulocytes did not incorporate [^{14}C]acetate at any point during the entire time course studied.

The losses of [^{14}C]acetate activity during chase periods provide an indication of the histone deacetylase activities for the four acetylated histones in both erythrocytes and reticulo-

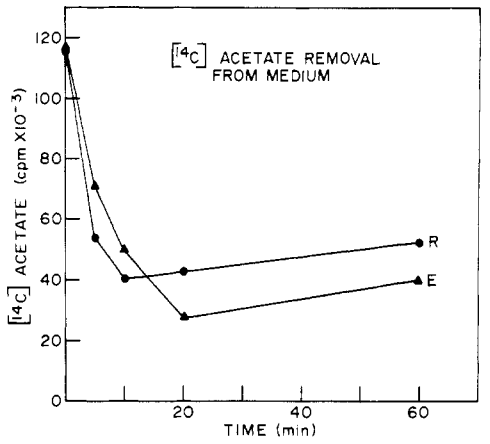

FIGURE 7: Removal of [^{14}C]acetate from medium by erythrocytes (E) and reticulocytes (R). Acetate removal was monitored by determining the radioactivity remaining in 0.01-ml aliquots of cell suspension supernatants after various times of incubation.

cytes. As shown in Figures 5 and 6, the erythrocytes exhibited significantly higher deacetylase activities for all the acetylated histones than did the reticulocytes.

As a control, the uptake of [^{14}C]acetate into both cell types was determined (Figure 7). By the end of the 60-min pulse period, reticulocytes had taken up 56% of the added label while erythrocytes had taken up 64% of the label. In each incubation mixture, the cells accounted for only 28% of the total volume indicating that there was active transport and/or utilization of acetate in each cell type. The relatively small amount of ^{14}C label returned to the medium between 20 and 60 min was probably due to the lysis of some cells and/or the conversion of some of the [^{14}C]acetate to diffusable products, *e.g.*, $^{14}CO_2$ (Schjeide *et al.*, 1964).

Since histone F2b had previously been reported not to be acetylated either in calf thymus (Vidali *et al.*, 1968) or in mouse mammary gland (Marzluff and McCarty, 1970), and only slightly acetylated in human lymphocytes (Desai and Foley, 1970), it was important to demonstrate that the apparent significant acetylation of this histone in both avian erythrocytes and reticulocytes (Figures 4, 5, and 6) was not an artifact due to possible contamination with small amounts of the relatively highly labeled arginine-rich histone F3. Therefore, a sample of histones F1, F2c, and F2b (obtained from a reticulocyte population after a 90-min incubation in the presence of [1-^{14}C]acetate) was oxidized and then chromatographed on Sephadex G-100 as described under Materials and Methods. As shown in Figure 8, histones F1, F2c, and F2b could be readily resolved by this method. It is important to note that all of the F3 dimer (F3-S-S-F3) contaminant in the preparation cochromatographed with histone F2c. Furthermore, analytical gel electrophoresis gave no evidence of any F3 monomer (F3-SH) contaminant in the F2b-containing pool III (Figure 8).

In order to provide additional proof of the absence of any contaminating histone F3 and to localize the site(s) of acetylation within histone F2b, the ^{14}C-labeled F2b was digested with cyanogen bromide and the digest was chromatographed on Sephadex G-50. As demonstrated in Figures 9 and 10, the avian histone F2b can be cleaved into N- and C-terminal half-molecules similar to those of calf thymus and pea bud histone F2b (Iwai *et al.*, 1970; Li and Bonner, 1971; Johns *et al.*, 1972). It is apparent from Figures 9 and 10 that no F3 histone contaminant was present in the F2b preparation,

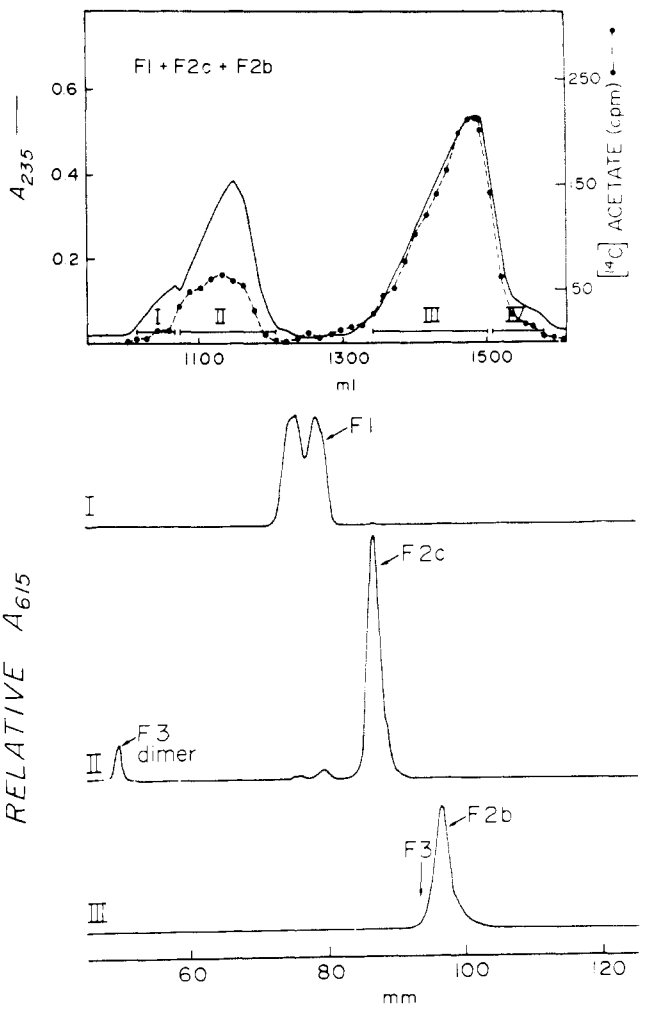

FIGURE 8: Gel filtration and electrophoresis of histones F1, F2c, and F2b. Avian reticulocytes were incubated for 90 min in the presence of [1-^{14}C]acetate, and the histones F1 + F2c + F2b were extracted, oxidized, and chromatographed on Sephadex G-100 as described under Materials and Methods. Top: gel filtration; radioactivity was determined by counting 4% aliquots of alternate fractions. Note the constant specific activity (cpm/ODU) within peak III. Bottom: gel electrophoresis of peaks I, II, and III performed at pH 2.8. Note the absence of histone F3 in peak III.

since none of the major F3 cyanogen bromide peptide (CNBr-1) could be detected by gel electrophoresis. Furthermore, the distribution of radioactivity shown in Figure 9 in conjunction with thermolysin digestion of the isolated F2b-N fragment followed by paper chromatography[3] indicates that the avian F2b histone contains at least two major sites of acetylation located within the basic N-terminal region of the molecule.

Discussion

When a developing avian erythroid cell reaches the reticulocyte stage, RNA synthesis has become considerably reduced relative to that of the earlier erythroblast stage (Cameron and Prescott, 1963; Sherrer *et al.*, 1966). A significant amount of RNA synthesis is detectable, however, in the immature populations of erythroid cells studied here, which consist predominantly of reticulocytes. This synthesis is apparently directed toward the manufacture of several types of RNA, including the 10S globin mRNA (Pemberton and Baglioni, 1972). In contrast to the reticulocyte populations,

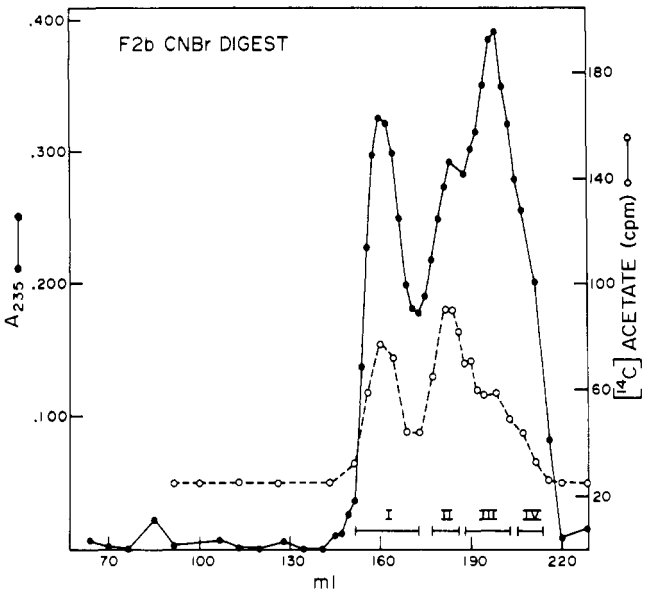

FIGURE 9: Gel filtration of histone F2b cyanogen bromide digest. Histone F2b (peak III, Figure 8) was digested with cyanogen bromide, and the digest was chromatographed on Sephadex G-50 as described under Materials and Methods. Radioactivity was determined by counting 4% aliquots of alternate fractions.

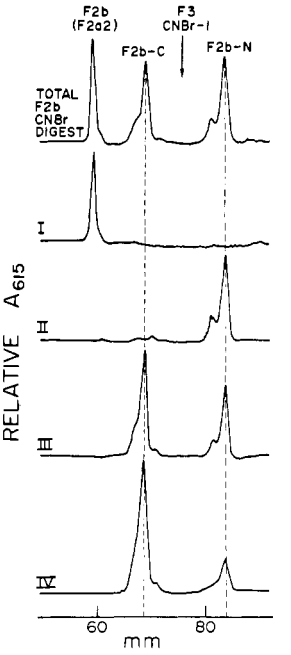

FIGURE 10: Gel electrophoresis of histone F2b cyanogen bromide peptides. Roman numerals refer to the various protein pools obtained by gel filtration (Figure 9). Electrophoresis was performed at pH 3.2 for only 5 hr. Arrow denotes the position of the major F3 cyanogen bromide peptide (CNBr-1) when electrophoresed under identical conditions.

the populations of mature avian erythrocytes studied here proved to be inactive in RNA synthesis.

In view of these findings, one might expect to find differences in the chromatin composition and/or structure as the cell matures to the inactive erythrocyte stage. Nevertheless, examination of the chromatin of these cells failed to reveal any detectable differences between reticulocyte and erythrocyte populations in either the relative amounts of the various histones or in the histone:DNA ratios. Certain changes in the chromatin were observed, however, in histone acetylation (Figures 5 and 6). Although the magnitudes of acetate incorporation into the histones of both cell populations were in the same order, F2a1, F3 > F2b > F2a2, the overall magnitudes of acetate incorporation were significantly higher in erythrocytes than reticulocytes.

We have also examined the specificity of internal acetylation in several of the histone fractions. Distinct differences exist between reticulocytes and erythrocytes in the loci of acetylation in both histones F2a1 and F3 (manuscript in preparation). In addition, cyanogen bromide and thermolysin digestions of histone F2b from the avian red blood cells have revealed at least two major sites of acetylation within the basic N-terminal half of the molecule, which contains the presumed DNA binding site.

The observed acetylation of histone F2b in reticulocytes and erythrocytes is in contrast to findings in other cell systems. Histone F2b has recently been found to be only slightly acetylated in calf thymus (Marzluff *et al.*, 1972b) and human lymphocytes (Desai and Foley, 1970), and is not acetylated in mouse mammary gland (Marzluff and McCarty, 1970). These differences imply the existence of some degree of tissue and/or species specificity of histone acetylation.[4]

For each of the four histones examined in the present study, the acetylation process appears to be readily reversible as indicated by the results of the pulse-chase experiments (Figures 5 and 6). In particular, the rates of deacetylation were significantly higher for each of the four acetylated histones in the mature erythrocytes than in immature reticulocytes. These findings suggest the possibility that increases in histone deacetylation which accompany maturation of the erythroid cell may serve to increase the net positive charge of particular histone molecules, thereby increasing the condensation (Kernell *et al.*, 1971) and/or template repression of the chromatin.

Some correlations have also been observed in other cell systems between levels of histone deacetylation and the template activity of chromatin. For example, Libby (1970) detected higher deacetylase activity in rat liver than in hepatoma, and Pogo *et al.* (1968) observed apparently higher deacetylase activities in normal rat liver than in the regenerating liver following partial hepatectomy. Recently, Boffa *et al.* (1971) demonstrated correlations of histone deacetylase activities with the active synthesis of proteins in 11- and 16-day chick embryos.

The reticulocyte and erythrocyte histones F1 and F2c are unique, in that neither demonstrated significant internal acetylation during pulse-labeling studies. Several other chemical similarities of these two histones are the following: (1) both are lysine rich, containing 25.9 and 24.0 mol % lysine, respectively (Sanders and McCarty, 1972); (2) they have similar molecular weights of 22,000 and 18,400, respectively (Bustin and Cole, 1970; Greenaway, 1971); (3) both are selectively extractable from chromatin in 5% $HClO_4$;[3] (4) both have acetylated amino termini of *N*-acetylserine (Rall and Cole, 1971) and *N*-acetylthreonine,[5] respectively; and finally

[4] It is important to note that during the preparation of this manuscript, Candido and Dixon (1972a) reported significant levels of acetylation in trout testis histone F2b, which appears to contain two to four major sites of acetylation. As in the avian red blood cell F2b, these acetylation sites are located within the basic N-terminal half of the molecule (Candido and Dixon, 1972b).

[5] W. Cieplinski and R. C. Huang, personal communication.

(5) both can be phosphorylated on internal serine residues (Seligy and Neelin, 1971; Balhorn *et al.*, 1972) or methylated on internal histidine residues to yield 3-methylhistidine (Gershey *et al.*, 1969). These similarities between the lysine-rich histones F1 and F2c, along with the observation that neither undergoes reversible acetylation, lend support to the hypothesis that these histones may serve different functions from the other four histones.

It is also possible that histone F2c plays a specific role in avian erythroid cell development and differentiation, apart from the role of histone F1. Studies with chick embryos have demonstrated that F2c synthesis is initiated at days 5–6 followed by a progressive increase during early embryonic development. Little or no changes occur, however, in the relative proportions of the other five histones (Moss *et al.*, 1972). Recent studies of the bone marrow of anemic adult chickens have revealed similar increases of histone F2c during both the early and middle stages of erythroid cell development (Billett and Hindley, 1972).

Regarding the possible biochemical functions of histone acetylation, Allfrey was among the first to hypothesize that the acetylation of particular histones, *e.g.*, the arginine-rich histones F2a1 and F3, may be an early event in the activation for RNA synthesis from previously repressed gene loci (Allfrey, 1970; Allfrey *et al.*, 1964). According to this hypothesis, the reduction in net positive charge following the acetylation of lysine ϵ-amino groups would lead to weakened interactions between the histones and DNA, thereby inducing changes in the fine structure of the chromatin and allowing transcription to occur. More recently, Dixon and coworkers have suggested that histone acetylation and deacetylation may function first in facilitating the binding of certain newly synthesized histones to DNA and then in "locking" the histones into place in the major groove of the DNA helix (Louie and Dixon, 1972; Candido and Dixon, 1972a).

In contrast to the predictions of the hypotheses mentioned above, readily detectable levels of histone acetylation were found in mature avian erythrocytes. Furthermore, the significant acetylation of histone F2b has been reported here—a finding which is also surprising since this histone has been reported not to be acetylated in several other tissues. Even if it is assumed that the process of histone acetylation facilitates either gene expression or the correct binding of histones to newly synthesized DNA, it remains to be explained why a nondividing erythrocyte that is apparently completely inactive in both RNA and protein synthesis still demonstrates extensive histone acetylation and deacetylation activity. Thus it is apparent that no plausible theory is yet available to explain fully the role of histone acetylation and deacetylation in the process of cellular differentiation and the control of gene expression.

Acknowledgments

We thank Dr. William F. Marzluff for his helpful advice during the course of this work. We are indebted to Dr. Kenneth McCarty, Jr., for preparing the electron micrographs of reticulocytes and erythrocytes.

References

Allfrey, V. G. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 1447.

Allfrey, V. G., Faulkner, R., and Mirsky, A. E. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 786.

Allfrey, V. G., Littau, V., and Mirsky, A. E. (1963), *Proc. Nat. Acad. Sci. U. S. 49*, 414.

Attardi, G., Parnas, H., and Attardi, B. (1970), *Exp. Cell Res. 62*, 11.

Attardi, G., Parnas, H., Hwang, M-I. H., and Attardi, B. (1966), *J. Mol. Biol. 20*, 145.

Balhorn, R., Chalkley, R., and Granner, D. (1972), *Biochemistry 11*, 1094.

Benjamin, W. B. (1971), *Nature (London), New Biol. 234*, 18.

Billett, M. A., and Hindley, J. (1972), *Eur. J. Biochem. 28*, 541.

Boffa, L. C., Gershey, E. L., and Vidali, G. (1971), *Biochim. Biophys. Acta 254*, 135.

Bollum, F. J. (1966), *Procedures Nucl. Acid Res. 1*, 298.

Bonner, J., Huang, R. C., and Gilden, R. V. (1963), *Proc. Nat. Acad. Sci. U. S. 50*, 893.

Brecher, G. (1949), *Amer. J. Clin. Pathol. 19*, 895.

Bustin, M., and Cole, R. D. (1970), *J. Biol. Chem. 245*, 1458.

Cameron, I. L., and Prescott, D. M. (1963), *Exp. Cell Res. 30*, 609.

Candido, E. P. M., and Dixon, G. H. (1972a), *J. Biol. Chem. 247*, 3868.

Candido, E. P. M., and Dixon, G. H. (1972b), *Proc. Nat. Acad. Sci. U. S. 69*, 2015.

Ceriotti, G. (1955), *J. Biol. Chem. 214*, 59.

Choules, G. L., and Zimm, B. H. (1965), *Anal. Biochem. 13*, 336.

Cohn, E. J., Hughes, W. L., and Weare, J. H. (1947), *J. Amer. Chem. Soc. 69*, 1753.

DeLange, R. J., Fambrough, D. M., Smith, E. L., and Bonner, J. (1969), *J. Biol. Chem. 244*, 319.

DeLange, R. J., and Smith, E. L. (1971), *Annu. Rev. Biochem. 40*, 279.

DeLange, R. J., Smith, E. L., Fambrough, D. M., and Bonner, J. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1145.

Desai, L. S., and Foley, G. E. (1970), *Arch. Biochem. Biophys. 141*, 552.

Gershey, E. L., Haslett, G. W., Vidali, G., and Allfrey, V. G. (1969), *J. Biol. Chem. 244*, 4871.

Greenaway, P. J. (1971), *Biochem. J. 124*, 319.

Greenaway, P. J., and Murray, K. (1971), *Nature (London), New Biol. 229*, 233.

Iwai, K., Ishikawa, K., and Hayashi, H. (1970), *Nature (London) 226*, 1056.

Johns, E. W., and Butler, J. A. V. (1962), *Biochem. J. 82*, 15.

Johns, E. W., Forrester, S., and Riches, P. L. (1972), *Arch. Biochem. Biophys. 152*, 287.

Kernell, A. M., Bolund, L., and Ringertz, N. R. (1971), *Exp. Cell Res. 65*, 1.

Langan, T. A. (1969), *J. Biol. Chem. 244*, 5763.

Levintow, L., and Darnell, J. E. (1960), *J. Biol. Chem. 235*, 70.

Li, H., and Bonner, J. (1971), *Biochemistry 10*, 1461.

Libby, P. R. (1970), *Biochim. Biophys. Acta 213*, 234.

Lingrel, J. B., and Borsook, H. (1963), *Biochemistry 2*, 309.

Louie, A. J., and Dixon, G. H. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1975.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem 193*, 265.

Lucas, A. M., and Jamroz, C. (1961), Atlas of Avian Hematology, Washington, D. C., U. S. Department of Agriculture Monograph.

Marks, P. A., Rifkind, R. A., and Danon, D. (1963), *Proc. Nat. Acad. Sci. U. S. 50*, 336.

Marzluff, W. F., and McCarty, K. S. (1970), *J. Biol. Chem. 245*, 5635.

Marzluff, W. F., McCarty, K. S., and Turkington, R. (1969), *Biochim. Biophys. Acta 190*, 517.
Marzluff, W. F., Miller, D. M., and McCarty, K. S. (1972b), *Arch. Biochem. Biophys. 152*, 472.
Marzluff, W. F., Sanders, L. A., Miller, D. M., and McCarty, K. S. (1972a), *J. Biol. Chem. 247*, 2026.
Moss, B. A., Joyce, W. G., and Ingram, V. M. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 417A.
Ogur, M., and Rosen, G. (1950), *Arch. Biochem. 25*, 262.
Oliver, D., Balhorn, R., Granner, D., and Chalkley, R. (1972), *Biochemistry 11*, 3921.
Ord, M. G., and Stocken, L. A. (1966), *Biochem. J. 98*, 888.
Paik, W. K., and Kim, S. (1970), *J. Biol. Chem. 245*, 88.
Panyim, S., and Chalkley, R. (1969a), *Biochemistry 8*, 3972.
Panyim, S., and Chalkley, R. (1969b), *Arch. Biochem. Biophys. 130*, 337.
Patterson, M. S., and Green, R. C. (1965), *Anal. Chem. 37*, 854.
Pemberton, R. E., and Baglioni, C. (1972), *J. Mol. Biol. 65*, 531.
Pogo, B. G. T., Allfrey, V. G., and Mirsky, A. E. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 805.
Pogo, B. G. T., Allfrey, V. G., and Mirsky, A. E. (1967), *J. Cell Biol. 35*, 477.
Pogo, B. G. T., Pogo, A. O., Allfrey, V. G., and Mirsky, A. E. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 1337.
Purkayastha, R., and Neelin, J. M. (1966), *Biochim. Biophys. Acta 127*, 468.
Rall, S. C., and Cole, R. D. (1971), *J. Biol. Chem. 246*, 7175.
Rasmussen, P. S., Murray, K., and Luck, J. M. (1962), *Biochemistry 1*, 79.
Sanders, L. A., and McCarty, K. S. (1972), *Biochemistry 11*, 4216.
Schjeide, O. A., McCandless, R. G., and Munn, R. J. (1964), *Growth 28*, 29.
Seligy, V. L., and Neelin, J. (1971), *Can. J. Biochem. 49*, 1062.
Sherrer, K., Marcaud, L., Zajdela, F., London, I., and Gros, F. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1571.
Short, E., Warner, H. R., and Koerner, J. (1968), *J. Biol. Chem. 243*, 3342.
Vidali, G., Gershey, E. L., and Allfrey, V. G. (1968), *J. Biol. Chem. 243*, 6361.
Wangh, L., Ruiz-Carrillo, A., and Allfrey, V. G. (1972), *Arch. Biochem. Biophys. 150*, 44.

Structural Studies on the Glycolipids from the Envelope of the Heterocyst of *Anabaena cylindrica*†

Fernand Lambein‡ and C. Peter Wolk*

ABSTRACT: Four glycolipids were isolated and purified from heterocysts of *Anabaena cylindrica*. The structures of these lipids have been studied by mass spectrometry, infrared spectroscopy, and nuclear magnetic resonance spectrometry. The position of the bond between the sugar and the aglycone was determined by permethylation of the intact lipid, followed by hydrolysis, trimethylsilylation, and mass spectrometry. A C-26 and a C-28 polyhydroxy alcohol are glycosylated at their terminal hydroxyl. A C-26 and probably a C-28 hydroxy fatty acid are glycosylated at their carboxylic group.

Certain blue-green algae contain unusual lipids (Nichols and Wood, 1968) which are localized largely or wholly in a laminated layer of the envelope that surrounds the wall of differentiated cells called heterocysts (Walsby and Nichols, 1969; Wolk and Simon, 1969; Winkenbach *et al.*, 1972). The more polar of these lipids are nonsaponifiable glycolipids (Nichols and Wood, 1968; Walsby and Nichols, 1969), the aglycone moieties of which, from *Anabaena cylindrica*, were identified by Bryce *et al.* (1972) as 1,3,25-trihydroxyhexacosane and probably 1,3,25,27-tetrahydroxyoctacosane.

We have fractionated the "heterocyst lipids" from the same alga into four distinct glycolipids. Lipids III and IV consist of a hexose bound by a glycosidic linkage to the terminal hydroxyls of the long-chain polyhydroxy alcohols described by Bryce *et al.* (1972). Lipid I consists of hexose bound to the carboxyl group of 25-hydroxyhexacosanoic acid. Lipid II appears to be similar structurally to lipid I.

Material and Methods

Centrifugal pellets of heterocysts isolated from *A. cylindrica* Lemm. (Wolk, 1968) were extracted three times with five volumes of chloroform–methanol (2:1, v/v) at room temperature. The pooled extracts, concentrated under vacuum, were shaken with chloroform and water in a separatory funnel. The lipids of the heterocyst envelope stayed largely at the interface. The aqueous and interface layers were extracted five times with 50 ml of chloroform. The lipid material at the interface was separated from the water layer by filtration through sintered glass and was then dissolved with chloroform–methanol (2:1) or with hot methanol.

The interface lipids were separated by repeated chromatography on a 21 × 0.5 in. column of a mixture of silicic acid and Celite (1:2, w/w). The lipids were applied to the column in a minimum volume of chloroform–methanol, washed with 200 ml of chloroform, and eluted with 100-ml volumes of chloroform–methanol mixtures containing increasing concentrations (2, 4, 5, 6, 8, and 10%) of methanol. The eluate

† Contribution from the MSU/AEC Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48823. *Received September 11, 1972.* This work was supported in part by the University of Ghent, Ghent, Belgium, and by a fellowship from North Atlantic Treaty Organization to F. L., and in part by the U. S. Atomic Energy Commission under Contract AT(11-1)-1338.

‡ Present address: Laboratory of Physiological Chemistry, Faculty of Medicine, State University of Ghent, Ghent, Belgium.