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ACETYLATION OF HUMAN FETAL HEMOGLOBIN OCCURS THROUGHOUT ERYTHROID CELL MATURATION

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The biosynthesis of human acetylated fetal hemoglobin (Hb F_1) has been examined by incubating the following cell types with [^{3}H]leucine: (a) burst-forming unit erythroid cells cultured from umbilical cord mononuclear cells, (b) infant bone marrow, (c) umbilical cord blood, and (d) peripheral blood cells from adults with elevated fetal hemoglobin. Newly synthesized Hb F_1 was 18–20% that of Hb F_0 in burst-forming unit erythroid cells which were immature, mature, or in an intermediate state of development. In infant marrow and in infant and adult peripheral blood the extant Hb F_1 comprised 10.8 ± 1.8% of the total Hb F. In marrow cells the specific radioactivity (cpm/mg) of Hb F_1 was 1.4–2.0-times greater than that of Hb F_0. In peripheral blood cells these ratios were slightly greater. [^{3}H]Leucine-labeled infant bone marrow, umbilical cord blood, and adult peripheral blood cells were subjected to density gradient ultracentrifugation. The ratios of specific radioactivity for Hb F_1/Hb F_0 increased from 1.0–1.8 in the lightest cell zone to 5.2–9.0 in the more dense cells. Thus the biosynthesis of Hb F_1 is enhanced in cells which are more mature than those responsible for the bulk of hemoglobin synthesis, and the acetylation of Hb F provides a marker of erythroid cell maturation.

Introduction

Human fetal hemoglobin (Hb F) is composed of two α- and two γ-polypeptide chains. Analysis of Hb F, isolated from the blood of a variety of fetal and adult sources, consistently shows that 10–20% is acetylated. The acetylated form, Hb F_1, differs in structure from the unacetylated component, Hb F_0, only by the presence of acetyl groups at the amino terminus of the γ-chains [1]. We reported previously [2] that, in the circulating reticulocytes of the newborn and in those adults with elevated fetal hemoglobin, the rate of biosynthesis of acetylated fetal hemoglobin was higher than that expected from the relative amounts of this hemoglobin existing in the blood. Further analysis by density gradient ultracentrifugation of peripheral blood cells from an adult with $\delta^\circ\beta^\circ/\beta^+$-thalassemia showed that much of the Hb F_1 synthesis occurred in a population of erythroid cells heavier than, and probably more mature than the cells responsible for most of the hemoglobin synthesis.

In the present work we show that there are substantial amounts of Hb F_1 synthesis in human fetal bone marrow and burst-forming unit erythroid cultured cells, both of which include significant numbers of the most immature cells capable of hemoglobin synthesis. In addition we show that elevated Hb F_1 synthesis is enhanced in

the more dense fraction from ultracentrifugation of fetal bone marrow cells, umbilical cord blood cells, and peripheral blood cells from a β°-thalassemic adult. Structural analyses show that the newly synthesized Hb F_1 is genuine and does not represent a newly synthesized non-hemoglobin contaminant.

Materials and Methods

Biosynthesis of hemoglobin in infant bone marrow, umbilical cord blood, and in adult peripheral blood. Newborn umbilical cord blood and peripheral blood from two adults with elevated levels of Hb F were collected in EDTA and chilled to 4°C. The blood hemoglobin composition of the newborn was: 78% F_0, 8% F_1, 14% A. The hematologic disorders and blood hemoglobin compositions of the two adult donors were: C.G., doubly heterozygous for $\delta^{\circ}\beta^{\circ}$ and β^{+}-thalassemia, 80% F_0, 11% F_1, 8% A, 2% A_2; S.O., homozygous for β°-thalassemia, 84% F_0, 13% F_1, 3% A_2. Bone marrow aspirates were obtained from the posterior iliac crest of the following three infants: B.D., a newborn with a chromosomal abnormality but without a hematologic disorder, 74% F_0, 7% F_1, 19% A; G.A., a 16-week-old infant with severe hemolytic anemia of unknown origin, 11% F_0, 1% F_1, 88% A; E.K., a 36-day-old infant with a myeloproliferative disorder associated with Down's syndrome, 17% F_0, 2% F_1, 82% A. The blood or marrow cells were washed three times in modified saline and incubated at 37°C with L-[4,5-^{3}H]leucine (60–65 Ci/mmol) (New England Nuclear, Boston, MA) for 60 min as described previously [3]. In some experiments, the washed ^{3}H-labeled cells were separated into zones of increasing density by centrifugation into a discontinuous gradient of Dextran 40 (Sigma, St. Louis, MO) [2,4]. Cells located at the top, bottom (pellet), and at the interfaces of the gradient zones were harvested and washed with cold saline solution.

Hemolysates from the ^{3}H-labeled and washed cells were analyzed for absorbance at 540 nm (total hemoglobin), for total protein ^{3}H-radioactivity by trichloroacetic acid precipitation, and for the relative amounts and synthetic rates of Hb F_1 and Hb F_0 by chromatography on columns of Bio-Rex 70 (Bio-Rad Labs, Richmond, CA) as described previously [2,5]. Samples (0.4–50 mg Hb) of each marrow cell lysate were passed first through a column (1.2 × 50 cm) of Sephadex G-100 fine (Pharmacia Fine Products, Piscataway, NJ), in an attempt to separate non-hemoglobin proteins from hemoglobin by molecular sieving, prior to Bio-Rex 70 chromatography. Because of the low amount of hemoglobin in the chromatographed lysate from the least dense zone of bone marrow cells, an aliquot from each eluted fraction was reacted with tetramethylbenzidine (Tridon Chem. Co., Haupauge, NY) essentially as described [6].

Biosynthesis of hemoglobin in burst-forming unit erythroid cells. Fetal hemoglobin synthesis was also examined in burst-forming unit erythroid cells grown in culture from mononuclear cells isolated from the umbilical cord blood of a normal newborn [7]. Depending upon the intensity of red color and colony appearance, the erythroid bursts were classified into the following three categories, representative of their maturation-hemoglobinization status: (a) Young bursts, having no visible hemoglobinization (i.e., no pink or red color) in any of their subcolonies. (b) Intermediate, showing color (pink or orange) in all or some of their subcolonies. (c) Mature bursts, all subcolonies showed intense red color. The bursts were lifted individually from the plates using ultrathin pipettes and pooled according to their maturation class. These cells were incubated with [^{3}H]leucine for 6 h at 37°C. The ^{3}H-labeled cell lysates were passed through Sephadex G-100 [8], after which unlabeled umbilical cord lysate containing 21 mg of hemoglobin was added as carrier, and these mixtures were analyzed by Bio-Rex chromatography.

Analysis of globin polypeptide chains. The distribution of ^{3}H-radioactivity among the globin chains of certain hemolysates or purified hemoglobin samples was determined by chromatography on CM-cellulose in 8 M urea [2,9].

Tryptic peptide analysis of newly synthesized Hb F_1 and Hb F_0. Washed red cells from an adult donor (C.G.) with β-thalassemia intermedia were incubated with 87.2 μM [^{3}H]phenylalanine (46.7 Ci/mmol) (New England Nuclear) for 60 min at 37°C in a protein synthesis reaction mixture [3] depleted of nonradioactive phenylalanine. The newly-synthesized [^{3}H]Hb F_1 and [^{3}H]HbF_0 were isolated by chromatography on Bio-Rex 70 [2],

and globin chains were separated on CM-cellulose in 8 M urea [2,9]. Approx. 5 mg of isolated γ- and γ^{Ac} chains in 5 ml of 0.5% ammonium bicarbonate, pH 8.5, were digested with 0.05 ml of trypsin (Worthington Biochemical Corp., Freehold, NJ) (1 mg/ml in 0.001 N HCl) for 6 h at 23°C. The digest was heated for 5 min at 90°C and lyophilized. About 2 mg of the lyophilized peptides were suspended in 0.23 ml of 10% acetic acid, and after centrifugation, 200 μl of the supernatant were analyzed by high performance liquid chromatography (HPLC) on a Bondapak C18 column (Waters Associates, Milford, MA) as described previously [10]. The absorbance of each eluted fraction (1.5 ml) was determined at 220 nm, and radioactivity was determined by counting in 10 ml of ACS scintillant (Amersham, Arlington Heights, IL).

Analysis of acetate in newly synthesized Hb F_1 and Hb F_0. Blood cells from C.G. were incubated with 5.7 mM [^{3}H]acetate (spec. act., 2 Ci/mmol) and 91.4 μM [^{14}C]leucine (spec. act., 312 mCi/mmol) (New England Nuclear) for 60 min at 37°C, after which the ^{3}H- and ^{14}C-labelled cells were separated into five zones by density gradient ultracentrifugation [2,4]. Radioactive globins from newly synthesized Hb F_1 and Hb F_0 from cell hemolysates from each gradient zone were mixed with excess nonradioactive F_1 globin (30–50 mg of $\alpha\gamma^{Ac}$-dimer) containing a known amount (1.0–1.5 μmol) of protein acetate. The mixture was digested in a sealed tube with 0.5 ml of 2 N HCl in methanol at 100°C for 4 h [11]. The methyl acetate formed was recovered by vacuum distillation, and the amount of acetate in the distillate was determined by colorimetric assay [12]. The fraction of ^{3}H-radioactivity present as distillable acetate was determined by suspending samples of both distillate and residue in 0.5 ml of 1 N NaOH and counting these suspensions in 15 ml of a dioxane-based scintillation system [13].

Results

Biosynthesis of hemoglobin in infant bone marrow. In infant bone marrow cells the rate of biosynthesis of the acetylated fetal hemoglobin Hb F_1 was higher than that expected from the relative amounts of this component of Hb F existing in the cells (Table I). The F_1/F_0 specific radioactivity ratios of 1.4, 1.9, and 2.0 were similar to those we reported previously [2] for adult peripheral and umbilical cord cells labeled in a similar manner. After centrifugation into zones of increasing densities of a Dextran 40 medium, the ^{3}H-labeled bone marrow cells of one infant (E. K.) had elevated F_1/F_0 specific radioactivity ratios and thus enhanced preferential synthesis of Hb F_1 in the more dense cells, a finding previously observed for the peripheral blood reticulocytes of a β-thalassemic adult with 90% fetal hemoglobin [2]. However, the specific radioactivity ratio of Hb F_1/Hb F_0 was no less than unity in the least dense zone of marrow cells, responsible for 49% of the total composite synthesis of new hemoglobin, although containing less than 2% of the existing hemoglobin (Table III). Thus, even in those marrow cells which are most active in hemoglobin synthesis and probably most immature, there was substantial synthesis of acetylated fetal hemoglobin. The specific radioactivity ratio for Hb F_1/Hb F_0 in the heaviest cell fraction was 5.2.

Biosynthesis of hemoglobin in burst-forming unit erythroid cells. To confirm the finding of Hb F_1 in younger erythropoietic cells, colonies of burst forming unit erythroid cells in various stages of maturation were incubated with [^{3}H]leucine. Table II shows that there was substantial synthesis of Hb F_1 during each of the three stages of maturation represented by progressively increased amounts of cellular hemoglobin. The low numbers of cells in these cultures precluded an accurate measurement of hemoglobin amount and, hence, of specific radioactivity. Nevertheless, the finding that Hb F_1 synthesis represented about 15% of total fetal hemoglobin synthesis is similar to that above for the bone marrow cells and those we previously reported for adult peripheral and umbilical cord blood cells [2].

Synthesis of acetylated fetal hemoglobin in peripheral blood. The ^{3}H-labeled red cells from newborn umbilical cord blood and from the peripheral blood of a β°-thalassemic adult (S.O.) were also separated by density gradient ultracentrifugation. In each case 82% of the radioactivity due to newly synthesized hemoglobin was in the least dense cell zone, with the remainder being distributed in the heavier zones (Table III).

TABLE I

BIOSYNTHESIS OF Hb F_1 AND Hb F_0 IN BONE MARROW CELLS

The amounts and specific radioactivities of hemoglobin components were determined after Bio-Rex 70 chromatography of ^{3}H-labeled bone marrow lysates. The ^{3}H-labeled cells of E.K. were separated into four populations of increasing density by ultracentrifugation for 90 min at 35000 rpm into a discontinuous gradient consisting of 28%, 24%, and 20% Dextran 40.

Donor and gradient zone	mg Hb F_1 / mg Hb F_0	cpm/mg Hb F_1	cpm/mg Hb F_0	cpm/mg Hb F_1 / cpm/mg Hb F_0
B.D.	0.10	1110000	590000	1.89
G.A.	0.09	208000	145000	1.43
E.K.	0.10	163000	81400	2.01
1, light	0.12	1690000	1670000	1.0
2	0.10	154000	118000	1.3
3	0.11	143000	38100	3.8
4, heavy	0.10	104000	20100	5.2

TABLE II

HEMOGLOBIN SYNTHESIS IN BURST-FORMING UNIT ERYTHROID CELLS

The total radioactivity of separated hemoglobin components was determined after Bio-Rex 70 chromatography of ^{3}H-labeled burst-forming unit erythroid cell lysates.

Degree of maturation	cpm in Hb A	cpm in Hb F_1	cpm in Hb F_0	cpm Hb F_1 / cpm Hb F_0
Immature	6740	2750	14600	0.19
Intermediate	56100	15800	79000	0.20
Mature	59000	13400	75800	0.18

TABLE III

DISTRIBUTION OF THE TOTAL HEMOGLOBIN AND PROTEIN ^{3}H-RADIOACTIVITY AMONG CELLS OF VARIOUS DENSITIES AFTER SEPARATION IN A DEXTRAN 40 GRADIENT

Donor and gradient zone	^{3}H-radioactivity in zone (cpm)	Percent of total radioactivity	Amount of hemoglobin in zone (mg)	Percent of total hemoglobin
Bone marrow (E.K.)				
Zone 1, light	2570000	48.7	0.7	1.7
Zone 2	1030000	19.6	6.2	14.8
Zone 3	1080000	20.5	23.4	55.7
Zone 4, heavy	596000	11.3	11.8	28.1
Umbilical cord blood				
Zone 1, light	44400000	82.4	69.6	21.2
Zone 2	5520000	10.2	114.8	35.0
Zone 3	2130000	4.0	77.8	23.7
Zone 4, heavy	1840000	3.4	65.8	20.1
β-Thal. (S.O.)				
Zone 1, light	22300000	82.3	20.3	12.4
Zone 2	1820000	6.7	37.2	22.6
Zone 3	1330000	4.9	41.6	25.3
Zone 4	958000	3.5	38.5	23.4
Zone 5, heavy	694000	2.6	26.5	16.1

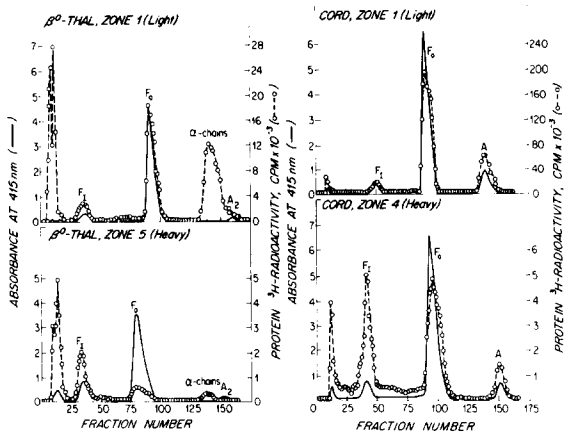

Fig. 1. Bio-Rex 70 chromatography of hemolysates from light and heavy cell zones after density gradient ultracentrifugation. Umbilical cord blood cells from a normal newborn and peripheral blood cells from a β^0-thalassemic (S.O.) were incubated with [^{3}H]leucine for 1 h at 37°C and were then separated in a Dextran 40 gradient in the ultracentrifuge. An aliquot from the lysate of each gradient zone was subjected to chromatography on Bio-Rex 70 (1.2×20cm) at 4°C as described in Materials and Methods. In each case zone 1 is the least dense. For the umbilical cord blood zone 4 is the most dense, while for S.O., the most dense zone is 5. Absorbance at 415 nm (———) and protein ^{3}H-radioactivity (O---O) of each chromatographic fraction were determined. Recovery of absorbance at 415 nm was from 96 to 100%. Recovery of radioactivity was from 86 to 94%.

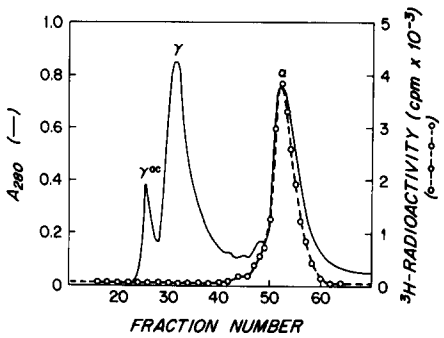

Fig. 2. Elution pattern from the separation of the globin chains of a newly synthesized protein fraction in the lightest erythroid cells of a β^0-thalassemic adult. Fractions 130–150 from the Bio-Rex chromatography of the ^{3}H-labeled hemolysate of S.O. shown in Fig. 1, upper panel, were pooled and added to an excess (40 mg of hemoglobin) of unlabeled hemolysate from the same individual. The mixture was converted to globin, and the α, γ, and γ^{Ac} globin chains were separated by chromatography on a CM-cellulose column (1.2×18 cm) in 8 M urea [2,9]. The distribution of ^{3}H-radioactivity among the globin chains was determined by adding each eluted fraction (3 ml) to 10 ml of ACS counting fluid (Amersham) and counting in a liquid scintillation counter: 87% of the ^{3}H-radioactivity applied to the column was recovered in the fractions.

Fig. 1 shows the chromatographic patterns of hemolysates from the light and heavy zones of red cells from umbilical cord blood and β^0-thalassemic blood. In each case the radioactivity due to Hb F_0 is greater than Hb F_1 in the lightest cell zone, whereas in the heavy cell zone there is at least as much radioactivity in Hb F_1 as in Hb F_0. The ratio of specific radioactivity Hb F_1/Hb F_0 increases markedly with higher cell density, similar to results obtained on peripheral blood cells from an individual (C.G.) with thalassemia intermedia [2].

Analysis of globin polypeptide chains. In light cells from S.O., one of the major radioactive fractions is due to free α-chains (Fig. 2). This free α-chain peak is diminished or absent in lysates from the most dense cells from S.O., and also is virtually absent in the cord blood lysates (Fig. 1).

In cells from S.O. there were sizable decreases in the syntheses of both α- and non-α-chains with increasing cell density, with the decrease in α-chain synthesis occurring more rapidly during cell maturation than the decrease in total γ-chain synthesis (Table IV). The non-α/α-chain synthesis ratio of the total hemolysate increased from 0.28 in the lightest cell zone to 1.67 in the heaviest cells, a finding consistent with the decrease in the amount of newly synthesized free α-chains (Figs. 1, 2). Moreover, Table IV shows that, although total γ (acetylated and unacetylated) chain synthesis decreased during cell maturation, there was an increased relative synthesis of γ^{Ac}-chains, i.e., a higher fraction of total newly synthesized γ-chains was being acetylated. The distribution of radioactivity between the α- and non-α-chains of the chromatographically isolated Hb F_1 and Hb F_0 were consistent with the data obtained from the total hemolysates. Table IV shows that the γ/α synthesis ratio of the isolated Hb F_0 decreased from 0.96 in the lightest cell zone to 0.51 in the heaviest zone, showing a relative increase in specific radioactivity of the precursor α-chain pool compared to that of the γ-chain pool in the denser cells. The radioactivity of the γ^{Ac}-chains of Hb F_1

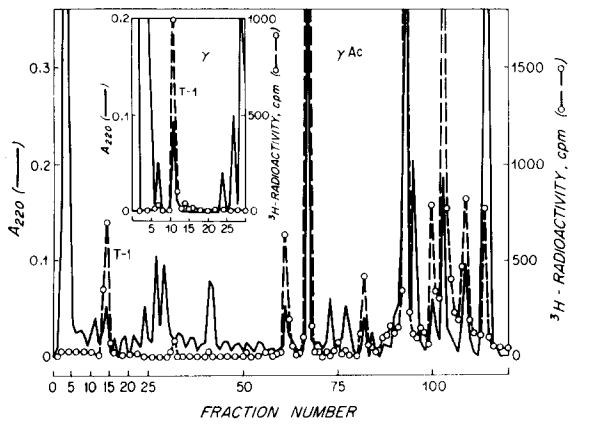

Fig. 3. Separation of acetylated γ-chain (γ^{Ac}) peptides by high performance liquid chromatography (HPLC). Blood cells from a donor with β-thalassemia intermedia (C.G.) were incubated with [^{3}H]phenylalanine, and the ^{3}H-labeled Hb F_1 and Hb F_0 were separated by chromatography of the cell soluble phase on a Bio-Rex 70 column. The acetylated γ-chains were isolated from the [^{3}H]Hb F_1 and digested with trypsin, and the soluble peptides were analyzed by HPLC on a μBondapak C18 column. The peptide amount and radioactivity of each eluted fraction were determined by measurement of the absorbance at 220 nm and by counting in a liquid scintillant, respectively. The inset shows the elution profile for the first 30 fractions from a similar analysis of the γ-chain peptides isolated from the ^{3}H-labeled Hb F_0; the absorbance and radioactivity elution profile for the remainder of the γ-chain chromatogram was identical to that shown for the γ^{Ac} peptides. The elution position of the N-terminus (T-1) tryptic peptides for both the γ^{Ac} and γ (inset) chains are shown. Six tryptic peptides of the human γ-chain have phenylalanine residues [14]; our finding of more than six peptides labeled with ^{3}H-radioactivity probably occurred because of incomplete digestion with trypsin.

was higher than that of the α-chains, suggesting that during hemoglobin assembly, some of the newly synthesized γ^{Ac}-chains combined with pre-existing unlabeled α-chains, an event that probably occurred because of a smaller relative pool size of γ^{Ac}-chain precursors.

Tryptic peptide analysis of newly synthesized Hb F_1 and Hb F_0. To provide additional evidence that the ^{3}H-labeled Hb F_1 represented de novo synthesis of genuine fetal hemoglobin acetylated at the N-terminus of the γ-chain, we analyzed the peptides obtained after tryptic digestion of the γ-chains. Washed blood cells from (C.G.) were incubated with [^{3}H]phenylalanine instead of [^{3}H]leucine, because of the presence of phenylalanine and absence of leucine in the γ-chain N-terminal tryptic peptide T-1 [14].

The HPLC elution profile obtained after separation of the γ^{Ac} peptides from the newly synthesized Hb F_1 (Fig. 3) is similar to that reported by Wilson et al. [10] for γ tryptic peptides. Analysis of the γ-chain peptides, isolated from the newly synthesized Hb F_0, resulted in an absorbance and radioactivity elution profile identical to that of the acetylated γ-chain peptides, except that a [^{3}H]phenylalanine-labeled peptide in Fraction 11 (see Fig. 3 inset) replaced that in Fraction 14. Amino acid analysis of these two ^{3}H-labeled peptides confirmed their identity as γ T-1 peptides (data not shown). These results are consistent with the finding that γ^{Ac}- and γ-chains are identical in primary structure expect for the N-terminal acetyl group of the former [1]. In this experiment, the specific radioactivity of the chromatographically purified [^{3}H]Hb F_1 was 3.1-times that of the [^{3}H]Hb F_0. The specific radioactivity of the γ^{Ac} T-1 peptide (cpm per A_{220} nm unit) subsequently isolated by HPLC was 3.8-times that of the corresponding γ T-1 peptide. This finding suggests that the enhanced relative synthesis of Hb F_1 is authentic, and that the results cannot be explained by the presence of a putative contaminant, e.g., a newly synthesized non-hemoglobin protein, in the Hb F_1 component.

Analysis of acetate in newly synthesized Hb F_1 and Hb F_0. To further confirm the biochemical identity of newly synthesized Hb F_1, we incubated cells from donor C.G. with sodium [^{3}H]acetate and [^{14}C]leucine and separated them into five zones of increasing density in a Dextran 40 gradient. The [^{14}C]leucine specific radioactivity of the Hb F_1 and Hb F_0 was entirely consistent with previous findings [2].

Table V shows that about 30 and 10% of the Hb F_1 ^{3}H-radioactivities of the heaviest and lightest zones of cells, respectively, were recovered as distilled methyl acetate. As expected, none of the Hb F_1 ^{14}C-radioactivities were recovered in the distillate (data not shown). When additional samples of these radioactive F_1 globins were analyzed by chain separation chromatography ón CM-cellulose in 8 M urea, most of the ^{3}H-radioactivity eluted with acetylated γ-chains, although a substantial minority eluted with the α-chains. These

TABLE IV

RELATIVE AMOUNTS OF NEWLY SYNTHESIZED α- AND NON-α-CHAINS IN HEMOLYSATES AND HEMOGLOBINS OF ERYTHROID CELLS OF INCREASING DENSITY

The ^{3}H-labeled globin chains from hemolysates, Hb F_1, and Hb F_0 from S.O., C.G., and umbilical cord blood cells from density gradient ultracentrifugation were separated by CM-cellulose chromatography. The amount of radioactivity (cpm) associated with each chain type represents that calculated for the total number of cells in each density zone. Data were obtained in a similar manner from a sample of cells (control) which were incubated with [^{3}H]leucine but not separated in a Dextran gradient.

Donor and gradient zone	Total lysate				Hb F_0	Hb F_1
	α	γ	γ^{Ac}	cpm non-α / cpm α	cpm γ / cpm α	cpm γ^{ac} / cpm α
S.O., control	3783000	814400	454400	0.34	0.70	2.73
1 light	10535000	2346000	612400	0.28	0.96	2.27
3	256400	109900	281600	1.54	0.68	5.17
5 heavy	180100	49530	229900	1.56	0.51	4.19
C.G., control	8631000	2168000	880900	0.46	–	–
1 light	55273000	10942000	2631000	0.33	1.08	3.14
3	409900	396200	563700	2.63	–	4.94
5 heavy	358700	394000	542400	2.86	0.84	4.77
Newborn No. 1, control	1274000	678600	76400	0.91	1.30	1.45
1 light	10797000	4128000	601700	0.81	1.26	1.42
2	1550000	689900	306500	1.05	–	–
3	350600	261300	242700	1.79	–	–
4 heavy	291800	203600	152300	1.67	1.11	1.83

results, as well as earlier work [15] indicate that a significant fraction of the [^{3}H]acetate precursor was converted by the blood cells to ^{3}H-labeled amino acids which were incorporated into globin chains. This finding provides an explanation for the failure to recover more than 30% of the Hb F_1

TABLE V

RECOVERY OF ACETATE FROM ACETYLATED GLOBIN SAMPLES

Hb F_1 and Hb F_0 were isolated from [^{3}H]acetate and [^{14}C]leucine labeled cells (C.G.) which had been separated by density gradient ultracentrifugation. Radioactive Hb F_1 globin from the top (lightest), middle, and bottom (heaviest) cell zones and radioactive Hb F_0 globin from the top zone were mixed with 30–50 mg of nonradioactive Hb F_1 globin (1–1.5 μmoles of $\alpha\gamma^{Ac}$ dimer), and the mixture was digested with methanolic HCl. The methyl acetate recovered is expressed as a percentage of the initial amount of $\alpha\gamma^{Ac}$ dimer. The total recoveries of ^{3}H- and ^{14}C-radioactivities of both distillate and residue ranged from 85 to 115% of those in the radioactive globin added to the digestion mixture. The ^{3}H-radioactivity in the distillate is expressed as a percentage of the total ^{3}H-cpm in the digestion mixture.

^{3}H, ^{14}C-labeled hemoglobin	Gradient zone of cells	Carrier F_1 globin acetate		Globin ^{3}H cpm	
		Total μmol	% Distilled [a]	Total	% Distilled
Hb F_1	top	1.47	67	4478	9.8
Hb F_1	middle	1.38	62	990	23.6
Hb F_1	bottom	1.13	73	515	31.2
Hb F_0	top	0.95	55	14266	0.9

[a] Analyses of similar amounts of unacetylated Hb F_0 or Hb A globins gave distillate values identical to those of the methanolic HCl reagent 'digested' without added globin. These 'blank' values were consistently 0.15–0.20 μmol of acetate and have been subtracted from the Hb F_1 values shown.

^{3}H-radioactivity in the distilled acetate fraction. In summary, the results of the dual-labeled experiments are consistent with enhanced synthesis of authentic Hb F_1 in the denser blood cells.

Discussion

The acetylation event occurs early in the life of a protein molecule, perhaps during growth of the nascent polypeptide chain [15–17]. The acetyltransferase enzyme is probably bound to the ribosomes [18], the organelles responsible for production of individual polypeptide chains. Kasten-Jolly and Taketa [19] reported recently that the N-terminal acetylation of the β-chain of cat Hb occurred when the nascent chain was about 30 amino acids long. A similar mechanism of γ-chain acetylation may occur in human erythroid cells. Human fetal hemoglobin is unique, because only a minority of the γ-chains are acetylated. This incomplete acetylation may occur because a growing polypeptide chain with glycine as the N-terminal amino acid residue, e.g. the nascent γ-chain, is less than an ideal substrate for the acetyltransferase enzyme. Jornvall [20] emphasized the role of the type of N-terminal amino acid in specificity of the substrate for acetylation, and found that serine and alanine were preferable to glycine. Indeed, the normal N-terminal valine of the β-chain of adult human hemoglobin is replaced by alanine in the variant Hb Raleigh, and all of these abnormal β-chains are acetylated at the N-terminus [21].

Our experiments show that the biosynthesis of authentic γ-acetylated Hb F_1 was increased markedly in those cells which were more dense, and probably more mature than the cells responsible for the bulk of hemoglobin synthesis.

The following data suggest that the more dense cells from ultracentrifugation in Dextran 40 are older cells. When red blood cells from adult or fetal donors are subjected to ultracentrifugation in a discontinuous gradient of Dextran 40, there is a continual decrease in numbers of reticulocytes and in mean corpuscular volume, and an increase in mean corpuscular hemoglobin concentration as the cell density increases [4,22]. Moreover, the lightest cells have a greater percentage of nucleated erythroid cells and markedly greater synthetic activity.

In addition to its synthesis in adult and fetal peripheral blood, Hb F_1 is also synthesized in burst-forming unit erythroid cells and infant bone marrow cells, erythroid cells which are relatively more immature. In addition, the results from our current experiments are consistent with the earlier finding of a marked increase in the synthesis of Hb F_1 relative to Hb F_0 in the more mature cell fractions. These experiments, performed on a variety of erythroid cells suggest that this is a universal phenomenon which is not confined to the cells of a single β-thalassemic individual.

Two events occurring in the older erythroid cells of our studies may enhance the probability that a nascent γ-chain will be acetylated: (a) hemoglobin synthesis is diminished in reticulocytes compared to younger erythroid cells and is nonexistent in mature circulating red cells. For example, there was a decrease of both α- and non-α-chain synthesis in the heavier ^{3}H-labeled erythropoietic cells in our experiments. Past studies showed that this decline in hemoglobin synthesis was directly associated with a disappearance of ribosomes in the older erythropoietic cells [23,24]. The ribosomes are probably degraded into their constituent components [25], one of which is the acetyltransferase enzyme. The resulting elevated acetyltransferase activity in the cell soluble phase may contribute to acetylation of the γ-chains being synthesized on the few remaining active polyribosomes. (b) Our results suggested that there was a selective decrease in the amount of free α-chains in the older reticulocytes. During assembly of the hemoglobin tetramer, soluble α-chains may interact with the complementary nascent non-α-chain partners and aid in their completion and release from the polyribosome fraction [26]. Such interaction of soluble α-chains with nascent γ-chains in the human erythroid cells of our work may interfere with the acetyltransferase enzyme and effectively remove the growing γ-chain from the acetylation substrate pool. Our previous finding that the completed Hb F_0 tetramer is not an intermediate in Hb F_1 synthesis [2] is consistent with the proposal that the αγ-dimer, once formed, cannot serve as an acetylation substrate. A decrease in the concentration of soluble α-chains, competitors of the acetyltransferase enzyme for the nascent γ-chains, would enhance the acetylation event in older reticulocytes.

Two other mechanisms may contribute to the

relatively enhanced synthesis of Hb F_1 in more mature cells. N-terminal acetylation may protect the γ-chain against proteolysis. Alternatively, and more likely, as erythroid cells mature, the competition between γ- and γ^{Ac}-chains for combination with the more limiting amounts of α-chains (Table IV) is greatly enhanced. The greater negative charge of the γ^{Ac}-chain relative to the γ-chain may favor a more rapid combination with α-chains to form the stable $\alpha\gamma^{Ac}$-dimer. Recent findings [27–30] have indicated that electrostatic interactions are an important determinant of hemoglobin assembly. Individuals heterozygous for positively charged β-globin variants have a significantly lower proportion of abnormal hemoglobin, relative to Hb A, than those with negatively charged variants. these differences become more pronounced when alpha thalassemia is also present. Kinetic experiments employing isolated chains indicate that the rate of assembly of the tetramer is influenced by alterations in surface charge on the chains. A similar conditions may occur in the mature erythroid cells of the present study, in which the relatively more negatively charged γ^{Ac}-chains may compete more efficiently for α-chains than the unacetylated γ-chains, in an environment of a diminished α-chain pool.

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