The Biosynthesis of Human Hemoglobin A_{1c}

SLOW GLYCOSYLATION OF HEMOGLOBIN IN VIVO

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A B S T R A C T Hemoglobin A_{1c}, the most abundant minor hemoglobin component in human erythrocytes, is formed by the condensation of glucose with the N-terminal amino groups of the β-chains of Hb A. The biosynthesis of this glycosylated hemoglobin was studied in vitro by incubating suspensions of reticulocytes and bone marrow cells with [^{3}H]leucine or ^{59}Fe-bound transferrin. In all experiments, the specific activity of Hb A_{1c} was significantly lower than that of Hb A, suggesting that the formation of Hb A_{1c} is a posttranslational modification. The formation of Hb A_{1c} in vivo was determined in two individuals who were given an infusion of ^{59}Fe-labeled transferrin. As expected, the specific activity of Hb A rose promptly to a maximum during the 1st week and remained nearly constant thereafter. In contrast, the specific activity of Hb A_{1c} and also of Hbs A_{1a} and A_{1b} rose slowly, reaching that of Hb A by about day 60. These results indicate that Hb A_{1c} is slowly formed during the 120-day life-span of the erythrocyte, probably by a nonenzymatic process. Patients with shortened erythrocyte life-span due to hemolysis had markedly decreased levels of Hb A_{1c}.

INTRODUCTION

The diverse biological functions of many proteins depend upon posttranslational modifications (1). Because the biosynthesis and structure of human hemoglobin are so well understood, this protein is ideally suited for the study of such structural alteration. Fortunately, human hemoglobin is less heterogeneous than that of other mammals. About 90% of hemoglobin of adults and children above the age of 6 mo is Hb A ($\alpha_2\beta_2$). Hb A_2 ($\alpha_2\delta_2$) and

Hb F ($\alpha_2\gamma_2$) comprise about 2.5 and 0.5% of the total, respectively. The synthesis of these minor components is controlled by δ- and γ-chain genes. In contrast, the other minor hemoglobin components found in normal adult hemolysate may be posttranslational modifications of Hb A. When human hemolysate is chromatographed on cation exchange resins, three negatively charged minor hemoglobin components are eluted before the main Hb A peak (2, 3). Hemoglobins A_{1a}, A_{1b}, and A_{1c} comprise approximately 1.6, 0.8, and 4% of the total hemoglobin of adult human erythrocytes, respectively. Thus, Hb A_{1c} is the most abundant minor hemoglobin component. Holmquist and Schroeder (4) showed that Hb A_{1c} is structurally identical to Hb A except that an unidentified group was linked to the terminal amino group of the β-chain by means of a Schiff base. Bookchin and Gallop (5) demonstrated that both β-chains of Hb A_{1c} were attached to a hexose. Recently, we have established the presence of glucose on β-A_{1c} and have presented evidence indicating that this moiety is attached to the N-terminal amino group by a unique ketoamine linkage (6), formed by a rearrangement of the Schiff base.

Interest in Hb A_{1c} has been considerably enhanced by the fact that this glycoprotein is increased about twofold in patients with diabetes mellitus (7–9). Other qualitative and quantitative abnormalities of glycoproteins have been described in both clinical and experimental diabetes (10) and may provide new insights into the pathogenesis of the disease and its complications. An understanding of the biosynthesis of hemoglobin A_{1c} is essential in determining its biological relevance both in normal individuals and in those with diabetes. In this paper, we present both in vitro and in vivo evidence that the formation of hemoglobin A_{1c} is a relatively slow process, probably nonenzymatic in nature, which pro-

ceeds continuously throughout the life-span of the erythrocyte.

METHODS

Specimens of blood and bone marrow were collected in heparin from normal adult volunteers and from selected patients with hemolytic anemias. Hemolysates were prepared by the method of Drabkin (11) and were analyzed for the presence of minor hemoglobin components A_{1a}, A_{1b}, and A_{1c} by the method of Schnek and Schroeder (12) as modified by Trivelli et al. (8). Columns were eluted with Developer no. 6 (12). Under these conditions, negatively charged nonheme protein appears in the initial eluate followed by Hbs A_{1a}, A_{1b}, and A_{1c}. Hb A was eluted by the addition of 0.3 M phosphate, pH 6.4. Approximately 800 mg of hemoglobin was analyzed on 2×30-cm Bio-Rex 70 columns at room temperature with a flow rate of 45 ml/h (Bio-Rad Laboratories, Richmond, Calif.). For experiments that required the preparation and analysis of much larger amounts, approximately 5 g of hemoglobin was applied to a 5×50-cm preparative column operated at a flow rate of 120 ml/h. These columns all gave a reproducible and satisfactory isolation of Hb A_{1c}. Special care was needed to separate Hb A_{1a} from Hb A_{1b}. If the column was overloaded or if the flow rate was too rapid, these two components merged into one peak. Column effluent was monitored at 540 nm. For more precise quantitation, hemoglobin peaks were converted to the cyanmet derivative. Optical density was read at 540 and 421 nm in 10- and 2-mm cuvettes with a Gilford recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin. Ohio). The following mM (heme) extinction coefficients were used (13): 10.99 (540 nm) and 122.5 (421 nm). From the volume and optical density of the hemoglobin peaks, the percent of hemoglobin A_{1a}, A_{1b}, and A_{1c} was determined. In some experiments, dilute hemoglobin solutions were concentrated by pressure filtration before spectrophotometric and radioactivity measurements.

In vitro studies. Suspensions of bone marrow or reticulocyte-rich blood were incubated with tritium and ^{14}C-labeled compounds obtained from New England Nuclear, Boston, Mass. 3 ml of autologous plasma containing 45 μCi of [^{3}H]-leucine or [^{14}C]glucose and 4.5 mg of unlabeled glucose was mixed with 1 ml of packed cells and incubated at 37°C with gentle shaking. At selected time intervals, aliquots were removed and washed four times with ice-cold saline. Hemolysate was then passed through Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) equilibrated with the same developer used in the chromatographic separation of the hemoglobins. In this way, hemoglobin was readily separated from labeled compounds of both lower and higher molecular weight. The labelled hemoglobins were then analyzed on Bio-Rex 70 as described above. In one experiment, globin subunits were prepared from column-purified hemoglobins (14). ^{14}C and ^{3}H radioactivity was determined on a liquid scintillation counter (Isocap 300, Searle Analytic Inc., Des Plaines, Ill.). To minimize color quenching, hemoglobin solutions were bleached with 10% H_2O_2 as described previously (15). Counts per minute were corrected for error due to chemiluminescence by comparison with an external standard curve. Specific activity was determined from counts per minute and optical density at 540 (hemoglobin) or 280 nm (globin).

Suspensions of bone marrow were also incubated with plasma containing ^{59}Fe-bound transferrin. 30 μCi of [^{59}Fe]-ferrous citrate (Mallinkrodt Inc., St. Louis, Mo.) was added

slowly to heparinized plasma in amounts that did not exceed transferrin binding capacity. At selected time intervals, aliquots of these cell suspensions were also washed four times, lysed, and dialysed against the chromatography developer. Measured amounts of concentrated purified hemoglobins were counted in a Baird model 530 gamma scintillation counter (Baird Atomic, Inc., Bedford, Mass.).

In vivo studies. Two individuals were given intravenous infusions of autologous heparinized plasma containing 5 and 20 μCi of [^{59}Fe]citrate prepared as described above. In the first patient, iron kinetics were measured as part of a general evaluation of erythropoiesis and erythrocyte lifespan. The studies were performed in compliance with the guidelines of the Human Subjects Committee of the Peter Bent Brigham Hospital. Both individuals gave fully informed consent. After the infusion of [^{59}Fe]transferrin, 40-ml blood specimens were withdrawn every 10–20 days. Approximately 4 g of hemoglobin was applied to a preparative Bio-Rex column to obtain sufficient amounts of the minor components for significant measurements of radioactivity. Samples containing 15–250 mg of hemoglobin were each counted for 25 min on the 4th day after collection and also for 100 min after the last day of collection. Radioactivity measurements were corrected for the natural decay of the isotope and for the effect of small changes in sample volume on counting efficiency. The counting error on these samples was estimated to be 5% or less. From the corrected counts per minute of the sample and its volume and hemoglobin concentration, the specific activity was determined.

RESULTS

In vitro studies. Incorporation of [^{3}H]leucine into hemoglobins of reticulocyte-rich blood is shown in Fig. 1 and Table I. Chromatography of the hemolysate on Bio-Rex 70 revealed a front-running radioactive peak which overlapped with Hb A_{1a} — A_{1b}. Measurement of absorbances at 540 and 280 nm (not shown) indicated that this labeled peak coincided with the negatively charged nonheme protein (2). There was no radioactivity peak coincident with the elution of hemoglobin A_{1c}. The counts per minute associated with Hb A_{1c} probably represented a trailing edge from the highly radioactive nonheme protein. Thus the specific activities of Hb A_{1c} calculated from these data and shown in Table I are likely to be artifactually increased. To document this, globin was prepared from this A_{1c} peak and separated into α- and β-chains. The specific activity of these sub-

TABLE I

Biosynthesis of Hb A_{1c} and Hb A In Vitro

Cell suspension	Labeled precursor	Time	Sp act	
			Hb A_{1c}	Hb A
		min	*cpm/mg*	
Reticulocyte	[^{3}H]Leucine	60	~1,660*	1,520
		120	~3,500*	3,600
Marrow	^{59}Fe	90	2.04	6.97
	[^{3}H]Leucine	90	865	2,625

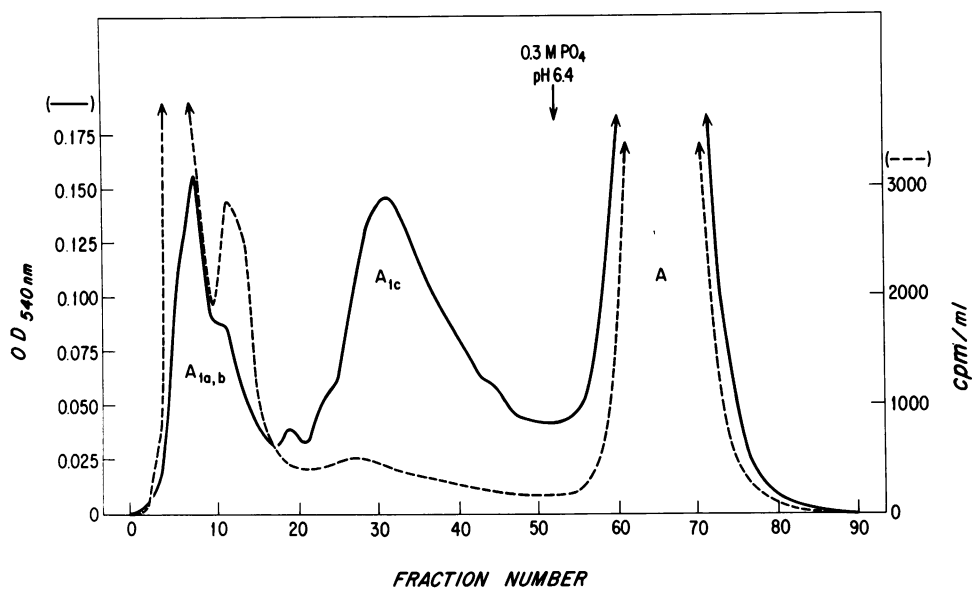* Specific activity is artifactually elevated. See Results.

FIGURE 1 Incubation of reticulocyte-rich blood suspension with [³H]leucine. Optical density
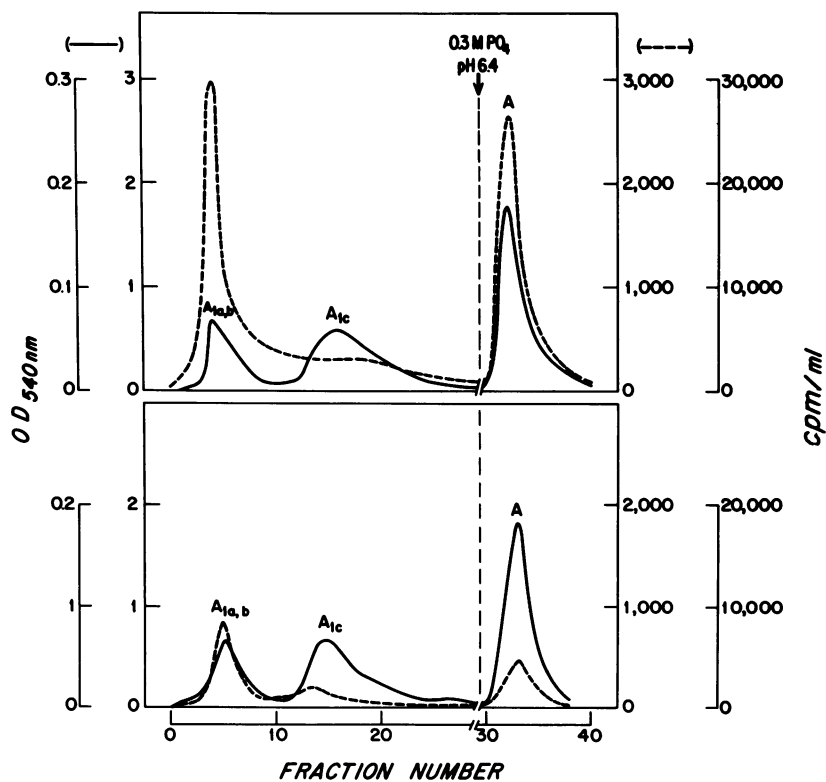(——) and radioactivity (- - -) of eluate from Bio-Rex 70 column are shown.

FIGURE 2 Incubation of suspension of normal bone marrow with [³H]leucine (above, A)
and [¹⁴C]glucose (below, B). Optical density (——) and radioactivity (- - -) of eluate from
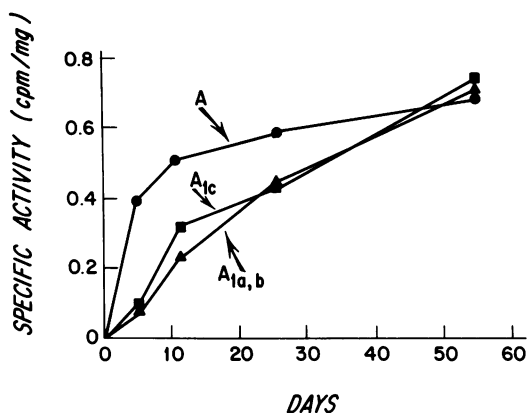Bio-Rex 70 column are shown. The scale for hemoglobin A has been expanded 10-fold.

FIGURE 3 In vivo labeling of hemoglobins. Specific activities of Hb A, Hb A₁c, and Hbs A₁a and A₁b are shown after infusion of [⁵⁹Fe]transferrin into a patient recovering from pure erythrocyte aplasia.

was threefold that of Hb A₁c. Furthermore, as shown in Fig. 2A, there was again no radioactivity peak for Hb A₁c. The radioactivity in this region probably also represents the trailing edge of a heavily labeled nonheme protein. Similar results were also obtained when [⁵⁹Fe]-transferrin was employed in the marrow incubation (Table I). An acidic (negatively charged) ⁵⁹Fe-labeled nonhemoglobin peak was also seen. These data rule out the possibility that Hb A₁c is synthesized preferentially in younger erythroid precursors.

Marrow suspensions were also incubated with [¹⁴C]-glucose (Fig. 2B). The amount of radioactivity employed (45 µCi) was comparable to that in the [³H]leucine incubation (Fig. 2A). No significant radioactive peaks were associated with Hb A₁c. Similar results were obtained with reticulocyte incubations. The radioactivity associated with the Hb A peak in Fig. 2B probably represents the incorporation of labeled amino acids derived from metabolized glucose.

In vivo studies. Patient L. W. was studied during a period when she was recovering from pure erythrocyte aplasia. Plasma iron turnover was 0.84 mg/kg per day (normal 0.42 mg/kg per day), and erythrocyte incorporation of ⁵⁹Fe was 42% in 10 days. The half survival time of autologous ⁵¹Cr-labeled erythrocytes was 22 days (normal 25–40 days). As shown in Fig. 3, ⁵⁹Fe became rapidly incorporated into Hb A. The specific activity of Hb A remained nearly constant during the 60-day observation period. In contrast, the specific activity of Hb A₁c rose in a nearly linear fashion and reached the specific activity of Hb A by day 50. Because the radio-
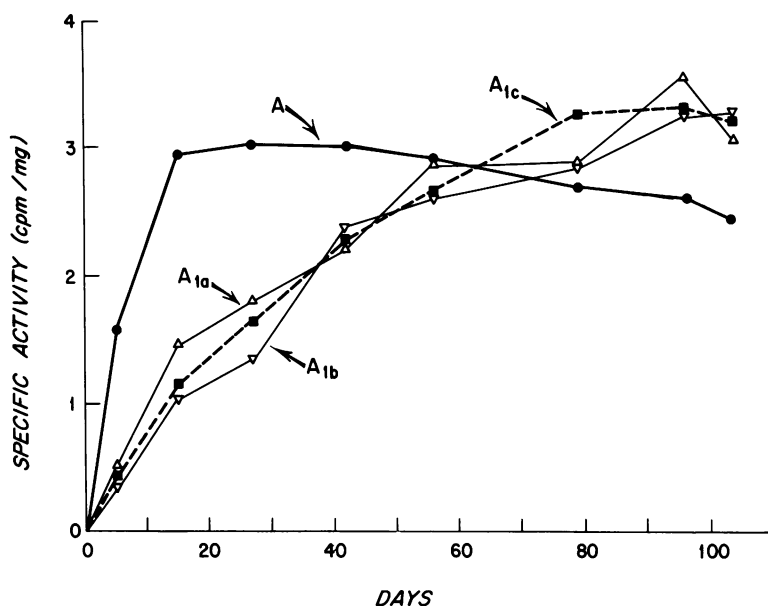
units was only 19 and 17%, respectively, of the unfractionated hemoglobin. This result indicates that the radioactivity associated with the Hb A₁c peak does not reflect incorporation of [³H]leucine into globin subunits. Thus, the true specific activity of Hb A₁c was considerably lower than that estimated from the hemoglobin separated on Bio-Rex 70.

One possible explanation for these results is that Hb A₁c is synthesized primarily in marrow erythroblasts and that, like Hb A₃ (16), its synthesis declines as the erythroid cells mature to the reticulocyte stage. To test this possibility, incubations of marrow suspensions were performed. As Table I shows, after incubation with [³H]leucine for 90 min, the specific activity of Hb A

FIGURE 4 In vivo labeling of hemoglobins A₁a(△-△), A₁b(▽-▽), A₁c(■- - -■), and A(●-●) in a normal individual, after the infusion of [⁵⁹Fe]transferrin.

5 —
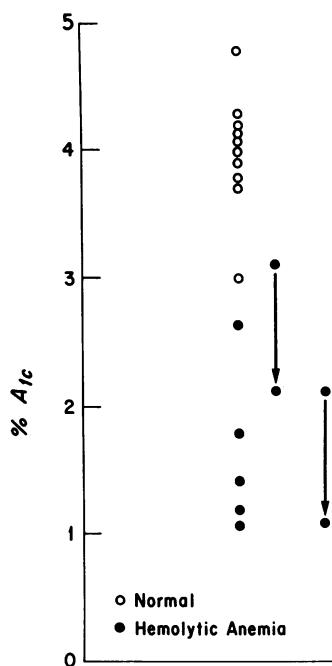
4 —

3 —

% A₁c

2 —

1 —

o Normal

● Hemolytic Anemia

0 —

FIGURE 5 Levels of Hb A₁c in patients with hemolytic anemia (●) and in normal individuals (○). Two specimens were corrected for contamination with Hb F (arrows).

activity of Hb A₁ₐ and A₁ᵦ was low in this study these two minor components were combined. The specific activity of mixtures of Hb A₁ₐ and Hb A₁ᵦ paralleled that of Hb A₁c. These results may be influenced by the fact that this patient had some degree of ineffective erythropoiesis. This study was repeated in a normal subject (Fig. 4). Because four times as much ⁵⁹Fe was infused, Hb A₁ₐ and Hb A₁ᵦ contained sufficient radioactivity that their specific activities could be quantitated separately. As expected, the specific activity of Hb A rose to a maximum within 10 days after infusion of ⁵⁹Fe and fell slowly thereafter because of a small degree of random destruction of erythrocytes.[1] The specific activities of Hb A₁ₐ, Hb A₁ᵦ, and Hb A₁c again rose in nearly linear fashion and reached that of Hb A by day 60. Thereafter, the specific activities of these minor hemoglobins exceeded that of Hb A. No measurements were obtained after day 100 because reutilization of ⁵⁹Fe from senescent erythrocytes makes interpretation of such specific activity data unreliable.

As Fig. 5 shows, the content of Hb A₁c in erythrocytes of patients with hemolytic anemia and shortened erythrocyte life-span was approximately half the hemoglobin A₁c levels in normal individuals. The Hb A₁c peak of two of these patients was analyzed by electro-

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[1] The fall in specific activity of Hb A cannot be due to slow conversion to Hb A₁c.

phoresis and found to be contaminated with a substantial amount of Hb F. In contrast, no significant amount of Hb F was detected in the Hb A₁c peak from three normal individuals. Hb A₁ₐ and A₁ᵦ were also significantly reduced in these patients.
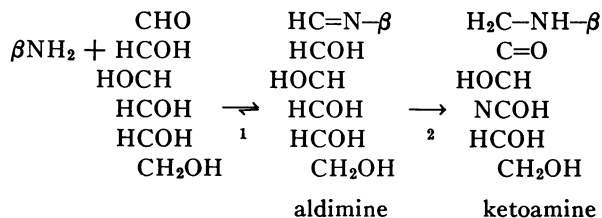
DISCUSSION

These experiments demonstrate that the conversion of Hb A to Hb A₁c is a slow posttranslational event, occurring continuously throughout the 120-day life-span of normal erythrocytes. This conclusion is supported by the low levels of Hb A₁c in patients with hemolysis (17 and Fig. 5). Our in vitro studies are also consistent with this conclusion. Whatever radioactivity that co-chromatographed with Hb A₁c in suspensions of reticulocytes incubated with [³H]leucine appeared to be largely in nonglobin protein. This finding explains the conflicting results of Holmquist and Schroeder (18). They found that the specific activities of Hb A₁c and Hb A did not differ significantly after incubation of human reticulocytes with [¹⁴C]valine. The rather marked fluctuations in the specific activity of Hb A₁c which they observed were probably due to variable amounts of labeled nonglobin protein that were isolated with Hb A₁c. In our marrow cell incubations, we were able to demonstrate a significantly lower rate of incorporation of [³H]leucine and ⁵⁹Fe into Hb A₁c, compared to Hb A. However, it is likely that at least part of Hb A₁c radioactivity in these preparations was also due to the presence of acidic nonglobin proteins that co-chromatograph with Hb A₁c.

Because of the uncertainties involved in these in vitro studies, it was important to obtain precise data on the biosynthesis of Hb A₁c in vivo. After the labeling of a cohort of erythrocytes with ⁵⁹Fe, a slow and nearly linear increase in the specific activity of Hb A₁c was observed. The labeling pattern of the other minor components Hb A₁ₐ and Hb A₁ᵦ was similar to that of Hb A₁c. The extremely slow conversion of Hb A to Hb A₁c suggests a nonenzymatic process. There are only a few examples of nonenzymatic modification of proteins that are known to occur in vivo (1). These include the formation of most disulfide bonds, the conversion of NH₂-glutamine to pyroglutamine and the deamidation of glutamine and asparagine residues.

Hemoglobin A₁c is the product of the chemical condensation of hemoglobin and glucose (6), reactants that are present in high concentration within the erythrocyte. The reaction is favored by the fact that the N-terminal amino groups of hemoglobin have relatively low pKₐ (19), making them very effective nucleophiles at physiological pH. Dixon (20) has shown that the aldehyde of glucose and the amino group of valyhistidine can form a reversible Schiff base linkage when incubated for 30 h at pH 6.2, 50°C in the absence of enzyme or

cofactors. Our studies on human Hb A_{1c} (6) suggest that the hemoglobin-glucose Schiff base (aldimine) linkage rearranges to form a more stable ketoamine attachment:

CHO	HC=N—β	H$_2$C—NH—β
βNH$_2$ + HCOH	HCOH	C=O
HOCH	HOCH	HOCH
HCOH $\rightleftharpoons$	HCOH $\longrightarrow$	NCOH
HCOH ^{1}	HCOH ^{2}	HCOH
CH$_2$OH	CH$_2$OH	CH$_2$OH
	aldimine	ketoamine

Reaction 1 is probably rate limiting and reaction 2 essentially irreversible at physiological pH. If so, then measurement of the rate of formation of Hb A_{1c} in erythrocytes should provide an estimate of the velocity (v) of the condensation reaction (k_1). Since erythrocytes contain 320 mg/ml hemoglobin or 10 mM hemoglobin ($\alpha\beta$ dimer) of which about 4% is Hb A_{1c}, the time-averaged rate of Hb A_{1c} synthesis, in mM/day, is:

$$v = \frac{SA\ A_{1c}\ (t)}{SA\ A\ (t)} \times \frac{10 \times 0.04 \cdot}{t}$$

If one assumes totally irreversible synthesis of A_{1c},

$$v = k[A][Glc].$$

The data on Fig. 4 give a value for dA_{1c}/dt of 7.1 nmol/ml per day. If mean erythrocyte glucose concentration is 4 mM (21) and hemoglobin ($\alpha\beta$ dimer) is 10 mM, then $k = 1.78 \times 10^{-4}$ mM^{-1} day $^{-1}$. It will be of interest to compare this rate constant with that obtained in vitro by incubating [^{14}C]glucose with purified hemoglobin under carefully controlled conditions. The fact that hemoglobins A_{1a} and A_{1b} have the same labeling patterns as A_{1c} suggests that they, too, are posttranslational modifications of Hb A. We are currently studying the structure of these two minor components.

As shown in Fig. 4, the specific activities of hemoglobins A_{1a}, A_{1b}, and A_{1c} reached that of Hb A by day 60, half the life span of the erythrocyte. If hemoglobin A were slowly and irreversibly converted to a minor component A_x during the 120-day life-span of erythrocytes, the predicted specific activity pattern after a cohort label would be that shown in Fig. 6. Our experimental data shown in Fig. 4 follow this theoretical plot with a crossover observed at day 60. The rise in specific activity of Hb A_{1c} that was observed experimentally deviated slightly from linearity and may indicate that the glycosylation of hemoglobin is reversible to a slight degree (Fig. 6 and Appendix). The failure to observe a continued rise in the specific activity of Hb A_{1a}, A_{1b}, and A_{1c} above that of Hb A after day 90 may be due in part to reversible glycosylation and in part to reutilization of ^{59}Fe that accompanies the senescence of the la-
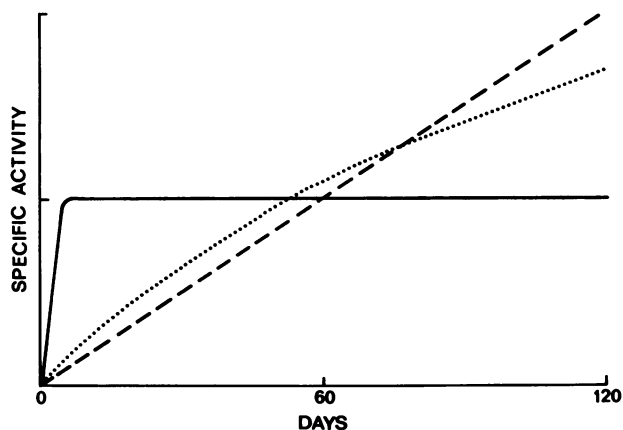

FIGURE 6 Theoretical plot of the specific activity of posttranslationally-modified minor hemoglobin components after the infusion of a cohort label such as [^{59}Fe]transferrin. If the posttranslational modification is slow and irreversible, a linear increase in specific activity with time will be observed (- - -). If the reaction is slightly reversible, a curvilinear plot (. . . .) will be obtained. If the reaction is readily reversible, the specific activity plot will resemble that of unmodified hemoglobin A (———). The experimental data shown in Fig. 4 for Hbs A_{1a}, A_{1b}, and A_{1c} fit best with a slow reaction that is only slightly reversible (. . . .). A detailed kinetic analysis is included in an Appendix.

beled cohort. Very little of this reutilized iron would appear as Hb A_{1c}. Our in vivo data are consistent with earlier reports (22, 23) that hemoglobin "A_3" prepared by starch block electrophoresis increased specific activity as ^{59}Fe-labeled erythrocytes aged in vivo. This poorly resolved electrophoretic component contains Hb A_{1a} and A_{1b} and perhaps some Hb A_{1c}.

These biosynthesis studies help to explain the elevated levels of Hb A_{1c} found in patients with diabetes mellitus. From the kinetic considerations developed above, the rate of formation of Hb A_{1c} should be directly proportional to the time-averaged concentration of glucose within the erythrocyte. Thus, the level of Hb A_{1c} in the diabetic should be a reflection of the adequacy of control, over a sustained time period. There is reason to believe that many of the complications of diabetes are attributable to the cumulative effects of hyperglycemia (24–26). If so, serial measurement of Hb A_{1c} may prove useful in monitoring diabetics and may provide an independent assessment of the adequacy of control.

Recently, Koenig and Cerami (27) have demonstrated an elevation in an acidic minor hemoglobin component in a strain of diabetic mice. Although the structure of this component has not yet been determined, it is likely to be a glycoprotein. ^{59}Fe labeling experiments indicate that the formation of this minor component like human Hb A_{1c} is a posttranslational event. The glycosylation of hemoglobin in man and mouse raises the question of

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what other proteins may be similarly modified during sustained periods of hyperglycemia. If ketoamine linkages analogous to that in Hb A_{1c} are found in other proteins, their detection may be complicated by their relative resistance to acid hydrolysis. An alternative analytical approach such as reduction with [^{3}H]borohydride may prove useful in demonstrating this type of protein modification.

APPENDIX

Kinetic analysis of the post-translational modification of Hb A to Hb A_x (footnote 2).
Hb A condenses with x to form A_x:

$$A + x \underset{k_2}{\overset{k_1}{\rightleftharpoons}} A_x,$$
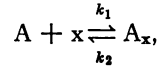
so

$$\frac{dA_x}{dt} = k_1[A][x] - k_2[A_x]. \tag{1}$$

If only a small proportion (approx. 4%) of the hemoglobin is modified, [A] and [x] remain nearly constant; therefore,

$$k_1[A][x] = k_3.$$

At time t:

$$[A_x] = \frac{SA\ A_x(t)}{SA\ A(t)} \times 10 \times 0.04, \tag{2}$$

$$= 0.4y, \tag{3}$$

where

$$y = \frac{SA\ A_x(t)}{SA\ A(t)}. \tag{4}$$

Thus,

$$\frac{dy}{dt} = \frac{1}{0.4}\frac{dA_x}{dt} = 2.5k_3 - k_2 y. \tag{5}$$

Upon integrating:

$$k_2 t = \ln\frac{2.5k_3}{2.5k_3 - k_2 y}, \tag{6}$$

or,

$$y = 2.5\frac{k_3}{k_2}(1 - e^{-k_2 t}). \tag{7}$$

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^{2} Since the experiments described involve a cohort label, A_x is the concentration of Hb A_{1c} in a developing line of erythrocytes. The use of the expression (SA $A_x[t]$)/(SA A[t]) rests on the safe assumption that all the Hb A in a cell is synthesized early in the life-span of the cell, and that the cohort and average Hb A concentrations are essentially equal. Thus (1)/(SA A[t]) is a normalizing factor; and (SA $A_x[t]$)/(SA A [t]) reflects the progressive increase in the concentration of Hb A_x in the cohort with respect to its average concentration. The factor of 0.4 is merely a scaling factor representing the average Hb A_{1c} concentration of 0.4 mM.

If the reaction is essentially irreversible, ($k_3 \gg k_2[A_x]$), then

$$y = 2.5k_3 t, \tag{8}$$

and the function is linear ([- - -] in Fig. 6).
If the reaction is readily reversible ($k_3 \cong k_2[A_x]$), then

$$y = 2.5\frac{k_3}{k_2}, \tag{9}$$

and the function is constant with time, assuming the form of a rectangle ([——] in Fig. 6).
If the reaction is slightly reversible ($k_3 \geq k_2[A_x]$), the function has the exponential form of Eq 7 ([$\cdots$] in Fig. 6).
The total amount of A_x synthesized per cell over a 120-day life-span, under each set of initial conditions, is proportional to the area under the corresponding curve, i.e.,

$$\int_0^{120} A_x\ dt. \tag{10}$$

The mean (time-averaged) concentration of A_x per cell is

$$\frac{1}{120}\int_0^{120} A_x\ dt, \tag{11}$$

which for A_{1c} is approximately 0.4 mM. All three areas must thus be equal to 48. This provides estimates for k_2 and k_3 under the three sets of initial conditions. Thus:

Reversible (Eq 9):

$$48 = \int_0^{120}\frac{k_3}{k_2}\ dt = \frac{k_3}{k_2}t\bigg|_0^{120} = 120\frac{k_3}{k_2}, \tag{12}$$

$$\left(\frac{k_3}{k_2}\right)_{rev.} = 0.40\ mM. \tag{13}$$

Irreversible (Eq 8):

$$48 = \int_0^{120} k_3 t\ dt = \tfrac{1}{2}k_3 t^2\bigg|_0^{120} = 7{,}200\ k_3, \tag{14}$$

$$k_{3\ irrev.} = 6.67\ \mu M\ day^{-1}. \tag{15}$$

Slightly reversible (Eq 7):

$$48 = \int_0^{120}\frac{k_3}{k_2}(1 - e^{-k_2 t})dt = \frac{k_3}{k_2}t\bigg|_0^{120} + \frac{k_3}{k_2}e^{-k_2 t}\bigg|_0^{120}. \tag{16}$$

This equation does not permit explicit solution for k_2 and k_3. Rather, these parameters must be chosen to satisfy the equation and the initial condition that $k_3 \geq k_2$. Appropriate values for such a fit are:

$$k_2 = 0.010\ day^{-1},$$

$$k_3 = 9.6\ \mu M\ day^{-1}.$$

Care must be taken not to confuse the k_2 and k_3 calculated from the various initial conditions, as they will in general not be similar.

The experimental data shown in Fig. 4 fit best the condition that the reaction is slightly reversible.

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