The Biosynthesis of Human Hemoglobin A1c

SLOW GLYCOSYLATION OF HEMOGLOBIN IN VIVO

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ABSTRACT Hemoglobin A1c, the most abundant minor hemoglobin component in human erythrocytes, is formed by the condensation of glucose with the N-terminal amino groups of the \( \beta \)-chains of Hb A. The biosynthesis of this glycosylated hemoglobin was studied in vitro by incubating suspensions of reticulocytes and bone marrow cells with \(^{[H]}\)leucine or \(^{57}Fe\)-bound transferrin. In all experiments, the specific activity of Hb A1c was significantly lower than that of Hb A, suggesting that the formation of Hb A1c is a posttranslational modification. The formation of Hb A1c in vivo was determined in two individuals who were given an infusion of \(^{57}Fe\)-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 A1c and also of Hbs A1b and A1c rose slowly, reaching that of Hb A by about day 60. These results indicate that Hb A1c 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 A1c.

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 A1c (\( \alpha_2 \delta_2 \)) 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 \( \beta \) - and \( \gamma \)-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 A1b, A1c, and A2 comprise approximately 1.6, 0.8, and 4% of the total hemoglobin of adult human erythrocytes, respectively. Thus, Hb A1c is the most abundant minor hemoglobin component. Holmquist and Schroeder (4) showed that Hb A1c is structurally identical to Hb A except that an unidentified group was linked to the terminal amino group of the \( \beta \)-chain by means of a Schiff base. Bookchin and Gallop (5) demonstrated that both \( \beta \)-chains of Hb A1c were attached to a hexose. Recently, we have established the presence of glucose on \( \beta\)-A1c 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 A1c has been considerably enhanced by the fact that this glycoprotein is increased about two-fold 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 A1c 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 A1c is a relatively slow process, probably nonenzymatic in nature, which pro-

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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 Aα, Aβ, and Aε 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 Aα, Aβ, and Aε. 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 Aα. Special care was needed to separate Hb Aε from Hb Aβ. 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 cyanomet 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 Aα, Aβ, and Aε 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 14C-labeled compounds obtained from New England Nuclear, Boston, Mass. 3 ml of autologous plasma containing 45 μCi of [3H]leucine or [14C]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 labeled hemoglobins were then analyzed on Bio-Rex 70 as described above. In one experiment, globin subunits were prepared from column-purified hemoglobins (14). 14C and 3H 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% H2O2 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 55Fe-bound transferrin, 30 μCi of [55Fe]ferrous citrate (Malinkrodt 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 dialyzed 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 [57Fe]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 [57Fe]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 [3H]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 Aα – Aε. 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 radioactive peak coincident with the elution of hemoglobin Aβ. The counts per minute associated with Hb Aε probably represented a trailing edge from the highly radioactive nonheme protein. Thus the specific activities of Hb Aε calculated from these data and shown in Table I are likely to be artfactually increased. To document this, globin was prepared from this Aε peak and separated into α- and β-chains. The specific activity of these sub-

<table>
<thead>
<tr>
<th>TABLE I</th>
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<tr>
<td><strong>Biosynthesis of Hb Aα and Hb A In Vitro</strong></td>
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<table>
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<tr>
<th>Cell suspension</th>
<th>Labeled precursor</th>
<th>Time (min)</th>
<th>Hb Aα</th>
<th>Hb A</th>
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<tbody>
<tr>
<td>Reticulocyte</td>
<td>[3H]Leucine</td>
<td>50</td>
<td>~1,650*</td>
<td>1,260*</td>
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<tr>
<td></td>
<td></td>
<td>120</td>
<td>~3,500*</td>
<td>3,000*</td>
</tr>
<tr>
<td>Marrow</td>
<td>[55Fe]</td>
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<td>2.04</td>
<td>6.97</td>
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<td>[3H]Leucine</td>
<td>90</td>
<td>855</td>
<td>2.625</td>
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* Specific activity is artifactually elevated. See Results.

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

Figure 2 Incubation of suspension of normal bone marrow with $[^3]H$]leucine (above, A) and $[^3]C$]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.

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units was only 19 and 17%, respectively, of the unfractionated hemoglobin. This result indicates that the radioactivity associated with the Hb A1s peak does not reflect incorporation of [1H]leucine into globin subunits. Thus, the specific activity of Hb A1s was considerably lower than that estimated from the hemoglobin separated on Bio-Rex 70.

One possible explanation for these results is that Hb A1s is synthesized primarily in marrow erythroblasts and that, like Hb A (16), its synthesis declines as the erythrocyte cells mature to the reticulocyte stage. To test this possibility, incubations of marrow suspensions were performed. As Table I shows, after incubation with [1H]leucine for 90 min, the specific activity of Hb A was threefold that of Hb A1s. Furthermore, as shown in Fig. 2A, there was again no radioactivity peak for Hb A1s. The radioactivity in this region probably also represents the trailing edge of a heavily labeled nonhemoglobin peak. Similar results were also obtained when [15Fe] transferrin was employed in the marrow incubation (Table I). An acidic (negatively charged) 15Fe-labeled nonhemoglobin peak was also seen. These data rule out the possibility that Hb A1s 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 [1H]leucine incubation (Fig. 2A). No significant radioactive peaks were associated with Hb A1s. 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 15Fe was 42% in 10 days. The half survival time of autologous 55Cr-labeled erythrocytes was 22 days (normal 25-40 days). As shown in Fig. 3, 15Fe 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 A1s rose in a nearly linear fashion and reached the specific activity of Hb A by day 50. Because the radio-
activity of Hb A\textsubscript{10} and A\textsubscript{2} was low in this study these two minor components were combined. The specific activity of mixtures of Hb A\textsubscript{10} and Hb A\textsubscript{2} paralleled that of Hb A\textsubscript{10}. 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 \textsuperscript{57}Fe was infused, Hb A\textsubscript{10} and Hb A\textsubscript{2} 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 \textsuperscript{57}Fe and fell slowly thereafter because of a small degree of random destruction of erythrocytes.\textsuperscript{1} The specific activities of Hb A\textsubscript{10}, Hb A\textsubscript{2}, and Hb A\textsubscript{14} 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 utilization of \textsuperscript{57}Fe from senescent erythrocytes makes interpretation of such specific activity data unreliable.

As Fig. 5 shows, the content of Hb A\textsubscript{10} in erythrocytes of patients with hemolytic anemia and shortened erythrocyte life-span was approximately half the hemoglobin A\textsubscript{10} levels in normal individuals. The Hb A\textsubscript{10} peak of two of these patients was analyzed by electrophoresis 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 A\textsubscript{10} peak from three normal individuals. Hb A\textsubscript{10} and A\textsubscript{2} were also significantly reduced in these patients.

**DISCUSSION**

These experiments demonstrate that the conversion of Hb A to Hb A\textsubscript{10} 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 A\textsubscript{10} 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 A\textsubscript{10} in suspensions of reticulocytes incubated with [\textsuperscript{3}H]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 A\textsubscript{10} and Hb A did not differ significantly after incubation of human reticulocytes with [\textsuperscript{14}C]valine. The rather marked fluctuations in the specific activity of Hb A\textsubscript{10} which they observed were probably due to variable amounts of labeled nonglobin protein that were isolated with Hb A\textsubscript{10}. In our in vitro studies, we were able to demonstrate a significantly lower rate of incorporation of [\textsuperscript{3}H]leucine and \textsuperscript{57}Fe into Hb A\textsubscript{10}, compared to Hb A. However, it is likely that at least part of this Hb A\textsubscript{10} radioactivity in these preparations was also due to the presence of acidic nonglobin proteins that co-chromatographed with Hb A\textsubscript{10}.

Because of the uncertainties involved in these in vitro studies, it was important to obtain precise data on the biosynthesis of Hb A\textsubscript{10} in vivo. After the labeling of a cohort of erythrocytes with \textsuperscript{57}Fe, a slow and nearly linear increase in the specific activity of Hb A\textsubscript{10} was observed. The labeling pattern of the other minor components Hb A\textsubscript{10} and Hb A\textsubscript{14} was similar to that of Hb A\textsubscript{10}. The extremely slow conversion of Hb A to Hb A\textsubscript{10} 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 NH\textsubscript{2}-glutamine to pyroglutamine and the deamidation of glutamine and asparagine residues.

Hemoglobin A\textsubscript{10} 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 pK\textsubscript{a} (19), making them very effective nucleophiles at physiological pH. Dixon (20) has shown that the aldehyde of glucose and the amino group of valhistidine can form a reversible Schiff base linkage when incubated for 30 h at pH 6.2, 50°C in the absence of enzyme or

\textsuperscript{1}The fall in specific activity of Hb A cannot be due to slow conversion to Hb A\textsubscript{10}.

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

\[
\begin{align*}
\beta \text{NH}_2 + \text{HCHO} &\rightarrow \text{CHO} = \text{N} - \beta \quad \text{H}_2\text{C} - \text{NH} - \beta \\
\text{HOCH} &\rightarrow \text{HOCH} \quad \text{HOCH} \\
\text{HCOH} &\rightarrow \text{HCOH} \quad \rightarrow \text{NCOH} \\
\text{CH}_2\text{OH} &\rightarrow \text{CH}_2\text{OH} \quad \text{CH}_2\text{OH} \\
\text{aldimine} &\rightarrow \text{ketoamine}
\end{align*}
\]

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\textsubscript{\textalpha} in erythrocytes should provide an estimate of the velocity (\(v\)) of the condensation reaction (\(k\)). Since erythrocytes contain 320 mg/ml hemoglobin or 10 mM hemoglobin (\(\alpha\beta\) dimer) of which about 4% is Hb A\textsubscript{\textalpha}, the time-averaged rate of Hb A\textsubscript{\textalpha} synthesis, in mM/day, is:

\[
v = \frac{SA_{\text{A\textalpha}}(t)}{SA_{\text{A}}(t)} \times 10 \times 0.04 \times \frac{1}{t}
\]

If one assumes totally irreversible synthesis of A\textsubscript{\textalpha},

\[
v = k[A][\text{Glc}].
\]

The data on Fig. 4 give a value for \(dA_{\text{A\textalpha}}/dt\) 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 \(k = 1.78 \times 10^4 \text{mM}^{-1} \text{day}^{-1}\). 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\textsubscript{\textalpha} and A\textsubscript{\textbeta} have the same labeling patterns as A\textsubscript{\textbeta} 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\textsubscript{\textalpha}, A\textsubscript{\textbeta}, and A\textsubscript{\textgamma} 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\textsubscript{\textalpha} 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\textsubscript{\textalpha} 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\textsubscript{\textalpha}, A\textsubscript{\textbeta}, and A\textsubscript{\textgamma} above that of Hb A after day 90 may be due in part to reversible glycosylation and in part to utilization of \(^{57}Fe\) that accompanies the senescence of the labeled cohort. Very little of this reutilized iron would appear as Hb A\textsubscript{\textalpha}. Our in vivo data are consistent with earlier reports (22, 23) that hemoglobin "A\textsubscript{\textalpha}" prepared by starch block electrophoresis increased specific activity as \(^{57}Fe\)-labeled erythrocytes aged in vivo. This poorly resolved electrophoretic component contains Hb A\textsubscript{\textalpha} and A\textsubscript{\textbeta} and perhaps some Hb A\textsubscript{\textgamma}.

These biosynthesis studies help to explain the elevated levels of Hb A\textsubscript{\textalpha} found in patients with diabetes mellitus. From the kinetic considerations developed above, the rate of formation of Hb A\textsubscript{\textalpha} should be directly proportional to the time-averaged concentration of glucose within the erythrocyte. Thus, the level of Hb A\textsubscript{\textalpha} 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\textsubscript{\textalpha} 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. \(^{57}Fe\) labeling experiments indicate that the formation of this minor component like human Hb A\textsubscript{\textalpha} 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 $A_{1s}$ 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 $[3H]$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$_s$ (footnote 2).

Hb A condenses with x to form A$_x$:

$$A + x \xrightarrow{k_1} A_x,$$

so

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

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

$$k_1[A][x] = k_2.$$

At time $t$:

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

where

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

Thus,

$$\frac{dy}{dt} = \frac{1}{0.4} \frac{dA_x}{dt} = 2.5k_3 - k_2y. \quad (5)$$

Upon integrating:

$$k_2 t = \ln \frac{2.5k_3}{2.5k_3 - k_2y}, \quad (6)$$

or,

$$y = 2.5 \frac{k_3}{k_2} (1 - e^{-k_2t}). \quad (7)$$

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

$$y = 2.5k_3, \quad (8)$$

and the function is linear ($[- -]$ in Fig. 6).

If the reaction is readily reversible $(k_2 \approx k_3[A_x])$, then

$$y = 2.5 \frac{k_3}{k_2}, \quad (9)$$

and the function is constant with time, assuming the form of a rectangle ($[- -]$ in Fig. 6).

If the reaction is slightly reversible $(k_2 \approx k_3[A_x])$, the function has the exponential form of Eq 7 ($[- -]$ 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.,

$$\int_0^{120} A_x \, dt. \quad (10)$$

The mean (time-averaged) concentration of A$_x$ per cell is

$$\frac{1}{120} \int_0^{120} A_x \, dt, \quad (11)$$

which for A$_{1s}$ is approximately 0.4 mM. All three areas must thus be equal to 48. This provides estimates for $k_3$ and $k_5$ under the three sets of initial conditions. Thus:

Reversible (Eq 9):

$$48 = \int_0^{120} \frac{k_3}{k_2} \, dt = \frac{k_3}{k_2} \bigg|_0^{120} = \frac{k_3}{k_2}, \quad (12)$$

Irreversible (Eq 8):

$$48 = \int_0^{120} k_3 \, dt = \frac{1}{2}k_3^2 \bigg|_0^{120} = 7,200 \, k_3, \quad (14)$$

$$k_{3,irrev.} = 6.67 \, \mu M \, day^{-1}. \quad (15)$$

Slightly reversible (Eq 7):

$$48 = \int_0^{120} \frac{k_3}{k_2} (1 - e^{-k_2t}) \, dt = \frac{k_3}{k_2} \bigg|_0^{120} + \frac{k_3}{k_2} e^{-k_2t} \bigg|_0^{120}. \quad (16)$$

This equation does not permit explicit solution for $k_3$ and $k_5$. Rather, these parameters must be chosen to satisfy the equation and the initial condition that $k_2 \geq k_5$. Appropriate values for such a fit are:

$$k_2 = 0.010 \, day^{-1}, \quad k_5 = 9.6 \, \mu M \, day^{-1}.$$

Care must be taken not to confuse the $k_3$ and $k_5$ 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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* Since the experiments described involve a cohort label, A$_x$ is the concentration of Hb A$_{1s}$ 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$_{1s}$ concentration of 0.4 mM.
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