Kinetic Analysis of the Nonenzymatic Glycosylation of Hemoglobin*

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The rate constants have been derived for (a) the condensation of glucose with hemoglobin to form the labile Schiff base intermediate, pre-AI; (b) the dissociation of this complex to hemoglobin and glucose; (c) the rearrangement of this complex to form the stable ketoamine, Hb AIc. These measurements required the purification of commercially available D-[14C]glucose in order to remove a rapidly reacting contaminant. The initial condensation reaction rate (k′1) was measured by incubating column purified Hb AIc for up to 8 h under physiologic conditions with purified D-[14C]glucose in the presence of cyanoborohydride which traps the Schiff base and reduces it to a stable adduct. A parallel incubation utilizing Hb AIc revealed the contribution of the β-NH2-terminal amino group (k2) to the overall value for k′. The reverse reaction rate (k−1) was determined from incubations carried out in the absence of cyanoborohydride. The rate of the Amadori rearrangement (k3) was determined from longer (6-21 days) incubations under identical conditions, followed by chromatographic isolation of Hb AIc. This value for k3 agrees well with one we previously obtained from in vitro data.

These experiments provide direct chemical evidence for an aldime precursor in the nonenzymatic glycosylation of protein. Furthermore, the use of these rate constants provides a reasonable estimate of the distribution of the labile aldime (pre-AI) and the stable ketoamine (Hb AIc) in normal and diabetic red cells. This information is useful in the interpretation of measurements of glycosylated hemoglobin in diabetic patients.

A variety of proteins undergo nonenzymatic modification by forming covalent linkages with glucose. The aldehyde function of glucose condenses with amino groups to form a reversible Schiff base or aldime linkage which is capable of rearranging to a more stable ketoamine. The best understood example of such a modification is hemoglobin AIc, the most abundant minor component in normal human red cells. Hb AIc is identical in structure to the major component, Hb A, except that glucose is attached to the NH2-terminus of the β chain by a ketoamine linkage (1-4). The steps in the synthesis of Hb AIc are as shown in Scheme 1. Hb AIc is formed slowly and nearly irreversibly during the 120-day life span of the red cell (5). The extent to which Hb AIc accumulates depends on the average concentration of glucose in the plasma during the preceding 2-3 months. Thus, Hb AIc has proved to be a reliable index of diabetic control (6-9) and is measured routinely in many diabetes clinics.

In order to gain a better understanding of the formation of glycosylated hemoglobin, we have completed a kinetic analysis. Specifically, we have devised methods to determine the individual rate constants for the formation (k1) and dissociation (k−1) of the aldime and for the conversion of the aldime to the ketoamine (k3). These experiments permit a much clearer understanding of the chemical mechanism for the formation of Hb AIc. This information should be useful in the clinical interpretation of glycosylated hemoglobin.

**MATERIALS AND METHODS**

Red cell hemolysates were prepared as previously described (15), gassed with carbon monoxide, and chromatographed on Bio-Rex 70 cation exchange resin (Bio-Rad, Inc.). Glycosylated hemoglobin components (Hbs AIc, AIA, Ain, and Aib) were eluted by the method of McDonald et al. (10). The major component (Hb AIc) was eluted by a linear NaCl gradient from 0.1-1.0 M. Column fractions of Hb AIc were pooled, concentrated by pressure filtration (Amicon PM-10 membrane), and dialyzed versus Krebs Ringer phosphate buffer, pH 7.3.

Uniformly labeled D-[14C]glucose (New England Nuclear) was purified by preincubation with hemolysate for 4 h at 37°C in the presence of 20 mM NaCNBH3 (Aldrich). After incubation the unreacted D-[14C]glucose was separated from the hemolysate by pressure filtration (Amicon PM-10 membrane). The D-[14C]glucose solution was eluted through a cation exchange resin (Dowex 50W-X2, 200-400 mesh, H+ form) followed by an anion exchange resin (Dowex 1-X8, 200-400 mesh, formate form) and then lyophilized. Alternatively, the D-[14C]glucose was purified by thin layer chromatography as described below.

Purified Hb AIc was incubated with purified and unpurified D-[14C]glucose in a sterile solution of Krebs Ringer phosphate buffer, pH 7.3, at 37°C. Incubations were carried out either in the presence of 20 mM NaCNBH3 or without NaCNBH3. Concentration of glucose ranged between 15 and 50 mM. Aliquots were removed from the incubation solution at specific time intervals and unbound glucose removed by rapid passage through Sephadex G-25 (Pharmacia). Incorporation of D-[14C]glucose into Hb AIc was determined by measuring the hemoglobin concentration and radioactivity of each gel-filtered sample. To a 0.9-mL aliquot was added 1.5 mL of a 1:1 solution of Protosol (New England Nuclear) and isopropanol, 0.2 mL of 30% hydrogen peroxide, and 10 mL of Liquisint cocktail (National Diagnostics, Somerville, NJ). Samples were counted for 10 min on an Isocap/300 liquid scintillation counter (Searle Analytic Corp.). Counts were corrected using a standard quench curve.

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Purified and unpurified d-[14C]glucose was chromatographed by thin layer chromatography (MN 300 CM-cellulose plates, Analtech, Inc.) in butanol:pyridine:water (6:4:3). Autoradiograms of the plates were prepared using Kodak XR-5 film. Glucose was measured enzymatically by the glucose oxidase assay and colorimetrically by the ferricyanide test.

CALCULATION OF RATE CONSTANTS

The second order rate constant for the formation of the labile aldmine \( [H = G] \) can be calculated from incubations of purified d-[14C]glucose and hemoglobin in the presence of cyanoborohydride (Figs. 1 and 3). During the first several hours, the reaction obeys pseudo-zero order kinetics since only a trivial proportion of each reactant is consumed. \( k'1 \) can be calculated from the slope of the linear progress curve and the glucose concentration \([G]\).

\[
\frac{d[H=G]}{dt} = k'1[H][G] = (k_1 + k_\alpha + k_\beta + k_{\alpha} + k_{\beta} + \ldots) \frac{[H][G]}{[G]} \quad (1)
\]

where \( k'1 \) is the overall rate of condensation of glucose at several sites on hemoglobin (14, 15) as determined experimentally. \( k_1 = \frac{k}{k'} \) is the rate of reaction at the \( \beta\)-NH\(_2\) terminus while \( k_\alpha \) is the rate at the \( \alpha\)-NH\(_2\) terminus and \( k_{\alpha} \), \( k_{\beta} \) etc., are the rates at certain lysine residues.

\[
k'1 = \frac{\Delta[H=G]}{[H][G]} \quad (2)
\]

The first order rate constant \( (k_{-1}) \) for the dissociation of glucose from the aldmine complex \( [H=G] \) can be calculated from incubations of purified d-[14C]glucose with hemoglobin in the presence of cyanoborohydride. As shown in Fig. 4, the reaction comes to equilibrium at approximately 6 h:

\[
\frac{d[H=G]}{dt} = k'1[H][G] - k_{-1}[H=G] - k_0[H=G] = 0 \quad (3)
\]

Since \( k_{-1} \gg k_0 \) (see below), at equilibrium (e)

\[
k_{-1} = k'1 \frac{[H][G]}{[H=G]} \quad (4)
\]

The rate at which the aldmine at the \( \beta\)-NH\(_2\) terminus rearranges to the more stable ketoamine (Hb \( A_0 \)) can be calculated from the data shown in Fig. 5 where the formation of Hb \( A_0 \) is linear over 16 days. Since the concentration of glucose is constant, the concentration of aldmine \( [H=G] \) rapidly reaches a constant level, just as in the incubation shown in Fig. 4.

\[
\frac{d[H]}{dt} = k_0[H=G], \quad (5)
\]

\[
k_2 = \frac{k_{-1} \Delta[H=G][H]}{k'1[G] \Delta t} \quad (6)
\]

RESULTS

Determination of \( k_1 \)—In order to measure the rate at which hemoglobin forms the aldmine (Schiff base) linkage with glucose \( (k'1) \), it was necessary to devise incubation conditions in which all of the adduct formed was trapped so that the reverse reaction (the dissociation of the aldmine \( k_{-1} \)) could not take place. Cyanoborohydride readily reduces Schiff base linkages but not ketone groups at neutral pH (12, 13) and is also a less denaturing reducing agent than borohydride. We showed that during a 5 h incubation of glucose with 20 mM NaCNBH\(_3\) under conditions subsequently employed in the experiments described below, there was no loss of glucose, as determined by enzymatic assay. The rate of incorporation of d-[14C]glucose into hemoglobin was identical in 10 and 20 mM CNBH\(_3\). Therefore, at 20 mM all of the Schiff base that was formed during the incubation was reduced by cyanoborohydride before dissociation could take place. These preliminary experiments established the conditions for the reliable determination of \( k'1 \).

Incubations were initially carried out using unpurified d-[14C]glucose. Incorporation of \(^{14}\text{C}\) by hemoglobin in these experiments showed a highly reproducible curvilinear function with time (Fig. 1). The moles of glucose incorporated/mol of \( \alpha\beta \) dimer began to plateau at a point far below saturation of reaction sites. This result could not be explained by straightforward kinetic analysis. It seemed likely that the curvilinear function was due to inhomogeneity of the d-[14C]glucose. The data could be explained by a small amount of radioactive contaminant that reacted rapidly with the hemoglobin. As shown in Fig. 2, autoradiography following thin layer chromatography of the unpurified d-[14C]glucose revealed small amounts of streaking both above and below the spots corresponding to \( \alpha\) and \( \beta\) glucose. No such heterogeneity was observed when purified d-[14C]glucose was chromatographed (Fig. 2). Subsequently, hemoglobin incubations were carried out with purified d-[14C]glucose. As shown in Fig. 1, linear incorporation was observed, and indicates that the purified d-[14C]glucose was homogeneous. d-[14C]Glucose purified by thin layer chromatography also gave linear incorporation. From the data in Fig. 1, a \( k'1 \) of 0.7 \( \times 10^{-3} \) mm\(^{-1}\) h\(^{-1}\) was determined. Replicate experiments invariably revealed linear progress curves up to at least 8 h (Fig. 4). In 9 determinations, the mean value for \( k'1 \) was 0.9 \( \pm 0.2 \times 10^{-3} \) mm\(^{-1}\) h\(^{-1}\) (± S.D.).

The reliability of this approach to measuring \( k'1 \) was corroborated by two parallel experiments in which incubations of purified d-[14C]glucose in the presence of unlabeled cyanoborohydride were compared with unlabeled glucose in the

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\(^1\) S. Kornfeld, personal communication.

\(^2\) Sigma Technical Bulletin 510: 7-73.
presence of [3H]cyanoborohydride. Somewhat lower values for $k_1'$ were obtained with [3H]CNBH$_3$, perhaps because of a small tritium isotope effect.

These experiments do not distinguish among the sites on the hemoglobin molecule that are capable of forming ketoamine linkages with glucose. In normal red cells, about 4% of hemoglobin (αβ dimers) is glycosylated at the β-NH$_2$ terminus. In addition we have shown that about 8% of hemoglobin (αβ dimers) contains ketoamine-linked glucose at certain lysines as well as the NH$_2$ terminus of the α chain (14, 15). Therefore $k_1'$ determined from the above experiments is a composite representing the sum of the rates of adduct formation at several sites. We attempted to localize the sites of glycosylation involved in measurement of $k_1'$ (e.g. Figs. 1, 3, and 4) by structural analysis. Unfortunately, a prohibitively large amount of purified D-[14C]glucose was required to achieve levels of radioactivity adequate for determination of sites of glycosylation. Partial information on the sites of glycosylation was obtained by comparing the reactivities of Hb A$_c$ and Hb A$_{1c}$, which has blocked β-NH$_2$-terminal amino groups. As shown in Fig. 3, the rate of condensation of D-[14C]glucose with Hb A$_{1c}$ was $\frac{3}{5}$ that of Hb A$_c$. The contribution of the β-NH$_2$ terminus to the rate of condensation of glucose with hemoglobin is comparable to the ratio of Hb A$_{1c}$ to total glycosylated hemoglobin in normal red cells. Thus, we estimate that the rate of condensation of the β-NH$_2$ terminus ($k_1'$) is $0.3 \times 10^{-3}$ mm$^{-1}$ h$^{-1}$.

In order to apply this kinetic analysis to in vivo conditions we determined the effect of pH and hemoglobin concentration on $k_1'$. The rise in $k_1'$ with increasing pH is not surprising since glucose condenses only with nonprotonated amino groups. However, as shown in Fig. 4, the slope of $k_1'$ versus pH is less steep than what would be expected from the pH values of the reactive amino groups and probably reflects additional pH dependent factors. As Table I shows, $k_1'$ was not significantly affected by increasing the concentration of hemoglobin to about two-thirds the level found in normal red cells. This experiment was done under solvent conditions that mimic the milieu inside the red blood cell.

**Determination of $k_{-1}$**—The reverse reaction $H=\text{G} \rightarrow \text{H} + \text{G}$ can be examined by incubations of purified D-[14C]glucose and hemoglobin A$_c$ in the absence of cyanoborohydride. As shown in Fig. 5 the incorporation of glucose into hemoglobin was (a) curvilinear, (b) lower than that in the presence of NaCNBH$_3$, and (c) level after 4 h. From this progress curve, $k_{-1}$ could be calculated as explained above (see "Calculation of Rate Constants"). The curve shown in Fig. 4 is a theoretical plot based on a $k_{-1}$ value of 0.35 h$^{-1}$. There is excellent agreement between this theoretical plot and the experimental points. In a second determination of $k_{-1}$ a value of 0.30 h$^{-1}$ was obtained.

**Determination of $k_2$**—The rate of synthesis of the stable ketoamine form of Hb A$_{1c}$ was measured by sterile incubations of D-[14C]glucose with Hb A$_c$ in the absence of NaCNBH$_3$ and separation of the Hb A$_{1c}$ and Hb A$_c$ by cation exchange chromatography (10). The results of two of these incubations are shown in Fig. 6 for two concentrations of glucose (15 and 50 mM). In view of the length of the chromatographic separation and our estimate of $k_{-1}$, it is likely that nearly all of the aldime form of Hb A$_{1c}$ was lost during elution through the cation exchange column. Therefore, the rate of formation of the ketoamine, Hb A$_{1c}$, can be calculated from these data. No

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2 H. F. Bunn and P. J. Higgins, Science, in press.
These incubations were done in a buffer designed to simulate as closely as possible the intracellular milieu of the human red blood cell. The final composition of the incubation mixture was: carboxyhemoglobin A\textsubscript{c} 0.7-6.7 mM (aff); K\textsuperscript{+}, 126 mM; Na\textsuperscript{+}, 13 mM; Mg\textsuperscript{2+}, 3.5 mM; Cl\textsuperscript{−}, 120 mM; HCO\textsubscript{3}−, 13 mM; PO\textsubscript{4}\textsuperscript{3−}, 3 mM; pCO\textsubscript{2}, 40 mm of Hg; pH 7.2. The values for \( k_{1}' \), obtained with this buffer (0.87 ± 0.05 × 10\textsuperscript{-3} mm\textsuperscript{-1} h\textsuperscript{-1}) were not different from those determined from other experiments utilizing Krebs ringer phosphate (0.9 ± 0.2 × 10\textsuperscript{-3} mm\textsuperscript{-1} h\textsuperscript{-1}).

### TABLE I

**Effect of hemoglobin concentration on the rate of condensation with glucose (\( k_{1}' \))**

| Hemoglobin concentration (mg/ml) | \( k_{1}' \) × 10\textsuperscript{-3} m M\textsuperscript{-1} h\textsuperscript{-1} |
|---------------------------------|---------------------------------|
| 23                              | 0.89                            |
| 58                              | 0.80                            |
| 115                             | 0.86                            |
| 214                             | 0.92                            |

Fig. 5. Incorporation of purified D-[\textsuperscript{14}C]glucose into hemoglobin in the presence (●●●●) and absence (○○○) of 20 mM NaCNBH\textsubscript{3}. Conditions were the same as those in Fig. 1 except that the glucose concentration was 14 mM. ●●●● gives a value for \( k_{1}' \) of 0.7 × 10\textsuperscript{-3} mm\textsuperscript{-1} h\textsuperscript{-1}. The lower curve was calculated by integrating Equation 3 using a value for \( k_{1}' \) of 0.7 mm\textsuperscript{-1} h\textsuperscript{-1} and value of \( k_{-1} \) of 0.35 h\textsuperscript{-1}.

Fig. 6. Rate of formation of Hb A\textsubscript{c} during prolonged incubation of Hb A\textsubscript{c} with glucose in absence of NaCNBH\textsubscript{3}. Conditions are described in Fig. 1. These data give values for \( k_{2} \) of 0.0055 h\textsuperscript{-1} (15 mm glucose) and 0.0044 h\textsuperscript{-1} (50 mm glucose).

methemoglobin or precipitation was observed during these prolonged incubations. Results from six experiments are shown in Table II. We obtained a mean value for \( k_{2} \) of 0.0055 ± 0.0010 h\textsuperscript{-1}. This *in vitro* rate is in good agreement with a value of \( k_{2} \) of 0.0060 h\textsuperscript{-1} calculated from our *in vivo* iron kinetic data (5). Viewed another way, the three rate constants which we have derived predict that normal human red cells having an average glucose concentration of 5 mM and surviving 120 days would have a level of Hb A\textsubscript{c} (ketoamine) of 3.7% compared with a measured value of 4%.

### DISCUSSION

These experiments provide direct chemical evidence that nonenzymatic glycosylation of hemoglobin involves the initial formation of a reversible aldime (Schiff base) precursor which slowly rearranges to a stable ketoamine. Although the values we have obtained for the individual rate constants are reproducible and internally consistent, a number of considerations limit the application of these results to the *in vivo* phenomenon. First, it was necessary to remove a rapidly reacting contaminant from the D-[\textsuperscript{14}C]glucose. Recently, Trueb et al. (16) have reported the presence of variable amounts of this contaminant in [\textsuperscript{14}H- and [\textsuperscript{14}C]-labeled glucose preparations from a number of commercial suppliers. In this report, we describe two ways to purify the D-[\textsuperscript{14}C]glucose in order to obtain a compound that gives reliable second order kinetics. Second, the conditions we have used depart somewhat from those existing in the circulating red cell. Our hemoglobin preparations were fully saturated with carbon monoxide and had a final concentration 15% that of the erythrocyte. However, \( k_{1}' \) was not appreciably affected by increasing hemoglobin concentrations to a value approaching that of the red cell (Table I). It is unlikely that the other two rate constants are affected by hemoglobin concentration. The buffer contained inorganic phosphate rather than the organic phosphate 2,3-diphosphoglycerate which is very important in mediating hemoglobin function. Finally, the “on” and “off” rates we have obtained for the aldime (\( k_{1}' \) and \( k_{-1} \)) involve more than one reactive site. Even though the NH\textsubscript{2} terminus of the \( \beta \) chain is favored, other sites on the hemoglobin molecule are also glycosylated (14, 15), although more slowly. The experiment depicted in Fig. 3 shows that the contribution of the \( \beta \)-NH\textsubscript{2} terminus to \( k_{1}' \) is roughly proportional to the ratio of Hb A\textsubscript{c} to total glycosylated hemoglobin in red cells. This experiment permits an estimation of the rate of condensation at the \( \beta \)-NH\textsubscript{2} terminus (\( k_{1} \)). Likewise, the value we have obtained for \( k_{2} \) pertains only to the rate of rearrangement at the \( \beta \)-NH\textsubscript{2} terminus in contrast, we have no direct measurement of \( k_{-1} \) at this site, but assume that this rate is similar at all reactive sites.

Despite these reservations, the rate constants we have obtained are likely to be reasonably close to those which pertain *in vivo* and provide insights into the mechanisms responsible for the nonenzymatic glycosylation of hemoglobin. The results can be summarized as shown in Scheme 2. During the incubation of hemoglobin with glucose, the labile aldime (\( H\textrightleftharpoons G \)) increases within a few hours to reach an equilibrium...
plateau (Fig. 5). During this time, only a minute amount of ketoamine (HG) is formed since the rate of the rearrangement (k2) is only 1/60 that of the rate of dissociation of H\(\rightleftharpoons\)G back to hemoglobin and glucose (k\(-1\)). Likewise, in the circulating erythrocyte, at any given concentration of glucose, there is a rapidly equilibrating level of hemoglobin in the aldime form. The approximate proportion of this labile adduct can be calculated from these rate constants. We estimate that, in normal red cells with an average glucose concentration of 5 mM, about 0.5% of the total hemoglobin is pre-A1c (aldime) or about 10% of the total Hb A1c (ketoamine and aldime). In diabetic red cells, the amount of the labile pre-A1c should be proportional to the level of blood glucose and therefore can vary widely depending on the degree of control. Several investigators have reported a prompt fall in glycosylated hemoglobin upon institution of rigorous diabetic control \(^4\) (17-20). This finding can be readily explained by a rapid decrease in pre-A1c.

The rate constants derived from the present in vitro incubations are in full quantitative agreement with our previous in vivo study (5) and corroborate that Hb A1c, in contrast to pre-A1c, is formed slowly and in an almost linear fashion. \(^5\) This kinetic pattern indicates that, unlike pre-A1c, the formation of Hb A1c is nearly irreversible.

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\(^4\) The measurement of glycosylated hemoglobin in these studies includes both the ketoamine and aldime forms of Hb A1c.

\(^5\) In the present work, as well as in our previous in vivo study (5), column-purified Hb A1c contained little, if any, pre-A1c.
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