colder than the Salmon Springs one; the maximum temperature probably averaged 1° to 2°C lower.

Before now, finite ages of the climatic changes interpreted from the Hoh-Kalaloch stratigraphic sequence were not established earlier than pollen assemblage zone 5. The chronology, controlled by 29 ^{14}C ages, was finite only to about 43,000 years ago. Extension of the chronology to zones 6 and 7—back to approximately 75,000 years ago—now provides additional time planes not only for continental and intercontinental correlation but also for correlation with the marine pollen stratigraphy, which in the Northeast Pacific is tied in with the oxygen isotopic stages (*19*). The continental and marine pollen zonation has been correlated with the oxygen isotopic stratigraphy through stage 6 (*20*).

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The Glycosylation of Hemoglobin: Relevance to Diabetes Mellitus

H. Franklin Bunn, Kenneth H. Gabbay, Paul M. Gallop

cells is covalently linked to glucose, resulting in the formation of a chromatographically distinct minor component designated by Allen *et al.* (*5*) as hemoglobin A_{Ic} (Hb A_{Ic}) (see Fig. 1). Interest in Hb A_{Ic} was considerably enhanced by the discovery that there is a two- to threefold increase in this glycoprotein in patients with diabetes mellitus (*6*). In this article we review the structure and the biosynthesis of glycosylated hemoglobin and then consider its relevance to the pathogenesis, diagnosis, and management of diabetes.

The extraordinary diversity among proteins is considerably enhanced by posttranslational modifications (*1*). A wide variety of proteins owe many of their functional properties to the covalent attachment of carbohydrates at certain residues in the polypeptide chain. Such modifications often provide enhanced stability or solubility, or both. Protein glycosylation is particularly important in maintenance of the integrity of plasma membranes and in facilitating the secretion of proteins into the extracellular space. These specific modifications are generally under precise enzymatic control. In contrast, certain proteins may undergo nonenzymatic glycosylation. This phenomenon depends on the presence of a high concentration of the free sugar and often requires nonphysiologic incubation conditions. For example, the "browning" reaction is well recognized in the dairy industry. When milk is heated for prolonged periods, carbonyl groups on sugars combine with amino groups on proteins such as casein to form Schiff base adducts, resulting in the formation of a heterogeneous, poorly soluble brown product (*2*). This type of reaction can also modify small proteins such as insulin (*3*) and oligopeptides (*4*).

Recently, attention has been focused on the nonenzymatic glycosylation of human hemoglobin. Unlike the browning reaction, the glycosylation of hemoglobin takes place under physiologic conditions, at a specific site on the protein. Normally, about 5 percent of hemoglobin in a population of normal human red

Minor Components of Human Hemoglobin

Human hemoglobin is less heterogeneous than that of most other mammals. In adults and children above the age of 6 months, about 90 percent of their hemoglobin is Hb A ($\alpha_2\beta_2$), a tetramer composed of two pairs of unlike polypeptide chains, each attached to the prosthetic heme group. The α and β

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0036-8075/78/0407-0021$01.75/0

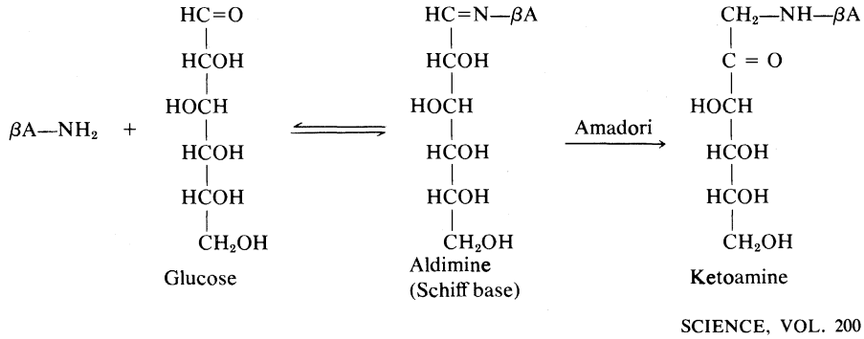
chains are coded by separate genes. Hemoglobin A_2 ($\alpha_2\delta_2$) and Hb F ($\alpha_2\gamma_2$) comprise about 2.5 percent and 0.2 percent of the total. The synthesis of these two minor components is controlled by two other globin chain genes (δ and γ). In contrast, the other minor hemoglobin components found in human red cells are posttranslational modifications of Hb A. When human hemolysate is chromatographed on a cation exchange resin, several negatively charged minor components are eluted before the main Hb A peak. Figure 1 shows an elution pattern on Bio-Rex 70 resin, as developed by the method of McDonald and her associates (*7*). Nonhemoglobin protein appears in the void volume followed by four minor hemoglobin components which have been designated A_{Ia_1}, A_{Ia_2}, A_{Ib}, and A_{Ic} (*7*). These hemoglobins comprise 0.2, 0.2, 0.4, and 3 percent of the hemoglobin, respectively. In this system Hb F is eluted close to Hb A_{Ic}. It is usually present in amounts too low to be detectable as a discrete peak on the chromatogram. Hemoglobin A_2 ($\alpha_2\delta_2$) is more positively charged than the main component (Hb A) and, therefore, is eluted later.

Summary. Glucose reacts nonenzymatically with the NH_2-terminal amino acid of the β chain of human hemoglobin by way of a ketoamine linkage, resulting in the formation of hemoglobin A_{Ic}. Other minor components appear to be adducts of glucose 6-phosphate and fructose 1,6-diphosphate. These hemoglobins are formed slowly and continuously throughout the 120-day life-span of the red cell. There is a two- to threefold increase in hemoglobin A_{Ic} in the red cells of patients with diabetes mellitus. By providing an integrated measurement of blood glucose, hemoglobin A_{Ic} is useful in assessing the degree of diabetic control. Furthermore, this hemoglobin is a useful model of nonenzymatic glycosylation of other proteins that may be involved in the long-term complications of the disease.

Structural Studies

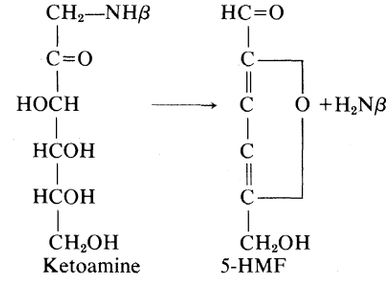
Holmquist and Schroeder (*8*) showed that Hb A_{Ic} was identical to Hb A except that an unidentified group was attached to the NH_2-terminal valine of the β chain by a linkage reducible by sodium borohydride, presumably a Schiff base. Subsequently, Bookchin and Gallop (*9*) established the stoichiometry of binding (two groups per hemoglobin tetramer) and examined the structure of the blocking group by mass spectrometry. They reduced the linkage in Hb A_{Ic} with [^{3}H]borohydride and, after acid hydrolysis, recovered a tritiated *N*-alkylated valine. This material was reacted with acetic anhydride in order to make it sufficiently volatile for analysis by mass spectrometry. The spectrum was consistent with the acetylated derivative of *N*-(1-deoxyhexitol)valine. In full support of this analysis, *N*-(1-deoxygalactitol)-valine was synthesized and showed a mass spectrum virtually identical to the compound isolated from the borohydride-reduced hemoglobin. Although these results established that a hexose was linked to the NH_2-terminal group of the β chain, they provided no information about its identity, or the nature of its linkage.

Subsequently, using mild acid hydrolysis, we were able to isolate reducing sugar from Hb A_{Ic} with a 20 to 30 percent yield (*10*). Glucose and mannose in a 3 : 1 ratio accounted for nearly all of the reducing sugar. The recovery of mannose was unexpected since the red cell contains little if any of this aldohexose. In order to work out the nature of the sugar-protein linkage, we treated Hb A_{Ic} with tritiated borohydride, and oxidized the isolated β chain, containing 95 percent of the radioactivity, with periodate. If the sugar were linked to the protein as an aldimine (Schiff base), [^{3}H]formaldehyde would be formed. Instead, nearly all the radioactivity was recovered as [^{3}H]formic acid. This finding suggests that the second carbon atom of the sugar was tritiated rather than the first. Taken together, these results suggest that in the red cell, glucose reacts initially with the NH_2-terminal amino group of the β chain to form an aldimine linkage, which subsequently undergoes an Amadori rearrangement to form the more stable ketoamine linkage:



The scheme is consistent with the experimental results cited above. Upon conversion of the ketoamine to the aldimine during acid hydrolysis, there is racemization at the second carbon atom. Therefore, both glucose and its C-2 epimer mannose are formed. The relatively high stability of the ketoamine linkage to hydrolysis probably explains why only a 20 to 30 percent yield of sugar was obtained. Treatment with mild acid also appears to promote the dehydration of the sugar group to form 5-hydroxymethyl furfural (5-HMF).

Recently, two other studies have provided independent structural evidence which fully supports the above reaction mechanism. Flückiger and Winterhalter (*11*) treated Hb A_{Ic} with 0.4*N* oxalic acid at 100°C and obtained a good yield of 5-HMF. Formation of 5-HMF (identified by the characteristic spectrum of the adduct formed with thiobarbituric acid) is consistent with the ketoamine linkage:



These reactions may provide the basis for a practical colorimetric determination of Hb A_{Ic} (*11*).

Koenig *et al.* (*12*) have analyzed the structure of Hb A_{Ic} by proton nuclear magnetic resonance (NMR). They obtained the β chain NH_2-terminal dipeptide (R-Val-His; when R is a radical and Val and His are valine and histidine, respectively) from borohydride-reduced Hb A_{Ic} and compared its gas chromatographic behavior and its proton NMR spectrum to synthetic model compounds. Their analyses indicated that the naturally derived material consisted of glucitol (sorbitol) and mannitol valylhistidines, providing strong support for the Amadori rearrangement.

Biosynthesis of Glycosylated Hemoglobins

The structural analysis of hemoglobin A_{Ic} indicated that it is formed by the condensation of glucose and hemoglobin. It was important to determine at what stage in red cell maturation and hemoglobin production this modification takes place and whether the process is enzymatically mediated. After either human reticulocytes or marrow were incubated with radioactively labeled amino acids, the specific activity of Hb A_{Ic} was considerably lower than that of Hb A, indicating that the modification was a late posttranslational event (*13*). The kinetics of the conversion of Hb A to Hb A_{Ic} in vivo was demonstrated by injecting a bolus of ^{59}Fe-bound transferrin to a normal volunteer and following the specific radioactivity of the major and minor hemoglobins over a period of 100 days (*13*). As shown in Fig. 2, the specific activity of the major component, Hb A, increased to a maximum by day 15 and then remained nearly constant during the next 80 days. This pattern is entirely consistent with normal erythropoiesis in which a cohort of red cells is labeled and remains viable until their senescence at about 100 to 130 days. In contrast, the specific activity of Hb's A_{Ia}, A_{Ib}, and A_{Ic} increased gradually over the life-span of the red cell, reaching that of Hb A by approximately day 60. Thereafter, the specific activities of these minor components exceeded that of Hb A. These results indicated that these minor components are formed slowly, continuously, and nearly irreversibly during the 120-day life-span of the red cells. Such a conclusion is consistent with earlier electrophoretic experiments on Hb A_3 (*14*) as well as studies on an acidic minor hemoglobin component in normal and diabetic mice (*15*). The fact that Hb A_{Ic} accumulates throughout the red cell's life-span explains why young red cells, isolated by a density gradient, have lower amounts of Hb A_{Ic} than old red cells (*16*) and why patients with a shortened red cell life-span (hemolytic anemia) have much less Hb A_{Ic} compared to normal individuals (*13, 17*).

The fact that hemoglobin in the circulating red cell is glycosylated so slowly suggests that the process is a nonenzymatic condensation of two abundant reactants, glucose and hemoglobin. If so, it should be possible to prepare Hb A_{Ic} by incubating a mixture of glucose and Hb A. Such an experiment, in which [^{14}C]glucose is used, results in the incorporation of radioactivity into hemoglobin (*11, 18, 19*). Furthermore, the fact that the rate of incorporation was the same whether purified Hb A or crude hemolysate was used (*11, 19*) supports the contention that the reaction is not mediated by a red cell enzyme. Flückiger and Winterhalter (*11*) incubated purified Hb A with 55 m*M* [^{14}C]glucose for 8 to 18 hours at 37°C and demonstrated the formation of a minor hemoglobin peak which had the same chromatographic behavior as authentic Hb A_{Ic}. Nearly all the radioactivity in this component was at the NH_2-terminal amino group of the β chain. Furthermore, the synthetic component formed the same colored product with the thiobarbituric acid as that formed from authentic Hb A_{Ic}, indicating that the Amadori rearrangement had taken place. We have confirmed these results using a more physiological concentration of glucose (15 m*M*) and a longer period of incubation (21 days). Hemoglobin A_{Ic} is not the only labeled product of the incubation (*11, 19*). Most of the radioactivity incorporated into hemoglobin cochromatographs with the leading edge of Hb A and is distributed among several peptides of the α and β chains (*19*). It is likely that several sites on the hemoglobin molecule besides the NH_2-terminal amino group of the β chain can form adducts with glucose. Recently we have found that the leading edge of the native Hb A peak, when eluted from a cation exchange resin, contains carbohydrate detected by the calorimetric test described above (*11*). This component is also increased in diabetic red cells.

Nature of the Other Minor Components

In subsequent incubation experiments, we examined the reactivity of hemoglobin A with the sugars and sugar phosphates that are contained within red cells (*20*). Hemoglobin reacted with glucose 6-phosphate (G6P) at a rate 20 times faster than that with glucose. Furthermore, the reaction between hemoglobin and G6P was less readily reversible. Structural analysis of [^{14}C]G6P-hemoglobin showed that about 95 percent of the radioactivity occurred at the NH_2-terminal amino acid of the β chain. Thus, the interaction of hemoglobin with G6P was much more specific than that with glucose. The reactivity with G6P was maximal when hemoglobin was deoxygenated and stripped of organic phosphates. The addition of 2,3-diphosphoglycerate reduced the reaction rate considerably. Glucose 6-phosphate appears to serve as

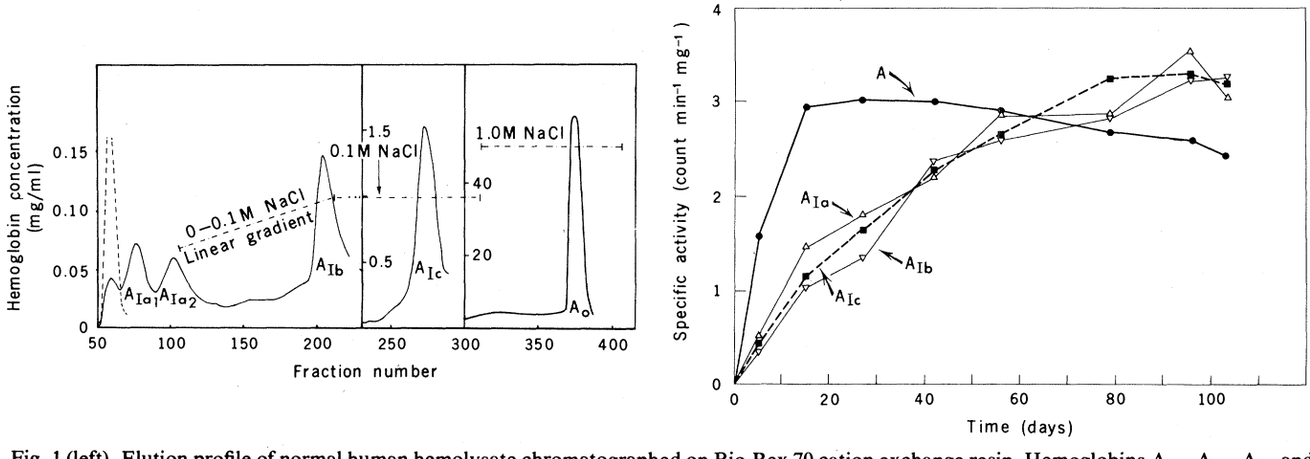


Fig. 1 (left). Elution profile of normal human hemolysate chromatographed on Bio-Rex 70 cation exchange resin. Hemoglobins A_{Ia_1}, A_{Ia_2}, A_{Ib}, and A_{Ic} are posttranslational modifications of Hb A (A_o). Nonhemoglobin protein is shown by the dashed line. For experimental details see (*7*). Fig. 2 (right). Biosynthesis of glycosylated hemoglobins in vivo. A normal human volunteer was given an injection of ^{59}Fe-bound transferrin. At selected time intervals the hemolysate was chromatographed on a large preparative Bio-Rex 70 column. The specific activities of these minor components reflect the rates at which they are formed from Hb A. [From Bunn *et al.* (*13*)]

Table 1. Posttranslational modifications of human hemoglobin.

Hemoglobin	Percentage of total Hb*		Modified subunit	Ratio of phosphate to $\alpha\beta$ dimer	Proposed structure†
	Normal	Diabetic			
A_{Ia_1}	0.19 ± 0.02	0.20 ± 0.03	β	2	$\alpha_2(\beta\text{-}N\text{-FDP})_2$?
A_{Ia_2}	0.19 ± 0.4	0.22 ± 0.04	β	1	$\alpha_2(\beta\text{-}N\text{-G6P})_2$
A_{Ib}	0.48 ± 0.15	0.67 ± 0.3	β	0	?
A_{Ic}	3.3 ± 0.3	7.5 ± 2.0	β	0	$\alpha_2(\beta\text{-}N\text{-Glc})_2$
A	96	80 to 90	None	0	$\alpha_2\beta_2$

*Mean ± 1 standard deviation. †FDP, fructose 1,6-diphosphate; G6P, glucose 6-phosphate; Glc, glucose.

an affinity label with its aldehyde group forming a covalent Schiff base linkage with the NH_2-terminal amino group of the β chain. This interaction is shown schematically in Fig. 3. Workers in other laboratories (*21*) have confirmed that sugar phosphates form adducts with hemoglobin more rapidly than does glucose. In addition to G6P, hemoglobin reacts readily with fructose 6-phosphate, fructose 1,6-diphosphate (FDP), ribose 5-phosphate, ribulose 5-phosphate, and glucuronic acid but not with glucose 1-phosphate or glucose 1,6-diphosphate (*20, 21*). Thus, the rapid formation of the adduct requires the presence of an aldehyde or a ketone group separated from a negatively charged group (PO_4^{3-} or COO^-).

Since sugar phosphates that are normally present in the red cell can interact with hemoglobin to form a stable and specific covalent linkage, it was important to ascertain whether any of the more negatively charged minor components (A_{Ia_1}, A_{Ia_2}, or A_{Ib}) in normal red cells might be adducts with these compounds. In support of this contention, phosphate analysis of these three components revealed 2, 1, and 0 phosphate groups, respectively, per $\alpha\beta$ dimer (see Table 1). Electrophoresis of canine-human hybrid hemoglobins indicated that all of these minor components had normal α chains and negatively charged β chains. Thus, Hb A_{Ia_1} has two phosphate groups per β chain, Hb A_{Ia_2} has one, and Hb A_{Ib} has none. All three components appear to contain carbohydrate as determined by the thiobarbituric acid test (*11*). It seems likely that these minor hemoglobin components are adducts of sugar metabolites found in normal human red cells (Table 2). Using the high resolution chromatographic system shown in Fig. 1, we have found that synthetic FDP-hemoglobin partially cochromatographs with Hb A_{Ia_1}, while synthetic G6P-hemoglobin cochromatographs precisely with Hb A_{Ia_2}. Thus Hb's A_{Ia_1} and A_{Ia_2} are probably adducts of Hb A with FDP and G6P, respectively. The ^{31}P nuclear magnetic resonance spectrum of synthetic G6P-hemoglobin is apparently identical with that of Hb A_{Ia_2}. The structure of Hb A_{Ib} is unknown. The modification is on the β chain but not at the NH_2-terminal amino acid (*22*). Data of Krishnamoorthy *et al.* (*23*) indicate that β^A may be converted to $\beta^{A_{Ib}}$ by deamidation.

Diabetic patients with two- to threefold increases in Hb A_{Ic} have normal amounts of Hb's A_{Ia_1} and A_{Ia_2} and, from analyses in our laboratories, normal amounts of red cell G6P and FDP. Thus it is most unlikely that G6P-hemoglobin (Hb A_{Ia_2}) is a precursor of Hb A_{Ic}. The simplest interpretation of these results is that the amounts of all the glycosylated hemoglobins are determined by the concentrations of various sugars in the red cell and the rates at which they react with hemoglobin. Thus, the concentration of G6P in the normal red cell is 1/200th that of glucose, but it reacts with hemoglobin at least ten times more rapidly than glucose. The ratio of Hb A_{Ia_1} to Hb A_{Ic} of about 1:10 is consistent with these findings.

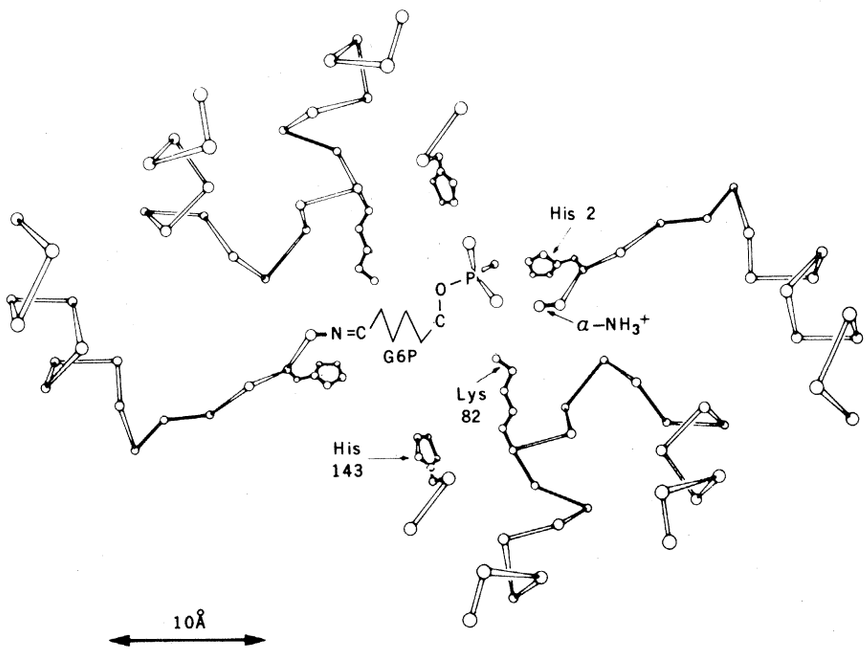


Fig. 3. Diagramatic representation of the sites of covalent and electrostatic interaction of glucose 6-phosphate and deoxyhemoglobin. [Redrawn from Arnone (*24*); see Haney and Bunn (*2*)]

Effect of Glycosylation on Hemoglobin Function

Since hemoglobin is glycosylated at a specific site, these derivatives, both natural and synthetic, provide an excellent opportunity to explore structure-function relationships. As already discussed, Hb's A_{Ia_1}, A_{Ia_2}, and A_{Ic} are modified at the NH_2-terminal amino group of the β chains, a site normally involved in the binding of organic phosphates (*24*). 2,3-Diphosphoglycerate (2,3-DPG) is an important regulator of intracellular hemoglobin function. Within mammalian red cells, 2,3-DPG is present in high concentrations that are approximately equimolar with the concentration of hemoglobin tetramer. The 2,3-DPG polyanion binds more strongly to deoxyhemoglobin than to oxyhemoglobin, thereby causing a marked reduction in the affinity of hemoglobin for oxygen. Its negatively charged groups form salt bonds with positively charged residues on the two β chains at the entrance to the central cavity of the hemoglobin molecule, including the NH_2-terminal amino groups. If this site is blocked by a covalent attachment such as a hexose (*25*), or an acetyl (*25*) or carbamyl (*26*) group, the reactivity of hemoglobin with 2,3-DPG is markedly reduced. In comparison to Hb A, the oxy-

gen affinity of Hb A_{Ic} is much less responsive to the addition of 2,3-DPG (*25*). In like manner, synthetic G6P-hemoglobin has a similarly decreased reactivity with 2,3-DPG (*20*). McDonald and her colleagues (*27*) have examined oxygen equilibria of Hb's A, A_{Ia_1}, A_{Ia_2}, A_{Ib}, and A_{Ic} as well as the kinetics of the binding of carbon monoxide with deoxyhemoglobin. They have found that Hb's A_{Ia_1} and A_{Ia_2} have a low affinity for heme ligands and decreased cooperativity between subunits. Like Hb A_{Ic} they also show decreased interaction with organic phosphates. It is likely that the covalently bound phosphates on Hb's A_{Ia_1} and A_{Ia_2} enhance the stability of the deoxy or T conformation (*28*), thereby lowering oxygen affinity. It will be interesting to compare the functional properties of naturally occurring Hb's A_{Ia_1} and A_{Ia_2} with synthetic FDP- and G6P-hemoglobins. Differences between the oxygen binding of these minor hemoglobins and the major component (Hb A) may affect the interpretation of experiments done on unfractionated hemolysate. In particular, the presence of these minor components may have a significant effect on measuring the binding of the first and fourth oxygen molecules to the hemoglobin tetramer (K_1 and K_4).

Since Hb A_{Ic} has decreased reactivity with 2,3-DPG, red cells of diabetic patients might be expected to bind oxygen abnormally. As predicted by oxygen equilibria in dilute solution, the oxygen affinity of diabetic red cells is slightly greater than that of normal red cells having a comparable amount of 2,3-DPG (*29*). Normal individuals have a P_{50} (*30*) of about 26 mm-Hg, whereas diabetics may have a P_{50} of 24 to 26 mm-Hg (*29*). Obviously there is considerable overlap between the two groups. Despite arguments to the contrary (*31*) such a small displacement in the oxygen dissociation curve is unlikely to have any physiological significance. Much greater shifts in the oxygen dissociation curve (P_{50} = 15 mm-Hg) encountered in patients with mutant hemoglobins have no apparent deleterious effect on tissue oxygenation and are unassociated with any significant clinical manifestations (*32*). Even under conditions of stress, such as ketoacidosis or infection, this slight abnormality in diabetics is unlikely to have any significant effect on oxygen or carbon dioxide transport.

Modifications at the NH_2-terminal amino acid of the β chain have variable effects on the propensity of sickle hemoglobin (Hb S) to polymerize. Sickling is markedly impaired by selective carbamylation of the β chains of Hb S (*33*), whereas modification at the same site with pyridoxal phosphate has a much smaller effect (*34*). We have found that the adduct of Hb S with G6P has about the same tendency to polymerize as unmodified Hb S. The sickling of the β-*N*-glucose adduct (Hb S_{Ic}) has not yet been examined. However, Abdella *et al.* (*35*) have shown that incubation of Hb S with high concentrations of glucose followed by borohydride reduction causes a small but significant decrease in sickling. Under these conditions, hemoglobin is almost certainly modified at a number of sites on both the α and β chains. Nigen and Manning (*36*) observed a much more significant inhibition of sickling with DL-glyceraldehyde. This reagent reacts with the ϵ-amino groups of lysines in preference to NH_2-terminal amino groups.

Table 2. Sugars in normal human red cells.

Compound	Concentration (μM)	Reactivity with hemoglobin
Glucose	4100	+
Glucose 6-phosphate	28	++
Glucose 1,6-diphosphate	150	0
Fructose 6-phosphate	10	++
Fructose 1,6-diphosphate	5	++
Ribose 5-phosphate	~ 10	++

Glycosylated Hemoglobin and Diabetes Mellitus

The recognition that increased amounts of a minor hemoglobin component exist in patients with diabetes antedated its identification as hemoglobin A_{Ic}. Huisman and Dozy (*37*) in 1962 first observed a two- to threefold increase in hemoglobin A_{Iabc} in four diabetic patients treated with tolbutamide. Attempts to reproduce this phenomenon in vitro by incubating red blood cells and hemolysates with tolbutamide were unsuccessful. In a remarkable independent study, Rahbar (*6*) surveyed 1200 patients at Tehran University hospitals and found two patients who showed an abnormal pattern on agar gel electrophoresis; both patients were diabetics. He examined another 47 diabetic patients and detected the same abnormal pattern in each case. Subsequently, he and his colleagues (*38*) found that the hemoglobin component present in diabetic subjects had the same chromatographic and electrophoretic properties as hemoglobin A_{Ic} and observed a twofold increase in chromatographically separated hemoglobin A_{Ic} in a small number of diabetic patients. Trivelli *et al.* (*39*) in a larger study showed a twofold increase of hemoglobin A_{Ic} over values found in normal subjects. These increased levels were not related to the age of the patients, duration of disease, type of therapy, or the presence of the complications of diabetes mellitus. No attempt was made to relate the increase in hemoglobin A_{Ic} to blood glucose concentrations. Paulsen (*40*) found similarly increased amounts of hemoglobin A_{Ic} in children with overt insulin-dependent diabetes and normal amounts in nine children with asymptomatic hyperglycemia, and suggested that these alterations may be specific genetic markers for diabetes mellitus. This proposal was disproved by the findings of Tattersall *et al.* (*41*) who measured hemoglobin A_{Iabc} in identical twins concordant and discordant for diabetes. The mean values for the proportion of hemoglobin A_{Iabc} in discordant twins differed markedly in the two members of the pair, with increased amounts in the diabetics and normal amounts in their nondiabetic twins. In concordant twins with juvenile-onset diabetes, no marked differences were observed between members of twin pairs. Four pairs of identical twins concordant for maturity-onset diabetes showed lower mean values for Hb A_{Iabc} but did not show marked intrapair differences. These authors proposed the abnormal increase in hemoglobin A_{Iabc} found in diabetes mellitus to be a manifestation of a metabolic abnormality of diabetes, rather than a genetic marker.

Findings analogous to those in humans were reported by Koenig and co-workers (*15, 42*) in genetically as well as in chemically induced diabetic mice. They showed that adult diabetic mice (C57BL/KsJ-*db*/*db*) have increased amounts of a minor fast-moving hemoglobin component compared to wild-type nondiabetic mice. This increased component had similar chromatographic mobility to human hemoglobin A_{Ic} and contained sodium borohydride reducible linkages on the β chains. They demonstrated that the *db*/*db* animals which have normal amounts of mouse hemoglobin A_{Ic} at weaning show an increase approximately 4 weeks after the onset of hyperglycemia. Mouse hemoglobin A_{Ic} is also formed by a postsynthetic modification of hemoglobin A throughout the life of the red cell in the circulation, and the rate of synthesis is greater in the diabetic than the nondiabetic mouse. The amount of hemoglobin A_{Ic} was normal in the nondiabetic obese C57BL/6J-*ob*/*ob* mouse and only a transient increase was noted in the transiently diabetic C57BL/6J-*db*/*db* mouse. These findings ruled out a di-

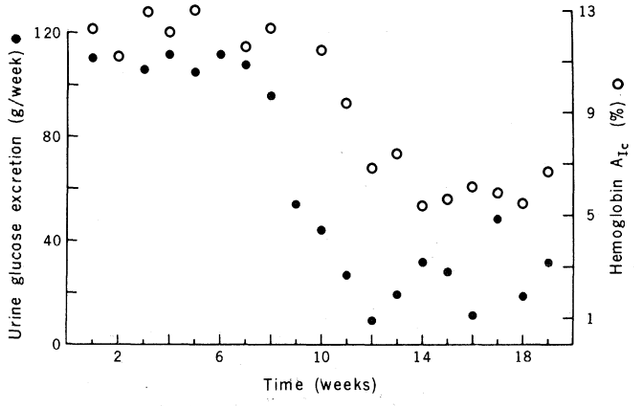


Fig. 4 (left). Relation between Hb A_{Ic} and urine glucose excretion in a diabetic patient following the institution of strict control. [From Koenig *et al.* (*43*)]

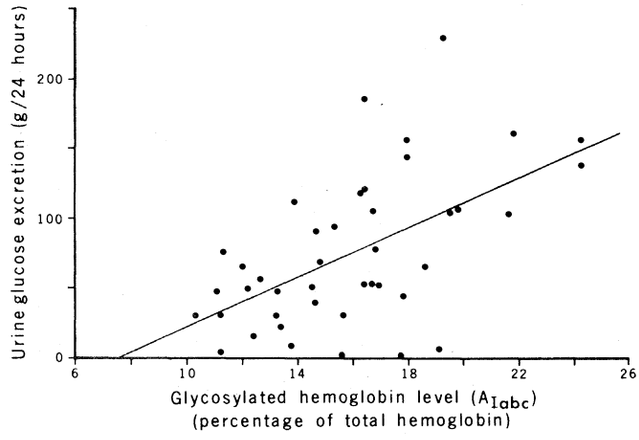


Fig. 5 (right). Correlation of hemoglobins A_{Iabc} with the amount of glucose excreted in the urine over a 24-hour period 2 months prior to the determination of glycosylated hemoglobin levels. [From Gabbay *et al.* (*44*)]

rect relation between either the *ob* or *db* gene and hemoglobin A_{Ic} levels. The increased hemoglobin A_{Ic} levels in nonobese mice with chemically induced diabetes strengthened the concept that the increase in hemoglobin A_{Ic} is secondary to the diabetic state. Again, these authors found no relation between the increase in hemoglobin A_{Ic} to the severity of the hyperglycemia, duration of diabetes, or age or weight of the mouse, and proposed that a humoral factor in the diabetic mouse stimulates the synthesis of hemoglobin A_{Ic}.

The above observations were clarified by the establishment of the structure of hemoglobin A_{Ic} as a glucose adduct of hemoglobin A by way of a Schiff base and a subsequent Amadori rearrangement. The stability of the ketoamine configuration along with the kinetic data in vivo (*13, 15, 42*) and the measurements of Hb A_{Ic} in young and old red cells (*16*) made it clear that hemoglobin A_{Ic} accumulates throughout the life of the circulating red cell. Therefore, the cumulative amount of hemoglobin A_{Ic} should be directly proportional to a time-averaged concentration of glucose within the erythrocyte. In a study of the relation of hemoglobin A_{Ic} levels to the development of capillary basement membrane thickening in the skeletal muscle of diabetic patients, Koenig and co-workers (*15, 42*) demonstrated that the hemoglobin A_{Ic} concentration correlated significantly with both the maximal response to a glucose tolerance test and to the fasting blood glucose in a group of diabetic patients treated with diet, oral hypoglycemics, or insulin. There was no correlation between hemoglobin A_{Ic} levels and muscle capillary basement membrane thickness; this lack of correlation was thought to result from the fact that a period of several years is required for the development of detectable basement membrane thickening.

Two approaches have been used to demonstrate the relation of hemoglobin A_{Ic} concentration to diabetic control. In one approach, Koenig *et al.* (*43*) hospitalized five diabetic patients in "poor diabetic control" and achieved a marked improvement of control by careful regulation of diet, exercise, and administration of insulin. Amelioration of diabetic control, as measured by urinary glucose excretion, fasting, and postprandial blood glucose determinations, was accomplished after 1 to 2 months of hospitalization. The concentrations of both hemoglobin A_{Ic}, and to a lesser extent, hemoglobins A_{Ia+b} were reduced approximately 4 weeks after the attainment of improved blood glucose control (Fig. 4). During optimal diabetic control, the concentration of blood sugar during fasting was normalized to a mean of 84 mg per 100 milliliters and the hemoglobin A_{Ic} concentration decreased to 5.8 percent. Thus, hemoglobin A_{Ic} concentration appeared to reflect the mean blood sugar concentration over the previous weeks.

In a second approach, we measured total glycosylated hemoglobin components (Hb A_{Iabc}) in 220 diabetic patients for whom data on urinary glucose excretion were available for the 24-hour periods immediately prior, and at 1, 2, and 3 months prior to the hemoglobin determination (*44*). These relationships were examined by linear regression analysis (Fig. 5); it was found that total glycosylated hemoglobin levels were significantly correlated with the amount of glucose excreted in the 24-hour periods of urine collection. The highest correlation was found between the concentrations of glycosylated hemoglobin and the amount of glucose excreted in the urine collected 2 months prior to the hemoglobin determination. These data suggest that glycosylated hemoglobin levels indeed reflect integrated glucose concentrations over the previous few weeks and hence represent an index of long-term blood glucose control.

Recently, other groups have confirmed that amounts of Hb A_{Ic} provide an independent assessment of diabetic control. Gonen *et al.* (*45*) rated the state of control of their patients on an arbitrary scale and found a significant correlation between the concentrations of glycosylated hemoglobin and the clinical assessment of control ($r = .63$). Several clinics have reported a correlation in diabetics between the blood sugar of fasted patients and the amount of Hb A_{Iabc} (*45, 46*). The reduction in Hb A_{Ic} that occurs when diabetic women become pregnant (*47*) may be due to a reduction in hyperglycemia or perhaps to an influx of young red cells during pregnancy.

Conclusions

Considerable information is now available regarding the structure and biosynthesis of glycosylated hemoglobins. These findings have relevance to a number of areas in diabetes research. Glycosylated hemoglobins provide an index of the patient's average blood glucose concentration over a long time period. This index is not affected by short-term fluctuations in blood sugar (hour to hour) and hence gives a relatively precise reflection of the state of blood glucose control in diabetes. Therefore, it is now possible to estimate more accurately and with greater sensitivity the degree of glucose intolerance, particularly in borderline cases. A diagnostic use for this determination is thus suggested which may enhance or supplant a variety of glucose

tolerance tests which are known to be variable in mild cases of diabetes. Furthermore, glycosylated hemoglobin determinations, by providing an accurate and objective estimate of the effectiveness of diabetic therapy, may lead to improvements in the control of blood glucose in diabetics. They represent an accurate technique to evaluate new ways of controlling blood glucose. The feasibility of using Hb A_{Ic} as a clinical test has been enhanced by the recent development of improved methods, including semiautomated high-pressure liquid chromatography (*48*), gel electrofocusing (*49*), radioimmunoassay (*50*), and the colorimetric test (*11*). All of these approaches have the potential for rapid analysis of multiple specimens.

Serial determinations of glycosylated hemoglobins should also enable prospective studies to be done to evaluate the relation of blood glucose control in diabetics to the development of various complications in the disease. This relation has been controversial both because of our inability to achieve adequate control under usual clinical circumstances, and to accurately measure control of blood sugar in diabetic subjects. It should be noted that the organs and tissues most affected by diabetic complications (for example, lens, peripheral nerves, kidney, retina, and blood vessels) are not insulin-dependent for glucose penetration, and hence achieve high intracellular glucose concentrations during periods of hyperglycemia. Increased intracellular glucose levels have been demonstrated to be responsible for the formation of some diabetic complications such as cataract and neuropathy by increased shunting of glucose metabolism into accessory pathways [such as the sorbitol pathway (*51*)] that are normally quiescent during periods of normoglycemia. The intracellular glycosylation of hemoglobin may thus represent another mechanism by which structural and enzyme proteins may be modified, resulting in altered function. Abnormal basement membrane thickening in diabetic glomeruli and capillary basement membranes may represent an analogous form of nonenzymatic glycosylation. The possibility that increased glucose concentrations per se directly contribute to the development of diabetic complications deserves thorough scrutiny.

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52. Supported in part by NIH grants AM18233, AM 15019, HL 20539, and AM 16754 and the Howard Hughes Medical Institute. Some of the recent information in this article was presented at a workshop on hemoglobin A_{Ic} in May 1977 under the joint sponsorship of the National Institute of Arthritis, Metabolism, and Digestive Diseases and the American Diabetes Association.