Glycosylated Hemoglobin in Human and Animal Red Cells
Role of Glucose Permeability

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SUMMARY

We have compared red cells from man and selected animals in order to determine the effect of glucose permeability on nonenzymatic glycosylation of hemoglobin. Glucose permeability was highest in the primates (human, baboon, rhesus monkey), lower in dogs and rabbits, and nearly zero in pigs. Glycosylation of hemoglobin was measured by three independent methods: cation-exchange chromatography on Bio-Rex 70 (Bio-Rad, Inc., Richmond, California), agar gel electrophoresis, and affinity chromatography. The colorimetric thiobarbituric acid test did not provide valid data on animal hemolysates. However, this test was useful for identifying glycosylated hemoglobin (HbA_{1c}) components isolated on Bio-Rex chromatography. In all animals tested, levels of HbA_{1c} (from Bio-Rex chromatography) and total glycosylated hemoglobin (from affinity chromatography) correlated well with glucose exposure, the product of intracellular glucose concentration, and red cell life span. These results indicate that nonenzymatic glycosylation of hemoglobin in mammals is determined by three major variables: mean plasma glucose concentration, red cell life span, and red cell glucose permeability. **DIABETES *31*:743–748, September 1982.**

Hemoglobin A_{1c} (HbA_{1c}) is formed by the nonenzymatic condensation of glucose and the α-amino group of the N-terminal valine residue of the β-chain.[1–6] In a similar manner glucose condenses with the α-amino group of the N-terminal valine residue of the α-chain as well as with ϵ-amino groups of certain lysine residues on α- and β-chains.[2] The initial aldimine form of HbA_{1c} (pre-A_{1c}) undergoes an Amadori rearrangement to a stable ketoamine linkage.[3–6] Levels of glycosylated hemoglobin accumulate slowly and continuously throughout the life span of the red cell.[7] Measurement of HbA_{1c} has proved to be useful in assessing diabetic control[8,9] since the formation of this hemoglobin component depends on the average concentration of glucose within the red cell over the preceding 2–3 mo.[9]

Several types of mammals can develop diabetes either spontaneously or following experimental manipulation, and have been used as models for the study of human diabetes.[10] Glycosylated hemoglobin has been determined in several mammals,[11–19] and although results are variable, it appears to be elevated in diabetic animals, just as in man. The levels of glycosylated hemoglobin would be expected to vary among species because of differences not only in red cell life span but also in red cell permeability to glucose.[20–22] These factors must be considered before any comparisons between animal models and humans can be made.

This report focuses on establishing the role of glucose permeability as a determinant of nonenzymatic glycosylation. We have found that the use of affinity chromatography in conjunction with other independent methods has been useful in establishing the relationship between glucose permeability and glycosylated hemoglobin. The interpretation of glycosylated hemoglobin measurements in animal models of diabetes depends on understanding all the independent variables that affect the extent of this posttranslational modification.

MATERIALS AND METHODS

Blood specimens from normal humans, baboons (*Papio anubis*), rhesus monkeys (*Macaca mulatta*), dogs (*Canis familiaris*), rabbits (*Oryctolagus cuniculus*), and pigs (*Sus scrofa*) were drawn into syringes containing either heparin or EDTA. Single specimens were obtained from multiple animals of a given species, and were random with respect to time of day and feeding. Protein-free extracts of an aliquot of whole blood were immediately prepared by adding 1 ml of blood to 2 ml 6% perchloric acid. Following centrifugation of another aliquot of blood, protein-free extracts of plasma were prepared. The hematocrit of each blood specimen was

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determined in duplicate immediately after blood was drawn. The glucose concentration of neutralized protein-free extracts was measured enzymatically by glucose-oxidase assay (Sigma technical bulletin *510*:7–73). Intracellular glucose was determined by the following formula:

$$[G]_{RBC} = [G]_P - \frac{[G]_P - [G]_{WB}}{Hct}$$

where $[G]_{RBC}$, $[G]_P$, and $[G]_{WB}$ are the concentrations of glucose in the red cell, plasma, and whole blood, respectively; Hct is the hematocrit.

Hemolysates were prepared as previously described,[23] gassed with carbon monoxide, and dialyzed in 0.05 M potassium phosphate buffer, pH 6.60, 6.70, or 6.80. Hemolysates were chromatographed on Bio-Rex 70 cation-exchange resin (Bio-Rad, Inc.). Human, dog, and pig hemolysates were chromatographed at pH 6.60 as previously described.[23] Hemoglobin (300 mg) was applied to 2.5 × 16-cm columns and eluted at 60 ml/h. Baboon and rhesus monkey hemolysates were chromatographed similarly at pH 6.70, except that the linear NaCl gradient used to elute rhesus monkey HbA_0 ranged from 0.1 to 0.5 M instead of from 0.1 to 1.0 M. Rabbit hemolysate was chromatographed at pH 6.80. Percentage of the total hemoglobin of each peak was determined from the absorbance of the column fractions. Column fractions of hemoglobin components were pooled and concentrated by pressure filtration (Amicon PM-10 membrane). Hemoglobin components were assayed for ketoamine-linked glucose by the thiobarbituric acid test.[5] In the comparison of hemolysates, each sample contained 10 mg of hemoglobin. Assays of individual chromatographic peaks contained 5–10 mg of hemoglobin. Following mild acid hydrolysis, released 5-hydroxymethylfurfural and thiobarbituric acid form a complex that absorbs at 443 nm.

Hemolysates (1 μl) were electrophoresed on agar gels at pH 6.3[24] and percent "fast" hemoglobins quantified with a Corning 720 scanning densitometer (Corning Medical Division, Medfield, Massachusetts). For human hemolysates this technique separates total HbA_1 from HbA_0.[24]

Hemolysates were chromatographed on GlycoGel-B (Pierce Chemical Company, Rockford, Illinois) glycoprotein affinity gel, which contains an aminophenyl boronic acid moiety bound to a 6% cross-linked agarose matrix.[25] The boronate group forms a reversible covalent attachment with vicinal hydroxyl groups on sugars. A hemolysate containing 25 mg hemoglobin was applied to a 0.9 × 7.0-cm column of GlycoGel-B and was eluted first with 40 ml of 0.25 M ammonium acetate, pH 8.5, to remove nonglycosylated hemoglobin. Glycosylated hemoglobin that adhered to the column was completely eluted with 40 ml of 0.2 M sorbitol in 0.25 M ammonium acetate, pH 8.5. Results of Mallia et al.[25] as well as experiments in this laboratory indicate that hemoglobin A_{1c} as well as the glycosylated portion of HbA_0 adheres strongly to the GlycoGel-B column under these conditions.

RESULTS

Glucose concentrations in whole blood, plasma, and red cells are shown in Table 1. The cell/plasma ratio, an indicator of red cell permeability to glucose, varied widely among the species tested; however, within a given species, the variation was small. Cell/plasma ratios of the primates (man, baboon, rhesus monkey) were not significantly different from one another ($P > 0.02$), while those of the dog, rabbit, and pig were significantly lower ($P < 0.001$). Pig erythrocytes had no significant amount of intracellular glucose.

Elution profiles of Bio-Rex 70 chromatography for each species are shown in Figure 1. For all animals, several negatively charged minor hemoglobins were isolated from the major hemoglobin component. Reaction of the pooled minor and major hemoglobin components with the thiobarbituric acid (TBA) test is summarized in Table 2. Minor components with A443 comparable to that of human HbA_{1c} were found in the dog (peak 3), rabbit (peak 3), baboon (peak 3), and rhesus monkey (peak 3) (shaded peaks, Figure 1); their percentage of the total Hb is summarized in Table 3. Although pig hemolysate contained minor components, none were TBA positive and therefore unlikely to be glycosylated. The TBA test on whole hemolysates varied widely among the species tested and did not agree with the chromatographic or electrophoretic data.

Agar gel electrophoresis of human and animal hemolysates effectively separated minor from major hemoglobin components (Figure 2). For human, baboon, rhesus monkey, and dog hemoglobins, resolution of "fast" components on agar gel was sufficient to permit their quantitation by scanning densitometry (Table 3). For human hemoglobin the "fast" hemoglobin includes HbA_{1a}, HbA_{1b}, and HbA_{1c}.[24] "Fast" hemoglobins of the rabbit and pig were not resolved well enough to be quantitatively measured. Minor hemoglobin components isolated by Bio-Rex chromatography were also run individually on agar gel. For human, baboon, rhesus monkey, and dog hemoglobin, the TBA-positive Bio-Rex peak (peak 3) coincided with the fast-migrating component

TABLE 1
Distribution of glucose in plasma and red cells

	N*	Glucose concentration (mM) (mean ± SD)			Cell/Plasma × 100 (mean ± SD)
		Blood	Plasma	Cell	
Human	8	4.1 ± 0.5	4.8 ± 0.6	3.2 ± 0.8	67.1 ± 14.3
Baboon	4	4.0 ± 0.2	4.4 ± 0.5	3.4 ± 0.4	77.0 ± 16.4
Rhesus monkey	4	3.0 ± 0.3	3.4 ± 0.5	2.4 ± 0.3	70.8 ± 12.8
Dog	8	3.2 ± 0.6	4.4 ± 1.1	1.5 ± 0.6	34.2 ± 12.9
Rabbit	10	4.8 ± 1.3	6.9 ± 2.2	1.2 ± 0.9	19.2 ± 4.4
Pig	4	3.1 ± 1.4	5.2 ± 2.2	0.2 ± 0.4	3.8 ± 5.0

* N is number of animals studied.

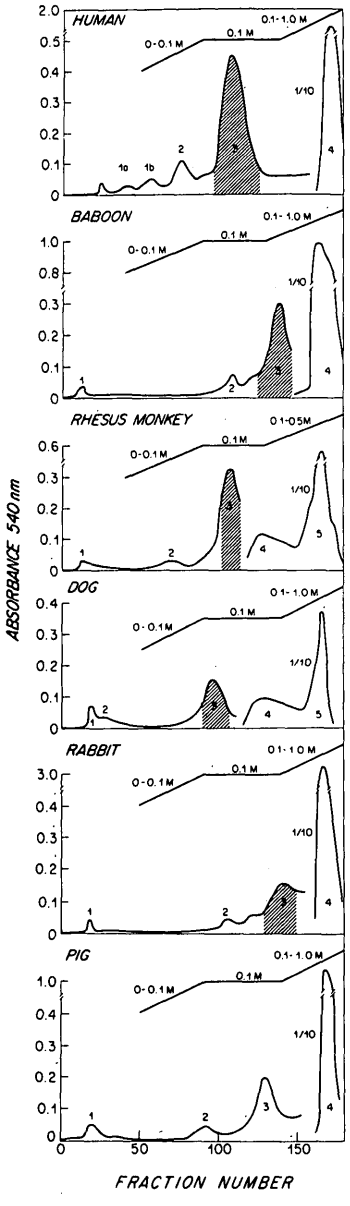


FIGURE 1. Bio-Rex 70 elution profiles of human and animal hemoglobin: human, pH 6.60; baboon, pH 6.70; rhesus monkey, pH 6.70; dog, pH 6.60; rabbit, pH 6.80; pig, pH 6.60. The TBA-positive peaks are shaded.

on the agar gel (data not shown). For rabbit hemoglobin, the TBA-positive Bio-Rex peak migrated only slightly faster than the major component and was not completely resolved. For the pig, the most abundant minor component (peak 3) and the major component had identical mobilities on agar gel.

From the results of Bio-Rex 70 chromatography, agar gel electrophoresis, and the TBA reaction, we conclude that peak 3 of the baboon, rhesus monkey, dog, and rabbit is HbA_{1c}. Pig hemoglobin contains no detectable HbA_{1c}.

To compare HbA_{1c} levels among animals and man, both the intracellular glucose levels (Table 1) and the erythrocyte survival need to be considered. Approximate red cell life spans of the animals studied in this report are: man, 120 days; baboon, 45 days; rhesus monkey, 95 days; dog, 100 days; rabbit, 60 days; pig, 90 days.[26] A combined parameter, here termed the glucose exposure [cell glucose (mM) × cell life span (days)], should be directly proportional to levels of HbA_{1c}, if these are the predominant factors affecting rate of glycosylation. The relationship between these two variables, as determined in the present study, is shown graphically in Figure 3. Percentage of HbA_{1c} in mammalian red cells, as determined by Bio-Rex chromatography, correlates well with their exposure to glucose ($r^2 = 0.91$).

Figure 4 illustrates a similar strong correlation between the formation of glycohemoglobin, as measured by affinity chromatography, and the degree of glucose exposure. The percentages of total glycohemoglobin from each animal are compared in Table 3 with percent HbA_{1c} from Bio-Rex chromatography and percent "fast hemoglobin" from agar gel electrophoresis. As can be seen from Table 3, total glycosylated hemoglobin correlates well with HbA_{1c}.

DISCUSSION

We have investigated the glycosylation of hemoglobin in selected mammals to gain a better understanding of the factors responsible for this important posttranslational modification. This information should be useful in interpreting measurements of HbA_{1c} in both experimental and clinical settings. In this study we selected mammalian species that have been used as animal models for research on diabetes,[12–15] and have a single major hemoglobin component. Other species such as the cat, guinea pig, rat, and certain strains of mice have two or more major hemoglobin components, owing to duplication of either the α- or β-chain genes or both. In these species glycosylated hemoglobins are difficult to measure and interpret.

This study enables a valid comparison of various methods for measuring glycosylated hemoglobins. As shown in Figure 1, cation-exchange chromatography adequately separated the major hemoglobin component from negatively charged minor components. In all animals except the pig, the most abundant minor component had a positive TBA test and was therefore glycosylated. Although we assume that these animal hemoglobins are analogous to human HbA_{1c}, we have no evidence that the site of glycosy-

TABLE 2
Measurement of ketoamine-linked glucose by the thiobarbituric acid test*

	Hemolysate	Peak 2	Peak 3	Peak 4	Peak 5
Human	0.005		0.056	0.001	
Baboon	0.004	0.015	0.043	0.001	
Rhesus monkey	0.002		0.038	0.002	0.000
Dog	0.003	0.014	0.037	0.001	0.001
Rabbit	0.003	0.013	0.036	0.002	
Pig	0.000	0.004	0.004	0.001	

* A443 nm/mg hemoglobin (mean of two determinations).

TABLE 3
Comparison of glycosylated hemoglobin by Bio-Rex chromatography, agar gel electrophoresis, and GlycoGel-B affinity chromatography

	N*	%HBA_{1c} (Bio-Rex)†	N*	% Fast Hb (agar gel electrophoresis)†	N*	% Total glycosylated Hb (GlycoGel-B)
Human	6	4.9 ± 0.8	6	6.7 ± 0.7	4	6.2, 7.1, 7.0, 7.4
Baboon	4	3.5 ± 0.9	4	4.3 ± 0.9	3	5.6, 3.4, 4.3
Rhesus monkey	4	3.3 ± 0.3	4	5.7 ± 0.4	2	5.5, 5.4
Dog	5	3.3 ± 0.6	7	6.1 ± 0.8	2	3.7, 3.0
Rabbit	4	1.4 ± 0.3	4	‡	2	2.7, 2.0
Pig	3	0.0	3	‡	2	0.3, 0.3

* N is number of animals tested.
† Mean ± 1 SD.
‡ "Fast hemoglobins" were not adequately resolved.

lation is the β-N terminus. Pig hemoglobin contained no glycosylated components. Agar gel electrophoresis, which is an effective way to measure total HbA_1 content in human hemolysates,[24] also separated "fast" from "slow" hemoglobins in the dog, baboon, and rhesus monkey. However, this method gave systematically higher levels of HbA_{1c} than the chromatographic analyses, probably owing to the fact that other minor hemoglobins comigrate with HbA_{1c} on agar gel.

Affinity chromatography is a new and potentially valuable assay for investigating glycosylated hemoglobins. Employing a solid matrix for attachment of the boronate group has clear-cut advantages over the use of free borate, which we described previously.[2] Brownlee et al.[27] used Affi-Gel 601 for the measurement of glycosylated amino acids, but glycosylated proteins cannot be separated on this resin. Mallia and his colleagues[25] have shown that the measurement of glycosylated hemoglobin using GlycoGel-B correlates well with HbA_{1c} in normal and diabetic humans. The GlycoGel-B resin binds hemoglobin glycosylated not only at the β-N terminus (HbA_{1c}) but also at other sites.[2] The estimate of the extent of glycosylation of normal human HbA_0 from the data on Table 3 is significantly lower than indirect measurements that we previously reported.[2,28] We are currently making a detailed evaluation of this affinity resin in order to determine the specificity and efficiency of binding of various glycosylated hemoglobins. This promises to be a powerful and versatile technique that can be readily applied to the study of nonenzymatically glycosylated proteins in other tissues. Since the resin binds all types of glycoproteins, it would be necessary to remove enzymatically linked carbohydrate before analysis.

Our results show that the permeability of cells to glucose is a major determinant of nonenzymatic glycosylation and must be considered in the interpretation of measurements of HbA_{1c} in mammals. Although this factor has not been considered in previous studies, it has an important bearing on nonenzymatic glycosylation of hemoglobin as well as on proteins in other tissues. In general, fetal cells are freely permeable to glucose.[29] However, during postnatal development, cells develop a permeability barrier to glucose of varying degree. The red cells of all mammals except primates acquire such a barrier early after gestation. Recently, Kondo and Beutler[30] showed that this alteration in membrane function correlated with the disappearance of band 4.5, a red cell membrane protein that is thought to be responsible for the facilitated diffusion of glucose.[31–33] The acquisition of a permeability barrier to glucose protects the cell against extensive nonenzymatic glycosylation. This protection may be important in limiting the modification of proteins within cells. Thus, glucose permeability is an important variable to assess in considering the extent to which

FIGURE 2. Separation of minor and major hemoglobins by agar gel electrophoresis at pH 6.3.

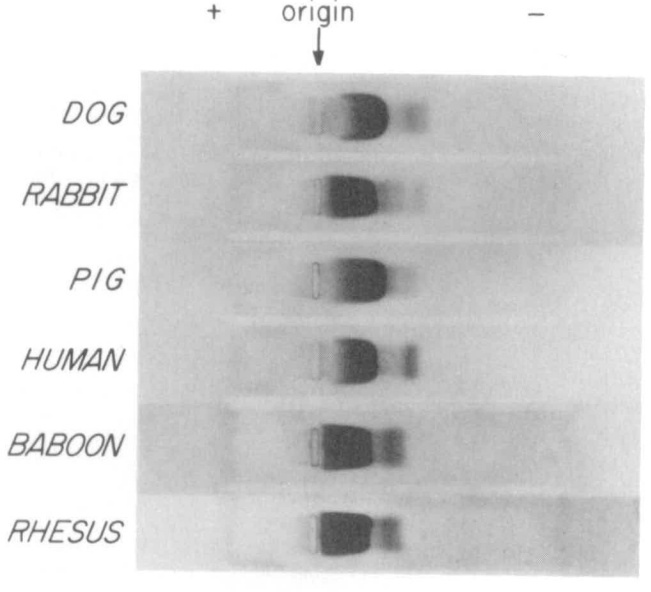
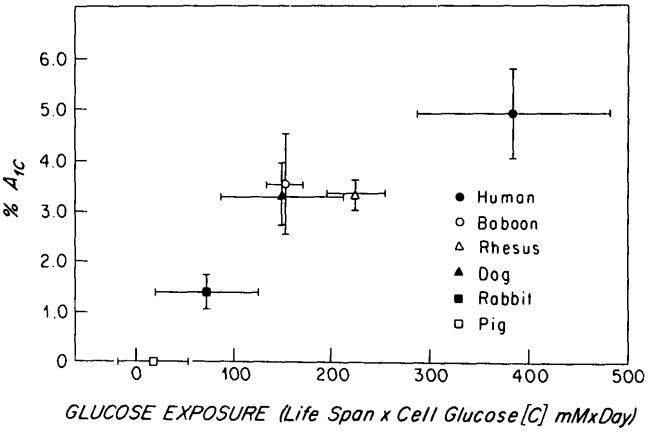


FIGURE 3. Correlation between percentage HbA_{1c} measured by Bio-Rex 70 chromatography and glucose exposure (red cell glucose × red cell life span). Each point represents the mean ± SD of the abscissa and ordinate; y = 0.12x + 0.67; r^2 = 0.91. The number of determinations is listed in Tables 1 and 3.



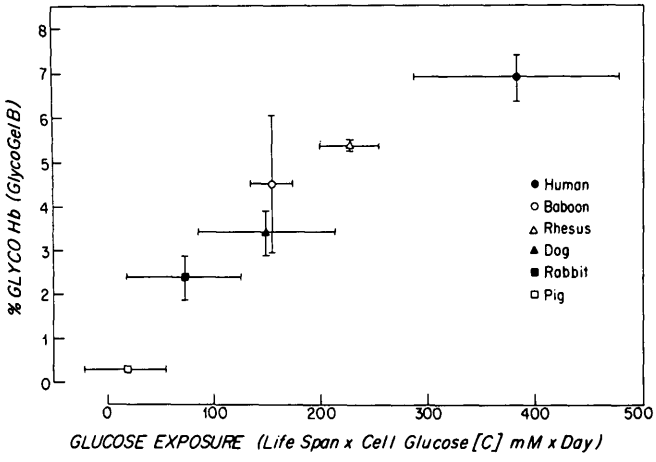


FIGURE 4. Correlation between total glycosylated hemoglobin measured by affinity chromatography on GlycoGel-B and glucose exposure. Affinity chromatography was performed as described in MATERIAL AND METHODS. The number of determinations is listed in Tables 1 and 3. The error bars on the abscissa represent mean ± SD while those on the ordinate represent range of determinations.

other proteins besides hemoglobin may be modified by nonenzymatic glycosylation, particularly in tissues that are responsible for the long-term complications of diabetes.

The distribution of blood glucose in plasma and red cells shown in Table 1 is consistent with previous observations.[20,21] In this group of animals the glucose permeability barrier ranged from negligible (primates) to complete (pig). The variability of the blood glucose levels may be due to differences in the nutritional status or the metabolic utilization of glucose in these animals.

We have considered two predominant factors (intracellular glucose concentration and red cell life span) that affect the synthesis and accumulation of HbA_{1c} in mammalian erythrocytes, and have combined them into a single parameter. Levels of HbA_{1c} in animals and man correlate well with glucose exposure as shown in Figure 3. The dog and baboon have nearly equal HbA_{1c} levels: the former has low red cell glucose and a relatively long cell life span; the latter has high red cell glucose and a shorter cell life span. Because red cell turnover rate can affect the level of HbA_{1c}, it is important to consider the clinical status of an animal when determining HbA_{1c}, since many diseases can shorten the life span of red cells.

Other factors that might also affect the rate of HbA_{1c} synthesis are (1) the presence of molecules such as 2,3-DPG[34] and CO_2, which interact with the β-NH_2 terminus; (2) oxygen saturation;[34] and (3) intracellular pH, which alters the charge of the amino group and hence its reactivity to glucose.[34,35] Red cell 2,3-DPG varies somewhat in diabetic patients whereas marked differences are noted among various mammals.[36] Recently, Smith and his colleagues[34] examined the effect of these variables on the function of HbA_{1c} in intact human red cells. They found that the synthetic rate was twofold higher in fully deoxygenated cells compared with fully oxygenated cells. Depletion of red cell 2,3-DPG caused a 30–50% decrease in synthetic rate whereas varying pH between 7.0 and 8.0 had no effect. Since much smaller changes in oxygen saturation and 2,3-DPG levels take place in vivo, these factors are unlikely to be major determinants of glycosylated hemoglobin.

Our observations on mammals support the simplest possible scheme for the glycosylation of hemoglobin: the bimolecular condensation of glucose and hemoglobin, unaffected by intracellular enzymes or cofactors. Therefore, levels of glycosylated hemoglobin can be predicted from a knowledge of in vitro rates of formation[35] and the intracellular exposure of hemoglobin to glucose. These principles can be extended to consideration of the nonenzymatic glycosylation of protein in other cells.

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