Glycosylation of Variant Hemoglobins in Normal and Diabetic Subjects

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The extent of in vivo glycosylation of variant hemoglobins was examined in individuals with S-, C-, and D-trait. Chromatographic estimates of glycosylation for nondiabetic individuals with S-trait were significantly lower than those for nondiabetic black subjects with normal hemoglobin ($P < 0.001$). However, chemical determinations of glycosylation (thiobarbituric acid or TBA technique) were similar for these groups ($P > 0.10$). The chromatographic elution pattern of hemoglobin S (HbS) was determined, and on this basis an adjustment procedure was performed for chromatographic data. A regression line was calculated for the relationship between chromatographic and colorimetric estimates of glycosylated hemoglobin in S-trait individuals with and without diabetes. The slope of this line was significantly different ($P < 0.001$) from that for the relationship in individuals with normal hemoglobin. However, after adjustment of chromatographic values from S-trait individuals, the slopes were similar ($P > 0.10$). Findings from individuals heterozygous for HbC and D were similar to those for individuals with S-trait. These data indicate that the extent of glycosylation of HbS, C, and D is similar to that of HbA in both the normoglycemic and hyperglycemic range. The TBA technique is the most direct method for determining the extent of glycosylation in individuals with HbS, C, or D. However, adjustment of column chromatographic values is feasible. DIABETES CARE 3: 590–593, SEPTEMBER–OCTOBER 1980.

Glycosylation of the hemoglobin molecule ($\alpha_2\beta_2$) occurs at the N-termini of the α and β chains and at the ϵ-amino groups of the lysine residues.[1,2] Glycosylation is increased in diabetic patients due to hyperglycemia, and its extent can be used as an index of long-term blood glucose control.[3] Glycosylation of the β-chain N-termini results in a sufficient change in charge to permit chromatographic separation of the resultant hemoglobin species (HbA_{1a+b+c} or HbA_1) on cation exchange chromatography. Chromatographic quantitation of the HbA_1 fraction is currently being used to estimate hemoglobin glycosylation in diabetic patients. Variant hemoglobins with altered charge properties (e.g., HbS, C, and D) have different chromatographic mobilities than HbA;[4] in patients with these abnormal hemoglobin traits, chromatographically determined estimates of glycosylation are misleading.[5]

To directly assess the extent of in vivo glycosylation of variant hemoglobins, we used a chemical procedure, the thiobarbituric acid method (TBA), which measures total glycosylation. In addition, indirect estimation of hemoglobin glycosylation was made by adjustment of chromatographic data on the basis of the altered mobilities of the variant hemoglobins. Data obtained by both methods indicate that the extent of glycosylation of the variant hemoglobins is comparable to that of HbA in both normoglycemic and diabetic subjects.

MATERIALS AND METHODS

Blood specimens were obtained from diabetic patients with and without hemoglobin variants, and from black nondiabetic parents of children with hemoglobinopathies followed in the Diabetes and Hematology Clinics of the Children's Hospital Medical Center. The parents are heterozygous for HbS, C, or D. Blood samples were also obtained from nondiabetic Caucasian and black volunteers with normal hemoglobin. Hemoglobin variants were identified by cellulose acetate and citrate agar electrophoresis;[6] the relative proportions were quantitated by densitometry after staining of cellulose acetate strips with Ponceau S.[6]

Blood samples were collected in EDTA tubes and the HbA_1 fraction quantitated by column chromatography ac-

cording to the method of Trivelli,[7] with several modifications.[1,8] Total glycosylation was measured on 10-mg aliquots of hemolysate hemoglobin by the TBA colorimetric technique, as previously described.[1] Results were expressed as OD_{443}/10 mg hemolysate hemoglobin. The mean value for nondiabetic subjects is 0.190 ± 0.022 (±SD, N = 27), while diabetic hemolysates have a mean value of 0.331 ± 0.082 (±SD, N = 152).

HbS was purified by column chromatography of a blood specimen from a patient homozygous for HbS. The HbS was dialyzed against 0.05 M Tris-HCl buffer, pH 7.4, and incubated with a 50-M excess of glucose 6-phosphate with 20 μCi of ^{14}C-labeled glucose 6-phosphate (New England Nuclear Corp., Boston, Massachusetts) for 21 h at 37°C. Free and electrostatically bound glucose 6-phosphate was removed by gel filtration on Sephadex G-25 in Developer 6, and the hemoglobin mixture was analyzed for HbS_{1a2} (G-6-P-HbS) content by Biorex-70 chromatography. An aliquot of the hemoglobin mixture was added to a HbAS hemolysate sample and also chromatographed on Biorex-70 to determine the elution position of HbS_{1a2}. The hemoglobin concentration was monitored spectrophotometrically at 415 or 540 nm and radioactivity was measured by counting 50-μl aliquots in a liquid scintillation counter.

Least squares regression was used for line fitting. Student's *t* tests were employed for significance testing.

RESULTS

Table 1 shows a comparison of column chromatographic and chemical measurement of hemoglobin glycosylation in nondiabetic black subjects with and without S-trait. The mean (±SD) percentage HbS in the S-trait patients was 38.9 ± 3.0%. The mean age of the two groups of subjects was virtually identical (29.2 ± 9.1 yr for normals and 28.9 ± 10.4 yr for individuals with S-trait). The mean HbA_1 values in black subjects with S-trait were significantly lower than in those with normal hemoglobin ($P < 0.001$). On the other hand, mean TBA values were not different ($P > 0.10$).

TABLE 1
Estimation of hemoglobin glycosylation by column chromatography and the TBA method (mean ± SEM) in nondiabetic black subjects with and without sickle cell trait

	Normal (N = 24)	S-trait (N = 13)	P value (2-sided)
Percent HbA_1	7.61 ± 0.13	5.41 ± 0.19	<0.001
TBA value (OD_{443})	0.203 ± 0.003	0.195 ± 0.005	>0.10

The lower chromatographic values obtained in S-trait subjects are a consequence of the retarded elution of the various variant hemoglobin components. Figure 1 shows the elution diagram of a hemolysate from an S-trait subject (HbAS) to which was added 5 mg of HbS previously incubated with ^{14}C-labeled glucose 6-phosphate (HbS_{1a2} or G-6-P HbS). In this system, HbS_{1a2} coelutes with HbA_{1a+b}, while HbS elutes after the main Hb peak. HbS_{1c} (analagous to HbA_{1c}) elutes with the main HbA peak. Similarly, HbC_{1c} elutes at the trailing edge of the main HbA peak (data not shown).

Figure 2A shows the relationship between colorimetric and chromatographic estimates of hemoglobin glycosylation determined on hemolysates from 22 normoglycemic and diabetic individuals with S-, C-, or D-trait. The regression lines for the paired values of the 18 S-trait individuals and for 224 normoglycemic and diabetic subjects with normal hemoglobin are also shown. The slope of the regression line for the

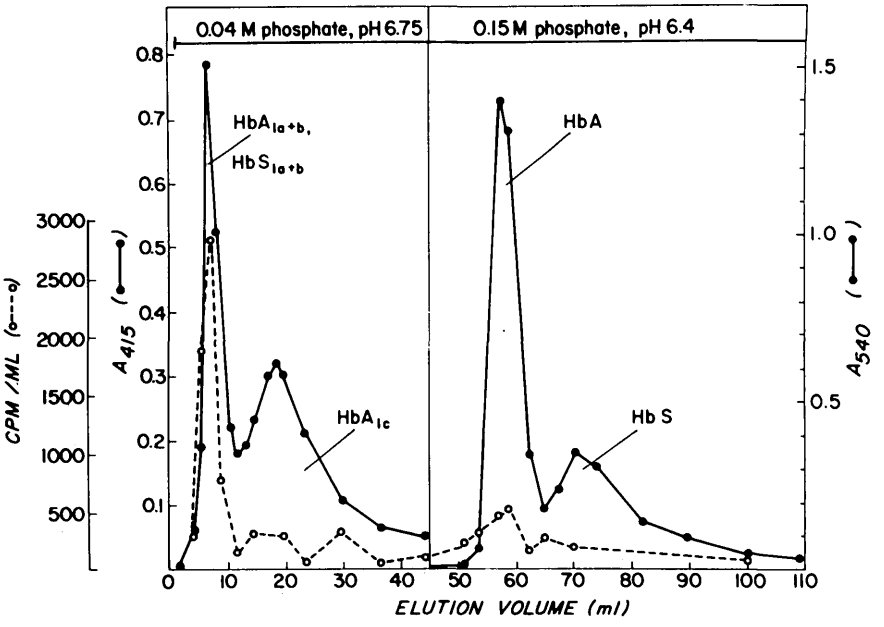

FIG. 1. Elution profile of hemolysate from an individual with sickle cell trait (HbAS). Added ^{14}C-labeled synthetic HbS_{1a2} (G-6-P HbS) coelutes with HbA_{1a+b}.

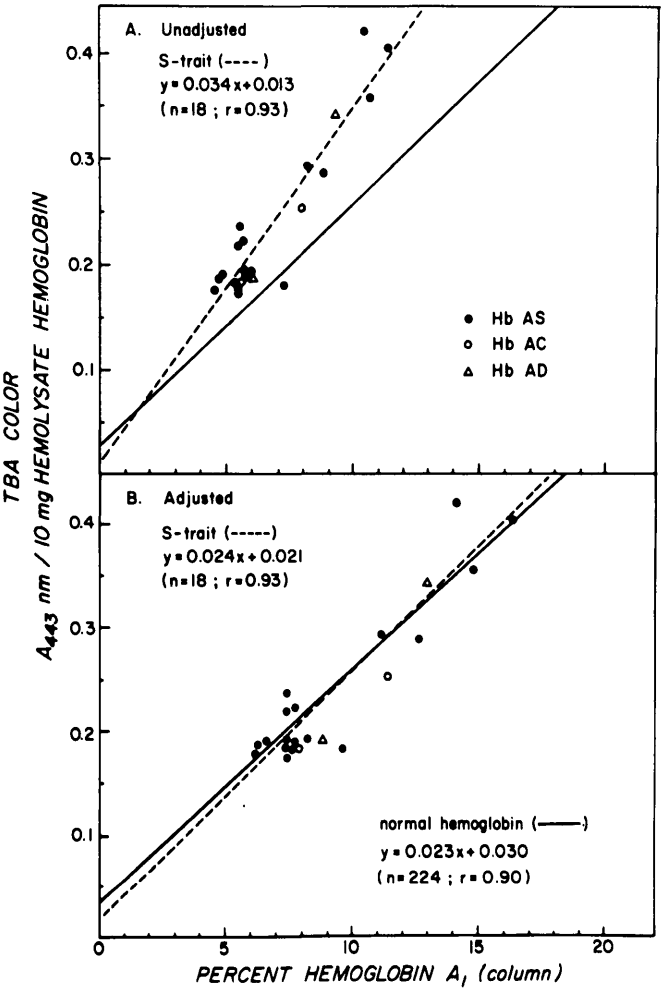

FIG. 2. Relationship between colorimetric and chromatographic values in nondiabetic and diabetic individuals heterozygous for hemoglobins S, C, and D. Regression lines were calculated for individuals with AS hemoglobin (dashed line) and individuals with normal hemoglobin (solid line). Note the difference in slopes in A (before adjustment) and the similarity in slopes in B (after adjustment).

values of the S-trait individuals was significantly different ($P < 0.001$) from that for values of subjects with normal hemoglobin. The correlation coefficients for the S-trait values and normal values were 0.93 and 0.90, respectively.

Figure 2B shows the same data plotted after adjustment (see Appendix) was made on the basis of the altered mobilities of the variant hemoglobins, i.e., HbS_{1a} and presumably HbS_{1b} coelute with HbA and HbS. The slopes of the two regression lines are almost identical ($P > 0.10$).

DISCUSSION

The results show that the extent of in vivo glycosylation of HbS is comparable to that of HbA and also suggest that this is true for HbC and HbD. These findings are based on direct quantitation of hemoglobin glycosylation by the TBA colorimetric technique. In this procedure, furfural compounds are generated from carbohydrate moieties on heating under acidic conditions and are quantitated colorimetrically with 2-thiobarbituric acid.

Chromatographic estimation of the glycosylated hemoglobin S, C, and D components gives low values. This underestimation of hemoglobin glycosylation is caused by their altered charge properties. Due to the more positive charge of hemoglobins S, C, and D compared with HbA, elution from the cation exchange resin is retarded. Consequently, as demonstrated for HbS, only HbX_{1a+b} (X = S, C, or D) elute in the first fraction collected during routine chromatographic analysis of glycosylated hemoglobins (Figure 1). HbX_{1c}, on the other hand, elutes with the main HbA and HbX fraction. Adjustment of the observed chromatographic results with a formula based on these findings (Appendix) yields calculated values virtually identical to those expected from the direct TBA measurement of glycosylation (Figure 2B).

Our adjustment of the chromatographic results differs from the procedure proposed by Aleyassine,[5] who assumed that HbS_{1a+b} elute with HbS_{1c} in the main hemoglobin fraction; our data show that this assumption is incorrect. Applied to our data, that correction procedure overestimates glycosylated hemoglobin by several percentage points in the hyperglycemic range.

Crucial to the clinical interpretation of these glycosylated hemoglobin values is the red cell life span in patients heterozygous for HbS, C, or D. The erythrocyte life span in HbAS subjects is not significantly different from that of normal homozygous HbA subjects.[9]

Our data indicate that the extent of glycosylation of three common variant hemoglobins is comparable to that of HbA; consequently, glycosylated hemoglobin levels, as measured directly by the TBA method, can be a clinically useful index of long-term blood glucose control in diabetic patients heterozygous for hemoglobinopathies.

APPENDIX

In order to adjust column chromatographic values obtained on samples from individuals with sickle cell trait, two assumptions have been made, that (1) HbS_{1b} elutes in the first fraction in our chromatographic system and (2) the proportion of the glycosylated hemoglobin species is identical for HbA and HbS. Since HbS_{1a+b} elute in the first fraction with HbA_{1a-c}, adjustment is only necessary for HbS_{1c}. Equation (1) describes this adjustment:

$$1/1 - HbS\ (Obs - a_T T_1) = T_{1c} \qquad (1)$$

where HbS = proportion of HbS; Obs = observed percentage of minor hemoglobin; $T_1 = HbA_{1a-c}$ and HbS_{1a-c}; $T_{1c} = HbA_{1c} = HbS_{1c}$; and a_T = proportion of T_1 that is HbA_{1a+b} and HbS_{1a+b}. Note that the expression $(Obs - a_T T_1)$ is equivalent to HbA_{1c}. It is also evident that:

$$T_1 - a_T T_1 = T_{1c} \qquad (2)$$

Rearranging Eqs. (1) and (2) yields:

$$1/1 - \text{HbS}\ (\text{Obs} - a_T T_1) = T_1 - a_T T_1 \qquad (3)$$

If the proportion of HbS is known, and a_T is estimated from the regression line describing the relationship between HbA_{1a+b} and HbA_{1c},[6] Eq. (3) can be solved for T_1. We have used a_T values of 0.30 and 0.24 for nondiabetic and diabetic individuals, respectively. The proportion of HbS can be directly determined or 0.40 used as a first approximation. Inserting the above estimates for HbS and a_T into Eq. (3) reduces the adjustment to 1.39 × Obs for nondiabetic and 1.44 × Obs for diabetic individuals with S-trait.

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