Characterization of Glycosylated Hemoglobins

RELEVANCE TO MONITORING OF DIABETIC CONTROL AND ANALYSIS OF OTHER PROTEINS

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ABSTRACT Boronate affinity chromatography and ion exchange chromatography were used to measure the levels of glycosylated hemoglobins in normal and diabetic hemolysates, as well as the distribution of glucose adducts on α-NH_2-valine and ϵ-NH_2-lysine residues.

When analyzed by ion exchange chromatography on BioRex 70 resin, the Hb A_{Ic} peak comprised 4.4±0.6% of 15 normal hemolysates and 9.1±2.1% of 15 diabetic hemolysates. The "Hb A_{Ic}" was rechromatographed on GlycoGel B boronate affinity resin that binds vicinal hydroxyl groups of covalently linked sugars. Only 70±5% of the hemoglobin adhered to the resin. Analysis by the thiobarbituric acid colorimetric test confirmed that the affinity resin effectively separated glycosylated from nonglycosylated hemoglobin. When corrected for nonglycosylated contaminants, the mean level of Hb A_{Ic} in normal hemolysates was 2.9±0.4%, a value considerably lower than those previously reported. In addition to Hb A_{Ic}, 5.2±0.5% of the remaining hemoglobin (Hb A_0) was glycosylated. In diabetics, glycosylated A_0 was increased in parallel with Hb A_{Ic}. After reduction with [^{3}H]borohydride and acid hydrolysis, glycosylated amino acids were first purified on Affi-Gel boronate affinity resin and then analyzed by ion exchange chromatography. The glucose adducts on Hb A_0 were distributed as follows: α-chain N-terminal valine, 14%; α-chain lysines, 40%; β-chain lysines, 46%.

This study has revealed several pitfalls in the analysis of nonenzymatically glycosylated proteins. Peaks isolated by ion exchange chromatography or electrophoresis are likely to be contaminated by nonglycosylated proteins. Furthermore, both the thiobarbituric acid test and [^{3}H]borohydride reduction show variable reactivity depending upon the site of the ketoamine-linked glucose.

INTRODUCTION

Many proteins undergo nonenzymatic glycosylation when exposed to monosaccharides. The aldehyde or ketone function of the sugar condenses with amino groups to form a Schiff base linkage, which can slowly undergo an Amadori rearrangement to the more stable ketoamine (1–4). This posttranslational modification has been encountered in a wide variety of tissues and may be responsible for certain long-term complications of diabetes mellitus (5, 6). Among the various proteins that are known to undergo nonenzymatic glycosylation in vivo, human hemoglobin (Hb)[1] has been the most thoroughly investigated (1–6). Measurement of glycosylated Hb has been useful in assessing diabetic control (7–13). Furthermore, Hb may be considered a model protein that has provided insights into nonenzymatic glycosylation in other more complex tissues (5, 6). Hb A_{Ic} is the most abundant minor Hb component in human erythrocytes. According to estimates based primarily on ion exchange chromatography, Hb A_{Ic} comprises 4–6% of Hb in normal erythrocytes and is elevated approximately twofold in diabetics (5–14). There is convincing structural evidence that Hb A_{Ic} differs from the major component Hb A_0 by the attachment of glucose to the NH_2-terminus of the β-chains by means of a ketoamine linkage (1–4). In addition, a small proportion of Hb A_0, which we previously estimated to be 8% (15, 16), is also glycosylated at one of several sites including the N-terminus of the

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[1] *Abbreviations used in this paper:* Hb, hemoglobin; TBA test, thiobarbituric acid test.

α-chains as well as at certain lysine residues (β-lysine-66, α-lysine-61, and β-lysine-17) (15, 16). Because these glycosylated Hb components are present in small amounts and have been difficult to isolate by standard chromatographic and electrophoretic procedures, their proportions in the erythrocyte and sites of modification are uncertain. The isolation of glycosylated proteins (16–18) and amino acids (19) by boronic acid affinity chromatography has enabled much more precise quantification of these Hb components as well as better definition of the sites of glycosylation. Our measurements show that previous estimates of glycosylated Hb were much too high. Furthermore, our detailed analysis of Hb has identified a number of problems in the characterization of nonenzymatically glycosylated protein by conventional means. This information should be useful in investigating other proteins that may contribute to diabetic complications.

METHODS

Human hemolysate was prepared from freshly drawn blood of normal and diabetic donors. Before any chromatography step the hemolysates were saturated with CO and dialyzed vs. the appropriate buffer for at least 6 h at 4°C, to allow the dissociation of the labile aldimine-linked glucose (20–22).

Ion exchange chromatography. Lysates (20–100 mg) were applied to a column (0.9 × 18 cm) of BioRex 70 (Bio-Rad Laboratories, Richmond, CA) cation exchange resin, prepared and developed with phosphate buffers and a linear NaCl gradient as described (14). A larger (5.0 × 40 cm) preparative column of BioRex 70 was used to chromatograph hemolysates containing 2.0–2.3 g of Hb. Purified Hb fractions were concentrated by means of an Amicon pressure filtration device and a PM-10 membrane (Amicon Corp., Scientific Sys. Div., Lexington, MA).

Affinity chromatography. Lysates (6–10 mg), purified Hb A_0 (3–10 mg), and minor glycosylated Hb fractions from BioRex 70 (Hb A_{Ia-c}, 0.1–10 mg) were dialyzed vs. 0.25 M ammonium acetate, pH 8.5, and applied to a column (0.9 × 8 cm) of GlycoGel B (Pierce Chemical Co., Rockford, IL) (17). Nonglycosylated Hb was eluted from the column with either 40 or 60 ml of the starting buffer. Glycosylated Hb components that adhered to the resin were eluted with 0.2 M sorbitol in 0.25 M ammonium acetate, pH 8.5. The columns were developed at 15 ml/h and 4°C, and eluted Hb fractions were monitored at 415 or 540 nm. A larger (2.5 × 18 cm) preparative GlycoGel B column was eluted at 40 ml/h.

Certain Hb fractions from BioRex and GlycoGel chromatography were analyzed for ketoamine-linked glucose by means of the colorimetric thiobarbituric acid test (TBA) (15, 23). Each sample contained 5.0 mg Hb. Hb A_0,[2] which did not adhere to GlycoGel B, was used to correct for nonspecific color development. In certain experiments globin chains were separated by carboxymethyl cellulose cation exchange chromatography in 8 M urea (24).

[2] Hb A_0 is the major Hb component when hemolysate is analyzed by chromatography on BioRex 70 cation exchange resin.

Analysis of glycosylated amino acids. Glycosylated Hb samples were incubated for 30 min at 4°C in 0.05 M potassium phosphate, pH 7.0, with a 200-fold molar excess of [^{3}H]$NaBH_4$ (New England Nuclear, Boston, MA), diluted with nonradioactive $NaBH_4$ (Sigma Chemical Co., St. Louis, MO) to a final specific radioactivity of 160 mCi/mmol. The labeled Hb was then dialyzed vs. five changes of 4 liters distilled water over a 3-d period at 4°C. Each sample was dried under a stream of N_2 and then subjected to hydrolysis in 6 N HCl for 18 h at 105°C in an evacuated sealed tube. The ^{3}H-labeled hydrolysates were dried under a stream of N_2, dissolved in 0.05 M potassium phosphate, pH 9.0, and were passed through a 0.45 μm nitrocellulose filter (Schleicher & Schuell, Keene, NH), after which each was applied to a 1.5 × 12.5-cm column of Affi-Gel 601 (Bio-Rad Laboratories) boronic acid affinity chromatographic gel (19). The nonglycosylated amino acids in each hydrolysate were eluted with 0.05 M potassium phosphate, pH 9.0. The glycosylated amino acids were then eluted with 0.1 M HCl. The column was developed at room temperature with a flow rate of 45 ml/h. ^{3}H-Radioactivity was monitored in vials containing Liquiscint scintillation fluid (National Diagnostics, Somerville, NJ) by means of a Tracor Analytic BetaTrac 6895 liquid scintillation counter (Tracor Inc., Instrument Group, Austin, TX). Glycosylated amino acids from Affi-Gel 601 were chromatographed on a 0.9 × 20-cm column of Durrum DC-6A (Durrum Chemical Co., Sunnyvale, CA) cation exchange resin. The column was developed with a 200-ml linear gradient of 0.2 M pyridinium acetate, pH 3.1 to 1.5 M pyridinium acetate, pH 5.0. The column was operated at 200–400 psi and 55°C. The elution position of glucitol-lysine (N^ϵ[1-deoxy-D-glucit-1-yl]-L-lysine) was located by chromatographing a ^{3}H-labeled standard synthesized in our laboratory and analyzed by mass spectrometry. The elution position of glucitol-valine (N^α-[1-deoxy-D-glucit-1-yl]-L-valine) was located with a standard prepared by incubating [^{14}C]glucose (New England Nuclear) with L-valine in Krebs Ringer phosphate, pH 7.4, for 21 d at 37°C. The glucosyl-valine was

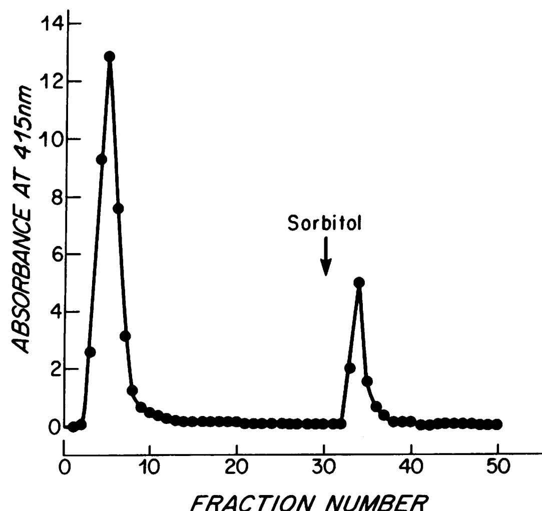

FIGURE 1 GlycoGel B boronic acid affinity chromatography of a diabetic erythrocyte hemolysate containing 10 mg Hb CO. The first peak, eluted in the void volume, consists of nonglycosylated Hb. The addition of sorbitol to the column permits the elution of glycosylated hemoglobin.

TABLE I
Percentage of Glycosylated Hb in Hemolysates

	Total glycosylated Hb	Glyco Hb A_0	Hb A_{Ic} (BioRex)	Hb A_{Ic} bound to GlycoGel B	Corrected Hb A_{Ic}
Normals	8.7	5.4	4.5	—	3.0
	8.2	5.1	4.2	—	2.8
	6.7	4.7	4.6	67.8	3.1
	8.2	5.2	4.1	—	2.7
	7.3	5.8	5.7	63.4	3.8
	7.0	5.6	4.2	65.7	2.8
	7.4	5.9	5.4	70.0	3.6
	7.4	4.0	3.8	71.7	2.5
	7.1	5.4	4.7	—	3.1
	7.6	5.2	4.6	—	3.1
	7.8	5.5	3.7	—	2.4
	7.1	5.4	4.3	64.2	2.8
	8.0	4.3	4.0	—	2.7
	6.9	4.6	3.4	—	2.3
	7.7	5.4	4.4	63.9	2.9
Mean±SD	7.5±0.5	5.2±0.5	4.4±0.6	66.7±3.0	2.9±0.4
Diabetics	18.3	9.7	8.5	86.2	7.3
	14.6	9.7	8.7	74.3	6.5
	14.7	8.2	7.9	70.2	5.6
	10.2	7.4	6.4	62.5	4.0
	9.8	6.0	6.4	68.1	4.4
	14.1	10.4	7.8	77.1	6.0
	16.9	10.9	8.5	85.3	7.2
	8.7	6.6	5.1	73.4	3.8
	16.9	9.3	8.6	71.8	6.1
	19.2	12.8	10.0	72.9	7.3
	23.6	14.6	11.8	78.7	9.3
	22.2	13.0	11.5	78.6	9.1
	20.1	12.7	11.8	71.2	8.4
	21.4	11.7	11.8	66.0	7.8
	21.2	14.0	11.4	71.5	8.1
Mean±SD	16.8±4.5	10.5±2.6	9.1±2.1	73.8±6.3	6.7±1.7

Hb from normal and diabetic hemolysates was subjected to chromatography on GlycoGel B boronic acid affinity gel and on BioRex 70 cation exchange resin. The Hb A_{Ic} and Hb A_0 components from BioRex 70 chromatography were rechromatographed on GlycoGel B. The percent glycosylated Hb from GlycoGel B chromatography is the percentage that adheres to GlycoGel in 0.25 M ammonium acetate and is released from the gel with the addition of 0.2 M sorbitol. For the normals seven Hb A_{Ic} samples from BioRex 70 chromatography were subjected to GlycoGel B chromatography. The percentage of Hb that adhered to GlycoGel B was determined for each sample, the values were averaged, and the average was multiplied times the individual values of percent Hb A_{Ic} from BioRex 70 to arrive at the percent corrected Hb A_{Ic} values. For the diabetic Hb A_{Ic} samples from BioRex 70, each was rechromatographed on GlycoGel B.

reduced with nonradioactive $NaBH_4$ (Sigma Chemical Co.) and was separated from [^{14}C]glucose by passage through a 1 × 100-cm column of Sephadex G-10-medium (Sigma Chemical Co.) in 10% acetic acid.

RESULTS

Separation of glycosylated Hb. Erythrocyte hemolysates from 15 normal and 15 diabetic donors were chromatographed on GlycoGel B boronic acid affinity resin (Fig. 1). As shown in Table I 7.5±0.5% (SD)[3] of Hb from normal hemolysates adhered to the resin. Diabetic hemolysates contained about twice as much adherent Hb. Experiments described below indicate

[3] Mean±1 SD will be used throughout this paper.

that all of the adherent Hb is glycosylated, while the nonadherent Hb contains no carbohydrate.

When analyzed on BioRex 70 ion exchange resin, a peak designated Hb A_{Ic} comprised 4.4±0.6% of normal hemolysates (Table I). Again, the level of Hb A_{Ic} among diabetics was twofold greater. When this Hb A_{Ic} peak was rechromatographed on GlycoGel B, 70±5% adhered to this affinity resin. To determine whether Hb A_{Ic} binds efficiently to GlycoGel B, we chromatographed two nondiabetic hemolysates on a large (5.0 × 40 cm) BioRex 70 column (Fig. 2) and sampled from the tube at the center of each Hb A_{Ic} peak. This Hb A_{Ic} was then rechromatographed on GlycoGel B. The adherence of these two Hb A_{Ic} samples to GlycoGel B was 95 and 92%, respectively. When the entire Hb A_{Ic} fraction from each large BioRex column was rechromatographed on GlycoGel B, 78 and 77% of the Hb adhered. Hemolysate, Hb A_0, and Hb A_{Ic} were assayed for ketoamine-linked hexose by means of the colorimetric TBA test (Table II). Samples of Hb A_{Ic} were assayed before and after chromatography on GlycoGel B. The color development for the

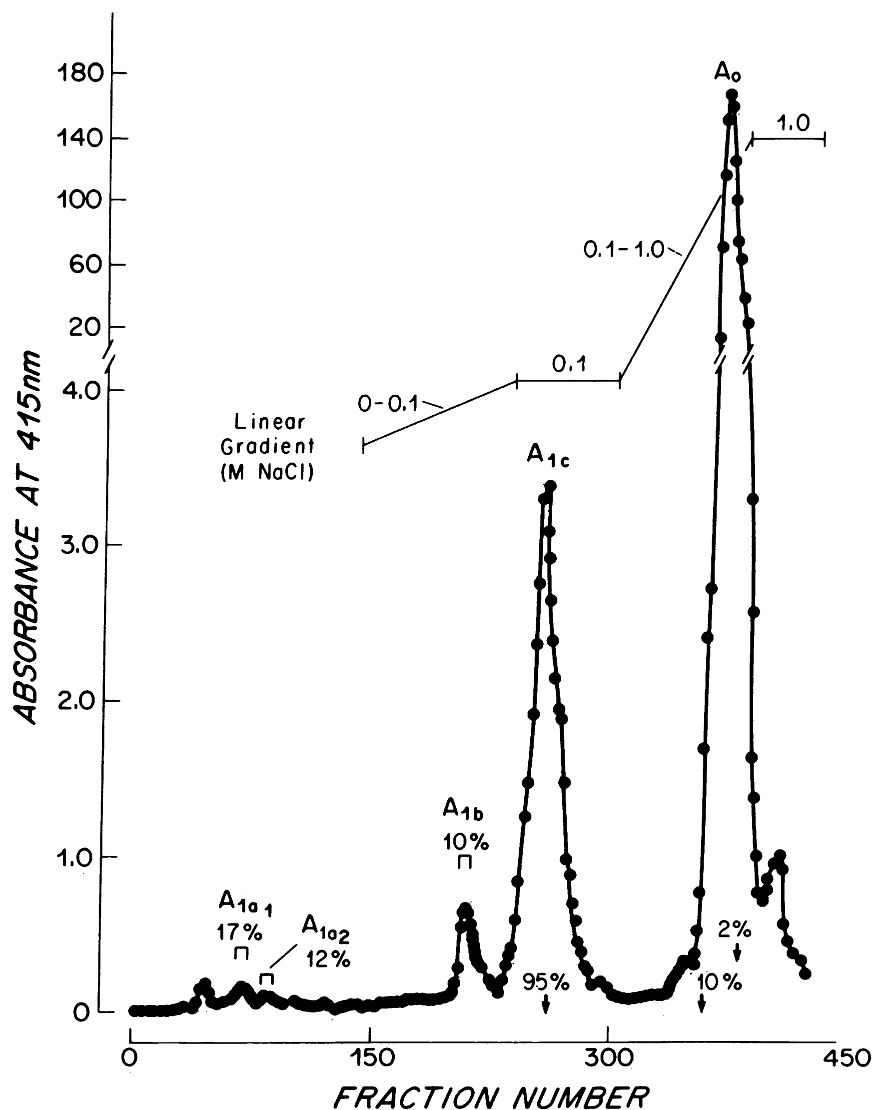

FIGURE 2 Preparative BioRex 70 chromotography of a nondiabetic hemolysate containing 2.3 g Hb CO. Flow rate 120 ml/h. Temperature, 4°C. The brackets indicate pooled fractions that were then rechromatographed on GlycoGel B. The arrows indicate individual fractions that were rechromatographed on GlycoGel B. The percentage of each fraction that adhered to GlycoGel B is shown.

Hb A_{Ic} obtained from the two-step purification was 30–33% higher than that obtained from Hb A_{Ic} peak that was not subjected to GlycoGel B chromatography.

Taken together, the chromatographic and colorimetric data indicate when hemolysates are analyzed by BioRex 70 chromatography, nonglycosylated Hb is eluted with authentic Hb A_{Ic}, and that purified authentic Hb A_{Ic} adheres efficiently to GlycoGel B. Therefore, we used the fractional yield from GlycoGel B chromatography to correct for the presence of contaminant in the Hb A_{Ic} isolated on BioRex 70. The corrected values for percent Hb A_{Ic} in normal individuals were 2.9±0.4%, compared with 6.7±1.7% in the diabetics.

Hemoglobin A_0 from the analytical BioRex 70 columns was also rechromatographed on GlycoGel B in order to determine the proportion of Hb A_0 that is glycosylated. Among normals, 5.2±0.5% of the Hb A_0 adhered to the GlycoGel B column, whereas twice as much diabetic Hb A_0 was adherent. Hb A_0 was assayed by the TBA test before and after GlycoGel B chromatography. As shown in Table II, glycosylated Hb A_0 provided much less efficient color development than Hb A_{Ic}. Hb A_0, which eluted from GlycoGel B in the column void volume, provided virtually no color development by the TBA test and is therefore, free of ketoamine-linked carbohydrate.

Fig. 3 is a plot of percentage of total glycosylated Hb and of glycosylated Hb A_0 vs. corrected percent Hb A_{Ic}. The data indicate that for both normal and diabetic specimens the total glycosylated Hb is ~2.4 times the corrected Hb A_{Ic}, while glycosylated Hb A_0 is 1.4 times Hb A_{Ic}. As a check on these values the total glycosylated Hb of a nondiabetic hemolysate,

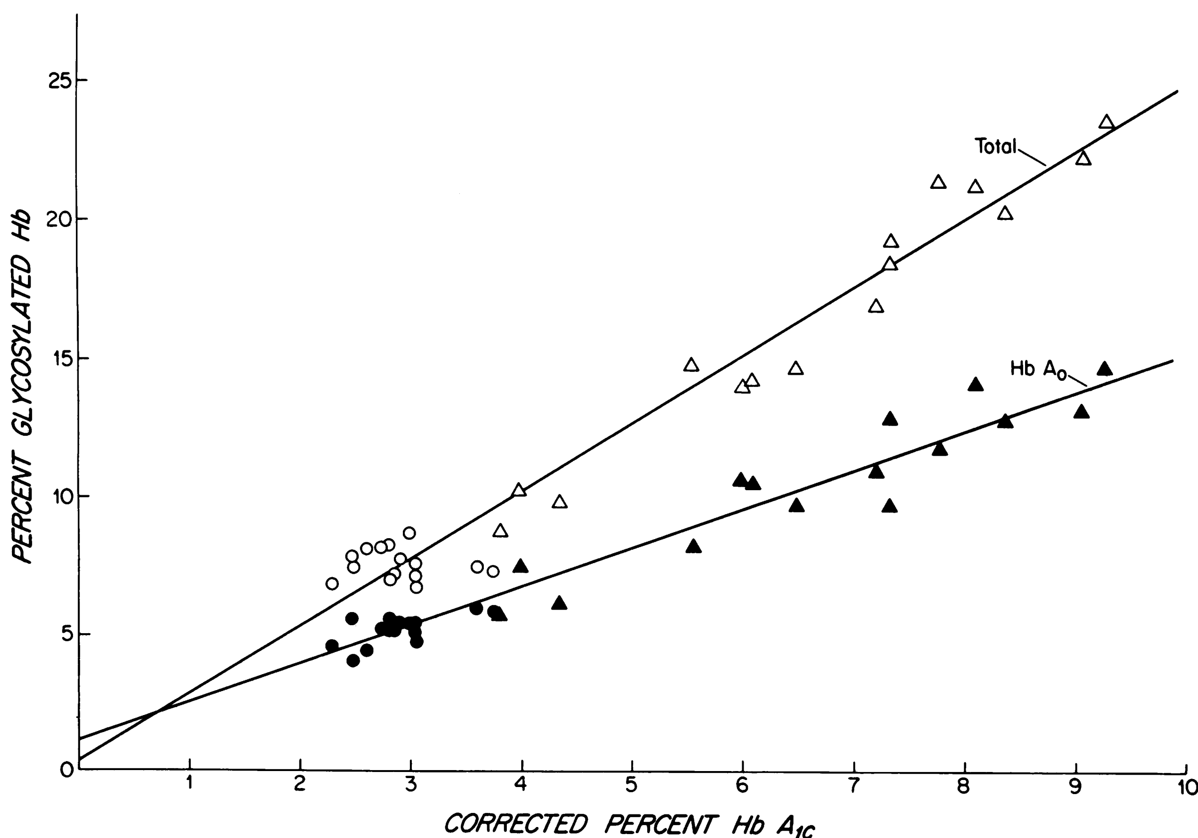

FIGURE 3 Percent glycosylated Hb from GlycoGel B boronic acid affinity chromatography, vs. percent corrected Hb A_{Ic}. The Hb A_{Ic} samples were from BioRex 70 chromatography followed by GlycoGel B chromatography. Glycosylated Hb from GlycoGel chromatography of nondiabetic hemolysates (○); glycosylated Hb from GlycoGel chromatography of diabetic hemolysates (△); glycosylated Hg A_0 from BioRex and GlycoGel chromatography of nondiabetic hemolysates (●); glycosylated Hb A_0 from BioRex and GlycoGel chromatography of diabetic hemolysates (▲). The formulae from linear regression analyses that fit the two sets of data are 2.44X + 0.39 (r = 0.99) for glycosylated Hb, and 1.40X + 1.09 (r = 0.98) for glycosylated Hb A_0 vs. Hb A_{Ic}.

TABLE II
*Analysis of Glycosylated Hb Samples by the Colorimetric TBA Test**

Hb species	Absorbance at 443 nm/mg Hb	
	Experiment 1 $n = 4$	Experiment 2 $n = 2$
Hb	0.009	0.010
Hb A_0	0.006	0.005
Hb A_{Ic} (BioRex only)	0.101	0.124
Glyco Hb (GlycoGel)	0.091	0.061
Glyco Hb A_0 (GlycoGel)	0.037	0.017
Hb A_{Ic} (BioRex + GlycoGel)	0.150	0.176
Hb (did not bind to GlycoGel)	0.009	
Hb A_0 (did not bind to GlycoGel)	0	
Hb A_{Ic} (did not bind to GlycoGel)	0.013	

* The colorimetric TBA test was conducted as described in the Methods section. Each sample contained 5.0 mg Hb. For each experiment the color generation due to the reaction of TBA with the Hb A_0 that did not bind to GlycoGel B was subtracted from each of the other values to correct for color development due to chromogens other than 5-hydroxymethylfurfural. A different nonglycosylated Hb A_0 sample was used in each experiment. The absorbance at 443 nm for nonglycosylated Hb A_0 was 0.015.

obtained from GlycoGel B, was rechromatographed on BioRex 70: 59±1.3% ($n = 5$) eluted from BioRex 70 as glycosylated Hb A_0, and the remainder as Hb A_{Ic}; therefore Hb A_0 was 1.4 times that of Hb A_{Ic} in good agreement with the data in Table I. The values for glycosylated Hb A_0 in nondiabetics may be slightly high relative to corrected Hb A_{Ic}. This is consistent with the fact that the line drawn from linear regression analysis for the plot of glycosylated Hb A_0 vs. corrected Hb A_{Ic} intersects slightly above the origin.

The other minor Hb A_I components from two nondiabetic donors were also rechromatographed on GlycoGel B. The percentages of Hb which bound to GlycoGel B were as follows: Hb A_{Ia_1}, 17 and 16%; Hb A_{Ia_2}, 12 and 20%; and Hb A_{Ib}, 9.5 and 15%. When synthetic glycose-6-phosphate Hb, which is thought to be the structure of Hb A_{Ia_2} (14, 25), was prepared by incubating Hb A_0 with glucose-6-phosphate (26), 89% of the purified Hb adhered to the GlycoGel B column. It appears that like Hb A_{Ic}, the Hb A_{Ia} and Hb A_{Ib} peaks from BioRex 70 were also contaminated with nonglycosylated Hb.

Analysis of glycosylated amino acids. Purified Hb components from two normal individuals were re-

TABLE III
Affinity Chromatography of [^{3}H]NaBH$_4$-labeled Acid Hydrolysates on Affi-Gel 601 Boronic Acid Affinity Gel

Hb species	Specific activity before hydrolysis	Specific activity after hydrolysis	Percent bound to Affi-Gel 601	Specific activity of amino acids bound to Affi-Gel 601
	dpm/mmol tetramer × 10^{-10}		*dpm*	*dpm/mmol tetramer × 10^{-10}*
Experiment 1				
Hemolysate	0.8	0.5	67	0.4
Glyco-Hb	13.0	6.1	62	3.8
Glyco-Hb A_0	13.5	6.4	70	4.5
Hb A_{Ic}	26.9	14.8	76	11.2
Experiment 2				
Glyco-Hb	9.6	5.1	70	3.6
Glyco-Hb A_0	8.0	4.4	65	2.8
Hb A_{Ic}	13.1	8.5	70	6.0

Glycosylated hemolysate from GlycoGel B chromatography, and glycosylated Hb A_{Ic} and Hb A_0 from BioRex 70 and GlycoGel B chromatography were treated with [^{3}H]NaBH$_4$ (Methods). The specific radioactivities were determined after extensive dialysis. These specific radioactivities are disintegrations per minute per millimole Hb tetramer (mol wt 64,500). The specific radioactivity was determined after acid hydrolysis. The hydrolyzed ^{3}H-labeled Hb were chromatographed on Affi-Gel 601 boronic acid affinity gel (Methods). From the percentage of disintegrations per minute which adhered to this affinity gel, the specific radioactivity was determined for the glycosylated amino acids relative to the Hb from which the amino acids were derived. The Hb samples in experiments 1 and 2 are the same Hb subjected to the colorimetric TBA test, as indicated in Table II.

duced with $[^3H]NaBH_4$. Following acid hydrolysis, amino acids were analyzed by Affi-Gel 601 boronic acid affinity chromatography (Table III and Fig. 4). The specific radioactivities of the dialyzed Hb fractions ranged from 0.8×10^{10} dpm/mmol tetramer for the unfractionated Hb sample, to 26.9×10^{10} dpm/mmol tetramer for Hb A_{Ic}. Because of nonspecific uptake of $[^3H]NaBH_4$, the specific radioactivities of the amino acids were 47–65% that of the Hb before hydrolysis. Of the seven samples that were applied to Affi-Gel 601 (Fig. 4) the recovery of radioactivity was 89–99%, of which 62–76% was associated with the bound (glycosylated) amino acids (Table III). The specific radioactivities for glycosylated amino acids from purified Hb A_{Ic} were higher than the corresponding values for glycosylated Hb A_0.

The glycosylated amino acids were analyzed by cation exchange chromatography to quantify the ^3H-radioactivity due to glucitol-valine and glucitol-lysine (Fig. 5 and Table IV). For the glycosylated amino acids obtained from Glyco-Hb A_0 the major glucitol-lysine fraction eluted with a peak at fraction 95, with a minor fraction at tube 115. Synthetic glucitol-lysine elutes as one symmetrical peak at fraction 95. The dual peak for glucitol-lysine has been regularly observed in our laboratory and may be the result of dehydration reactions of lysino-1-deoxysorbitol (glucitol-lysine) brought about by acid hydrolysis. Consistent with the proposed structure of Hb A_{Ic} (α_2 [β_2-*N*-Glc]$_2$), purified Hb A_{Ic} contained 7.4 times as much glucitol-valine as glucitol-lysine. In contrast, glycosylated Hb A_0 contained 6.6 times more glucitol-lysine than glucitol-valine (Table IV).

The α and β-chains of ^3H-reduced Glyco Hb A_0 and Hb A_{Ic} were separated on carboxymethyl cellulose (Table V). About 90% of the ^3H-radioactivity in Hb A_{Ic} was associated with the β-chains. In contrast, the α- and β-chains of Glyco Hb A_0 had approximately equal radioactivity. The amino acids from the α- and β-chains of Hb A_{Ic} and Glyco-Hb A_0 were then subjected to Affi-Gel 601 boronic acid affinity chromatography. Of the radioactivity applied, 95–100% was recovered, and 77±11% was bound. When these glycosylated amino acids were analyzed by Durrum DC-6A cation exchange chromatography, the β-chains of Hb A_{Ic} had a glucitol-valine:glucitol-lysine ratio of 12 (Table IV). In contrast, the β-chains from Glyco-Hb A_0 had a glucitol-valine:glucitol-lysine ratio of 0.07. The glucitol-valine in the Hb A_0 β-chains was unexpected and may represent a slight amount of contamination with either Hb A_{Ic} β-chains or with Hb A_0 α-chains.

DISCUSSION

Boronate affinity chromatography of protein and amino acids has enabled us to precisely measure the levels of glycosylated Hb in normal individuals and

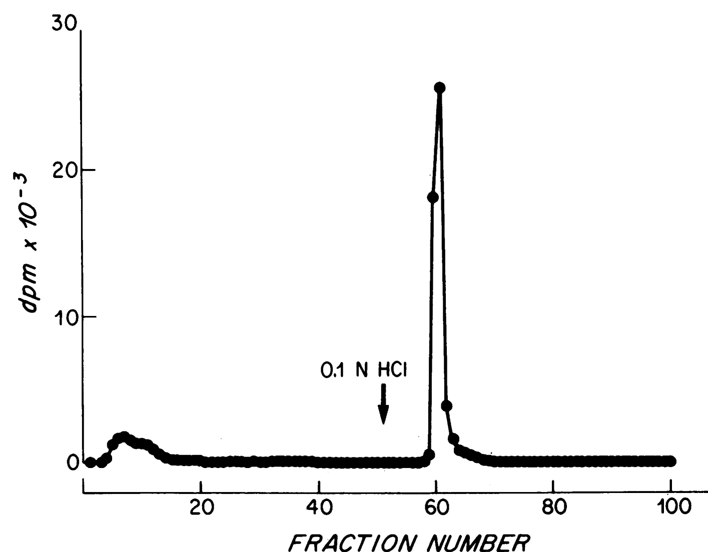

FIGURE 4 Affi-Gel 601 boronic acid affinity chromatography of an acid hydrolysate of $[^3H]NaBH_4$-labeled Hb A_{Ic}. The Hb A_{Ic} had been purified by BioRex 70 and GlycoGel B chromatography. The applied hydrolysate contained 2.6×10^6 dpm and corresponded to 1.14 mg Hb A_{Ic}. The recovered radioactivity was 2.5×10^6 dpm, which represents a 96% recovery. Of the recovered dpm, 76% was in the glycosylated amino acid peak, which was eluted after the addition of 0.1 N HCl.

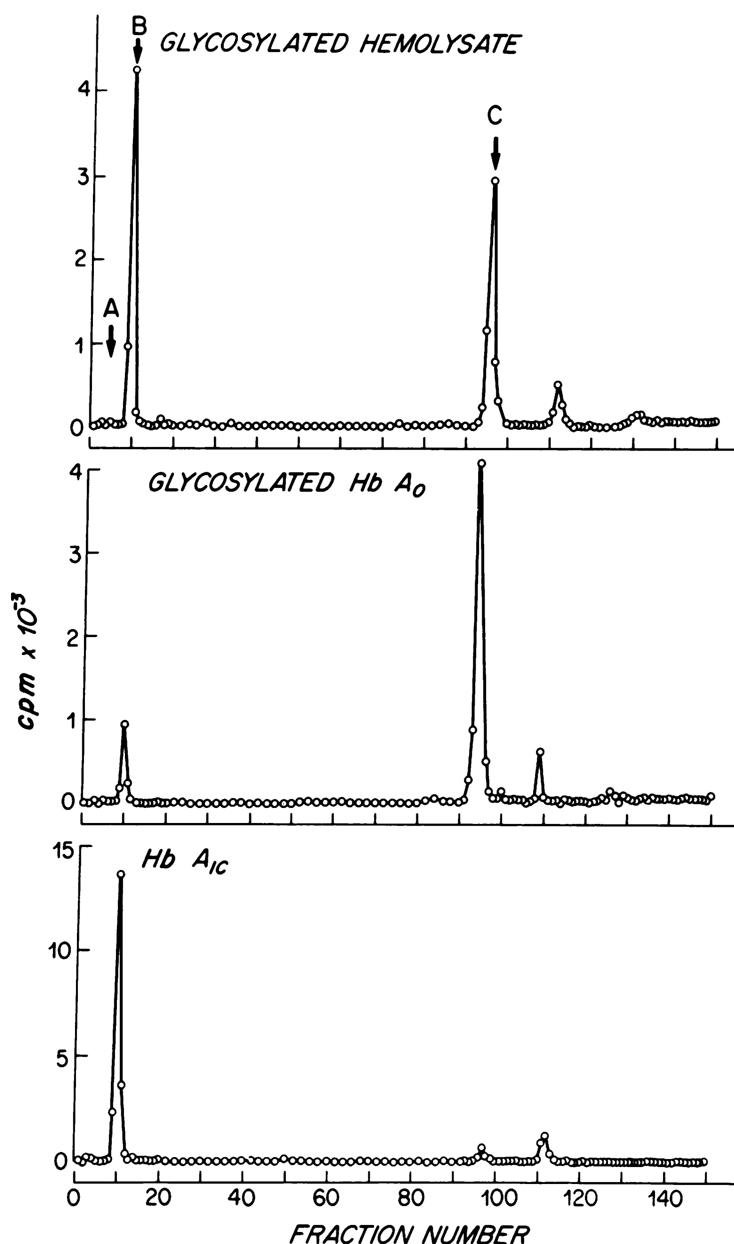

FIGURE 5 Cation exchange chromatography of glycosylated amino acids from acid hydrolysates of $[^3H]NaBH_4$-labeled glycosylated hemolysate, glycosylated Hb A_0, and purified Hb A_{Ic} from GlycoGel chromatography. The following compounds were used as markers: A, $[^{14}C]$glucose; B, $[^{14}C]$glucitol-valine; C, $[^3H]$glucitol-lysine.

diabetics as well as the distribution of glucose on α-NH_2-valine and ϵ-NH_2-lysine residues (Table VI). Analysis of Hb A_{Ic} by BioRex 70 chromatography gives falsely high estimates because of nonglycosylated Hb contaminants that coelute. Hb F usually comprises <0.5% of the total Hb, but occasionally is >1% in otherwise normal individuals. Additional minor Hb components that cochromatograph with Hb A_{Ic} can be found in uremics (27, 28), alcoholics (29, 30) and in some individuals with lead intoxication (31). Other minor Hb components are also likely to contribute to the "A_{Ic}" peak. Because of these nonglycosylated contaminants, the signal to noise ratio in chromatographic (and electrophoretic) measurements of Hb A_{Ic} is rel-

TABLE IV

Cation Exchange Chromatography on Durrum DC-6A of ^3H-labeled Glycosylated Amino Acids from Affi-Gel 601 Affinity Chromatography of Acid Hydrolysates

Hb species	Applied to column	Recovered from column	Glucitol-valine	Glucitol-lysine	Glucitol-valine / Glucitol-lysine
			dpm		
Glyco-Hb	114,000	81,800	27,300	33,500	0.81
	114,000	99,600	33,500	45,900	0.73
Glyco-Hb A_0	79,700	68,400	6,470	42,100	0.15
	79,700	68,900	7,160	47,900	0.15
Hb A_{Ic}	335,000	183,000	147,000	19,400	7.54
	335,000	312,000	231,000	31,900	7.24
Glyco Hb α	92,800	70,500	6,130	47,600	0.13
Glyco Hb β	98,100	81,400	46,100	20,300	2.27
Glyco Hb A_0 α	84,200	65,700	5,750	34,000	0.17
Glyco Hb A_0 β	36,200	31,200	1,130	17,000	0.07
Hb A_{Ic} α	19,500	14,800	2,740	5,770	0.47
Hb A_{Ic} β	108,000	94,500	74,000	6,280	11.8

The glycosylated Hb were subjected to acid hydrolysis followed by Affi-Gel 601 boronic acid affinity chromatography. The glycosylated amino acids from Affi-Gel 601 (Table III) were subjected to cation exchange chromatography on Durrum DC-6A cation exchange resin. The glycosylated amino acids subjected to cation exchange chromatography were from experiment 2 as indicated in Table II.

atively low and may contribute to the failure of some clinical surveys to find a significant correlation between patients' level of Hb A_{Ic} and their degree of diabetic control (32–34). Therefore, the clinical utility of this measurement would be considerably enhanced if the methods used were specific for Hb A_{Ic}.

When corrected for these nonglycosylated contaminants, the amount of Hb A_{Ic} in normal hemolysates is ~2.9%, a value considerably lower than estimates from a large number of laboratories (7–11, 32–34) but closer to the level of 3.3% that McDonald et al. (14) obtained for Hb A_{Ic} after chromatography and rechromatography on a high resolution BioRex 70 column.

In the initial structural analyses of Hb A_{Ic}, the number of β-N-terminal blocking groups was uncertain (1, 2). Furthermore, trypsin digestion of [^{3}H]$NaBH_4$ reduced $\beta^{A_{Ic}}$ yielded two labeled peptides (2). The major peptide was shown to have a hexose linked to the α-NH_2 valine of Tpl. Thus, there has been some uncertainty about the structural homogeneity of Hb A_{Ic}. Our analyses of glycosylated amino acids confirms the conclusion of Bookchin and Gallop (2) that Hb A_{Ic} contains two β-N-terminal sugar adducts per tetramer. We found that 88% of the glucose in Hb A_{Ic} is linked to the β-N-terminal valine. The remaining 12% is probably linked to the same lysine residues and α-1-valine as in Hb A_0. Because Hb A_{Ic} is an "aged" protein (35,

TABLE V

Separation of the Polypeptide Chains of ^3H-labeled Glycosylated Hb

Hb species	dpm applied	dpm recovered	dpm due to α-chains	dpm due to β-chains	dpm α-globin / dpm β-globin
Glycosylated Hb	2,170,000	1,910,000	604,000	1,020,000	0.59
Glycosylated Hb A_0	1,780,000	1,420,000	632,000	529,000	1.19
Hb A_{Ic}	2,670,000	2,300,000	211,000	1,670,000	0.12

The heme was removed from [^{3}H]$NaBH_4$-reduced samples of glycosylated Hb, glycosylated Hb A_0, and Hb A_{Ic}; and the globin was subjected to chromatography on carboxymethyl cellulose in 8 M urea, 0.05 M 2-mercaptoethanol, utilizing a sodium phosphate gradient. The Hb samples used in this experiment are the same ones that correspond to experiment 2 in Tables II and III.

TABLE VI
Distribution of Glycosylated Hb in Normal Individuals

Hb A_{Ic}	$\alpha_2(\beta\text{-1-Val-Glc})_2$		2.9%
Glyco Hb A_0	Total		5.2%
	$(\alpha\text{-1-Val-Glc})_2\beta_2$	0.7%	
	$(\alpha\text{-Lys-Glc})_2\beta_2$	2.1%	
	$\alpha_2(\beta\text{-Lys-Glc})_2$	2.4%	

These values were calculated from data in Tables I and IV.

36) it should have relatively more glycosylation at these sites than Hb A_0.

Analysis by affinity chromatography indicates that 5.2% of the major BioRex 70 component, Hb A_0, is nonenzymatically glycosylated in normal individuals. This estimate is lower than the 8–10% reported previously by our laboratory (15, 16). These earlier estimates were derived from less direct measurements. The amount of glycosylated Hb A_0 is increased in diabetic hemolysates in direct proportion to Hb A_{Ic} (Fig. 3; 14–16, 37). Therefore, the measurement of total glycosylated Hb by a boronate affinity resin provides a way of monitoring diabetic control that is more specific than the chromatographic or electrophoretic measurement of Hb A_{Ic}. The measurement of glycosylated Hb by affinity chromatography is quite precise (CV = 0.8–1.2%) (17) and yet it is as easy to perform as disposable ion exchange columns.

Interest in the nonenzymatic glycosylation of protein has been greatly stimulated by the possibility that this posttranslational modification may be responsible for the long-term complications of diabetes. Our studies on Hb have identified three pitfalls that pertain to the analysis of nonenzymatic glycosylation in other proteins:

Isolation of nonenzymatically glycosylated proteins. Conventional chromatography or electrophoresis cannot be relied upon to yield a purified preparation of nonenzymatically glycosylated protein. Boronate affinity chromatography is very effective in isolating all glycosylated proteins. In some cases, it may be necessary to first remove enzymatically linked sugars with less stable bonds, many of which can be cleaved by specific glycosidases.

[^{3}H]Borohydride labeling. Although [^{3}H]borohydride reduction has been very useful in identifying ketoamine-linked sugars, other sites on protein also take up radioactivity. This nonspecific labeling can be obviated by subsequent purification of the glycosylated protein by the boronate affinity resin. However, when the specifically labeled protein is analyzed, quantitative interpretation is limited by lack of uniformity of ^{3}H incorporation into different ketoamine linkages. As shown in Table III, the specific activity of purified Hb A_{Ic} was about twice that of purified Glyco-Hb A_0 even though both proteins would be expected to have 1 mol of ketoamine linkage per $\alpha\beta$-dimer. It is likely that the valine-linked ketoamine is more readily reduced by borohydride than the lysine-linked ketoamine. This difference may be overcome if larger concentrations of borohydride were used along with longer incubations but these steps would greatly enhance nonspecific uptake of label.

The TBA colorimetric test. This test provides a convenient way to detect the ketoamine linkage (23). However, other substances in the test sample may contribute to nonspecific color development. Furthermore, our analyses show that the color yield from ketoamine linked glucose depends markedly upon the site of attachment. α-NH_2 valine-linked glucose produced about eight times as much color as ϵ-NH_2 lysine-linked glucose. This conclusion is supported by previous indirect evidence (15). Since the bulk of nonenzymatic glycosylation of proteins other than Hb is on lysine residues, the lower color yield will greatly limit the sensitivity and the utility of this colorimetric test.

The approaches used here to characterize glycosylated Hb can be extended to the analysis of nonenzymatic glycosylation of other tissues, in the attempt to ascertain whether this posttranslational modification contributes to diabetic complications.

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