

# Nonenzymatic Glycosylation of Erythrocyte Membrane Proteins

## RELEVANCE TO DIABETES

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**ABSTRACT** Nonenzymatic glycosylation of proteins of the erythrocyte membrane was determined by incubating erythrocyte ghosts with [<sup>3</sup>H]borohydride. The incorporation of tritium into protein provides a reliable assay of ketoamine linkages. The membrane proteins from 18 patients with diabetes incorporated twice as much radioactivity as membrane proteins from normal erythrocytes. After acid hydrolysis, amino acid analysis showed that the majority of radioactivity was localized to glucosyllysine. Autoradiograms showed that all of the major proteins of the erythrocyte membrane, separated by electrophoresis on sodium dodecyl sulfate gels, contained ketoamine linkages. No protein bands in either normal or diabetic erythrocytes showed significant preferential labeling. Erythrocyte membranes from three patients with hemolytic anemia showed reduced incorporation of tritium from [<sup>3</sup>H]borohydride, indicating decreased nonenzymatic glycosylation. Two patients with diabetes and hemolytic anemia had incorporation of radioactivity similar to that of normal individuals. In these groups of patients the incorporation of tritium into erythrocyte membrane proteins correlated with levels of hemoglobin A<sub>1c</sub>. Thus the modification of membrane proteins like that of hemoglobin depends on blood glucose levels as well as erythrocyte age. These studies show that the enhanced nonenzymatic glycosylation of proteins in diabetics extends beyond hemoglobin to the proteins of the erythrocyte membrane and probably affects other proteins that have slow turnover and are exposed to high concentrations of glucose.

## INTRODUCTION

During the circulation of erythrocytes in vivo, hemoglobin is slowly glycosylated. Glucose reacts non-

enzymatically with the NH<sub>2</sub>-terminal residue of the β-chain (β-NA1 valine) to form hemoglobin A<sub>1c</sub> (1-3). Hemoglobin A<sub>1c</sub> is increased two- to threefold in patients with diabetes mellitus and provides a useful assessment of blood glucose control (4-6). In addition, glucose forms similar ketoamine linkages with the NH<sub>2</sub>-terminus of the α-chain and with certain lysine residues on the α- and β-chains (7). The glycosylation of lysine residues on human hemoglobin is also increased in patients with diabetes (7, 8). This type of post-translational modification has been proposed in the pathogenesis of the long-term complications of diabetes (9, 10). Therefore, it is important to determine what other proteins undergo nonenzymatic glycosylation. Since the reaction is very slow, the most likely candidates are proteins that have a long turnover time and are exposed to concentrations of glucose comparable to that in plasma. Glucose-lysine adducts have been demonstrated in bovine collagen (11), human albumin (12), and in the proteins of the human erythrocyte membrane (13). Furthermore, preliminary evidence indicates the presence of glucosyllysine in lens crystallin (14) and basic myelin protein (15).

We have investigated nonenzymatic glycosylation of the proteins of the human erythrocyte membrane. We have determined the distribution of glucosyllysine residues among the various membrane proteins of normal erythrocytes as well as erythrocytes of patients with diabetes and hemolytic anemia.

## METHODS

*Patient selection.* All patients with diabetes mellitus had two random serum glucose concentrations > 250 mg/100 ml, were not receiving oral hypoglycemic agents, had no clinical evidence of pancreatitis, had received no blood transfusions within the previous 2 mo, and had hemoglobin concentrations > 13 g/100 ml. In addition blood specimens were obtained on three patients with various types of hemolytic anemias and

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on two patients with diabetes and hemolytic anemia. Healthy laboratory personnel served as controls.

**Incubation of erythrocyte ghosts.** Erythrocyte ghosts were prepared according to the method of Dodge et al. (16) from freshly drawn heparinized venous blood from 18 normal individuals and 18 diabetic patients. Ghosts were stored overnight at 4°C in 5 mM sodium phosphate buffer pH 8. Equivalent amounts of normal and diabetic ghosts were incubated for 40 min at room temperature in 16 mM [<sup>3</sup>H]sodium borohydride (New England Nuclear, Boston, Mass.), adjusted to pH 7.4. Eight separate experiments were performed. Experiments 1 and 2 employed 10 mCi [<sup>3</sup>H]-NaBH<sub>4</sub>, whereas experiments 3 and 4 used 16 mCi, and experiments 5–8 used 25 mCi. The labeled ghosts were exhaustively dialysed in 50 mM sodium phosphate pH 6.8, with four exchanges over 24 h. Protein determinations were performed according to the method of Lowry et al. (17) after solubilizing aliquots of ghosts with .2% sodium dodecyl sulfate (SDS).<sup>1</sup> Absorbance was measured in a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 720 nm.

**SDS gel electrophoresis.** 25 μg of erythrocyte membrane protein was applied to 4% SDS polyacrylamide gels and electrophoresed according to the method of Fairbanks et al. (18). The gels were stained with Coomassie Blue, destained in 10% acetic acid, photographed, and then sliced with reference to the Coomassie Blue protein bands. The sliced gels were incubated at 37°C in 1 ml 50% H<sub>2</sub>O<sub>2</sub> until completely solubilized and bleached. 10 ml of liquid scintillation fluid (Liquiscint) was added and tritium incorporation was determined on a Searle Isocap 300 counter (Searle Radiographics Inc., Des Plaines, Ill.). In some experiments, the proteins of the erythrocyte membrane were also analyzed on polyacrylamide gel slabs as described by Laemmli (19).

**High-pressure amino acid chromatography.** Equivalent amounts of [<sup>3</sup>H]sodium borohydride reduced erythrocyte membrane proteins were hydrolysed in 6 N HCl at 110°C for 24 h. Amino acids were separated by high-pressure liquid chromatography on Durrum DC6A (Durrum Instrument Corp., Sunnyvale, Calif.) cation exchange resin with a pyridine acetate gradient described previously (7). Recovery of amino acids in the eluate was monitored by fluorescamine.

**Autoradiography.** SDS gels were washed twice in 100 ml dimethyl sulfoxide for 1 h with constant mixing. The gels were then placed in a mixture of 16 g 2,5-diphenyloxazole and 80 ml dimethyl sulfoxide for 3 h, and washed in distilled water for 1 h. The gels were dried and autoradiogrammed for 10 d at -40°C.

Hemoglobin A<sub>1c</sub> was determined by a modification (7) of the colorimetric method of Flückiger and Winterhalter (20), calibrated by chromatography of hemoglobin on Biorex 70 ion exchange resin (Bio-Rad Laboratories, Richmond, Calif.) (21).

## RESULTS

The treatment of erythrocyte membranes with [<sup>3</sup>H]-borohydride resulted in incorporation of radioactivity into both proteins and lipid. The various erythrocyte membrane proteins are effectively separated from labeled lipid by electrophoresis on SDS gels. As shown in Table I, comparison of incorporation of radioactivity into equivalent amounts of erythrocyte membrane pro-

tein from diabetic and normal subjects revealed that diabetic membrane protein consistently incorporated more tritium than did membrane protein from normal controls: diabetic/normal 1.9±0.32 SEM; range, 1.5–2.5. This difference was statistically significant at a *P* < 0.01 using a simple sign test. Since incorporation of tritium from [<sup>3</sup>H]BH<sub>4</sub> is considered a reliable detector of the glucosyl-ketoamine linkage these data suggest that diabetic erythrocyte membrane protein undergoes greater nonenzymatic glycosylation than that of normal controls.

To determine the specificity of borohydride incorporation, equivalent amounts (50 μg) of [<sup>3</sup>H]BH<sub>4</sub> reduced membrane protein were subjected to acid hydrolysis and analyzed by high-pressure liquid chromatography. In eight analyses, 72±5% of the applied radioactivity was recovered in the effluent. Fig. 1C and D show representative chromatographic profiles of <sup>3</sup>H radioactivity from a diabetic and a normal subject. The major radioactive peak eluted from the column at the same position as synthetic glucosyllysine. In addition, a less prominent radioactive peak was obtained before the elution of the glucosyllysine peak. A similar elution profile was also observed when poly-L-lysine was incubated with glucose, reduced with [<sup>3</sup>H]borohydride and hydrolysed (Fig. 1A). Less heterogeneity was observed on analyses of hemoglobin A<sub>0</sub>, which contains glucosyllysine residues at several sites on both the α- and β-chains (7). The radioactive prepeak obtained on erythrocyte membranes is likely to be a sugar amino acid adduct, since it adheres to Affi-Gel 601 (boronate gel; Bio-Rad Laboratories, Richmond, Calif.), a resin with strong affinity for compounds containing vicinal hydroxyl groups. Recent structural analyses of glycosylated albumin by Day et al. (22) indicate that the prepeak may be authentic glucosyllysine, whereas the major peak may be a dehydrated form of glucosyllysine. In contrast to the earlier results of Bailey et al. (13) we obtained higher yields of glucosyllysine and better resolution with acid hydrolysis than with alkaline hydrolysis.

These results indicate that incorporation of tritium from [<sup>3</sup>H]BH<sub>4</sub> into erythrocyte membrane proteins is due primarily to the ketoamine linkage between glucose and the ε-amino of lysine residues. In a comparison of four pairs of normals and diabetics, the amount of radioactivity in the glucosyllysine peak of the diabetic subjects was 2.2 times that of the normal controls. These results indicate that diabetic patients have enhanced nonenzymatic glycosylation primarily of lysine residues present in the proteins of erythrocyte membranes.

Erythrocyte membrane proteins were separated on Steck-Fairbanks SDS gels according to molecular size. Treatment with borohydride had no effect on the

<sup>1</sup> Abbreviation used in this paper: SDS, sodium dodecyl sulfate.

**TABLE I**  
*Incorporation of [<sup>3</sup>H]Borohydride into Erythrocyte Membrane Proteins*

Experiment No.	Normals	Diabetics	Diabetic + hemolytic anemia	Hemolytic anemia
<i>total cpm in membrane proteins*</i>				
1	2,200	4,800		
	2,100	4,500		
	3,200	5,000		
	3,600	5,400		
2	2,800	4,000		
	3,100	6,500		
3	8,000	24,000		
	8,000	16,000		
4	8,500	25,000		
	20,000	39,000		
5	22,500	37,000		
	30,000	40,000		
	26,000	38,000		
6	23,000	41,000		
	26,000	42,500		
7	48,000	68,000	55,000	35,000
8	21,000	39,000	30,000	14,000
	23,500	41,000		17,000

\* Equivalent amounts of normal and diabetic erythrocytes were incubated with 16 mM [<sup>3</sup>H]borohydride for 40 min. The amount of radioactivity employed varied between experiments (see text). After exhaustive dialysis, equivalent amounts (25 μg) of normal and diabetic erythrocyte membrane protein were applied to SDS gels. After electrophoresis and staining all of the protein bands except hemoglobin were sliced and counted.

pattern of the stained bands. Examination of autoradiograms of these gels revealed incorporation of radioactivity into each of the bands in approximate proportion to the amount of protein. Quantitation of borohydride incorporation into the separated proteins of normals and diabetics is shown in Fig. 2. For every protein band, the diabetic subjects had greater tritium incorporation indicating more nonenzymatically glycosylated protein. Thus, the diabetic membranes had increased incorporation compared to the normal controls without a selective increase in any one protein band. This conclusion was confirmed by analysis of [<sup>3</sup>H]borohydride reduced membrane proteins on SDS slab gels as described by Laemmli (19) (Fig. 3). Even with the enhanced resolution provided by this method no consistent selectivity in radiolabeling could be seen in either normal or diabetic erythrocyte membranes.

To assess the effect of erythrocyte lifespan on the nonenzymatic glycosylation of erythrocyte membrane

protein, we examined erythrocytes of patients with hemolytic anemia with and without coexistent diabetes. As shown in Fig. 4, patients with uncomplicated hemolytic anemia had decreased incorporation of [<sup>3</sup>H]borohydride into membrane proteins. Those with diabetes who were studied had the expected increase and those with coexisting diabetes and hemolysis had values close to those of normals. In all of these patients, there was good correlation between the <sup>3</sup>H labeling of membrane proteins and the corresponding level of hemoglobin A<sub>1c</sub>.

## DISCUSSION

Nonenzymatic glycosylation of hemoglobin involves the condensation of two abundant reactants within the erythrocyte: glucose and hemoglobin. The reaction takes place slowly and continuously throughout the cell's 120-d lifespan (23). Initially, this type of post-translational modification was considered to be

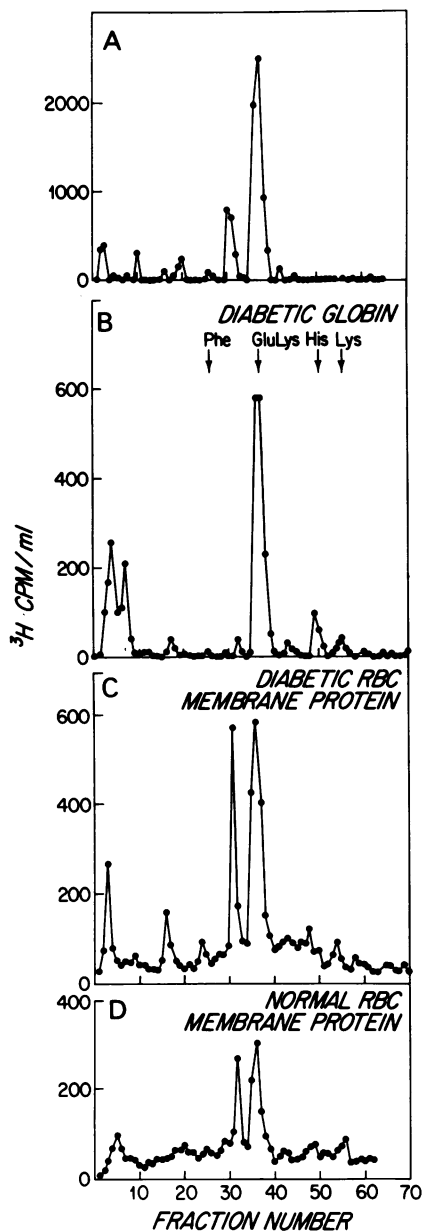


FIGURE 1 Elution profile of amino acid mixture obtained by acid hydrolysis of [ $^3\text{H}$ ]NaBH $_4$ -labeled proteins. A: Poly-L-lysine incubated with glucose. B: hemoglobin A from diabetic patient. C: erythrocyte (RBC) membrane from diabetic patient. D: erythrocyte (RBC) membrane from normal control. The ghosts from normal and diabetic erythrocytes were incubated with identical amounts of labeled borohydride and identical amounts of hydrolysed proteins were applied to the cation exchange column.

specific for the NH $_2$ -terminal amino group of the  $\beta$ -chain. Recently, however, it has become clear that glucose forms similar ketoamine linkages with a number of other amino groups in hemoglobin including

the NH $_2$ -terminal amino group of the  $\alpha$ -chain as well as several lysine residues on both the  $\alpha$ - and the  $\beta$ -chains (7, 24) ( $\beta$ -Lys 66,  $\alpha$ -Lys 61,  $\beta$ -Lys 17, and  $\alpha$ -Lys 40). The broader range of reactive sites on hemoglobin raises the question of whether other proteins are modified in a similar fashion.

The extent of nonenzymatic glycosylation of hemoglobin *in vivo* depends upon two independent variables: the average blood glucose concentration over the preceding 2–3 mo and the lifespan of the erythrocyte. Accordingly, glycosylated hemoglobin is increased in patients with diabetes and decreased in those with hemolysis (23, 25). The results presented here show that these two factors apply equally well to the nonenzymatic glycosylation of erythrocyte membrane proteins. Like hemoglobin, the proteins of the erythrocyte membrane are synthesized during erythropoiesis in the bone marrow and remain intact, with negligible turnover, throughout the cell's lifespan. We have shown that most if not all the membrane proteins separable on SDS gels are modified by ketoamine linkages with glucose. It was of interest to determine if any protein bands were preferentially labeled with borohydride. A possible candidate would be band 4.5, a protein which appears to be responsible for the facilitated diffusion of glucose across the erythrocyte membrane (26, 27). Therefore the transmembrane segment of this molecule would be expected to have enhanced contact with glucose. However no clear-cut preferential labeling was observed. According to current understanding of the structure of the erythrocyte membrane (28) certain proteins such as the anion channel (band 3) and glycophorin span the lipid bilayer, whereas others such as spectrin (bands 1 and 2), actin (band 5), and ankyrin (band 2.1) form a lattice that laminates the cytoplasmic surface of the lipid bilayer. Charged residues on these proteins such as lysine are likely to be exposed to the aqueous solvent on either the outer or inner surface of the membrane and should be exposed to nearly equivalent concentrations of glucose. Nonenzymatic glycosylation may also involve other residues, particularly at the NH $_2$ -terminus where the pK of the amino group is significantly lower than the  $\epsilon$ -amino of lysine. Our amino acid analyses do not permit the identification of other glycosylated residues.

This study as well as the structural analysis of hemoglobin cited above (7, 24) depends on the use of [ $^3\text{H}$ ]borohydride to identify ketoamine linkages. At neutral pH, borohydride has a high degree of specificity for aldehyde and ketone groups, converting them to the corresponding alcohols. The reagent is not reactive with esters, lactones, amides, or acid carbonyl groups. In acidic media borohydride can reduce indoles (29). Furthermore, under certain conditions, fatty acid esters can be reduced to fatty alcohols (30). As shown in Fig. 4,

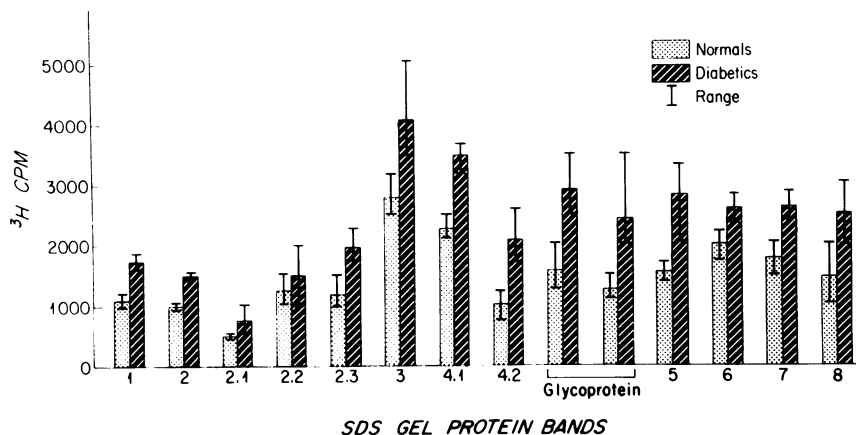


FIGURE 2 Comparison of radioactivity of protein bands from erythrocytes of three normal individuals (cross-hatched bars) and three diabetic patients (stippled bars). Fresh blood samples were collected on the same day. Equivalent amounts of [ $^3\text{H}$ ]NaBH $_4$  was added to each ghost preparation (Table I, experiment 5). The range of radioactivity values is shown for each band.

we did observe considerable incorporation of radioactivity into membrane lipids which were readily separable from proteins by electrophoresis on SDS gels. The fact that we obtained a high yield of glucosyllysine from preparation of [ $^3\text{H}$ ]borohydride reduced membrane protein attests to the specificity of the reagent under the experimental conditions that were employed.

Our results indicate that enhancement of nonenzymatic glycosylation in diabetics extends beyond hemoglobin to the proteins of the erythrocyte membrane. This phenomenon may have functional significance. Recently Peterson et al. (31) have shown that diabetics have a moderately shortened erythrocyte survival, which is corrected by the institution of rigid control.

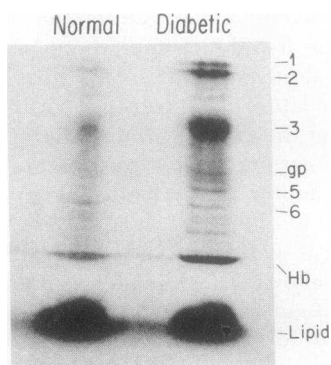


FIGURE 3 Autoradiograms of erythrocyte membranes of a normal individual and a diabetic patient after electrophoresis on slab gels as described by Laemmli (19). The two ghost preparations were incubated with identical amounts of labeled borohydride and identical amounts of protein were applied to the gels. Hb, hemoglobin.

Furthermore, McMillan et al. (32) have demonstrated that diabetic erythrocytes are less deformable than normal when drawn into a 2- $\mu\text{m}$  capillary pipette. It is uncertain whether the abnormal rheology of the membrane in diabetic erythrocytes is due to alterations in the composition of lipids in the bilayer or perhaps to post-translational modification of membrane proteins.

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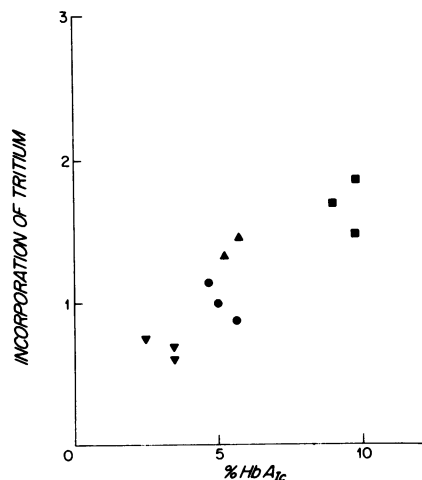


FIGURE 4 Comparison of the incorporation of [ $^3\text{H}$ ]NaBH $_4$  into erythrocyte membrane proteins with corresponding levels of Hemoglobin A $_{1c}$ : ●, normals; ■, diabetics; ▼, patients with hemolytic anemia; ▲, patients with diabetes and hemolytic anemia. The ordinate represents the ratio of radioactivity of patients to that of normal controls run simultaneously. Raw data are shown in Table I, experiments 7 and 8.

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