Nonenzymatic Glycosylation of Erythrocytic Proteins in Normal and Diabetic Subjects

Enzymes of Nucleoside and Nucleotide Metabolism

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SUMMARY

A modification of the technique of Glyco-Gel affinity column chromatography has been employed to separate glycosylated proteins from nonglycosylated proteins of hemolysates. When glycosylation in hemolysates of 11 type I diabetic subjects was compared with that from 7 normal subjects, significant increases were found in glycosylation of hemoglobin (Hb) (12.1 ± 6.0% versus 4.7 ± 0.5%) and purine nucleoside phosphorylase (PNP) (5.3 ± 3.0% versus 2.1 ± 0.5%). However, no differences were found for nucleoside diphosphokinase (NDPK) (1.5 ± 1.1% versus 1.0 ± 0.4%) and adenylate kinase (AMPK) (0.5 ± 0.4% versus 0.7 ± 0.2%). Linear relationships were seen between glycosylated Hb and glycosylated PNP (r = 0.97) or glycosylated NDPK (r = 0.81). On incubation of hemolysates from normal individuals with high glucose (1500 mg/dl or 83 mM) and $NaCNBH_3$ (20 mM), linear increases in the degrees of glycosylation were seen with time. After 18 h, the percentages of glycosylation of Hb, PNP, NDPK, and AMPK were increased from normal values to 31, 24, 11, and 3, respectively. When partially purified human erythrocytic PNP was incubated with various monosaccharides (20 mM) in the presence of $NaCNBH_3$ for 6 h, glycosylation increases of 2–5-fold were seen in the order ribose > mannose > galactose > glucose. DIABETES 1985; 34:251–55.

A number of proteins undergo nonenzymatic alterations through the formation of covalent linkages with glucose. The chemical reactions involved are, first, the reversible formation of a Schiff base between the aldehydic functional groups of glucose and N-terminal amino acids or the ε-amino groups of lysine residues in proteins. This is followed by a relatively slow, but essentially irreversible Amadori rearrangement, with formation of ketoamines (Figure 1A). In this process, the glucose moiety adds to the protein in the open-chain form, thus making available *cis* hydroxyl groups that may bind to boronate (Figure 1B). Evidence accumulates that such nonenzymatic glycosylations are increased both in rate and quantity in hyperglycemic states, such as diabetes mellitus. Previous studies have demonstrated that proteins glycosylated include: hemoglobin (Hb),[1,2] erythrocytic membrane proteins,[3] lens crystallin,[4] collagen,[5] serum proteins.[6] Recently, in vitro nonenzymatic glycosylations of rat brain tubulin[7] and of alcohol dehydrogenase[8] have also been reported.

Previously, we (K.C.A. and R.E.P.) have studied the behavior and properties of various enzymes of purine and pyrimidine metabolism, several of which occur in high concentration in human erythrocytes. In the present study, we have examined the glycosylation of three erythrocytic enzymes: purine nucleoside phosphorylase (PNP), adenylate kinase (AMPK), and nucleoside diphosphokinase (NDPK). In the present study, using boronic acid chromatography (Glyco-Gel B), we have shown that PNP and NDPK may be glycosylated and have demonstrated significant correlations between the concentrations of glycosylated hemoglobin and the amounts of glycosylated PNP and NDPK in the erythrocytes of normal and diabetic subjects. These findings support the concept that the nonenzymatic glycosylation reactions may occur with other proteins in erythrocytes, in addition to hemoglobin, in the presence of hyperglycemia. A preliminary report of these findings has been presented.[9]

MATERIALS AND METHODS

Blood specimens obtained from normal volunteers and patients with type I diabetes mellitus were anticoagulated with EDTA or sodium citrate. For isolation of erythrocytes, the whole blood was centrifuged at 132 × *g* for 10 min at 4°C, and the plasma and buffy coat were removed. The erythrocytes were washed twice by resuspension in approximately 10 vol of ice-cold buffered medium containing po-

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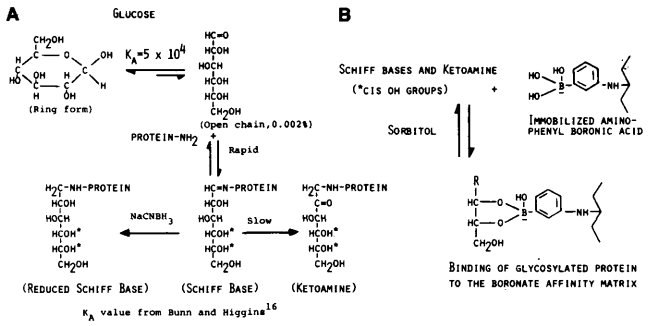

FIGURE 1. The nonenzymatic glycosylation of proteins via the formation of covalent linkages with glucose. Please see text for details.

tassium phosphate, 50 mM (pH 7.5); NaCl, 75 mM; $MgCl_2$, 2 mM; glucose, 10 mM; penicillin, 100 U/ml; and streptomycin, 10 μg/ml. The washed erythrocytes were finally suspended in the same medium to hematocrit values of about 40%. Aliquots of this suspension were diluted fivefold with cold potassium phosphate buffer, 50 mM (pH 7.5) and hemolyzed by freezing and thawing twice. The cell stromas were removed by centrifugation at 1350 × *g* for 20 min at 4°C and the hemolysates were used for boronic acid column chromatography or glucose incubation experiments.

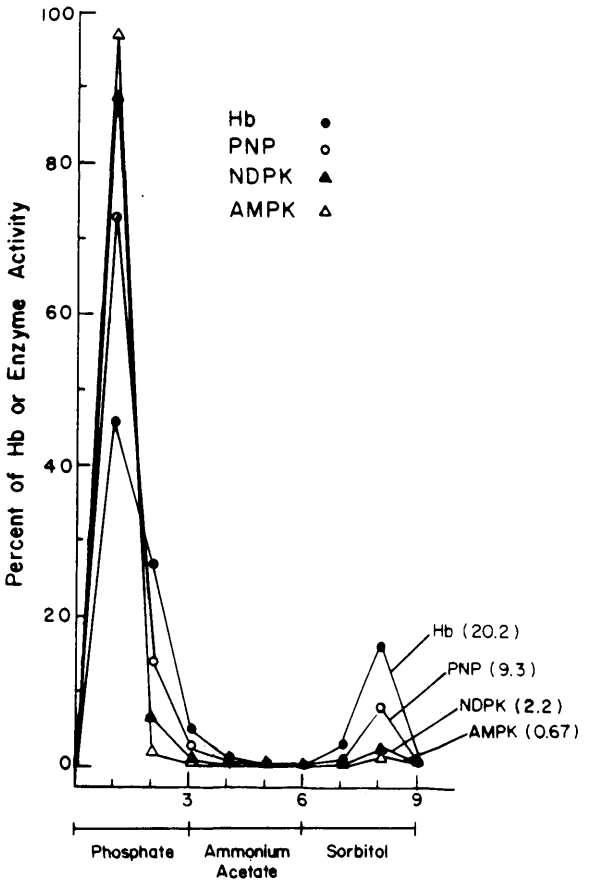

FIGURE 2. Separation of glycosylated from nonglycosylated proteins from a hemolysate sample of a diabetic subject. One-hundred microliters of the hemolysate (10%) was applied to the Glyco-Gel B column and the glycosylated proteins from nonglycosylated proteins were separated as described in the text. Fractions collected: phosphate buffer (6 ml), 1–3 (2 ml each); ammonium acetate (6 ml), 4–6 (2 ml each); and sorbitol (3 ml), 7–9 (1 ml each).

Enzyme assays. AMPK and NDPK activities were assayed by the coupled pyruvate kinase-lactate dehydrogenase method.[10] In the AMPK assay, the AMP concentration was 1 mM. PNP activity was measured by the coupled xanthine-oxidase method.[11]

Boronic acid (Glyco-Gel B) column chromatography. Prepacked Glyco-Gel B analytic columns were obtained from Pierce Chemical Co., Rockford, Illinois. Nonglycosylated and glycosylated erythrocytic proteins were separated on these columns by the following modification of the recommended procedures.[12] The columns were equilibrated with 6 ml of potassium phosphate buffer, 0.1 M (pH 7.5). The flow rate was adjusted to approximately 1 ml/3 min. The column flow was stopped and an aliquot (0.1 ml) of the hemolysate was added. The column was then washed with 6 ml of potassium phosphate buffer, 0.1 M (pH 7.5), to elute nonglycosylated proteins. The column was then equilibrated with 6 ml of ammonium acetate, 0.25 M (pH 8.5), and the glycosylated proteins were eluted with 3 ml of sorbitol, 0.2 M in Tris-buffer, 0.1 M (pH 8.5) (see Figure 2).

Equilibration of the columns with potassium phosphate buffer, 0.1 M, prevents nonspecific adsorption of enzymes such as PNP and NDPK to the boronic acid columns. It had been learned previously that these erythrocytic enzymes adsorb quantitatively to calcium phosphate gel and may be eluted with high concentrations of polyvalent anions such as phosphate and sulfate.[11]

HPLC analysis. The percentages of glycosylated hemoglobin A_{1c} (HbA_{1c}) were determined in dialyzed hemolysates by the technique of high-performance liquid chromatography.[13]

RESULTS

Figure 2 shows a typical separation of glycosylated from nonglycosylated proteins in a hemolysate sample from a diabetic subject. The percentages of glycosylated Hb, PNP, NDPK, and AMPK in this sample were 20.2, 9.3, 2.2, and 0.67, respectively. In all experiments, the recoveries of enzyme activities and hemoglobin from the column ranged from 95% to 100% of the added amounts. Table 1 shows the percentages of glycosylation of these proteins in hemolysates of 7 normal and 11 diabetic subjects. Two- to threefold increases in glycosylation of the Hb and PNP were seen in the hemolysates from the diabetic subjects. Figure 3 shows a linear relationship (r = 0.93) between glycosylated Hb values obtained from the Glyco-Gel columns and HbA_{1c} values determined by HPLC in dialyzed hemolysates of 11 diabetic subjects. Linear relationships were also seen between glycosylated Hb and glycosylated PNP (r = 0.97) or NDPK

TABLE 1
Percent of glycosylated hemoglobin or enzyme activity in hemolysates of normal and diabetic subjects (mean ± SD)

Proteins	Normal (N = 7)	Diabetic (N = 11)	P-value*
Hb	4.7 ± 0.5	12.1 ± 6.0	0.001
PNP	2.1 ± 0.5	5.3 ± 3.0	0.02
AMPK	0.7 ± 0.2	0.5 ± 0.4	NS
NDPK	1.0 ± 0.4	1.5 ± 1.1	NS

*Diabetic versus normal, based on Student's *t*-test (two-tailed).

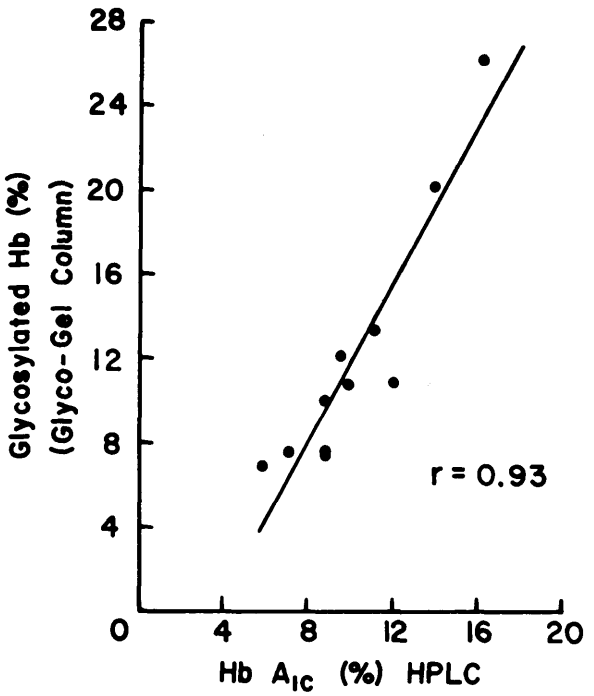

FIGURE 3. Relationship between the HbA_{1c} values measured by HPLC and glycosylated Hb values obtained by Glyco-Gel B columns in hemolysates from 11 different diabetic subjects.

(r = 0.81) (Figures 4 and 5). The activity of glycosylated AMPK in normal hemolysates was not significantly different from that of hemolysates from diabetic subjects.

To determine whether nonenzymatic glycosylation of these erythrocytic proteins occurs in vitro, freshly prepared hemolysates were incubated with 83 mM glucose (1500 mg/dl) in the presence of 20 mM sodium cyanoborohydride ($NaCNBH_3$). At 2, 6, and 18 h, aliquots (100 μl) of these hemolysates were chromatographed on Glyco-Gel B columns. Figure 6 presents results from three such experiments.

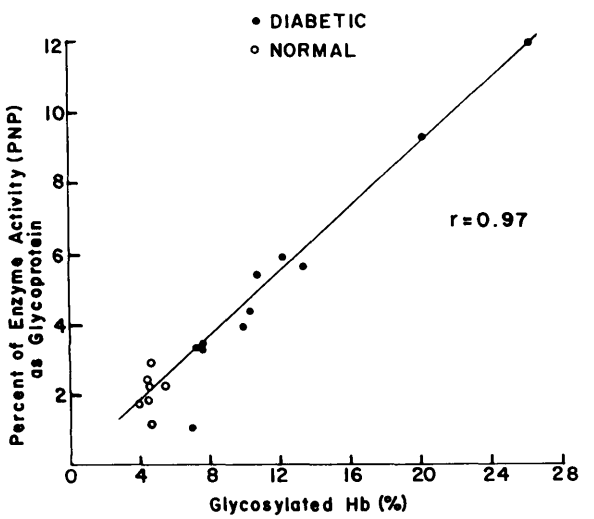

FIGURE 4. Relationship between glycosylated Hb and glycosylated PNP. Glyco-Gel B columns were employed to separate the glycosylated from the nonglycosylated proteins in hemolysates of 7 normal and 11 diabetic subjects.

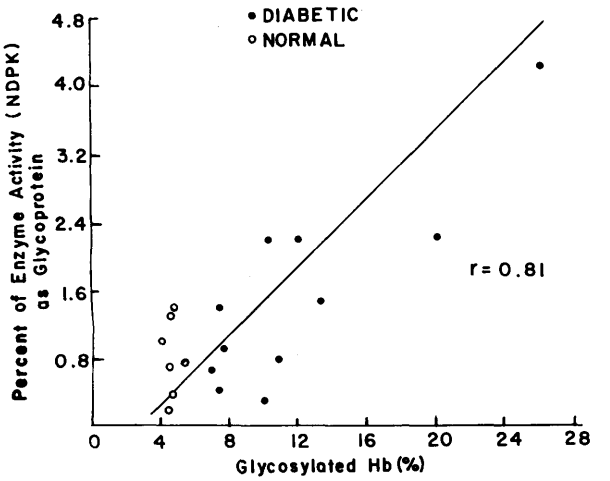

FIGURE 5. Relationship between glycosylated Hb and glycosylated NDPK. Glyco-Gel B columns were employed to separate the glycosylated from the nonglycosylated proteins in hemolysates of 7 normal and 11 diabetic subjects.

Decreases of 14–16% in the activities of PNP, NDPK, and AMPK were seen after 18 h of incubation with glucose and $NaCNBH_3$ in comparison with control hemolysates. No significant decrease in enzymatic activities was observed after 2 and 6 h. The percentages of glycosylation of Hb, PNP, NDPK, and AMPK, after 18-h incubation, were increased from normal values to 31.1 ± 3.7 (mean ± SD), 23.6 ± 5.2, 10.6 ± 2.9, and 3.1 ± 0.7, respectively. Under these conditions, approximately 10-fold increases in the glycosylation of PNP and NDPK were seen, whereas the increases in the glycosylation of Hb and AMPK were on the order of 4–6-fold. It should be noted that use of $NaCNBH_3$ in these experiments causes reduction of the newly formed Schiff bases to more stable secondary amines (Figure 1A). Thus, the re-

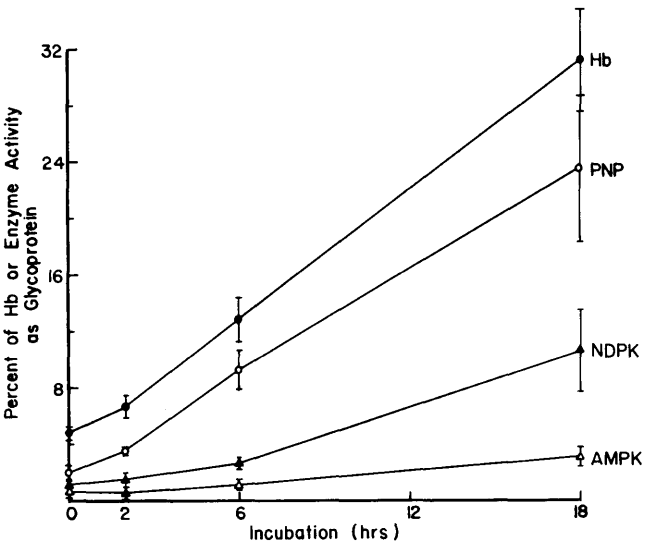

FIGURE 6. Effect of high-glucose concentration on glycosylation of human erythrocytic proteins. Freshly prepared hemolysates (10%) from normal subjects were incubated with glucose (1500 mg/dl or 83 mM) in the presence of $NaCNBH_3$ (20 mM) at 37°C. After 2, 6, and 18 h, aliquots (100 μl) were chromatographed on Glyco-Gel B columns to separate glycosylated from nonglycosylated proteins as described in the text. Each point represents mean ± SD of three separate experiments.

TABLE 2
In vitro nonenzymatic glycosylation of partially purified PNP from human erythrocytes

Monosaccharide (20 mM)	Glycosylation (%) (mean ± SD)	Carbonyl form* (%)
Control	2.2 ± 0.4	—
Glucose	3.7 ± 0.7	0.002
Galactose	5.0 ± 0.4	0.02
Mannose	7.4 ± 1.0	0.005
Ribose	11.8 ± 1.4	0.05

Partially purified human erythrocytic PNP (sp. act. 0.44 U/mg) was incubated at 37°C with a monosaccharide (20 mM) and $NaCNBH_3$ (20 mM); after 6 h, an aliquot (100 or 200 μl) was applied to a Glyco-Gel column. Glycosylated and nonglycosylated PNP were separated using the procedure described in the text.
*Bunn and Higgins.[19]

sults reflect principally the first step of the glycosylation phenomenon rather than the formation of ketoamines by the slow Amadori rearrangement.

On incubation of partially purified PNP from human erythrocytes with glucose, galactose, mannose, or ribose (20 mM), increases of 2–5-fold in the degrees of glycosylation were seen (ribose > mannose > galactose > glucose) (Table 2). This is in general agreement with the earlier findings of Bunn and Higgins, who showed that the rate of nonenzymatic glycosylation of HbAo by various monosaccharides correlates directly with the extent to which each sugar exists in the open-chain configuration.[19]

DISCUSSION

These studies demonstrate that certain human erythrocytic enzymes of purine metabolism may undergo nonenzymatic glycosylation reactions that are correlated with the amount of glycosylated hemoglobin found in the cell. Elevated levels of glycosylation were observed in erythrocytes from diabetic subjects and from hemolysates of nondiabetic subjects incubated with high concentrations of glucose. These findings support the concept that, in diabetes mellitus, nonenzymatic glycosylation of proteins may be a generalized phenomenon that is not confined to a few proteins.[1–8] These studies were facilitated by the use of boronic acid affinity columns in which *cis* hydroxyl residues of the open chain of the covalently linked glucose bind to the boronate. The glycosylated proteins then may be eluted with sorbitol. This technique could find wide application with other tissues and proteins.

The enzymes examined, PNP, NDPK, and AMPK, were selected because they occur at high activity in human erythrocytes, are readily assayed, and have been studied extensively in this laboratory.[10,11,14] The high level of glycosylation seen with PNP is of special interest. This enzyme is readily purified to homogeneity from human erythrocytes and currently is subjected to a detailed study of its structure by use of techniques such as x-ray crystallography, protein sequencing, etc.[14] Future studies will examine the effects of glycosylations on the structural parameters and behavior of the enzyme.

A point of interest is that there is considerable isoelectric heterogeneity in human erythrocytic PNP, which increases with erythrocytic aging.[15] Previously, we[15] and others[16] had speculated that deamidation of asparagine or glutamine residues might be responsible. Currently under study is the possible role of nonenzymatic glycosylations in this phenomenon. Another question is the influence of glycosylations on the functionality of these enzymes, i.e., are there effects on the kinetic parameters (K_m or V_{max}) of various substrates, or on the response to allosteric modifiers? As noted by Means and Chang,[17] the locus of the amino acid(s) glycosylated in the protein structure may be a major determinant of functional alterations. Thus, comparative studies of the effects of nonenzymatic glycosylations on both the structure and functions of these enzymes might yield useful insights.

Since erythrocytic PNP can undergo a relatively high degree of glycosylation, is found at high activity in erythrocytes, and is readily assayed by a variety of simple procedures,[11,18] PNP offers a potentially useful marker, in addition to HbA_{1c}, for monitoring the longitudinal status of patients with diabetes mellitus.

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REFERENCES

[1] Bookchin, R. M., and Gallop, P. M.: Structure of HbA_{1c}: nature of the N-terminal beta-chain blocking group. Biochem. Biophys. Res. Commun. 1968; 32:86–93.

[2] Bunn, H. F., Shapiro, R., McManus, M., Garrick, L., McDonald, M. J., Gallop, P. M., and Gabbay, K. H.: Structural heterogeneity of human hemoglobin A due to non-enzymatic glycosylation. J. Biol. Chem. 1975; 254:3892–98.

[3] McMillan, D. E., and Brooks, S. M.: Erythrocyte spectrin glycosylation in diabetes. Diabetes 1982; 31 (Suppl.):64–69.

[4] Monnier, V. M., and Cerami, A.: Nonenzymatic glycosylation and browning in diabetes and aging: studies on lens proteins. Diabetes 1982; 31:57–63.

[5] Robins, S. P., and Baily, A. J.: Age related changes in collagen: the identification of reducible lysine-carbohydrate condensation products. Biochem. Biophys. Res. Commun. 1972; 48:76–84.

[6] Kennedy, L., Mehl, T. D., Elder, E., Varghese, M., and Merimee, T.: Nonenzymatic glycosylation of serum and plasma proteins. Diabetes 1982; 31 (Suppl.):52–56.

[7] Williams, S. K., Howarth, N. C., Devenny, J. J., and Bitensky, M. W.: Structural and functional consequences of increased tubulin glycosylation in diabetes mellitus. Proc. Natl. Acad. Sci. USA 1982; 79:6546–50.

[8] Tsai, C. S., and White, J. H.: Activation of liver alcohol dehydrogenase by glycosylation. Biochem. J. 1983; 209:309–14.

[9] Parks, R. E., Jr., Agarwal, K. C., Widness, J. A., and Schwartz, R.: Nonenzymatic glycosylation of erythrocytic protein from normal and diabetic humans. Fed. Proc. 1983; 42:2037.

[10] Agarwal, R. P., Robison, B., and Parks, R. E., Jr.: Nucleoside diphosphokinase from human erythrocytes. *In* Methods in Enzymology. Colowick, S. P., and Kaplan, N. O., Eds. New York, Academic Press, 1978:376–86.

[11] Stoeckler, J. D., Agarwal, R. P., Agarwal, K. C., and Parks, R. E., Jr.: Purine nucleoside phosphorylase from human erythrocytes. *In* Methods in Enzymology. Colowick, S. P., and Kaplan, N. O., Eds. New York, Academic Press, 1978:530–38.

[12] Mallia, A. K., Hermanson, G. T., Krohn, R. I., Fujimoto, E. K., and Smith, P. K.: Preparation and use of a boronic acid affinity support for separation and quantitation of glycosylated hemoglobins. Anal. Lett. 1981; 14:649–61.

[13] Widness, J. A., Rogler-Brown, T. L., McCormick, K. L., Petzold, K. S., Susa, J. B., Schwartz, H. C., and Schwartz, R.: Rapid fluctuations in

glycohemoglobin (hemoglobin A_{1c}) related to acute changes in glucose. J. Lab. Clin. Med. 1980; 95:386–94.

[14] Stoeckler, J. D.: Purine nucleoside phosphorylase: a target for chemotherapy. *In* Developments in Cancer Chemotherapy. Glazer, R. I., Ed. Boca Raton, Florida, CRC Press, 1984:35–60.

[15] Agarwal, K. C., Agarwal, R. P., Stoeckler, J. D., and Parks, R. E., Jr.: Purine nucleoside phosphorylase. Microheterogeneity and comparison of kinetic behavior of the enzyme from several tissues and species. Biochemistry 1975; 14:79–84.

[16] Turner, B. M., Fisher, R. A., and Harris, H.: An association between the kinetic and electrophoretic properties of human purine-nucleoside-phosphorylase isozymes. Eur. J. Biochem. 1971; 24:288–95.

[17] Means, G. E., and Chang, M. K.: Nonenzymatic glycosylation of proteins: structure and function changes. Diabetes 1982; 31 (Suppl.):1–4.

[18] Stoeckler, J. D., and Parks, R. E., Jr.: Purine nucleoside phosphorylase: measurements of activity and use of inhibitors. *In* Methods in Adenosine Research. In press. Paton, D. M., *Ed. New York, Plenum Publishing* Corp., 1985.

[19] Bunn, H. F., and Higgins, P. J.: Reaction of monosaccharides with proteins: possible evolutionary significance. Science 1981; 213:222–24.