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Nonenzymatic glycosylation of peripheral nerve protein in diabetes mellitus

(boronic acid/affinity chromatography/NaB^3H_4/amino acid analysis)

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ABSTRACT **A new affinity chromatography system that selectively retains glycosylated amino acids has been utilized to determine the amount of nonenzymatic glycosylation present in peripheral nerve from diabetic and control rats and dogs. The mean value for glycosylated amino acids in diabetic rats was 2.8 times greater than the mean value in normal rats ($P < 0.001$). In diabetic dogs, mean values were 2.15 times greater than normal values ($P < 0.05$). Amino acid analysis of reduced, glycosylated amino acids previously isolated by affinity chromatography showed that glycosylated lysine and its hydrolysis rearrangement products were the major borohydride-reducible adduct present. In addition, another glycosylated product was noted to be present in major proportions. This radioactive product did not chromatograph with any of the available glycosylated amino acid standards. The finding that diabetes results in a nearly 3-fold increase of peripheral nerve glycosylation is consistent with a number of previous investigations in which glycosylation was measured in hemoglobin, serum albumin, and urinary amino acids and peptides from diabetics and normals. The results reported here provide evidence that increased nonenzymatic glycosylation is occurring in a tissue where physiological, morphological, and clinical degeneration characteristically develop as a result of diabetes mellitus.**

Diabetic neuropathy is characterized by a variety of morphological changes associated with decreased sensory and motor conduction velocities, including axonal degeneration and segmental demyelination. Although a number of biochemical alterations have been described in diabetic nerve, the ultimate sequence of events leading from insulin deficiency and its attendant hyperglycemia to the functional and structural manifestations characterizing clinical neuropathy remains to be elucidated (1).

Studies of glucose interactions with such proteins as hemoglobin, the lens protein α-crystallins, albumin, erythrocyte cell membrane, and collagen (2–6) have demonstrated that glucose covalently modifies the structural and functional properties of a number of proteins. Nonenzymatic glycosylation of certain susceptible proteins in such insulin-independent tissues of diabetics as nerve may play a role in the pathogenesis of some long-term complications.

In the past, extensive investigations of this hypothesis have been limited by methodological difficulties. Now, by utilizing a new affinity chromatography system, it is possible to selectively isolate and quantitate nonenzymatically glycosylated amino acids from complex biological samples, such as tissue homogenates (7). Utilizing this technique, we have determined the amount of nonenzymatic glycosylation in peripheral nerve from diabetic and control rats and dogs.

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MATERIALS AND METHODS

Female Sprague–Dawley rats (120–150 g; Taconic Laboratories, New York) were fasted for 18 hr and then injected through the tail vein with freshly prepared alloxan monohydrate (40 mg/kg of body weight) dissolved in 0.1 M sodium phosphate buffer (pH 3.0). Both normal rats and those treated with alloxan were maintained on Purina chow and water ad lib. The presence of diabetes was assessed by serum glucose determinations with a Stat Tek glucose analyzer (Biodynamics, Indianapolis, IN) 48 hr after alloxan injection and at weekly intervals thereafter. All diabetic animals had plasma glucose concentrations persistently greater than 300 mg/dl.

Three months after alloxan administration, 12 diabetic and an equal number of age- and sex-matched control rats were sacrificed by exposure to CO_2. The sciatic nerves were rapidly removed from each rat and placed in 0.1 M sodium phosphate buffer (pH 7.0) at 4°C. Under a dissecting microscope, the connective tissue sheaths, adipose tissue, and vascular remnants were removed. One sciatic nerve (30–50 mg wet weight) from each animal was then homogenized in 1 ml of ice-cold buffer with a glass Potter–Elvehjem homogenizer. The homogenates were then subjected to reduction with 1 mg of NaB^3H_4 (specific activity, 8 μCi/μmol; 1 Ci = 3.7×10^{10} becquerels) for 10 min at room temperature and an additional 50 min at 4°C. The reduced homogenates were dialyzed for nine changes (6–8 hr each) against 1000-fold excess of 0.01 M sodium phosphate buffer (pH 7.0). Dialyzed samples were then hydrolyzed in 6 M HCl in sealed evacuated tubes at 110°C for 12 hr. The acid was removed by evaporation, and the residual amino acids were dissolved in 4.0 ml of 0.025 M sodium phosphate buffer (pH 9.0). Aliquots (10%) of each sample were taken for determination of total amino acid content by the method of Moore and Stein (8). The remainder of each sample was then applied to a column (1.5 × 14 cm) containing *m*-aminophenylboronic acid immobilized on Bio-Gel P-6, previously equilibrated with 0.025 M sodium phosphate buffer (pH 9.0) containing 1 mM NaN_3. Fresh buffer was applied, and 4.0-ml fractions were collected at a flow rate of 20 ml/hr. After 70 fractions were collected, the buffer was removed, and the column was eluted with 0.025 M HCl as described (7). Seventy 2.0-ml fractions were collected. Aliquots (10%) of all fractions were counted in 7.0 ml of Hydrofluor (National Diagnostics, Sommerville, NJ). The amount of radioactivity was determined with a Packard Tricarb scintillation counter.

Segments of femoral nerves obtained from diabetic and age- and sex-matched control beagle dogs were provided by R. Engerman (University of Wisconsin). Diabetes had been induced by either pancreatectomy or alloxan, and the animals had been maintained in a poorly controlled diabetic state for 60 mo before sacrifice. Removal and preparation procedures were the same

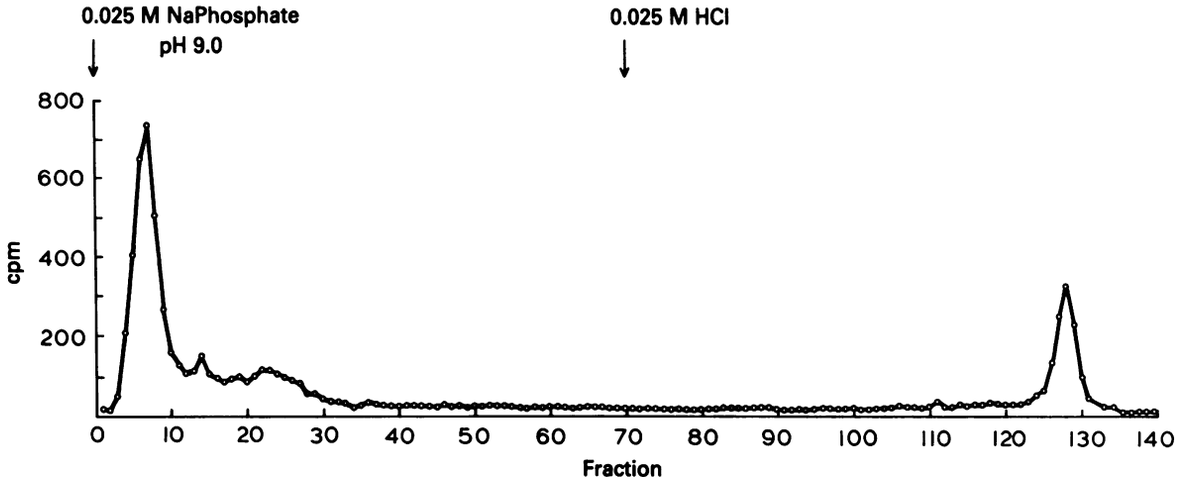

FIG. 1. Separation by affinity chromatography of glycosylated amino acids from reduced and hydrolyzed peripheral nerve homogenates. Buffer changes are indicated by arrows.

as those for rat sciatic nerves, except that the specific activity of the NaB^3H_4 used for reduction was 80 μCi/μmol because the wet weight of each sample was only 5–10 mg.

Peak tubes from the HCl column elutions were pooled and concentrated. Samples were applied to a Beckman Model 119C amino acid analyzer with stream division (90% to the fraction collector; 1.0-ml fractions), and aliquots (500 μl) were counted in 7.0 ml of Hydrofluor. The elution patterns were compared with chromatograms of reduced, hydrolyzed, glycosylated amino acid standards.

RESULTS

Reduced, hydrolyzed sciatic nerve homogenates separated on the affinity chromatography system gave essentially identical chromatograms to those obtained previously from reduced, hydrolyzed hemoglobin and from urinary amino acids and peptides (7). Washing the column with 0.025 M sodium phosphate buffer (pH 9.0) produced a large peak of nonspecific radioactivity in the void volume (nonglycosylated compounds), and a single sharp peak of radioactivity was eluted subsequently with 0.025 M HCl (Fig. 1). Comparison of the amount of glycosylated amino acids in sciatic nerves from diabetic rats with the amount from normal controls (Fig. 2) showed that the mean amount in diabetics (mean 1228 ± 309 cpm/μmol of amino acid) was 2.8 times ($P < 0.001$) greater than the mean value in normal animals (mean 438 ± 100 cpm/μmol of amino acid).

Similar results were observed in segments of femoral nerves from diabetic (mean 6060 ± 2900 cpm/μmol of amino acid) versus normal dogs (mean 2814 ± 897 cpm/μmol of amino acid), where diabetic values were 2.15 times ($P < 0.05$) greater than normal values.

In both rat and dog, amino acid analysis of glycosylated amino acids previously isolated by affinity chromatography from reduced, hydrolyzed sciatic nerve homogenates showed that glycosylated lysine and its hydrolysis rearrangement products constituted the major borohydride-reducible adduct present (Fig. 3, peak II). In addition, another glycosylated amino acid was noted to be present in major proportions (Fig. 3, peak I). The nature of this product is currently not known because the available glycosylated amino acid standards do not chromatograph with this radioactive product.

DISCUSSION

In diabetic neuropathy, the most significant morphologic change accompanying decreased conduction velocity is patchy demyelination. Similar peripheral nerve lesions are characteristically produced by the antisickling agent sodium cyanate (9). This agent, which retards the polymerization of hemoglobin S by carbamylation of the NH_2-termini of hemoglobin (10), presumably also carbamylates peripheral nerve proteins essential for normal structure and function. This similarity between the morphological findings of cyanate-induced neuropathy and those of diabetic neuropathy gave rise to the suggestion that glucose might undergo a chemical reaction similar to that of cyanate, with nerve proteins leading to the development of diabetic peripheral neuropathy. Therefore, these glucose–protein reactions were intensively investigated in a number of model systems with well-characterized purified proteins such as hemoglobin and the lens crystallins (2, 3). In all systems stud-

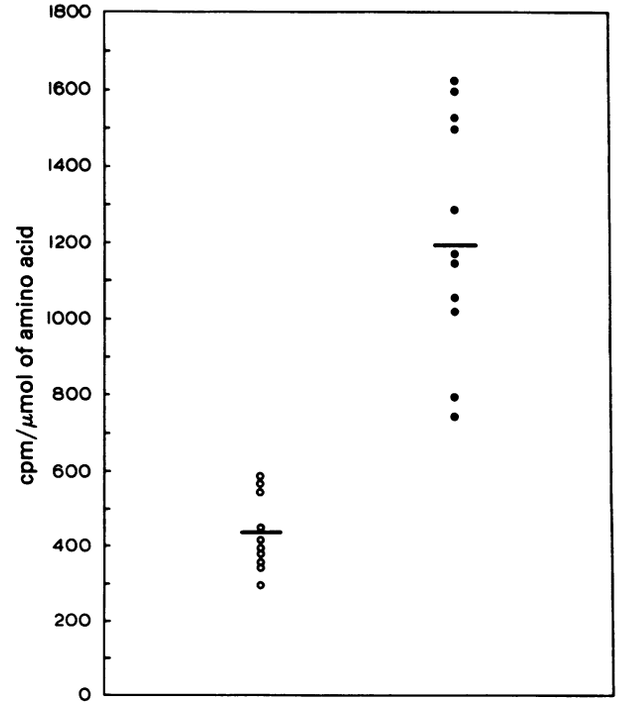

FIG. 2. Levels of glycosylated amino acids in peripheral nerve homogenates from normal (○) and diabetic (●) animals, expressed as cpm/μmol of total amino acid. Bar, mean in each group; P, <0.001 between normal and diabetic animals.

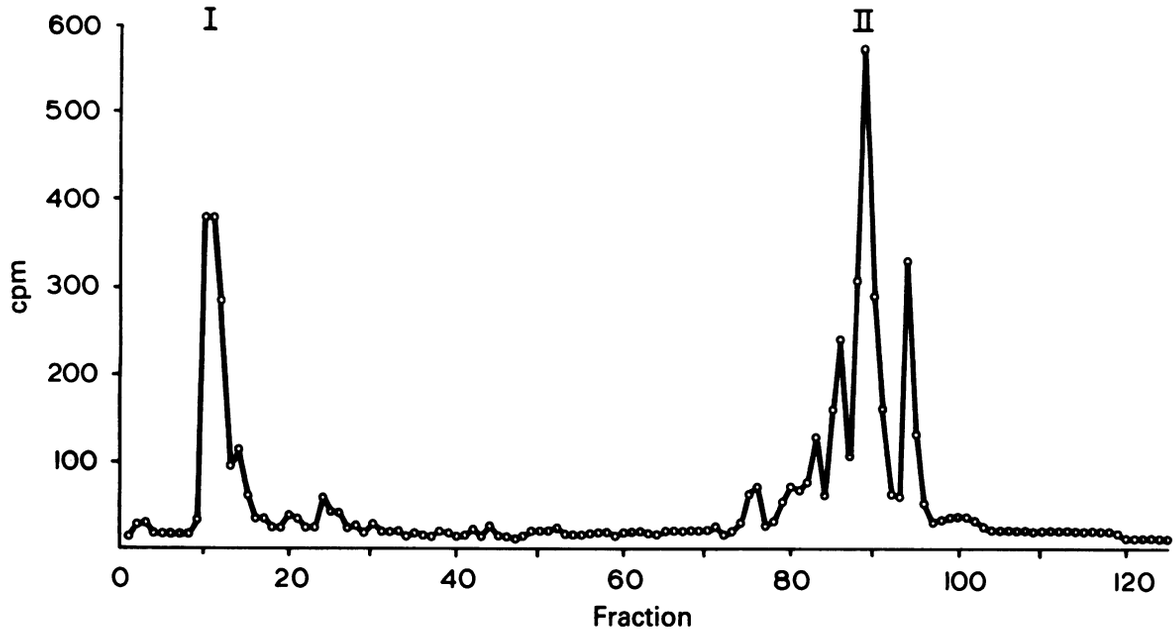

FIG. 3. Amino acid analysis of the glycosylated amino acids previously isolated by affinity chromatography from reduced, hydrolyzed sciatic nerve. Peaks: I, elution position of an unknown glycosylated product; II, elution position of reduced, hydrolyzed, glycosylated lysine and its hydrolysis rearrangement products.

ied, hyperglycemia increased the occurrence of nonenzymatic glycosylation. In both hemoglobin and crystallins, this excessive glucose attachment causes alterations in protein function.

The analytical methods developed for the study of nonenzymatic glycosylation of purified proteins were not applicable to complex tissues such as nerve, however, because nonspecific reduction of peptide bonds interfered with the resolution and quantitation of glycosylated adducts by amino acid analysis. To overcome this problem, we have developed a new method for measuring the amount of glycosylated amino acids found in complex biological samples (7).

In the present study, this affinity chromatography system was used to determine the amount of nonenzymatic glycosylation in peripheral nerve from diabetic and appropriate control rats and dogs. The finding that diabetes results in a greater than 2-fold increase of peripheral nerve glycosylation is consistent with a number of previous investigations in which glycosylation was measured in hemoglobin, serum albumin, and urinary amino acids and peptides from diabetics and normals (2, 4, 7). The results reported here provide evidence that excessive nonenzymatic glycosylation is occurring in a tissue where physiological, morphological, and clinical degeneration characteristically develop as a result of diabetes mellitus. Excessive nonenzymatic glycosylation of peripheral nerve in diabetes could involve primarily axonal proteins, myelin proteins, or various components of each.

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