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Structural and functional consequences of increased tubulin glycosylation in diabetes mellitus

(nonenzymatic glycosylation/microtubules/hyperglycemia/polymerization/glucose)

STUART K. WILLIAMS*, NANCY L. HOWARTH, JAMES J. DEVENNY, AND MARK W. BITENSKY†

Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06510

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ABSTRACT **The extent of *in vitro* nonenzymatic glycosylation of purified rat brain tubulin was dependent on time and glucose concentration. Tubulin glycosylation profoundly inhibited GTP-dependent tubulin polymerization. Electron microscopy and $NaDodSO_4$/polyacrylamide gel electrophoresis showed that glycosylated tubulin forms high molecular weight amorphous aggregates that are not disrupted by detergents or reducing agents. The amount of covalently bound NaB^3H_4-reducible sugars in tubulin recovered from brain of streptozotocin-induced diabetic rats was dramatically increased as compared with tubulin recovered from normal rat brain. Moreover, tubulin recovered from diabetic rat brain exhibited less GTP-induced polymerization than tubulin from nondiabetic controls. The possible implications of these data for diabetic neuropathy are discussed.**

Diabetes mellitus is often accompanied by the gradual development of diverse and multifocal pathology (1). Insulin and antibiotics have contributed substantially to the management of carbohydrate abnormalities and infection (2). However, deterioration in the microcirculation of retina and kidney and diabetic neuropathy continue to present serious clinical problems (3). It is still unclear whether these complications are uniquely the result of carbohydrate disturbances due to intermittent insulin deficiency or also reflect more subtle genetic or metabolic factors that are, at best, indirectly related to insulin levels (4).

Recent data have supported the hypothesis that increased nonenzymatic as well as enzymatic glycosylation of cellular components, as a result of the elevated glucose concentrations of diabetes, may play a contributory role in diabetic microangiopathy (5), and diabetic cataractogenesis (6). Modification, by the nonenzymatic addition of glucose, has now been reported for a variety of serum, cellular, and interstitial proteins and phospholipids (7–10), suggesting the possibility that unanticipated additional functional changes may yet be observed to result from nonenzymatic (or enzymatic) glycosylation.

We present here evidence for *in vitro* and *in vivo* glycosylation of brain tubulin as a consequence of exposure to elevated glucose concentrations. The *in vitro* nonenzymatic glycosylation of tubulin results in the formation of amorphous aggregates of tubulin and tubulin-associated proteins that remain insoluble in 8 M urea or $NaDodSO_4$. The *in vivo* formation of glycosylation-induced tubulin aggregates was found to compete with ordered tubulin polymerization in diabetic rats.

METHODS AND MATERIALS

Tubulin Isolation and Measurement. Tubulin was purified from male weanling Sprague–Dawley rat brain according to the procedure of Berkowitz *et al.* (11). Rats were sacrificed by occipital trauma and cervical dislocation. Brains were removed and weighed and 0.75 ml of purification buffer (11) [50 mM 4-morpholineethanesulfonic acid (Sigma)/2 mM EGTA/1 mM $MgSO_4$/4.0 M glycerol, pH 6.9] was added per gram of brain. Brains were homogenized in a loose-fitting motor-driven Teflon/glass homogenizer (10 strokes, 4°C; 10 sec at setting 4; 60 sec at setting 10). The homogenate was centrifuged at 23,000 × *g* at 4°C for 15 min, and the supernatant was collected and centrifuged at 100,000 × *g* for 70 min at 4°C. The resulting supernatant was brought to 1 mM GTP and incubated at 37°C for 45 min to enhance tubulin polymerization (11). Microtubules were collected by centrifugation at 100,000 × *g* for 45 min at 25°C and then suspended and depolymerized in 5 vol of cold purification buffer (11) without glycerol. The depolymerized tubulin was centrifuged at 100,000 × *g* for 30 min at 4°C. This tubulin was subjected to another cycle of polymerization/depolymerization and used immediately.

Tubulin was also prepared from male weanling Sprague–Dawley rat brain by using the vinblastine precipitation technique described by Feit *et al.* (12).

Tubulin concentrations were determined by using a [^{3}H]colchicine/charcoal assay as described by Sherline *et al.* (13). Rates of tubulin polymerization were determined spectrophotometrically by following the change in optical density at 350 nm (14).

D-[6-^{3}H]Glucose and Detection of Nonenzymatic Glycosylation. Protein samples (8 mg/ml) were incubated with D-[6-^{3}H]glucose and the time course of D-[6-^{3}H]glucose incorporation was determined by measuring the radioactivity associated with trichloroacetic acid-precipitable protein. The sample of D-[6-^{3}H]glucose used in these experiments had been tested previously (5) and found to be free of contaminants that have been associated with an apparent increase in the nonenzymatic glycosylation of proteins (15).

NaB^3H_4 Reduction. Protein samples were suspended in 0.1 M Tris·HCl (pH 7.4) and incubated with a 200 M excess of NaB^3H_4 for 40 min at room temperature (10). Samples were then dialyzed against 100 vol of distilled water (changed every 8 hr) for a minimum of 48 hr or until the dialysate exhibited background radioactivity.

Electron Microscopy. Microtubules formed *in vitro* were centrifuged at 40,000 × *g* for 60 min at 25°C. The pellets were fixed with 2.5% glutaraldehyde in 0.02 M phosphate buffer (pH 7.4) followed by 1% osmium tetroxide and dehydrated in a graded series of acetone solutions. Pellets were embedded in the Spurr low-viscosity embedding medium (16) and polymerized overnight at 60°C. Silver sections were cut with a diamond knife, counterstained with uranyl acetate (1 hr) and lead citrate

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* Present address: Dept. of Physiology, Jefferson Medical College, 1020 Locust St., Philadelphia, PA 19107.
† To whom reprint requests should be addressed at: Division of Life Sciences, Los Alamos National Laboratory, Los Alamos, NM 87545.

(10 min), and examined in a Philips 201 electron microscope.

***In Vivo* Studies with Diabetic Rats.** After a 48-hr fast, male Sprague–Dawley rats (150 g) were made diabetic with a single intraperitoneal injection (90 mg/kg of body weight) of streptozotocin in acid citrate solution (17). Elevated blood glucose, glucosuria, proteinuria, polyuria, and weight loss served as criteria for diabetes mellitus.

Protein Analysis. $NaDodSO_4$/polyacrylamide gel electrophoresis was done according to the procedure of Laemmli (18). Gels were stained with 0.025% Coomassie brilliant blue in 20% methanol/6% acetic acid/0.6% trichloroacetic acid and destained in 10% acetic acid. Autoradiography was done with Kodak Royal X-Omat x-ray film after gels were impregnated with EN^3HANCE (New England Nuclear). Protein concentrations were determined by using the Bio-Rad (Bio-Rad) protein assay.

Reagents. D-[6-^{3}H]Glucose, [^{3}H]colchicine, and NaB^3H_4 were obtained from New England Nuclear. GTP, colchicine, and vinblastine sulfate were obtained from Sigma.

RESULTS

Nonenzymatic Glycosylation of Purified Brain Tubulin. Two-cycle-purified rat brain tubulin monomers were incubated at three glucose concentrations. As expected, the resulting tubulin glycosylation was a function of both time and glucose concentration (Fig. 1). At the highest glucose concentration used (10 mg/ml), 3.6 mol of glucose was incorporated per mole of tubulin monomer after 24 hr. Since tubulin isolated by two cycles of polymerization/depolymerization contains both α and β subunits as well as microtubule-associated proteins (11), the specificity of this nonenzymatic glucose attachment was determined by analysis in a $NaDodSO_4$/acrylamide gel electrophoresis system. Treatment of two-cycle-purified tubulin with [^{3}H]glucose followed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography illustrated the time- and concentration-dependent nonenzymatic glycosylation of tubulin and tubulin-associated proteins (Fig. 2). Exposure to glucose had a negligible effect on the Coomassie blue-staining pattern of the α and β subunits and resulted in a time-dependent increase in the amount of stained protein in the high molecular weight regions and also in the appearance of high molecular weight Coomassie blue-staining material that did not enter the gels (Fig. 2A).

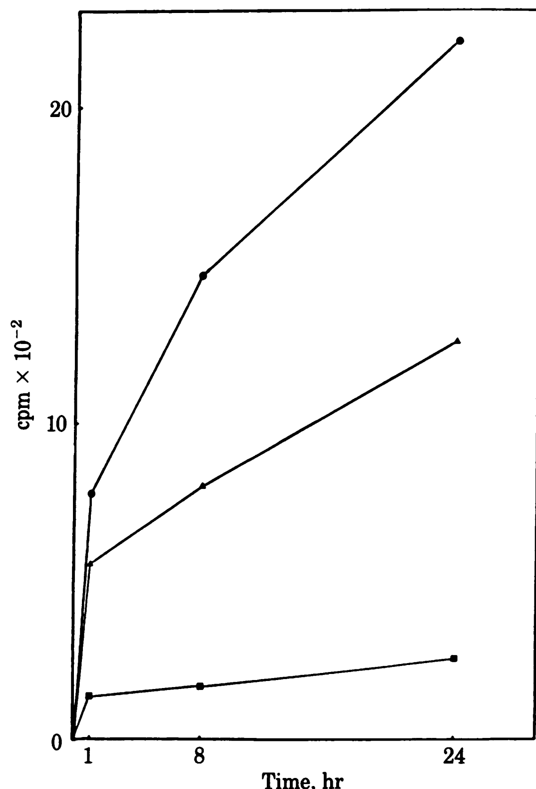

FIG. 1. Nonenzymatic glycosylation of rat brain tubulin. Two-cycle-purified rat brain tubulin (8 mg/ml) was incubated with D-[6-^{3}H]glucose at final glucose concentrations of 1 (■), 5 (▲), and 10 (●) mg/ml at 32°C. After 1, 8, and 24 hr, the amount of trichloracetic acid-precipitable protein was determined.

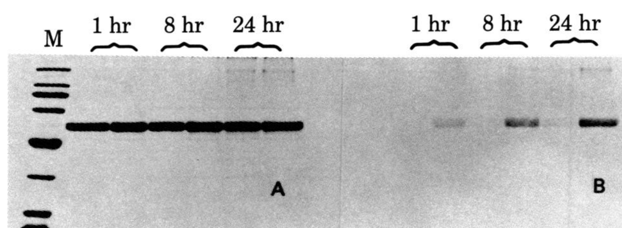

FIG. 2. (A) Effect of glucose treatment on Coomassie blue-staining pattern of tubulin subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. Two-cycle-purified rat brain tubulin (8 mg/ml) was incubated with D-[6-^{3}H]glucose for 1, 8, and 24 hr at final glucose concentrations of 1 (left lanes) and 10 (right lanes) mg/ml. The specific activities of both tritiated glucose solutions were identical. Lane M: molecular weight standards; serum albumin (69,000) is above and ovalbumin (43,000) is below the tubulin monomers. Under these gel conditions, resolution of the α and β subunits of tubulin is minimal. (B) Autoradiogram of gel shown in A.

Nonenzymatic Glycosylation of Tubulin and the Effect on Polymerization. To study the effects of nonenzymatic glycosylation on tubulin polymerization *in vitro*, samples of two-cycle-purified tubulin were incubated with various concentrations of glucose and glucose 6-phosphate (1 hr, 32°C). Samples were then centrifuged at 40,000 × *g* and the pellets and supernatants were assayed for tubulin. As shown in Table 1, exposure to higher concentrations of glucose and glucose 6-phosphate resulted in increased sedimentation of tubulin. We note that sedimentation of tubulin after exposure to glucose 6-phos-

Table 1. Tubulin sedimentation at 40,000 × *g* after *in vitro* nonenzymatic glycosylation and GTP-induced polymerization

	Tubulin not pelleted		Tubulin pelleted	
Conc., mg/ml	nmol	%	nmol	%
No Glc	0.161 ± 0.003	40.7	0.235 ± 0.004	59.3
(controls)	0.157 ± 0.007	39.7	0.239 ± 0.010	60.3
	0.161 ± 0.005	40.7	0.235 ± 0.008	59.3
D-Glc				
1	0.150 ± 0.006	37.9	0.246 ± 0.010	62.1
5	0.147 ± 0.004	37.1	0.249 ± 0.008	62.9
10	0.139 ± 0.007	35.1	0.257 ± 0.012	64.9
20	0.140 ± 0.008	35.4	0.256 ± 0.014	64.7
D-Glc-*P*				
1	0.143 ± 0.002	36.1	0.253 ± 0.004	63.9
5	0.093 ± 0.006	23.5	0.303 ± 0.020	76.5
10	0.054 ± 0.003	13.6	0.342 ± 0.020	86.4
20	0.022 ± 0.001	5.6	0.374 ± 0.016	94.4

Tubulin was prepared from weanling Sprague–Dawley rats. Samples (8 mg/ml) were incubated with glucose (Glc) or glucose 6-phosphate (Glc-*P*) at various concentrations (conc.) for 1 hr at 32°C. GTP was added (final concentration, 1 mM), and mixtures were incubated at 37°C for 45 min. Samples were then centrifuged at 40,000 × *g* for 30 min at 25°C. Results are mean ± SEM of three determinations. Assay was by the colchicine/charcoal method.

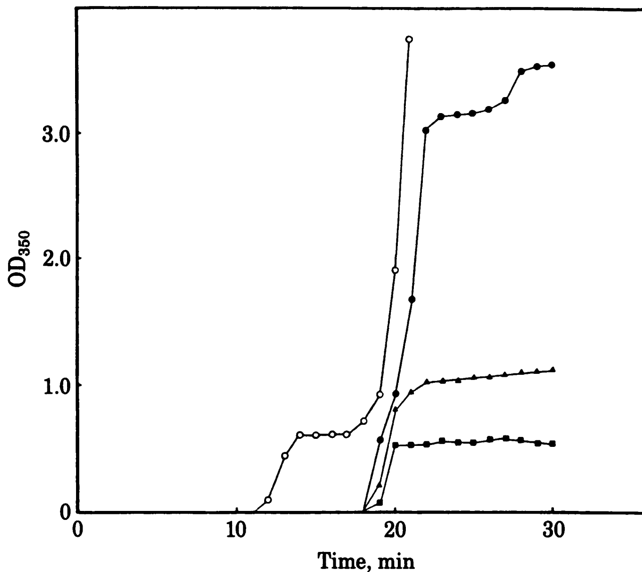

FIG. 3. Effect of glucose treatment on tubulin polymerization. Two-cycle-purified tubulin (8.0 mg/ml) was treated with glucose at final concentrations of 0 (○), 1 (●), 5 (▲), and 10 (■) mg/ml for 1 hr at 32°C. Polymerization was initiated with 1 mM GTP (0 time) and the increase in optical density was determined at 350 nanometers.

phate was more pronounced than that after exposure to glucose, especially at glucose concentrations greater than 5 mg/ml. Nonenzymatic glycosylation of hemoglobin is similarly augmented when glucose 6-phosphate rather than glucose is used (19).

These results initially suggested that nonenzymatic glycosylation might increase polymerization of tubulin into microtubules that readily sedimented at 40,000 × g. However, when the rate of tubulin polymerization was analyzed spectrophotometrically (14), the opposite results were observed. Tubulin treated with glucose for 1 hr exhibited an inhibition of polymerization that was dependent on glucose concentration (Fig. 3) but, when glucose (10 mg/ml) was added to unmodified tubulin immediately prior to addition of GTP, we found no significant impairment of tubulin polymerization.

We then investigated the possibility that increased sedimentation of tubulin due to treatment with high concentrations of glucose or glucose 6-phosphate may result not from microtubule formation but rather from formation of protein aggregates of sufficient mass to sediment at 40,000 × g. Lens protein aggregation as a result of nonenzymatic glycosylation has previously been demonstrated by Cerami (6). As shown in Table 2, when tubulin was treated first with [^{3}H]colchicine (which effectively inhibits ordered polymerization) and then with glucose 6-phosphate (10 mg/ml), a significant increase in the amount of tubulin

Table 2. Tubulin sedimentation at 40,000 × *g* after glucose 6-phosphate treatment (*in vitro*) in the presence of colchicine

	Tubulin not pelleted		Tubulin pelleted	
	nmol	%	nmol	%
Control	0.383 ± 0.011	97.2	0.011 ± 0.001	2.8
10 D-glucose 6-phosphate	0.310 ± 0.010	78.7	0.084 ± 0.001	21.3

Two-cycle-purified tubulin (8 mg/ml) was treated with 65 μM [^{3}H]colchicine. Samples were incubated with and without glucose 6-phosphate at 10 mg/ml, 1 mM GTP was added, and mixtures were incubated at 37°C for 45 min. Samples were then centrifuged at 40,000 × *g* for 30 min at 25°C. Results are mean ± SEM of three determinations.

sedimentation was observed. These data indicate that modification of tubulin by covalent attachment of glucose 6-phosphate results in the formation of sedimentable (nonmicrotubule) aggregates. Since the nonenzymatic glycosylation of tubulin monomers is also accompanied by a decrease in microtubule formation, this could reflect monomer sequestration by aggregates, a direct change in the polymerization potential of the glycosylated monomer, or some combination of both.

Electron Microscopic Examination of Glycosylated and Unmodified Tubulin. Freshly prepared tubulin monomers were incubated in the presence or absence of glucose 6-phosphate (final concentration, 10 mg/ml) for 2 hr at 37°C. The solutions were then made 1 mM in GTP, incubated for 45 min at 37°C, and centrifuged at 40,000 × g for 30 min at 25°C. The pellets were processed for electron microscopy (Fig. 4). Although the unmodified tubulin clearly produces the characteristic microtubule arrays (Fig. 4*A*), the glycosylated material consists of amorphous aggregates from which characteristic microtubules are conspicuously absent (Fig. 4*B*).

Effect of Hyperglycemia on Tubulin Polymerization *In Vivo*. To determine whether tubulin exhibits increased glycosylation *in vivo* as a result of diabetes, tubulin was purified from brain homogenates of control and diabetic rats by using the technique of vinblastine sulfate precipitation (because *in vivo* glycosylation might interfere with tubulin purification procedures that depend on polymerization). Tubulin isolated from rat brains by vinblastine precipitation exhibits a profound increase in the presence of reducing sugars covalently attached to tubulin and other (vinblastine) coprecipitated proteins as compared with controls (Fig. 5). This difference cannot be accounted for by differences in total protein or in tubulin monomer concentrations after the centrifugation step (Table 3). We note that vinblastine-precipitated tubulin preparations from diabetic rats showed increased amounts of high molecular weight Coomassie blue-staining material that did not enter the gels.

To determine the effect of hyperglycemia on tubulin glycosylation and tubulin polymerization, supernatants from equivalent amounts (wet weight) of rat brain were prepared (4°C)

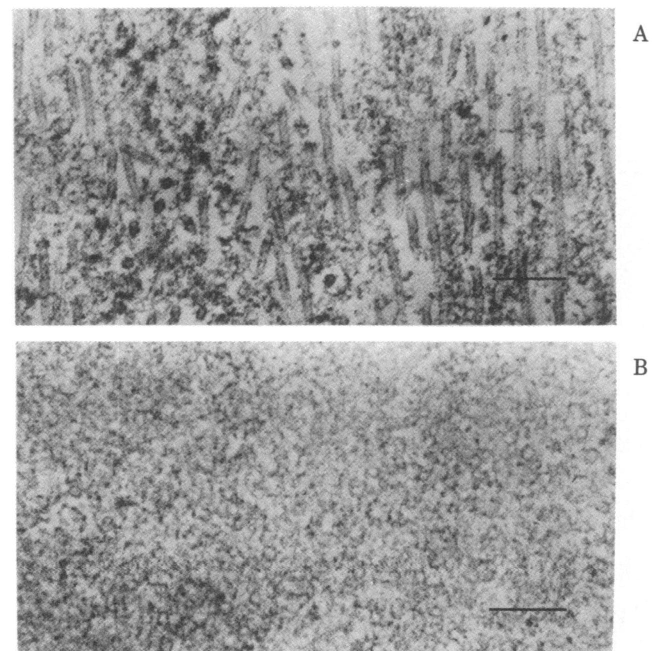

FIG. 4. (*A*) Neuronal tubulin/microtubules from unmodified tubulin. (*B*) Glucose 6-phosphate-treated tubulin. Bar = 100 nm.

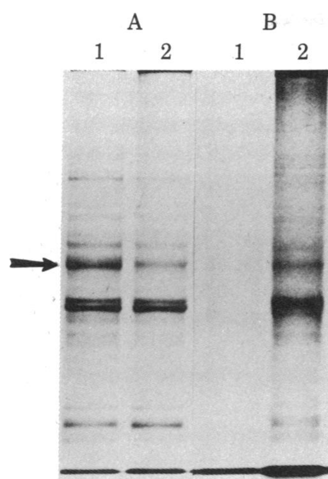

FIG. 5. (*A*) Polyacrylamide gel of vinblastine sulfate-purified NaB^3H_4-reduced tubulin samples from brains of control (lane 1) and diabetic (lane 2) rats. (*B*) The corresponding autoradiograms. Arrow: α- and β-tubulin monomers.

from control and diabetic rats by homogenization and centrifugation at 40,000 × *g* for 30 min. This step should sediment tubulin previously aggregated. However, because polymerization is inhibited or reversed at 4°C, nonaggregated tubulin monomers should remain in the supernatant. Supernatants were then adjusted to a total protein concentration of 8 mg/ml and treated with 1 mM GTP, and tubulin was polymerized at 30°C for 45 min. After sedimentation at 40,000 × *g*, homogenates from diabetic brains exhibited an average of 20% reduction in the amounts of pelleted tubulin (Table 3).

DISCUSSION

The role of microtubules in cellular activity and function is diverse and ranges from the participation of tubulin and microtubules in cellular neurosecretion (20) and axonal transport (21) to the maintenance of cellular morphology through the interaction of microtubules with other cytoskeletal and membrane elements (22). Covalent modification of tubulin might have a variety of functional manifestations. A number of reports (21–24) suggest that the neuropathy of diabetes mellitus may involve altered tubulin–microtubule function (25). Axonal shortening (26), decreased axonal flow (27), increased latency (28), and reduced conduction velocities (29), all features of diabetic neuropathy, could involve abnormalities of microtubule formation or altered (abnormal) tubulin interaction with other cytoskeletal components.

Our studies of *in vitro* nonenzymatic glycosylation indicate that tubulin and tubulin-associated proteins are readily modified by covalent attachment of glucose and glucose 6-phosphate in a concentration- and time-dependent manner. This nonenzymatic glycosylation results in the formation of high molecular weight aggregates here demonstrated by electron microscopy, by rapid sedimentation at 40,000 × *g*, and by their failure to penetrate acrylamide gels in the presence of $NaDodSO_4$ and dithiothreitol. Formation of such aggregates may not be unique to the glycosylated tubulin molecule; rather, they may be the common result of a process whereby nonenzymatic glycosylation can promote protein cross-linking, as described by Monnier *et al.* (6), for the aggregation of glycosylated lens protein. Such aggregate formation (either in the case of glycosylated tubulin or lens protein) is not reversed by treatment with reducing agents as well as urea and $NaDodSO_4$ (6). We also note that comparison of the data in Tables 1 and 2 (in particular those for incubation with glucose 6-phosphate at 10 mg/ml) leads to the suggestion that colchicine may partially inhibit the formation of tubulin aggregates after glycosylation.

In experiments in which two-cycle-purified tubulin monomers are glycosylated *in vitro*, it is apparent by morphologic and biochemical criteria that such material is prevented from polymerizing (at least in part) because of aggregate formation. In supernatants from centrifuged brain homogenates prepared from normal and diabetic rats, the situation is more complex.

The data show that tubulin monomers in supernatants from diabetic animals form less polymerized tubulin (by 20%) than tubulin from control animals. This finding was not a result of differences in either total protein concentration or initial tubulin monomer concentration (Table 3). The data suggest that there could be intermediate forms in the glycosylation of tubulin monomer that neither form sedimentable aggregates nor readily polymerize: alternatively, there may be a factor(s) whose presence in or absence from the cytoplasm of diabetic animals retards or inhibits ordered tubulin polymerization.

Table 3. Tubulin sedimentation at 40,000 × *g* in brain supernatants from control and diabetic rats

Animal	Blood glucose, mg/ml	Tubulin not pelleted		Tubulin pelleted	
		nmol	%	nmol	%
Control					
1	0.99	0.211 ± 0.008	48.8	0.221 ± 0.008	51.2
2	0.90	0.164 ± 0.013	47.0	0.185 ± 0.015	53.0
3	1.01	0.144 ± 0.018	43.1	0.190 ± 0.023	56.9
4	0.67	0.187 ± 0.007	47.2	0.209 ± 0.008	52.8
Diabetic					
1	3.98	0.154 ± 0.001	63.1	0.090 ± 0.001	36.9
2	2.89	0.242 ± 0.021	55.5	0.194 ± 0.017	44.5
3	3.10	0.251 ± 0.004	62.4	0.151 ± 0.002	37.6
4	4.03	0.223 ± 0.013	51.6	0.209 ± 0.013	48.4
5	3.68	0.320 ± 0.021	51.3	0.304 ± 0.020	48.7
6	3.10	0.340 ± 0.022	55.2	0.276 ± 0.018	44.8

Brains were removed from diabetic and age/weight-matched control animals, homogenized (10 strokes in a Teflon/glass homogenizer), and further disrupted in a Virtis Polytron (10 sec at setting 4, 90 sec at setting 10) at 4°C in tubulin purification buffer. Homogenates were centrifuged at 40,000 × *g* for 60 min at 4°C. Supernatants were adjusted to a total protein concentration of 8 mg/ml, treated with 1 mM GTP for 45 min at 37°C, and then centrifuged at 40,000 × *g* for 30 min at 25°C. Results are mean ± SEM of three determinations.

It has been found that tubulin lysine residues are required for tubulin polymerization (30). Moreover, nonenzymatic glycosylation involves the reaction of glucose either with the NH_2-terminal amino group or the ε-amino group of specific lysine residues. It is thus suggestive that the nonenzymatic glycosylation observed in our experiments involves lysine residues that are necessary for the polymerization of tubulin monomers.

It is possible that the observed *in vivo* glycosylation of tubulin may not result exclusively from a nonenzymatic reaction. However, because known enzymatic glycosylation reactions usually require either ATP or phosphorylated glucose (31), the *in vitro* glycosylation observed in our study is most likely to occur via nonenzymatic attachment. In the preparation from diabetic animals, there is a dramatic increase in the availability of reducing sugars for interaction with NaB^3H_4 (Fig. 5). This difference represents the full (*in vivo*) impact of an uncontrolled diabetic environment on the carbohydrate modification of (vinblastine-precipitable) brain proteins. We emphasize that these differences may reflect both enzymatic and nonenzymatic attachment of carbohydrates to these proteins.

In conclusion, the data presented here show the nonenzymatic glycosylation of α- and β-tubulins *in vitro* and *in vivo* under conditions of untreated diabetic hyperglycemia. This glycosylation leads to formation of stable amorphous tubulin aggregates and inhibits GTP-dependent tubulin polymerization. Such changes could compromise some aspects of neuronal function, especially those dependent on microtubule formation (23). In addition, the reported diminution or reversal of diabetic neuropathy and angiopathy by rigorous control of plasma glucose levels (32, 33) also supports a potential role for *in vivo* tubulin glycosylation in the pathogenesis of diabetic neuropathy.

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