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# Posttranslational Modifications of Nerve Cytoskeletal Proteins in Experimental Diabetes

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## Abstract

Axonal transport is known to be impaired in peripheral nerve of experimentally diabetic rats. As axonal transport is dependent on the integrity of the neuronal cytoskeleton, we have studied the way in which rat brain and nerve cytoskeletal proteins are altered in experimental diabetes. Rats were made diabetic by injection of streptozotocin (STZ). Up to six weeks later, sciatic nerves, spinal cords, and brains were removed and used to prepare neurofilaments, microtubules, and a crude preparation of cytoskeletal proteins. The extent of nonenzymatic glycation of brain microtubule proteins and peripheral nerve tubulin was assessed by incubation with $^{3}$H-sodium borohydride followed by separation on two-dimensional polyacrylamide gels and affinity chromatography of the separated proteins. There was no difference in the nonenzymatic glycation of brain microtubule proteins from two-week diabetic and nondiabetic rats. Nor was the assembly of microtubule proteins into microtubules affected by the diabetic state. On the other hand, there was a significant increase in nonenzymatic glycation of sciatic nerve tubulin after 2 weeks of diabetes. We also identified an altered electrophoretic mobility of brain actin from a cytoskeletal protein preparation from brain of 2 week and 6 week diabetic rats. An additional novel polypeptide was demonstrated with a slightly more acidic isoelectric point than actin that could be immunostained with anti-actin antibodies. The same polypeptide could be produced by incubation of purified actin with glucose in vitro, thus identifying it as a product of nonenzymatic glycation. These results are discussed in relation to data from a clinical study of diabetic patients in which we identified increased glycation of platelet actin. STZ-diabetes also led to an increase in the phosphorylation of spinal cord neurofilament proteins in vivo during 6 weeks of diabetes. This hyperphosphorylation along with a reduced activity of a neurofilament-associated protein kinase led to a reduced incorporation of $^{32}$P into purified neurofilament proteins when they were incubated with $^{32}$P-ATP in vitro. Our combined data show a number of posttranslation modifications of neuronal cytoskeletal proteins that may contribute to the altered axonal transport and subsequent nerve dysfunction in experimental diabetes.

**Index Entries:** Neurofilaments; tubulin; microtubules; actin; glycation; phosphorylation; cytoskeleton; diabetic neuropathy.

## Introduction

Diabetic neuropathy is probably the most common secondary complication of diabetes mellitus, yet its pathogenesis remains uncertain. Experimental diabetes in the rat is associated with early changes in axonal transport (Tomlinson and Mayer, 1984; Sidenius and Jakobsen, 1979; McLean et al., 1985; McLean and Meiri, 1981; Medori et al., 1988). Axonal transport both contains and is dependent on the axonal cytoskeleton (Hoffman and Lasek, 1975; McLean et al., 1983; Banks and Till, 1975; Schnapp et al., 1985). In streptozotocin (STZ)-induced diabetes in the rat and in the mutant diabetic mouse, slow axonal transport of cytoskeletal elements in both slow component a (containing tubulin and neurofilament proteins) and slow component b (containing tubulin and actin) is impaired (Medori et al., 1988; Macioce et al., 1989; Vitadello et al., 1985).

Glycation, the attachment of glucose to the lysine residues of proteins without the influence of an enzyme, is a widespread phenomenon in diabetes (Cohen, 1986). An almost threefold increase in the glycation of peripheral nerve protein occurs in diabetic rats and dogs, although the precise proteins undergoing glycation have not been characterized (Vlassara et al., 1981). Lysine residues of tubulin are essential for assembly into microtubules, and their substitution is known to prevent assembly (Mellado et al., 1982; Szasz et al., 1982). Although changes in the glycation of brain tubulin have been described in experimental diabetes (Williams et al., 1982), this finding has recently been disputed (Eaker et al., 1991).

Another posttranslational protein modification, phosphorylation, and the enzymes associated with it are known to be altered in the diabetic state (Greene and Lattimer, 1986; Schrama et al.,

1987; Simpson and Hawthorne, 1988; Gabbay et al., 1990). Nerve cytoskeletal proteins, particularly neurofilament (NF) proteins, are phosphorylated in vivo and in vitro by a number of different protein kinases (Avila et al., 1988; Ohta et al., 1987; Jones and Williams, 1982; Carden et al., 1985); phosphorylation of certain cytoskeletal proteins influences their interaction with other elements of the cytoskeleton (Eyer and Leterrier, 1988; Hisanaga and Hirokawa, 1980; Yamauchi and Fujisawa, 1988). Among the protein kinases that phosphorylate bovine neurofilament proteins in vitro is protein kinase $F_A$. This is a second messenger-independent kinase that is similar if not identical to glycogen synthase kinase 3 from rabbit muscle (Guan et al., 1991), the activity of which is modified during experimental diabetes (Soderling and Sheorain, 1985). This article summarizes the results from our laboratory on changes in the glycation or phosphorylation of nerve cytoskeletal proteins in experimental diabetes in the rat.

## Methods

### Induction of Diabetes

Diabetes was induced in female Wistar albino rats by ip injection of 50 mg/kg STZ (a gift from ICI Pharmaceuticals, Macclesfield, UK) in citrate buffer. An equal number of nondiabetic age-matched rats received buffer alone. Three d after administration of STZ, some rats also received twice daily sc injections of 25 U/100 g Semitard insulin (Novo, Copenhagen, Denmark) in the morning and Mixtard in the evening; doses were adjusted to maintain nonfasting whole blood glucose levels at around 5 mmol/L. Untreated diabetic animals typically maintained blood glucose levels of 15–25 mmol/L for between 2 and 6 wk.

### Purification of Neurofilament Proteins

Diabetic and nondiabetic rats were killed, and spinal cords removed. Cords were homogenized in MES buffer (0.1 mol/L 2[*N*-morpholino] ethanesulfonic acid, pH 6.8, containing 1 m*M* EGTA and 1 m*M* $MgCl_2$), and NFs were prepared by differential centrifugation according to the method of Eyer et al. (1989). They were stored at –80°C until required.

### Preparation of Rat Brain Cytoskeletal and Microtubule Proteins and Measurement of Glycation

Rat brains were homogenized in MES buffer *(above)* and centrifuged at 75,000*g* for 1 h at 4°C. The supernatant was incubated with a half vol of 100% glycerol and 0.5 m*M* GTP for 20 min at 37°C. These first-cycle-purified cytoskeletal proteins were collected by centrifugation at 85,000g for 1.5 h through 0.8*M* sucrose in MES buffer containing 0.5 m*M* GTP. The pellet was resuspended in MES buffer containing 0.5 m*M* sucrose and 1 m*M* GTP, incubated on ice for 30 min, and centrifuged at 100,000*g* for 30 min at 4°C. The resulting supernatant contained second-cycle-purified microtubule proteins. Glycation of these microtubule proteins was measured by resuspending in 0.1 m*M* sodium phosphate buffer, pH 7.0, and treating as for sciatic nerve *(see below)*. Pig brain microtubule proteins were prepared in a similar way, except that the buffer contained 100 m*M* piperazine-*N,N'-bis*(2-ethane)sulfonic acid (PIPES) instead of MES.

### Preparation of Rat Sciatic Nerve Tubulin and Measurement of Glycation

Rat sciatic nerves were homogenized in 0.1 m*M* sodium phosphate buffer, pH 7.0. Homogenates were incubated with 1 mg sodium borohydride ($NaB^3H_4$), SA 5–20 Ci/mmol (Amersham, UK) for 40 min at 21°C and dialyzed extensively against phosphate buffer to remove unbound radioactivity. The resulting proteins were then separated by two-dimensional polyacrylamide gel electrophoresis (PAGE) according to the method of O'Farrell (1975) and as described previously (McLean, 1985). Proteins were visualized

by silver-staining (Wray et al., 1981). Spots corresponding to α- and β-tubulin (McLean, 1985) were cut from the gel, proteins eluted with 30% hydrogen peroxide ($H_2O_2$) at 55°C, and hydrolyzed by heating at 110°C for 12 h in 6*M* HCl. The resulting amino acids were dissolved in 25 m*M* sodium phosphate buffer, pH 9.0, and the radiolabeled glycated amino acids separated from nonspecific amino acid-bound radioactivity by affinity chromatography on Affi-gel 601 (Bio-Rad). Both fractions were lyophilized and resuspended in 25 m*M* phosphate buffer, pH 9.0, and the radioactivity determined by liquid scintillation counting.

## Separation and Identification of Rat Brain Actin

First-cycle-purified cytoskeletal proteins from diabetic and nondiabetic rat brains were separated by two-dimensional PAGE, as above, or subjected to DNase affinity chromatography prior to electrophoresis, to aid identification of actin. In this method, the cytoskeletal preparation was passed through a column of Sepharose 4B (Pharmacia) coupled to bovine pancreas DNase. Actin, which binds selectively to DNase, was retained on the column and eluted with 3*M* guanidine-HCl (McLean, 1985). Actin was further identified with a specific antibody C4, reactive against a highly conserved epitope of actin (Lessard, 1988). For this purpose, portions of gels containing separated actin were transferred electrophoretically to nitrocellulose paper. The resulting Western blots were blocked with 3% bovine serum albumin in Tris-buffered saline (TBS: 10 m*M* Tris and 140 m*M* NaCl, pH 7.4) and incubated with C4 followed by horseradish peroxidase-conjugated antimouse secondary antibody (DAKO Ltd.). Actin was visualized by incubation with chloronaphthol (0.6 mg/mL 4–chloronaphthol in TBS with 20% methanol and 0.02 $H_2O_2$).

In some experiments, first-cycle-purified cytoskeletal proteins were incubated with 10 m*M* glucose 6–phosphate for 24 h at 37°C prior to separation by electrophoresis.

## Preparation of Human Platelet Actin

Thirty-one patients with insulin-dependent diabetes mellitus (IDD) and 31 age- and sex-matched nondiabetic patients were identified. Blood samples were taken, and platelets extracted according to the method of Harris and Crawford (1973). Platelets were homogenized in 10 m*M* Tris buffer, pH 7.5, containing 1 m*M* $Ca^{2+}$, sonicated at 30 W for 30 s, and centrifuged at 85,000*g* for 20 min at 4°C. The supernatant fraction was subjected to DNase affinity chromatography as described above. Purity of the prepared actin was assessed by SDS-PAGE. The extent of glycation of this platelet actin was measured as for tubulin *(above)*.

## Analysis of Phosphorylation of NF Proteins In Vivo and In Vitro

NF from rat spinal cord, prepared as above, were suspended in 4% sodium dodecyl sulfate (SDS), 10% β-mercaptoethanol and 20% glycerol and separated by SDS-PAGE on 7.5% gels. Gels were stained with silver (Wray et al., 1981) or blotted on to nitrocellulose. Western blots were incubated with monoclonal antibodies RT97, which reacts with a highly phosphorylated epitope of the high-mol-wt neurofilament protein NF-H (Haugh et al., 1986), and RMd09, which reacts with NF-H in its nonphosphorylated form (Lee et al., 1988). Blots were stained and visualized with chloronaphthol, as above.

In vitro phosphorylation was measured by incubation of 0.20 mg/mL NF proteins at 37°C with 0.5 μCi $^{32}$P-ATP (SA 5 Ci/mmol), as described previously (Eyer et al., 1989). Phosphorylated proteins were identified as NF by SDS-PAGE on 7.5% gels followed by autoradiography.

In some experiments, NFs were dephosphorylated by incubation with 20 U/mL acid phosphatase (potato type II, Sigma, Poole, UK) at 37°C for 2 h. Enzyme was removed by centrifugation prior to in vitro phosphorylation. Where appropriate, endogenous NF-associated protein kinase activity was removed from NF preparations from diabetic or nondiabetic animals, by incubation

with 0.8*M* KCl, according to the method of Toru-Delbauffe and Pierre (1983), and added back to the NF from either group of rats, again prior to in vitro phosphorylation.

### Measurement of Rat Brain Microtubule Assembly In Vitro

Two cycle-purified brain microtubule proteins from diabetic and nondiabetic rats were brought to the same protein concentration. GTP was added to a final concentration of 1 m*M*, and the resulting assembly of microtubule proteins into microtubules was determined by spectrophotometry as an increase in optical density of the solution at 350 nm (Gaskin et al., 1974). In some experiments, microtubule protein preparations were incubated with 20 m*M* D-glucose for 1 h, or D-6-$^3$H-glucose for up to 24 h at 30°C prior to measurement of assembly. Microtubule proteins radiolabeled in this way were precipitated with 5% trichloroacetic acid, and the incorporated radioactivity analyzed by liquid scintillation counting.

## Results

### Glycation of Rat and Pig Brain Microtubule Proteins and Rat Peripheral Nerve Tubulin and Its Effect on Assembly

The mean blood glucose of diabetic rats 14 d after injection of STZ was 19.0 ± 6.2 m*M* compared to that of nondiabetic rats at 6.0 ± 0.6 m*M* (mean ± SD, $n = 5$). There was no change in the overall extent of glycation of rat brain microtubule proteins. Glycation, expressed as dpm/μg protein, was 50 ± 15 in nondiabetic and 42 ± 8 in diabetic rats (mean ± SD, $n = 5$).

Two cycle-purified microtubule proteins were prepared from nondiabetic and 14-d diabetic rats. The yield of microtubule proteins, which is itself a measure of the extent of polymerization, was not significantly different between diabetic and nondiabetic animals, nor was there any significant difference between the two groups in the extent of assembly as measured spectrophotometrically, in line with the lack of change in glycation in vivo (Table 1).

When pig brain microtubule preparations were incubated with D-6-$^3$H-glucose for up to 24 h there was a linear incorporation of glucose up to approx 5.5 mol/mol tubulin (Fig. 1). Incorporation of glucose for 1 h, which was the longest period of incubation possible without affecting the subsequent stability of microtubule proteins, was without effect on microtubule assembly (Cullum et al., 1991). Identical data were obtained with rat microtubule proteins.

On the other hand, when the extent of glycation of sciatic nerve tubulin was expressed as disintegrations/min (dpm) in the glycated fraction as a percentage of the total radioactivity, as shown in Fig. 2, there was a significant increase in the extent of glycation of tubulin in the sciatic nerve of rats after 14 d of STZ-diabetes ($p < 0.005$).

### Glycation of Brain Actin

Two-dimensional PAGE analysis of brain cytoskeletal proteins from diabetic and nondiabetic rats showed the presence of two main groups of proteins that were originally tentatively identified as tubulins and actin (Fig. 3a). In animals that had been diabetic for 6 wk, an extra distinct polypeptide was noted with an apparent mol wt similar to actin and with a slightly more acidic isoelectric point (Fig. 3b). An identical result was obtained from animals that had been diabetic for only 2 wk (not shown). Insulin administration throughout the 6 wk of diabetes led to the disappearance of the extra polypeptide in 6-wk (Fig. 3c), but not 2-wk diabetic animals.

The tentative identification of actin on the gels was confirmed by DNase affinity chromatography (McLean, 1985) and by staining of Western blots with the antiactin antibody C4 (Fig. 4a). The diabetes-associated polypeptide also stained with the antiactin antibody (Fig. 4b). When brain cytoskeletal proteins from nondiabetic rats were incubated with glucose-6–phosphate for 24 h and

Table 1
Mean Increase in Optical Density ± SEM
as a Measure of Assembly of Rat Brain Microtubules
Prepared from Brains of 14-d STZ-Diabetic and Nondiabetic Rats

| | OD | SEM | *n* |
|---|---|---|---|
| Nondiabetic | 0.265 | 0.051 | 7 |
| Diabetic | 0.212 | 0.069 | 5 |

Microtubule proteins were prepared from brains of nondiabetic and 14-d STZ-diabetic rats, and the assembly into microtubules was followed spectrophotometrically at 37°C in the presence of 1 m*M* GTP.

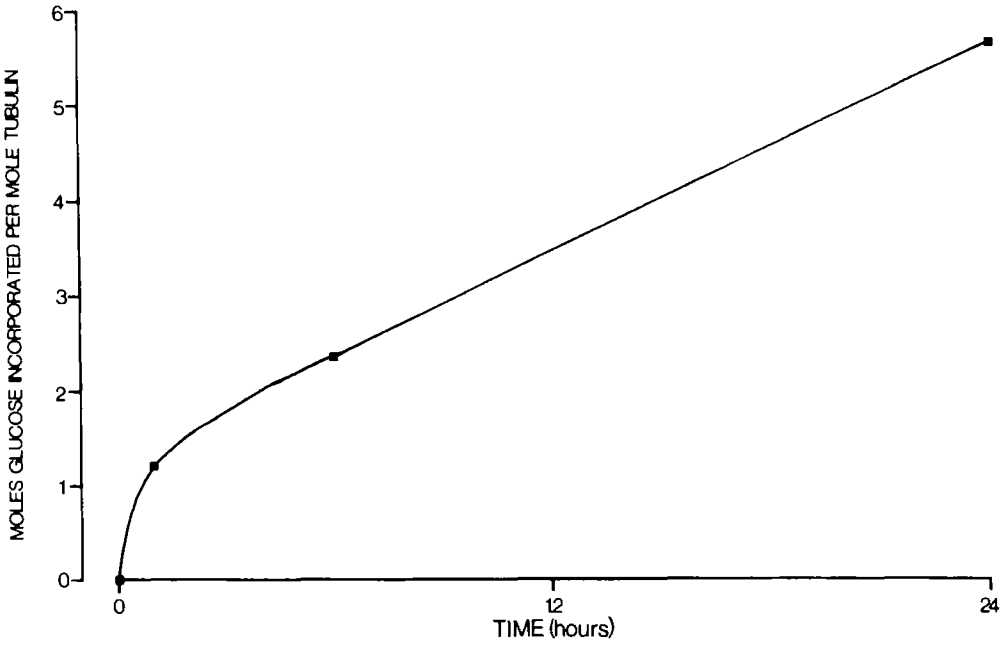


Fig. 1. Pig brain microtubule proteins were incubated with 10 mg/mL D-6-$^3$H-glucose (20 μCi/mmol) for up to 24 h at 30°C. Proteins were precipitated with 5% trichloroacetic acid and the protein-bound radioactivity analyzed by liquid scintillation counting.

separated by two-dimensional PAGE, a polypeptide similar in size and position to the one present in diabetic rat preparations appeared close to actin (Fig. 5). This polypeptide stained in an identical manner with antiactin antibody (not shown).

## Glycation of Human Platelet Actin

Actin from platelets of diabetic patients was significantly ($p < 0.05$) more glycated than that of matched control patients when all diabetic subjects were analyzed. The values, expressed as dpm/μg protein, were 88 ± 17 for nondiabetic subjects, compared with 125 ± 18 for diabetic subjects (mean ± SEM, $n = 48$).

## Phosphorylation of Rat Spinal Cord NF Proteins In Vivo and In Vitro

Figure 6 shows immunoblots of NF proteins from diabetic and nondiabetic rats stained with monoclonal antibodies to phosphorylated and nonphosphorylated forms of NF-H. The position

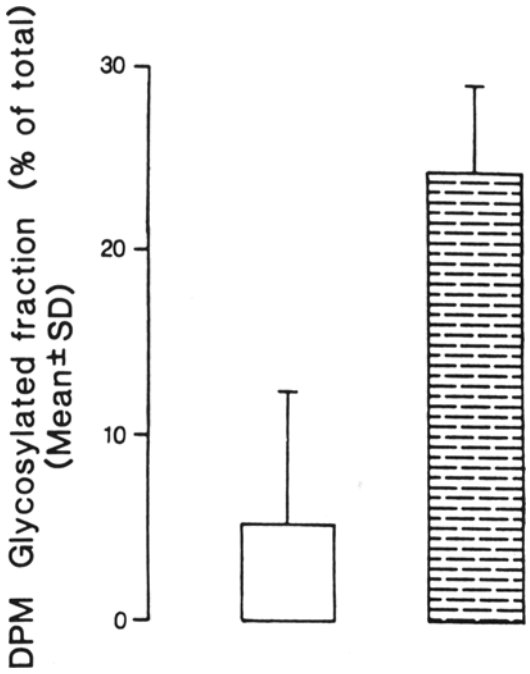


Fig. 2. Tubulin was purified from nondiabetic << and SlZ-diabetic⊟ rat sciatic nerve by two-dimensional PAGE, reduced with $NaB^3H_4$ and the glycated and nonglycated amino acids separated by phenylboronic acid chromatography. Glycation is expressed as dpm eluted in the fraction containing glycated amino acids, as a percentage of the total bound radioactivity. Results are means ± SD, $n = 5$ in each group.

of the more heavily stained band corresponded to a mol wt of 200 kDa when compared with mol wt markers run simultaneously (not shown). NF-H from diabetic rats was recognized more by the phosphate-dependent antibody than NF-H from nondiabetic rats. The opposite was true for the phosphate-independent antibody. The other, less heavily stained bands in Fig. 6 are believed to be NF-M (Fig. 6B) and a breakdown product of NF-H (Fig. 6A) (Pekiner and McLean, 1991).

If the same phosphorylation sites were involved, one would expect that a hyperphosphorylation in vivo would result in a reduced phosphorylation in vitro, i.e., the sites that already contained phosphate would be less likely to be radiolabeled when incubated with radiolabeled

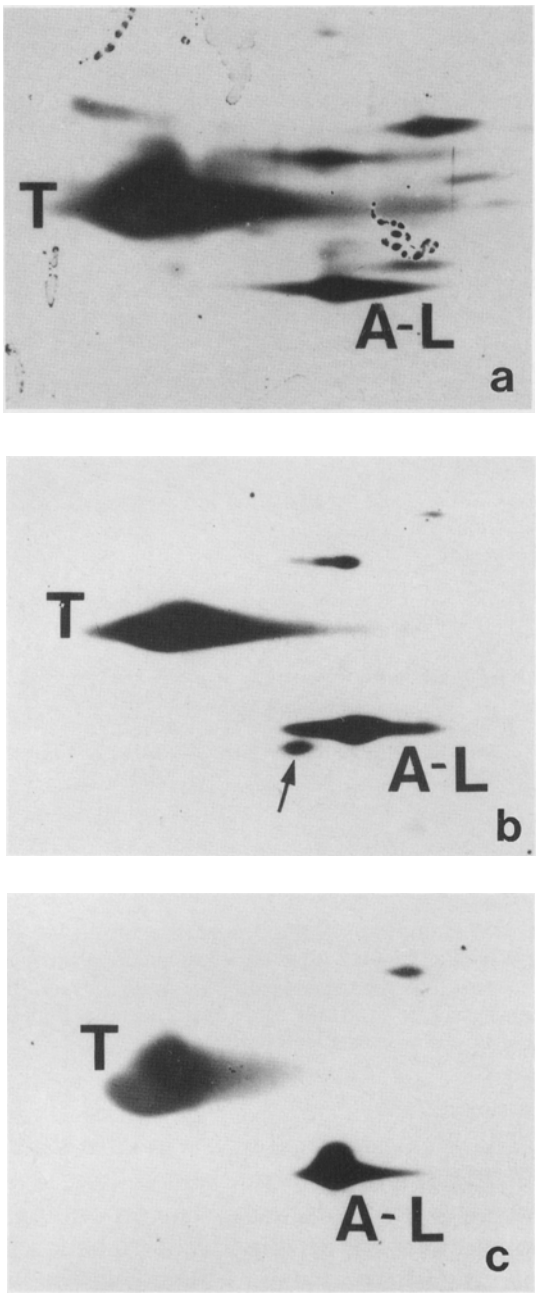


Fig. 3. Brain cytoskeletal proteins were prepared from (a) nondiabetic, (b) 6-wk STZ-diabetic rats, and (c) 6-wk STZ-diabetic rats that had been treated with insulin. Proteins were separated by PAGE on a 5–7% p*I* gradient in the first dimension and on a 6–14% gradient gel in the second dimension, and stained with silver. Tubulins (T) and the actin-like protein (A-L) are marked; the arrow in (b) points to the actin-related polypeptide found only in diabetic rats.

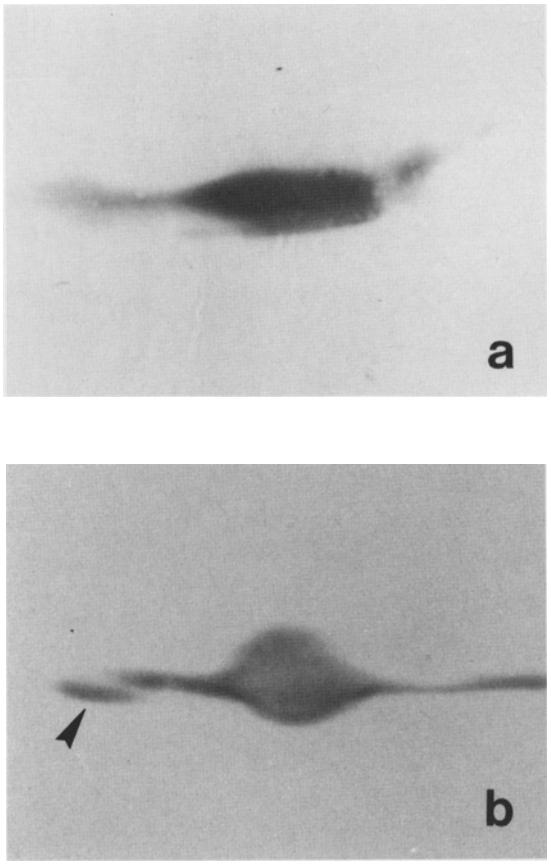


Fig. 4. Western blots of the preparations from Fig. 3(a) and (b) were incubated with antiactin monoclonal antibody C4, and reactive proteins visualized with horseradish peroxidase/chloronaphthol. Note in (b) that the diabetes-associated polypeptide stains with C4.

ATP. This was confirmed by the in vitro studies (Fig. 7), in which at 6 wk of diabetes there was a significant ($p < 0.05$) decrease in incorporation of phosphate into NF proteins from diabetic rats, compared with nondiabetics. Acid phosphatase pretreatment partially prevented this decrease in in vitro phosphorylation. However, extraction of protein kinase(s) from NF from diabetic rats and addition to NF from nondiabetic rats also resulted in some decrease in in vitro phosphorylation, implying that there was also a deficiency in the NF-associated kinase (for detailed results *see* Pekiner and McLean, 1991).

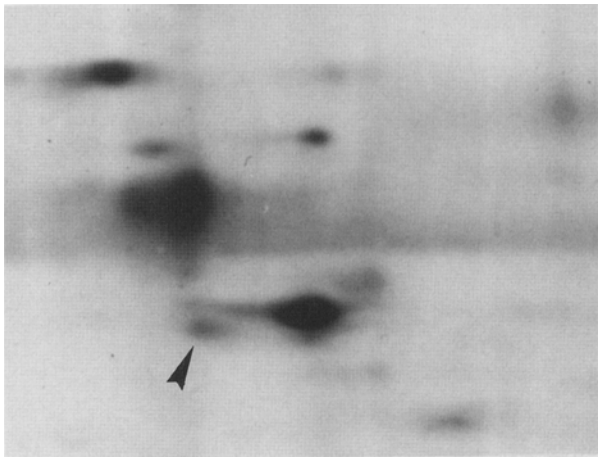

Fig. 5. Brain cytoskeletal proteins from nondiabetic rats were incubated with 10 m*M* glucose-6-phosphate for 24 h. Two-dimensional gels of the proteins were stained with silver. The actin-related polypeptide appeared in the glucose-6-phosphate-treated preparations, but not in preparations incubated without glucose-6-phosphate, which were identical to the one shown in Fig. 3(a).

## Discussion

We could find no evidence for an increased glycation of microtubule proteins in the brains of diabetic rats, or for an alteration in the ability of microtubule proteins to form microtubules in vitro, either from diabetic rats or after incubation with glucose. This was despite the fact that glucose could be incorporated into microtubule proteins over the time of incubation. Clearly, incubation with glucose over such a short time period does not simulate more than the very earliest stages of in vivo glycation, which involves both Schiff base formation and subsequent Amadori rearrangement to form a stable product (Brownlee et al., 1984).

The main reason for publicizing this negative data is the existence of a report by Williams et al. (1982), that showed an increased glycation of rat brain microtubule protein in STZ-diabetes and implied that even the early stages of glycation impair microtubule assembly. We have been unable to reproduce their findings in 2-wk

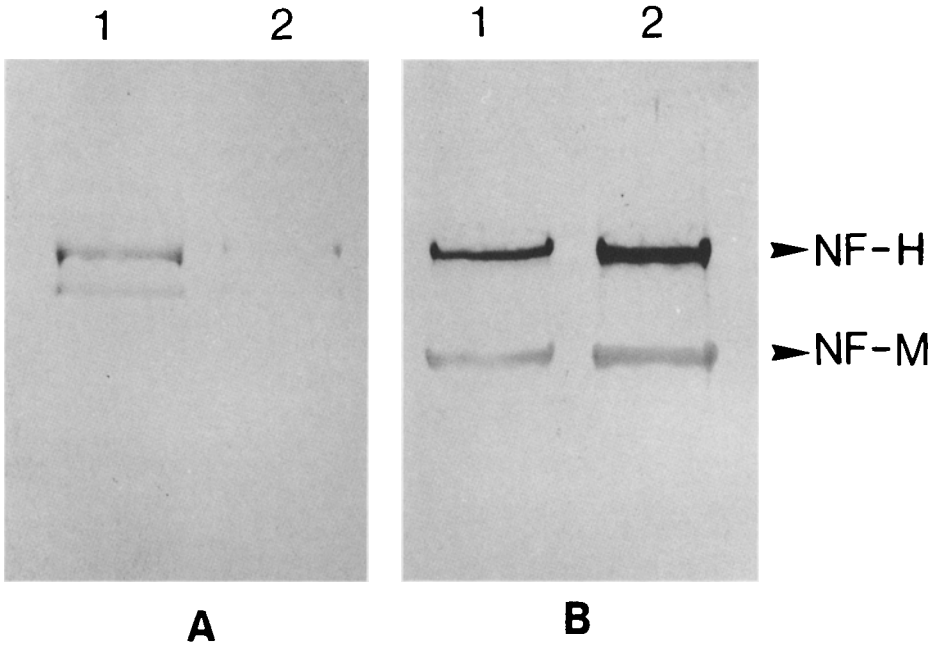


Fig. 6. NF proteins from nondiabetic (lane 1) and 6-wk STZ-diabetic (lane 2) rats were separated by SDS-PAGE on 7.5% gels. Western blots were incubated with (A) antibody RMd09 against predominantly nonphosphorylated NF-H, or (B) antibody RT97 against phosphorylated NF-H. Proteins were visualized with horseradish peroxidase/chloronaphthol.

diabetic rats. A recent report by Eaker et al. (1991), in which the experimental procedures of Williams et al. were more closely followed, showed equally negative data. Taken together, these experiments cast serious doubt on the role of glycation in modifying brain microtubule structure and function in experimental diabetes.

Nevertheless, the hypothesis that microtubule proteins may be altered by glycation in diabetes remains attractive, and our findings that glycation of rat sciatic nerve tubulin is increased in STZ-diabetes suggest that this may contribute to peripheral neuropathy. The integrity of axonal microtubules is known to be important for the maintenance of fast axonal transport (Banks and Till, 1975; Schnapp et al., 1985). Although the evidence that fast axonal transport is altered in experimental diabetes (Meiri and McLean, 1982) has been disputed (Whiteley et al., 1985; Abbate et al., 1991), we would still propose that tubulin glycation may contribute to the more clearly defined changes in slow transport (McLean and Meiri, 1981; Medori et al., 1988; Macioce et al., 1989; Vitadello et al., 1985; Larsen and Sidenius, 1989).

We also failed to find any evidence of altered electrophoretic mobility of brain tubulin in the one cycle-purified brain cytoskeletal preparation (Fig. 3) after up to 6 wk of diabetes. The most obvious change in these preparations was, however, the altered mobility of a component of brain actin that was apparently related to its glycation in an insulin-dependent manner. The apparent anomaly in the ability of insulin administration to prevent the appearance of the glycated actin after 6 wk, but not after 2 wk may be explained by the fact that insulin was only administered to the rats at the third d after injection of STZ, at a time when blood glucose would already be elevated. If the initial stages of glycation had already started by then (i.e., attachment of glucose), it is possible that they would proceed to completion

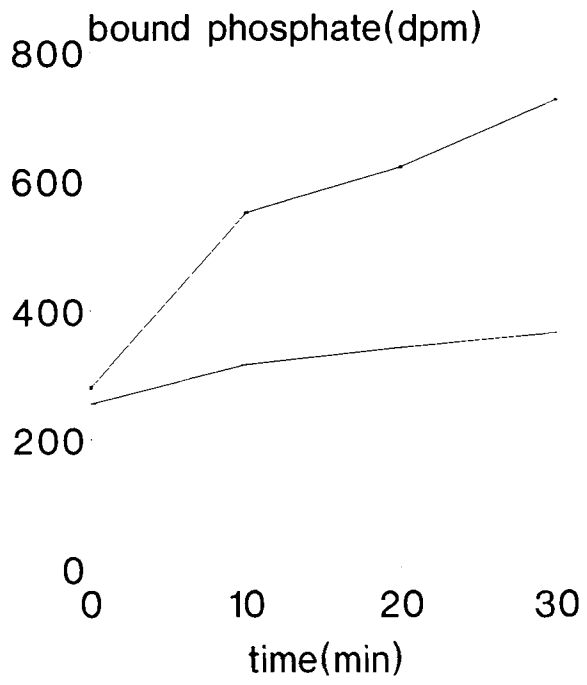


Fig. 7. Incorporation of $^{32}P$ into NF proteins from nondiabetic (•) and 6-wk STZ-diabetic (+) rats. NF proteins were incubated with $^{32}P$-ATP and the protein-bound radioactivity assayed at the times shown. Results are mean values from seven animals in each case.

even after lowering of blood glucose to normal levels. In the 6-wk diabetic animals, however, protein turnover could have taken place during the course of the insulin regime.

We know of no other evidence for glycation of actin. Actin is a major slowly transported protein in neurones (Hoffman and Lasek, 1975; Wray et al., 1981) as a component of the cytoplasmic matrix (Stossel, 1984; Faith and Lasek, 1988) and plays some role in transport mechanisms (Brady et al., 1984). How glycation would affect actin function is unclear, although unpublished work from this laboratory suggests that glycation in vivo does not influence the polymerization of G-actin to F-actin.

Owing to the inaccessibility of brain cytoskeleton from clinical diabetic patients, we investigated the glycation of platelet actin. The increased glycation we observed (*see* Results) is consistent with the observation of increased glycation of platelet protein in clinical diabetes (Sampietro et al., 1986), the importance of which has been discussed in detail (Yatscoff et al., 1987). Clearly, more work is necessary on the consequences of glycation for the structure and function of cytoskeletal proteins before further conclusions can be drawn.

The importance of the other posttranslational modification that we have examined, phosphorylation, is more apparent. NFs are phosphorylated by a large number of protein kinases, including $Ca^{2+}$-calmodulin-dependent protein kinase, microtubule-associated cyclic AMP-dependent kinase (Leterrier et al., 1981; Vallano, et al. 1985), protein kinase A, and protein kinase C (Sihag and Nixon, 1990), as well as the second messenger-independent NF-associated kinase, that is the main kinase involved in the in vitro phosphorylation observed in our work (Eyer and Leterrier, 1988; Eyer et al., 1989; Julien and Mushynski, 1981). Whatever the nature of the kinase that leads to the observed in vivo hyperphosphorylation, the in vitro studies would imply that the sites that are phosphorylated by the NF-associated kinase are altered in the diabetic state. This kinase is known to lead to phosphorylation of the carboxy terminal "tail" regions of the NF proteins (Sihag and Nixon, 1989,1990), which project outward from the core of the NF and are believed to be involved in the interactions between NFs and other elements of the cytoskeleton (Carden et al., 1985; Julien and Mushyski, 1982; Nixon and Sihag, 1991). A disruption of this interaction in experimental diabetes is likely to lead to a change in the normal relationship between cytoskeletal proteins in slow axonal transport and be manifested as a slowing of transport of NF proteins, as seen in radiolabeling studies (McLean and Meiri, 1981; Medori et al., 1988; Macioce et al.,1989; Vitadello et al., 1985). Since one of the main roles of NF is to maintain the caliber of large axons (Hoffman et al., 1985,1987), a disruption of the normal spatial organization of NF within the axon may lead to the impairment in radial growth of axons seen in diabetes (Iturriaga, 1985; Jakobsen and Sidenius,

1985). It is interesting to note that the inhibition of slow axonal transport resulting from intoxication of rats with β,-β'iminodipropionitrile (IDPN) is also associated with NF hyperphosphorylation (Watson et al., 1989), but in that case in vitro phosphorylation is increased (Eyer et al., 1989).

Altered activity of NF-associated kinase is consistent with the observation that this kinase leads to a pattern of phosphorylation of NF identical to that found with kinase $F_A$ or glycogen synthase kinase 3. The muscle substrate for this enyme, glycogen synthase, is hyperphosphorylated in vivo in alloxan-diabetic rabbits, with an associated decreased in vitro phosphorylation by the purified enzyme (Guan et al., 1991; Soderling and Sheorain, 1985). That work draws attention to the involvement of protein phosphatases, along with protein kinases and ATPases, in the regulation of phosphorylation. Our own studies are currently addressing the activities of these other enymes in experimental diabetes.

## Conclusion

Experimental diabetes leads to changes in posttranslational modifications of proteins in the nervous system. These include increased glycation of peripheral nerve tubulin and brain actin, and hyperphosphorylation of spinal cord neurofilament proteins. It is suggested that the altered activity of these proteins may contribute to the impairment of axonal transport and morphological alterations in neurons that are characteristic of experimental diabetic neuropathy.

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## References

Abbate S. L., Atkinson S. B., and Brueur A. C. (1991) Amount and speed of fast axonal transport in diabetes. *Diabetes* **40**, 117.

Avila J., Serrano L., Hernandez M. A., and Diaz-Nido J. (1988) Phosphorylation of neuronal microtubule proteins. *Protoplasma* **145**, 82–88.

Banks P. and Till R. (1975) A correlation between the effects of antimitotic drugs on microtubule assembly in vitro and the inhibition of axonal transport in noradrenergic neurones. *J. Physiol. (Lond.)* **254**, 283–294.

Brady S. T., Lasek R. J., Allen R. D., Yin H. -L., and Stossel T. P. (1984) Gelsolin inhibition of fast axonal transport indicates a requirement for actin microfilaments. *Nature* **310**, 56–57.

Brownlee M., Vlassara H., and Cerami A. (1984) Nonenzymatic glycosylation and the pathogenesis of diabetic complications. *Ann. Int. Med.* **101**, 527–537.

Carden M. J., Schlaepfer W. W., and Lee V. M.-Y. (1985) The structure biochemical properties and immunogenicity of neurofilament peripheral regions are determined by phosphorylation state. *J. Biol. Chem.* **260**, 9805–9817.

Cohen M. P. (1986) *Diabetes and Protein Glycosylation: Measurement and Biologic Relevance.* Springer, New York.

Cullum N. A., Mahon J., Stringer K., and McLean W. G. (1991) Glycation of rat sciatic nerve tubulin in experimental diabetes mellitus. *Diabetologia* **34**, 387–389.

Eaker E. Y., Angelastro J. M., Purich D. L., and Sninsky C. (1991) Evidence against impaired brain microtubule protein polymerization at high glucose concentrations or during diabetes mellitus. *J. Neurochem.* **56**, 2087–2093.

Eyer J. and Leterrier J. F. (1988) Influence of the phosphorylation state of neurofilament proteins on the interactions between purified filaments in vitro. *Biochem. J.* **252**, 655–660.

Eyer J., McLean W. G., and Leterrier J. F. (1989) Effect of a single dose of iminodipropionitrile in vivo on the properties of neurofilaments in vitro: comparison with the effect of iminodipropionitrile added directly to neurofilaments in vitro. *J. Neurochem.* **52**, 1759–1765.

Faith KR. and Lasek R. J. (1988) Two classes of actin microfilaments are associated with the inner cytoskeleton of axons. *J. Cell Biol.* **107**, 613–622.

Gabbay R. A., Siconolfi-Baez L., and Lebovitz H. E. (1990) Calcium-dependent protein kinase activity is decreased in diabetic sciatic nerve. *Endocrinology* **127**, 1194–1198.

Gaskin F., Cantor C. R., and Shelanski M. L. (1974) Turbidimetric studies of the in vitro assembly and disassembly of porcine neurotubules. *J. Mol.Biol.* **89**, 737–755.

Greene D. A. and Lattimer S. A. (1986) Protein kinase C agonists normalize decreased ouabain-inhibitable respiration in the diabetic rabbit nerve: implications for $Na^+$ $K^+$-ATPase regulation and diabetic complications. *Diabetes* **33**, 712–6.

Guan R. J., Khatra B. S., and Cohlberg J. A. (1991) Phosphorylation of bovine neurofilament proteins by protein kinase $F_A$ (glycogen synthase kinase 3) *J. Biol. Chem.* **266**, 8262–8267.

Harris G. L. A. and Crawford N. (1973) Isolation of pig platelet membranes and granules. Distribution and validiy of marker enzymes. *Biochim. Biophys. Acta* **291**, 701–709.

Haugh M. C., Probst A., Ulrich J., Kahn J., and Anderton B. H. (1986) Alzheimer neurofibrillary tangles contain phosphorylated and hidden neurofilament epitopes. *J. Neurol. Neurosurg. Psychiat.* **49**, 1213–1220.

Hisanaga S. and Hirokawa N. (1980) Dephosphorylation-induced interactions of neurofilaments with microtubules. *J. Biol. Chem.* **265**, 21852–21858.

Hoffman P. N. and Lasek R. J. (1975) The slow component of axonal transport. Identification of major structural polypeptides of the axon and their generality among mammalian nerves. *J. Cell Biol.* **66**, 351–366.

Hoffman P. N., Griffin J. W., and Price D. L. (1985) Control of axon caliber by neurofilament transport. *J. Cell Biol.* **101**,1332–1340.

Hoffman P. N., Cleveland D. W., Griffin J. W., Landes P. W., Cowan N. J., and Price D. L. (1987) Neurofilament gene expression: a major determinant of axonal caliber. *Proc. Natl. Acad. Sci. USA* **84**, 3472–3476.

Iturriaga R. (1985) Microtubule density and size of axons in early diabetes: implications for nerve cell homeostasis. *Exp. Neurol.* **88**,165–175.

Jakobsen J. and Sidenius P. (1985) Nerve morphology in experimental diabetes. *Clin. Physiol.* **5(Suppl 5)**: 9–13.

Jones S. M. and Williams R. C. Jr. (1982) Phosphate content of mammalian neurofilaments. *J. Biol. Chem.* **257**, 9902–9905.

Julien J. P. and Mushynski W. E. (1981) A comparison of in vitro and in vivo phosphorylated neurofilament polypeptides. *J. Neurochem.* **37**,1579–1585.

Julien J. P. and Mushynski W. E. (1982) Multiple phosphorylation sites in mammalian neurofilament polypeptides. *J. Biol. Chem.* **257**,10467–10470.

Larsen J. R. and Sidenius P. (1989) Slow axonal transport of structural polypeptides in rat early changes in streptozotocin diabetes and effect of insulin treatment. *J. Neurochem.* **52**, 390–401.

Lee V. M.-Y., Otvos L. Jr., Carden M. J., Hollosi M., Dietzschold B., and Lazzarini R. (1988) Identification of the major multiphosphorylation site in mammalian neurofilaments. *Proc. Natl. Acad. Sci. USA* **85**, 1998–2002.

Lessard J. L. (1988) Two monoclonal antibodies to actin: one muscle selective and one generally reactive. *Cell Motil. Cytoskel.* **10**, 349–362.

Leterrier J. F., Liem R. K. H., and Shelanski M. L. (1981) Preferential phosphorylation of the 15,0000 molecular weight component of neurofilaments by a cyclic AMP-dependent microtubule-associated protein kinase. *J. Cell Biol.* **90**, 755–760.

Macioce P., Filliatreau G., Figliomeni B., Hassig R., Thiery J., and Di Giamberardino L. (1989) Slow axonal transport impairment of cytoskeletal proteins in streptozotocin-induced diabetic neuropathy. *J. Neurochem.* **53**, 1261–1267.

McLean W. G. and Meiri K. F. (1981) Slow axonal transport of protein in rat sciatic nerves during early experimental diabetes. *J. Physiol. (Lond.)* **310**, 57.

McLean W. G., McKay A. L., and Sjostrand J. (1983) Electrophoretic analysis of axonally transported proteins in rabbit vagus nerve. *J. Neurobiol.* **14**, 227–236.

McLean W. G. (1985) Axonal transport and regeneration rate in nonmyelinated sensory nerve fibres. *Brain Res.* **333**, 255–260.

McLean W. G., Frizell M., and Sjostrand J. (1985) Pathology of axonal transport, in: Lajtha A. (ed.) *Handbook of Neurochemistry*, vol. 9. Plenum, New York pp. 67–86.

Medori R., Jenich H., Autilio-Gambetti L., and Gambetti P. (1988) Experimental diabetic neuropathy: similar changes of axonal transport and axonal size in different animal models. *J. Neurosci.* **8**, 1814–1821.

Meiri K. F. and McLean W. G. (1982) Axonal transport of protein in motor fibres of experimentally diabetic rats-fast anterograde transport. *Brain Res.* **238**, 77–82.

Mellado W., Slebe J. C., and Maccioni R. B. (1982) Tubulin carbamoylation. Functional amino groups in microtubule assembly. *Biochem. J.* **203,** 675–681.

Nixon R. and Sihag R. K (1991) Neurofilament phosphorylation: a new look at regulation and function. *Trends Neurosci.* **14,** 501–506.

O'Farrell P. H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250,** 4007–4021.

Ohta Y., Akiyama T., Nishida E., and Sakai H. (1987) Protein kinase C and cAMP-dependent protein kinase induce opposite effects on actin polymerizability. *FEBS Lett.* **222,** 305–310.

Pekiner C. and McLean W. G. (1991) Neurofilament phosphorylation in spinal cord of experimentally diabetic rats. *J. Neurochem.* **56,** 1362–1367.

Sampietro T., Lenzi S., Cecchetti P., Giampetro O., Cruschelli L., and Navales R. (1986) Nonenzymatic glycation of human platelet membrane proteins in vitro and in vivo. *Clin. Chem.* **32,** 1328–1331.

Schnapp B. J., Vale R. D., Sheetz M. P., and Reese T. S. (1985) Single microtubules from squid axoplasm support bidirectional movement of organelles. *Cell* **40,** 455–462.

Schrama L. H., Berti-Mattera L. N., and Eichberg J. (1987) Altered protein phosphorylation in sciatic nerves from rats with streptozotocin-induced diabetes. *Diabetes* **36,**1254–67.

Sidenius P. and Jakobsen J. (1979) Axonal transport in early experimental diabetes. *Brain Res.* **173,** 315–330.

Sihag R. K. and Nixon R. A. (1989) In vivo phosphorylation of distinct domains of the 70kilodalton neurofilament subunit involves different protein kinases. *J. Biol. Chem.* **264,** 457–464.

Sihag R. K. and Nixon R. A. (1990) Phosphorylation of the amino-terminal head domain of the middle molecular mass 145kDa subunit of neurofilaments. *J. Biol. Chem.* **265,** 4166–4171.

Simpson C. M. F. and Hawthorne J. N. (1988) Reduced $Na^+$-$K^+$-ATPase activity in peripheral nerve of streptozotocin-diabetic rats—a role for protein kinase C. Diabetologia **31,** 297–303.

Soderling T. R. and Sheorain V. S. (1985) Skeletal muscle glycogen synthase: hormonal regulation, in: Czech M. P. (ed.) *Molecular Basis of Insulin Action.* Plenum, New York, pp. 235–245.

Stossel T. P. (1984) Contribution of actin to the structure of the cytoplasmic matrix. *J. Cell Biol.* **99,** 15S-21S.

Szasz J., Burn R., and Sternlicht H. (1982) Effects of reductive methylation on microtubule assembly. *J. Biol. Chem.* **257,** 3697–3704.

Tomlinson D. R. and Mayer J. H. (1984) Defects of axonal transport in diabetes mellitus—a possible contribution to the aetiology of diabetic neuropathy. *J. Autonom. Pharmac.* **6,** 59–72.

Toru-Delbauffe D. and Pierre M. (1983) A rat brain protein kinase specifically phosphorylating neurofilaments. *FEBS Lett.* **162,** 230–234.

Vallano M. L., Buckholz T. M., and Delorenzo R. J. (1985) Phosphorylation of neurofilament proteins by endogenous calcium/calmodulin-dependent protein kinase. *Biochem. Biophys. Res. Commun.* **130,** 957–963.

Vitadello M., Filliatreau G., Dupont J., Hassig R., Gorio A., and Di Giamberardino L. (1985) Altered axonal transport of cytoskeletal proteins in the mutant diabetic mouse. *J. Neurochem.* **45,** 860–868.

Vlassara H., Brownlee M., and Cerami A. (1981) Nonenzymatic glycosylation of peripheral nerve proteins in diabetes mellitus. *Proc. Natl. Acad. Sci. USA* **78,** 5190–5192.

Watson D. F., Griffin J. W., Fittro K. P., and Hoffman P. N. (1989) Phosphorylation-dependent immunoreactivity of neurofilaments increases during axonal maturation and iminodipropionitrile intoxication. *J. Neurochem.* **53,** 1818–1829.

Whiteley S. J., Townsend J., Tomlinson D. R., and Brown M. (1985) Fast orthograde axonal transport in sciatic motoneurones and nerve temperature in streptozotocin-diabetic rats. *Diabetologia* **28,** 847–851.

Williams S. K., Howarth N. L., Devenny J. J., and Bitensky M. W. (1982) Structural and functional consequences of increased tubulin glycosylation in diabetes mellitus. *Proc. Natl. Acad. Sci. USA* **79,** 6546–6550.

Wray W., Boulikas T., Wray V. P., and Hancock R. (1981) Silver-staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118,** 197–203.

Yamauchi T. and Fujisawa H. (1988) Regulation of the interaction of actin filaments with microtubule-associated protein 2 by calmodulin-dependent protein kinase II. *Biochim. Biophys. Acta* **968,** 77–85.

Yatscoff R. W., Mehta A., Gerrard J. M., and Thliveris J. (1987) Glycation of platelet protein in diabetes mellitus: lack of correlation with platelet function. *Clin. Biochem.* **20,** 359–363.