Acta Neuropathol (Berl) (1981) 53:99–106

Acta Neuropathologica

Morphological and Morphometric Study of Peripheral Nerves from Rats with Streptozotocin-induced Diabetes Mellitus*

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Summary. One year after beginning of the experiment seven streptozotocin-injected Wistar rats and seven controls were fixed by whole-body perfusion, the nervus radialis was dissected and processed for light and electron microscopy. After light-microscopic study standard photographs of nerve cross sections were measured by means of a semiautomatic image analyzer. The following measurements were obtained: (1) surface of fibers, axons, and myelin sheaths; (2) ratio of myelin to axon surface; and (3) percent of endoneural space. Group means and standard errors were calculated, and cumulated class distributions were made. Ultrathin sections from all animals considered morphometrically were studied qualitatively for ultrastructural changes. The quantitative study revealed in the diabetics reduction of average myelin surface, increase of endoneural space, and reduction of myelin/axon ratio. The main ultrastructural findings were lesions of Schwann and mesenchymal cells, followed by less frequent and less severe changes in axons and endothelium. These results suggest a primary Schwann cell lesion was responsible for the observed myelin reduction.

Key words: Peripheral nerve – Rat – Streptozotocin diabetes – Morphometry – Morphology

It is well known that many diabetic patients suffer from a manifest neuropathy. Further, subclinical neurologic disturbances, generally consisting in a reduced conductivity of peripheral nerves, can be evidenced in practically all persons affected by diabetes. Neuropathy, therefore, according to the reviews by Bischoff (1977) and by Clements (1979), can be regarded as a frequent complication of diabetes mellitus. These functional disturbances may be accompanied by structural changes, usually described as axonal degeneration, segmental demyelination, Schwann cell lesions, and vasculopathy (Bischoff 1977; Clements 1979).

A reduced velocity of conduction could also be measured in nerves from animals with experimental diabetes (Sharma and Thomas 1974). Light-microscopic studies on peripheral nerves of experimental models for diabetes have repeatedly appeared in the literature, but with great disparity of results. Findings range from axonal dwindling, enlargement of endoneural space, reduction of nerve fiber diameter (Jakobsen 1976a, b; Powell et al. 1977; Jakobsen 1978; Clements 1979; Yagihashi et al. 1979) to no change at all (Sharma and Thomas 1974). This may in some cases result from differences in experimental conditions, such as duration of diabetes, type and number of animals and, for morphometric studies, methods of quantitation. As a consequence, comparisons among the various results are often difficult or impossible.

Ultrastructurally, different types of lesion have been reported in animals under various experimental conditions. Various lesions have been found, including duplication of basal lamina of vasa nervorum and Schwann cells, occurrence of cytoplasmic lamellar phospholipids in Schwann cells, degeneration of myelin, demyelination and remyelination, axonal degeneration, deposition of glycogen in several neural structures (Powell et al. 1977; Clements 1979; Yagihashi et al. 1979), and presence of endoneural edema (Jakobsen 1978). Other authors failed to detect any ultrastructural change (Sharma and Thomas 1974).

The above divergence of results and the lack of a morphologic and morphometric study of diabetes of long duration (12 months) induced by streptozotocin in prepuberal rats prompted us to untertake the experiments here reported.

* Supported by the Schweizer Nationalfonds grants nos. 3.198-0.77 and 3.552-0.79

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0001-6322/81/0053/0099/$1.60

Material and Methods

Male Wistar rats, Füllinsdorf strain, initially 100 g body weight (BW), were maintained two in a cage, under constant temperature and light conditions. Diabetes was induced by injecting into the tail vein, after 12 h of fasting, streptozotocin, at the dose of 8 mg/100 g BW, dissolved in Na-citrate buffered saline, pH 4.5. Control animals were not injected. One week later, blood glucose was measured (Dextrostix, Ames) and animals with levels of 175 mg/100 ml or more were considered diabetic. After 12 months the experiment was terminated. Under urethane anesthesia (150 mg/100 g BW), final blood samples were obtained from the jugular vein for glucose determination by a full enzymatic method (Boehringer, Mannheim, FRG). Immediately afterwards, the rat's abdomen was opened, the aorta cannulated at the iliac bifurcation, rinsed for 90 s with Ringer-procain (0.1%)-heparin (0.003%) solution, then the whole body perfused with 2% glutaraldehyde 1.5% paraformaldehyde K-phosphate buffered (pH 7.4) fixative solution (Rossi 1975). After 10 min perfusion (pressure 110 mm Hg) one forelimb was isolated, skinned, and maintained in the fixative solution for at least an additional 48 h.

Subsequently, under a stereomicroscope, the nervus radialis was carefully dissected free from the surrounding tissues, cut in comparable 1 – 2 mm long segments, rinsed, postfixed for 2 h in 2% S-collidin-buffered (pH 7.4) OsO_4 solution, dehydrated, infiltrated, and embedded in Spurr's low viscosity medium. Semithin and ultrathin sections were cut from the hardened blocks with an ultramicrotome (OmU2, Reichert) and stained with toluidine blue and with uranyl acetate and lead citrate, respectively.

After standard light-microscopic study, from semithin sections from one nerve of each animal, 12 randomized photomicrographs ($\times$400, oil immersion) were taken and negatives were projected (final magnification $\times$2,900) on the measuring tablet of a semiautomatic image analyzer (MOP AM-02, Kontron) interfaced with a table computer (HP-9815 A-001, Hewlett-Packard). By analyzing each nerve fiber present in the 12 fields (for a total of 250 – 350 fibers per rat) the following measurements were obtained: (1) cross section surface of fiber, axon, and myelin sheath; (2) ratio of myelin to axon surfaces; and (3) surface of endoneurium. After all measurements from one animal were achieved, the computer calculated averages ($\pm$ SD) per animal and made class distributions (20 classes). Seven controls and seven diabetic animals were studied morphometrically, group means and standard errors (SEM) were calculated, and cumulated distributions into classes were made. Significance of differences between groups was tested by means of analysis of the variance (sub-sampling).

Ultrathin sections from all animals considered morphometrically were studied qualitatively for ultrastructural changes by means of an electron microscope (EM 300, Philips).

Results

The light-microscopic study of toluidine blue-stained longitudinal and cross sections of the nerves (Fig. 1 a, b) from diabetic rats revealed, as compared to controls, a reduction in width of myelin sheaths and an increase in the endoneural space. No appreciable axonal change or demyelinated axon was observed.

By morphometric analysis it was found (Tables 1 – 3) that the surface of a nerve fiber in cross section, which in controls averages $95.4 \pm 19.6\,\mu m^2$, significantly ($P < 0.01$) decreases to $67.9 \pm 10.6\,\mu m^2$ in diabetics. This decrease clearly results from a reduction in thickness of the myelin sheath. The myelin surface in cross section, which measures $65.6 \pm 15.8\,\mu m^2$ in controls, is reduced significantly ($P < 0.002$) to $38.4 \pm 7.8\,\mu m^2$ in diabetics. The surfaces of axonal cross sections, on the contrary, were $29.9 \pm 4.7\,\mu m^2$ in controls and $29.5 \pm 3.3\,\mu m^2$ in diabetics, i.e., not significantly different. As a consequence, the surface ratio of myelin to axon, which was found to be 2.6 ± 0.4 in controls, was significantly ($P < 0.0001$) reduced to 1.3 ± 0.2 in diabetics. The endoneural space compensates for the loss of myelin; for unit surface of nerve cross section was 25.9% in the controls and became 42.1% in the diabetics. This increase is also significant ($P < 0.0001$).

The distribution in classes discloses in the diabetic group an increase in percentage of the smallest fibers (Fig. 2) and a strong increase in percentage of the small and decrease in the large myelin sheath surfaces (Fig. 4). Distribution of axon surfaces (Fig. 3) is practically the same in the two groups. As a consequence, in the diabetic rats the ratio of myelin to axon surface (Fig. 5) low values greatly outnumber high values.

By electron microscopy in all diabetic animals we observed changes in the Schwann cells (Fig. 7 a – d). Frequently, the ergastoplasm was dilated and contained granular material of low electron density (Fig. 7 a). In addition, almost all cells contained lipids, in structures delimited by unit membrane. Sometimes the lipids were homogeneous and of low electron density (Fig. 7 b), or appeared as myelin figures, with a periodical lamellar structure comparable to that of myelin (Fig. 7 c, d). Small amounts of lipids with low electron density could be exceptionally observed also in Schwann cells of controls.

Mesenchymal cells, filled with lipid containing vacuoles, comparable to those observed in Schwann cells, were frequently seen in diabetic animals (Fig. 7 e). These cells, found either disseminated among the nervous fibers or in perivascular position, sometimes bore clear signs of degeneration, such as degranulation and dilatation of the ergastoplasm (Fig. 7 f). In fibroblasts or endoneural mesenchymal cells of controls we very rarely saw intracytoplasmic lipids.

Lesions in axons were much less frequent than in Schwann and mesenchymal cells. They could be found both in diabetics and, with lesser frequency and severity, in controls. Glycogen accumulations, sometimes only partially delimited by unit membrane, were found to occupy most of the axonal cross section (Fig. 8 a). Penetration of the axonal cytoplasm by the Schwann cell was also seen. This results in invagination of the axolemma with sequestration of portions of the axonal cytoplasm, which often contain degenerating organelles (Fig. 8 b, c). The sequestrated axoplasm may further degenerate (Fig. 8 d).

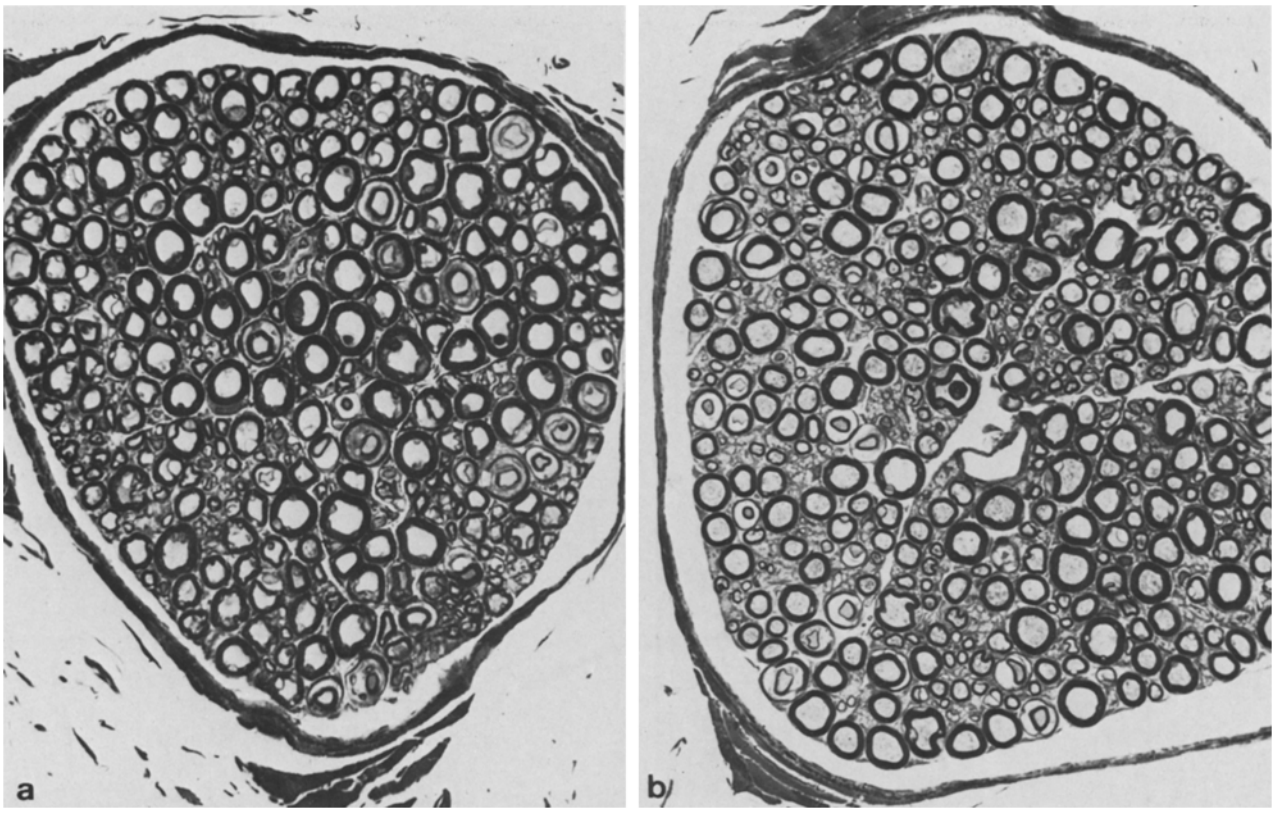

Fig. 1 a, b. Peripheral nerves, semithin sections, toluidine blue stain. **a** Control animal. **b** Myelin reduction and increased width of endoneural space in a diabetic animal. ×440

Table 1. Surfaces of fibers, axons (A), myelin sheaths (M) cross sections, M/A ratios, and percent of endoneurium. Individual values of control rats (mean ± SD)

Rat no.	Fibers (μm^2)	Axons (μm^2)	Myelin (μm^2)	M/A ratio	Endoneurium (%)
705.02	83.4 ± 68.02	27.0 ± 25.12	56.4 ± 47.44	2.7 ± 2.35	–
705.03	72.0 ± 54.05	27.6 ± 21.21	46.4 ± 36.40	2.1 ± 1.39	23.8
705.10	110.7 ± 77.77	28.5 ± 23.26	82.1 ± 57.19	3.3 ± 1.23	23.9
705.11	120.5 ± 96.39	35.8 ± 32.71	84.7 ± 67.60	2.9 ± 1.64	25.8
705.25	78.6 ± 62.47	25.2 ± 21.53	53.5 ± 43.90	2.6 ± 1.72	28.7
705.37	115.2 ± 74.57	36.6 ± 26.28	78.6 ± 51.76	2.4 ± 1.05	28.2
705.38	87.1 ± 65.20	30.6 ± 25.32	56.6 ± 42.04	2.1 ± 0.83	25.1

Table 2. Surfaces of fibers, axons (A), myelin sheaths (M) cross sections, M/A ratios, and percent of endoneurium. Individual values of diabetic rats (mean ± SD)

Rat no.	Fibers (μm^2)	Axons (μm^2)	Myelin (μm^2)	M/A ratio	Endoneurium (%)
705.09	77.3 ± 62.77	30.6 ± 25.72	46.7 ± 41.11	1.7 ± 1.43	39.1
705.15	54.6 ± 50.55	23.8 ± 21.30	30.9 ± 32.27	1.3 ± 1.18	45.3
705.21	67.6 ± 57.20	28.7 ± 23.04	38.9 ± 36.77	1.3 ± 0.84	45.3
705.22	85.3 ± 63.78	35.1 ± 25.57	50.2 ± 41.73	1.4 ± 0.82	39.7
705.41	64.3 ± 61.07	28.9 ± 27.29	35.4 ± 35.35	1.2 ± 0.42	40.8
705.42	68.1 ± 55.74	30.2 ± 23.74	37.8 ± 34.19	1.2 ± 0.60	41.5
705.48	58.4 ± 52.83	29.4 ± 25.90	29.1 ± 30.02	0.9 ± 0.49	43.0

Table 3. Final body weight and blood glucose, surfaces of fibers, axons (A), myelin sheaths (M) cross sections, M/A ratios, and percent of endoneurium of control and diabetic rats (group means ± SEM)

	No. of rats	Glucose mg/100 ml	Body weight (g)	Fibers (μm^2)	Axons (μm^2)	Myelin (μm^2)	M/A ratio	Endoneurium (%)
Controls	7	136 ± 5.2	510 ± 21.6	95.4 ± 19.57	29.9 ± 4.68	65.5 ± 15.74	2.6 ± 0.43	25.9 ± 2.11
Diabetics	7	551 ± 45.7	233 ± 29.5	67.9 ± 10.58	29.5 ± 3.33	38.4 ± 7.75	1.3 ± 0.24	42.1 ± 2.52
F[a]		–	–	9.62	0.00006	15.47	51.03	154.4
$P^a <$		–	–	0.01	n.s.	0.002	0.0001	0.0001

[a] Diabetic vs. controls, analysis of the variance (sub-sampling)

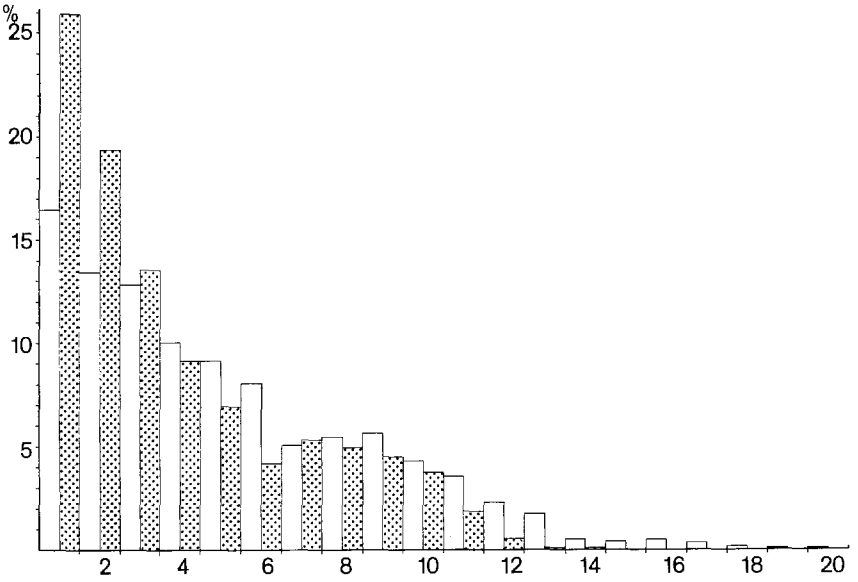

Fig. 2. Histogram of the surface distribution of nerve fiber cross sections from control (□) and diabetic (▩) animals. Class 1: 0–20 μm^2, class 20: 380 μm^2 or more

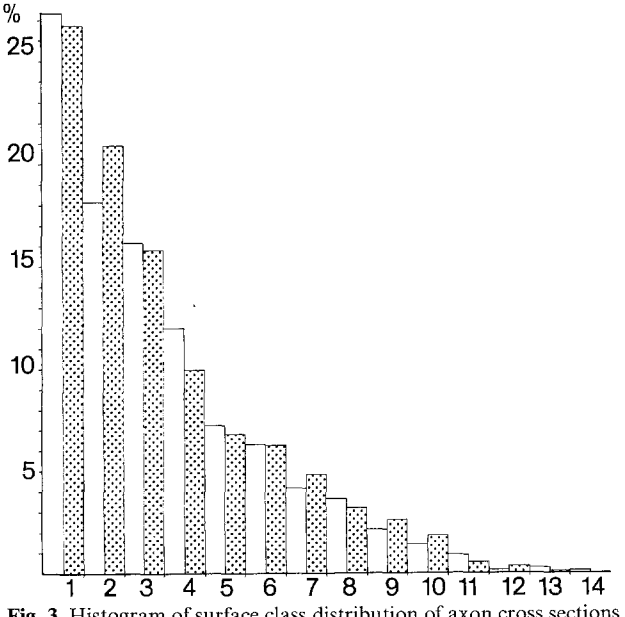

Fig. 3. Histogram of surface class distribution of axon cross sections from control (□) and diabetic (▩) animals. Class 1: 0–10 μm^2, class 20: 190 μm^2 or more

Lamellar accumulations of lipids were commonly observed in the endothelial cells of the endoneural vessels of diabetics.

Discussion

The occurrence of a morphologically well-characterized diabetic neuropathy is evidenced in our animal model by both qualitative and quantitative evaluations.

The quantitative study of the fiber cross section discloses in the diabetic a significant reduction of the average myelin surface, with the axonal surface practically the same as in the controls. The loss of myelin is compensated by an increase of the endoneural space so that the number of fibers per unit nerve surface remains equal in the two groups.

The size-class distribution of the nerve fibers indicates that a clear shift toward smaller fibers took place in the diabetics. Yet, from the distribution of the axons it appears that percentage class differences between diabetics and controls are negligible, since they

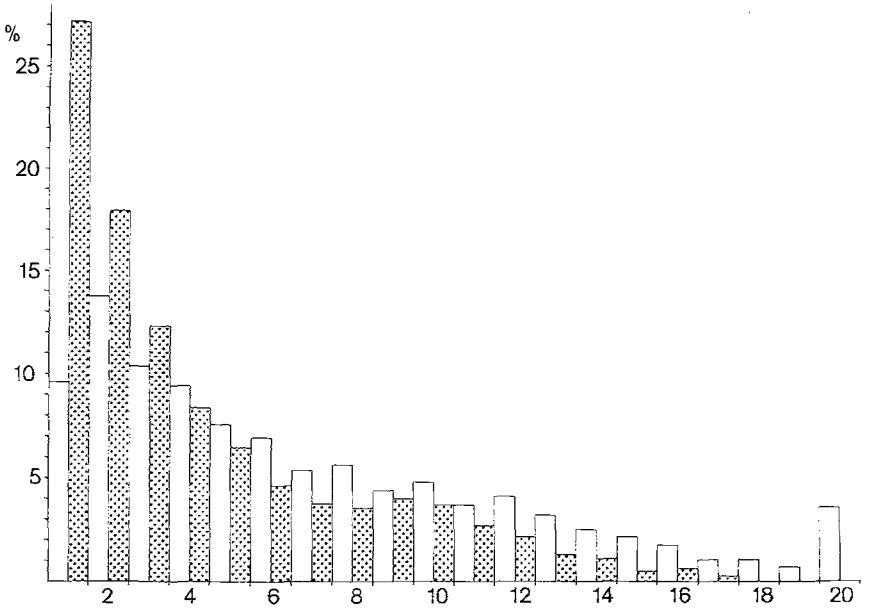

Fig. 4. Histogram of the surface class distribution of myelin sheath cross sections from control (□) and diabetic (▨) animals. Class 1: 0–10 μm², class 20: 190 μm² or more

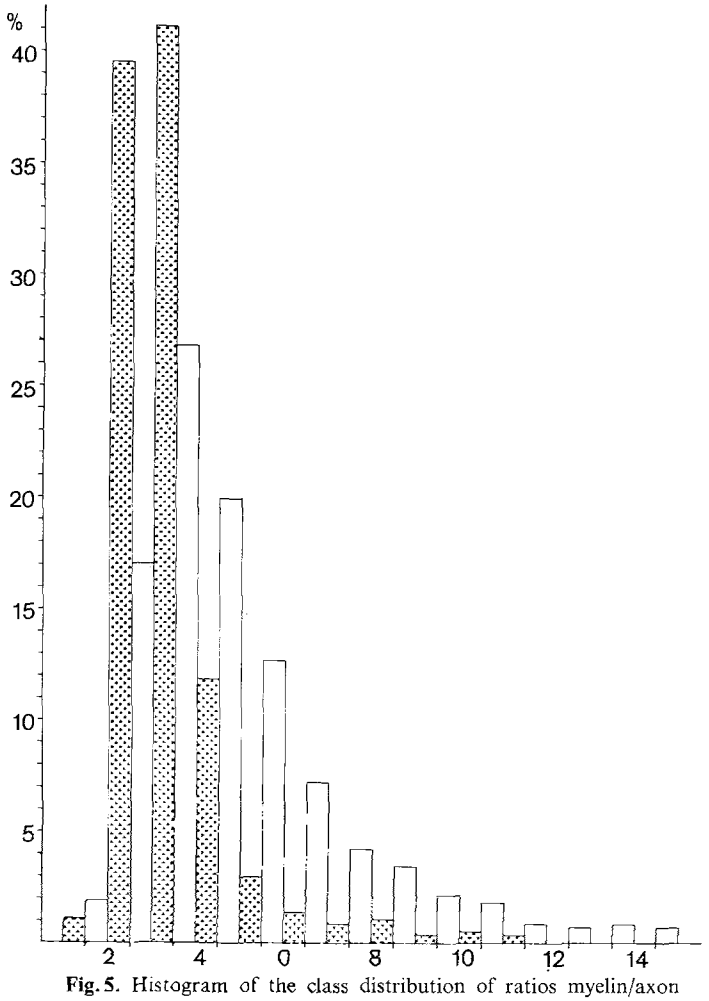

Fig. 5. Histogram of the class distribution of ratios myelin/axon surfaces from control (□) and diabetic (▨) animals. Class 1: 0–0.5, class 20: 9.5 or more

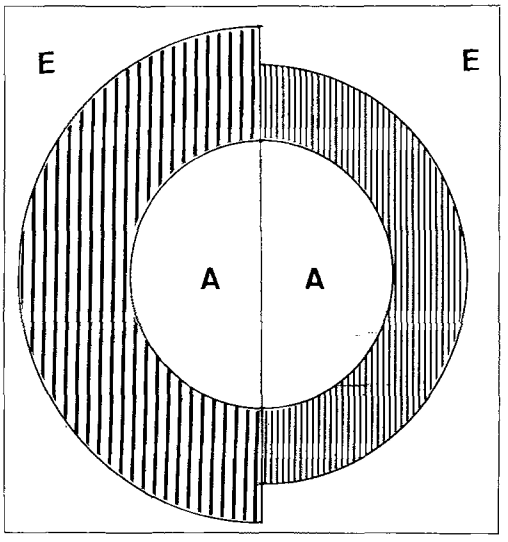

Fig. 6. Schematic representation of one half nerve fiber cross section of normal (*left*) and diabetic (*right*) animals. The myelin (*striped surface*) is reduced, the endoneuronal space (*E*) is increased, the axon (*A*) is unchanged. The surfaces are proportional to the real data

never exceed 2.7%. The shift toward smaller fibers is explained by the distributions of the myelin sheath surfaces and of the ratio of myelin to axon surfaces. From the former we can observe in the diabetics both a percentage increase of the thinner sheaths and a decrease of the wider sheaths, which results in a symmetric displacement of the myelin/axon ratio distribution to the left.

The light-microscopic findings fully agree with the quantitative data. In addition, they exclude the occurrence of a segmental demyelination at the time of autopsy or earlier.

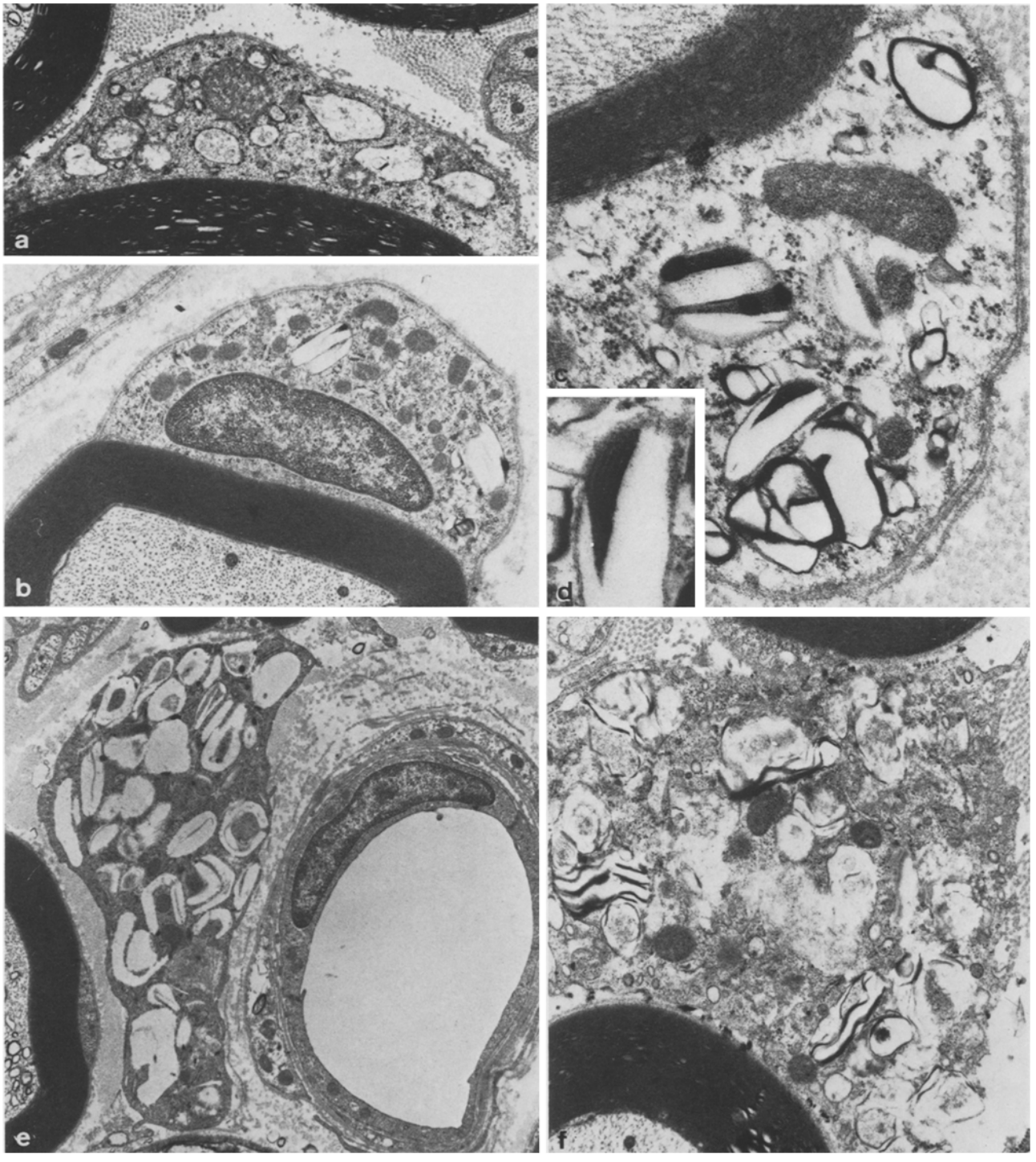

Fig. 7a–f. Changes of Schwann cells **(a–d)** and of mesenchymal endoneural cells **(e, f)**. **a** The ergastoplasm is dilated and contains granular material of low electron density. ×12,100. **b** Lipid structures of low electron density. ×14,800. **c** Myelin figures and lamellar lipid structures, ×34,000. **d** Detail of **c** showing a lamellar structure with periodicity similar to that of myelin. ×68,900. **e** Cytoplasm of mesenchymal cell filled with lipid containing vacuoles. ×12,100. **f** Cytoplasm of mesenchymal cell with lipid containing vacuoles and degenerating organelles. ×14,800

The main ultrastructural findings were the lesions of the Schwann and mesenchymal cells, followed in frequency and severity by those occurring in axons and in endothelium.

In Schwann cells of diabetics we observed dilatation of the ergastoplasm and accumulation of lipids. These, morphologically often resembled phospholipids. Both the frequency and the severity of these lesions suggest

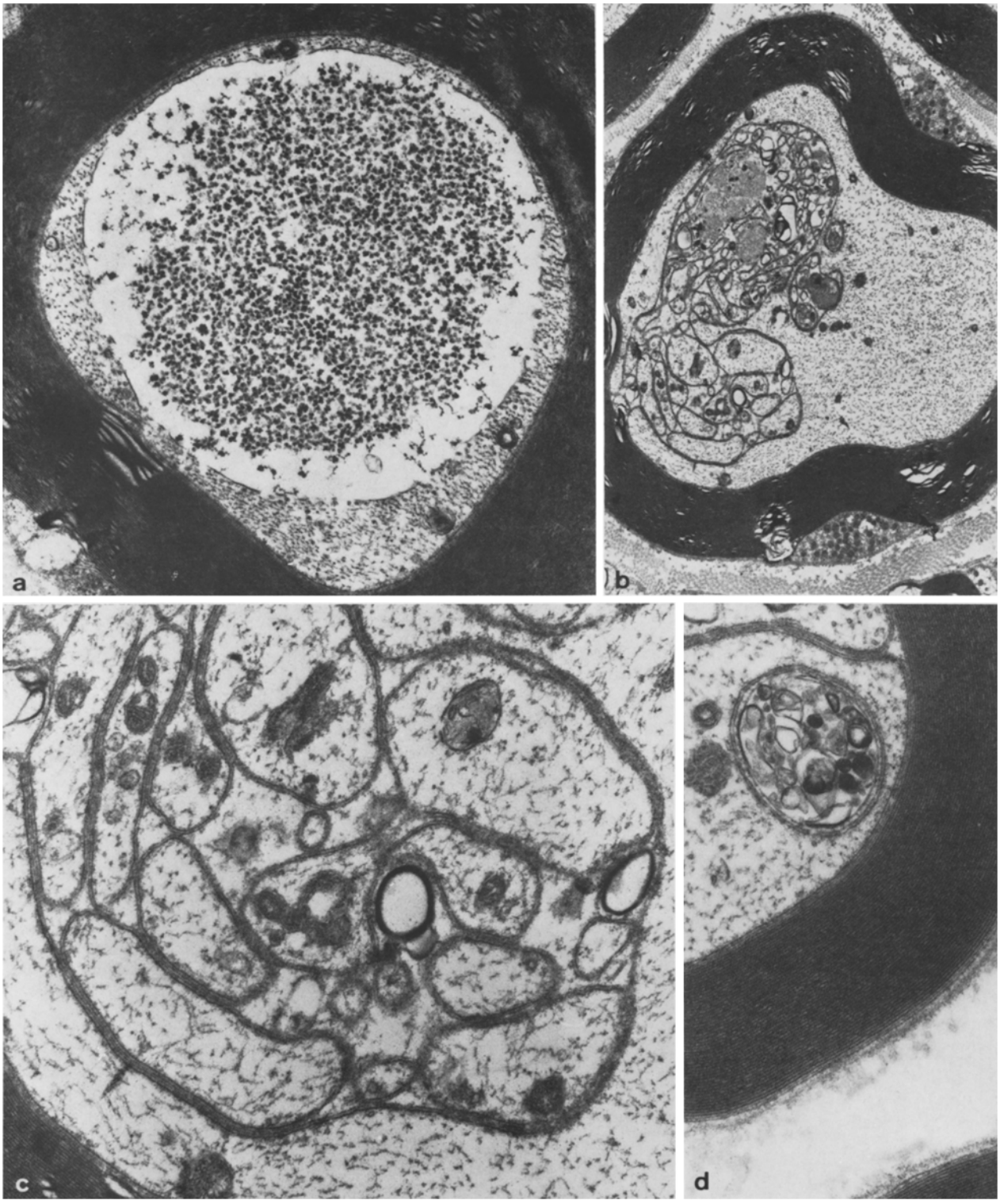

Fig. 8a—d. Changes in axons of diabetic animals. **a** Glycogen accumulation, partially delimited by unit membrane. × 24,400. **b** Portions of axonal cytoplasm sequestrated by Schwann cell membranes. × 8,400. **c** Detail from **b**. The axonal cytoplasm contains degenerated organelles. The intra-axonal myelin lamellae are formed by the Schwann cell. × 44,300. **a** Complete degeneration of the axoplasm sequestrated by a Schwann cell. × 34,300

that the reduction of the width of myelin sheaths that we measured quantitatively in the diabetics may be the consequence of a Schwann cell disturbance associated either with decreased myelin synthesis or with increased myelin catabolism, or both. Also the large accumulation of lipids in the mesenchymal cells might be related to an increased myelin catabolism. Yet, the accumulations of lipids in Schwann, mesenchymal and endothelial cells might be related to a more generalized lipid dysmetabolism, as reflected by the high levels of plasmatic triglycerides we measured in these animals (Riesen 1980, pers. commun.).

The axonal lesions observed in the diabetics are neither frequent nor severe. Further, they were present also in the controls, although to a somewhat lesser extent.

The glycogen accumulations found in axons are comparable to those we observed in encephalic neurons of the same diabetic subjects (Bestetti and Rossi 1980) and are apparently related to the alteration of carbohydrate metabolism occurring in diabetes mellitus. Similar findings have been frequently reported in various models of spontaneous or induced diabetes mellitus (Powell et al. 1977; Sima and Robertson 1979). In peripheral neuropathies of other etiology, where alterations of carbohydrate metabolism may occur, comparable glycogen accumulations have been seen (Powell et al. 1977; Sima and Robertson 1979). Phagocytosis of axoplasmic portions is a frequent finding in many types of spontaneous or induced neuropathies and would be considered as a change localized in isolated axoplasmic regions (Spencer and Thomas 1974).

According to Clements (1979), the most probable pathogenetic mechanisms which would lead to the diabetic neuropathy could be of (1) vascular, (2) axonal, or (3) Schwann cell origin. In our experimental model, comparable to the human condition of a juvenile diabetes with an early onset and long duration, the hypothesis that a Schwann cell dysfunction might be the primary pathogenetic factor seems to us the only one that could justify the observed reduction of the myelin surface. Yet, this hypothesis, although based on our findings, is in contrast with most of the data in the literature. A possible explanation might be found in the different experimental conditions we adopted for our studies. Jakobsen (1976a, b), who emphasizes the role of the axon in diabetic neuropathy, induced diabetes in adult rats which were studied 4 weeks later with techniques different from those we used. Sharma and Thomas (1974), who deny the existence of a morphologically detectable neuropathy in the streptozotocin-induced diabetes of the rat, examined only a few rats per time interval with controls which differed in age from diabetics, as criticized by Jakobsen (1976a, b). In a subsequent study of the same authors (Sharma et al. 1977) quantitative changes in diabetic animals were not found, but adult rats were employed and diabetes lasted only 5 weeks. In the study of Powell et al. (1977), a segmental demyelination was described, but the diabetogenic agent was alloxan, a substance with toxic properties greater than streptozotocin (Junod et al. 1967, 1969), adult rats were used and diabetes lasted 2 years.

In conclusion, in our experimental model we observed diabetic neuropathy, mainly characterized by lesions of the Schwann cells, together with reduction of width of the myelin sheath and increase of the endoneural space (Fig. 6).

Acknowledgements. We thank Miss D. Probst, Mrs. M. Balta, and Mr. G. Di Lullo for their technical assistance.

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Received September 5, 1980/Accepted October 3, 1980