

# Fast and Slow Axoplasmic Flow in Sciatic Nerve of Diabetic Rats

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## SUMMARY

**Accumulation of acetylcholinesterase (AChE) and choline acetylase (ChAc) activities proximal to a tie placed on the sciatic nerve was measured in control, untreated diabetic, and insulin-treated diabetic rats. In the diabetic animals AChE accumulation was reduced by about 20 per cent and ChAc accumulation by about 40 per cent. Insulin treatment eliminated the impairment. It remains an open question whether these reversible functional changes in rat have any counterpart in the diabetic neuropathy of man. DIABETES 24:1081-85, December, 1975.**

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The peripheral neuropathy of diabetes is characterized by symptoms and signs of sensory, motor, and, often, autonomic malfunction.<sup>1</sup> Evidence of a reduction of both motor<sup>2</sup> and sensory<sup>3</sup> conduction velocities has been obtained in patients, and conduction velocity depression has been found in experimentally induced diabetes as well.<sup>4-6</sup>

Although the clinical electromyographic signs have been attributed to reduced axoplasmic flow,<sup>1</sup> there is, so far as we can tell, no evidence supporting this attribution. Nonetheless, and despite the possibility that clinical and experimental diabetic neuropathies may ultimately prove to be quite dissimilar,<sup>6</sup> the present experiments were undertaken to determine whether there is a disturbance in axoplasmic flow in acute experimental diabetes in the rat and, if so, whether insulin treatment might prevent the disturbance.

## METHODS

Three similar experiments were performed in which diabetes was induced in male Holtzman rats by the

injection of streptozotocin 70 mg. per kilogram intravenously. Control animals received no treatment. The diabetic animals were maintained for three to four weeks, during which time they became hyperglycemic and lost weight. Serum glucose levels were monitored by an enzymatic fluorometric method<sup>8</sup> now and then during the illness and at the termination of the experiment. Insulin treatment was initiated ten to fourteen days after injection of streptozotocin and was continued for two weeks. It consisted of two daily subcutaneous injections of NPH insulin—2 I.U. in the morning and 6 I.U. in the evening. Serum levels of immunoreactive insulin and glucagon were measured in samples taken at the time of sacrifice by previously published methods.<sup>7</sup>

After three to four weeks the sciatic nerves of each animal were ligated under pentobarbital anesthesia (30-35 mg./kg. intraperitoneally). The wounds were closed and the rats allowed to survive for twelve hours (fourteen hours in experiment 1). In the second experiment, rectal temperatures were measured at hourly intervals for twelve hours and the average temperature for the experimental period obtained by integration of the values. To terminate the experiment, the animals were decapitated. The sciatic nerves were removed, placed on filter paper, frozen on solid carbon dioxide, and dried under vacuum at  $-35^{\circ}$  to  $-40^{\circ}$  C. The portion of each frozen-dried nerve just proximal to the ligature was cut into four 2-mm. segments, each of which was weighed on a quartz fiber (fishpole) balance<sup>8</sup> and homogenized in 90-100  $\mu$ L. 0.1 M phosphate buffer at pH 7.8, containing 0.05 per cent bovine serum albumin. The homogenates were stored at  $-80^{\circ}$  C. until assayed for acetylcholinesterase<sup>9,10</sup> and choline acetylase activity.<sup>10</sup> Accumulation of activity was calculated by subtracting the average activity of the two pieces farthest from the tie from that of each of the two pieces closest to the tie. Each difference was multiplied by the weight of the correspond-

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ing piece to obtain the accumulated enzyme activity. The sum of the accumulated enzyme activity in the two pieces closest to the tie was taken as the total enzyme accumulation.

In a group of control animals the dry weight of the piece of nerve closest to the tie was increased immediately by the tie itself. The specific activity of ChAc was slightly depressed (8 per cent) in the piece closest to the tie, but the specific activity of AChE was not changed appreciably, and the weight did not change further during the twelve-hour period after tying.

The accumulation of enzymes in rat sciatic nerve proximal to a ligation is nearly linear with time for twenty-four hours (Schmidt, Ross, and McDougal, unpublished). The rate of accumulation is, of course, affected both by the quantity of moving material and by the velocity at which it moves.

## RESULTS

Accumulation of acetylcholinesterase (AChE) in diabetic nerve was reduced 32 per cent in the first experiment (table 1).

Since the diabetic animals weighed appreciably less than the controls of the same age, younger control animals weighing the same as the diabetics were also used. The nerves of the younger animals weighed 72 per cent as much per unit length as those of the older ones in both control and diabetic rats (table 1), but the concentration of enzyme activity was greater. The total enzyme activity per unit length of nerve was not

significantly different in the two control groups. The accumulation of AChE activity at the tie was also the same in the two control groups.

In the second experiment the age control was retained, and some of the diabetic animals were treated with insulin.

A potential problem dealt with in this experiment was the hypothermia that occurs during anesthesia. If the hypothermia were greater in extent or longer in duration in the diabetic than in controls, then at least some of the differences in accumulation of enzyme activity might result from differences in body temperature.<sup>11</sup> Therefore, the body temperatures of all rats in experiment 2 were measured at intervals from the onset of anesthesia until the nerves were removed, more than twelve hours later. The average temperatures in controls, diabetics, and treated diabetics for this period were  $37.4 \pm 0.2$ ,  $36.9 \pm 0.2$  and  $37.7 \pm 0.1^\circ$  C., respectively. These data most likely eliminate hypothermia as an explanation of the results.

Choline acetylase (ChAc) activity was measured in experiment 2 in addition to AChE (figure 1). Again there was a reduction (22 per cent) in the amount of AChE activity accumulated at the tie in the diabetic animals. The reduction in accumulation of ChAc activity was even greater (41 per cent). There was a tendency toward a reversal of the diabetic effect with insulin. But this reversal was statistically significant only for ChAc, not for AChE.

Since some of the animals of experiment 2 became hypoglycemic during the twelve-hour test period, the

TABLE 1  
Accumulation of acetylcholinesterase fourteen hours after a tie in nerves of diabetic rats and of two types of control rats\*

	Acetylcholinesterase			Dry weight			Accumulation f (c-a) + e (b-a)  nmoles/nerve§/hr.
	Average pieces 4 & 3	Piece 2	Piece 1	Average pieces 4 & 3	Piece 2	Piece 1	
	a	b	c	d	e	f	
	mmoles/kg. dry wt./hr.			$\mu$ g.			
Age control	123 $\pm 11$	156 $\pm 9$	456 $\pm 31$	508 $\pm 29$	486 $\pm 13$	569 $\pm 45$	202 $\pm 14$
Weight control	187† $\pm 9$	236 $\pm 10$	677 $\pm 42$	371† $\pm 21$	360 $\pm 13$	392 $\pm 22$	206 $\pm 13$
Diabetic	120 $\pm 6$	130 $\pm 9$	356 $\pm 18$	511 $\pm 32$	518 $\pm 27$	560 $\pm 34$	138‡ $\pm 16$

\*There were three rats (six nerves) in each group. The animals were shown to be diabetic by elevated serum glucose levels,  $40 \pm 0.9$  mM. The body weights of the animals in the three groups were: age controls,  $330 \pm 10$  gm.; weight controls,  $194 \pm 4$  gm.; and diabetics,  $228 \pm 16$  gm.

†p  $\leq 0.003$  compared with age control.

‡p = 0.01 compared with age or weight control.

§Actually per 4 mm. segment.

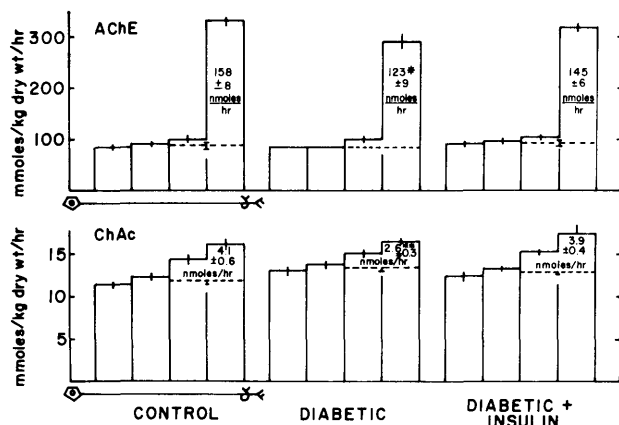


FIG. 1. The accumulation of AChE and ChAc activities twelve hours after a tie (†) was placed on the sciatic nerve in vivo in diabetic, insulin-treated diabetic, and control rats. At the time of sacrifice the body weights and the serum levels of glucose, insulin, and glucagon were as follows (mean  $\pm$  S.E.M.):

	Number of rats	Weights (gm.)	Glucose (mM)	Insulin ( $\mu$ U. IRI/ml.)	Glucagon (pg. IRG/ml.)
Control	(5)	503 $\pm$ 10	11.2 $\pm$ 1.3	75.5 $\pm$ 8.9	175 $\pm$ 15
Diabetic	(5)	292 $\pm$ 19	26.0 $\pm$ 3.0	8.1 $\pm$ 2.7	452 $\pm$ 105
Diabetic + Insulin	(7)	408 $\pm$ 14	2.8 $\pm$ 0.3	344. $\pm$ 68	260 $\pm$ 17

In the figure the cells of origin lie to the left. The width of each bar of the histograms represents 2 mm. of nerve. The vertical line at the top of each bar represents  $\pm$  S.E.M. Where none is shown, S.E.M. = 2 mmole/kg./hr. The dashed lines are drawn to indicate the mean activity of the two proximal pieces, which is subtracted from the activity of each of the two distal pieces. The differences so obtained are used to calculate the total accumulated activity, as described in Methods and shown in table 1. The result ( $\pm$  S.E.M.) is written in the appropriate histogram; \*different from control,  $p = 0.01$ ; \*\*different from control,  $p = 0.04$ ; and from diabetic + insulin,  $p = 0.017$ . For both enzymes, there were ten control and ten diabetic nerves and fourteen nerves from diabetic animals treated with insulin.

effect of hypoglycemia on movement of AChE was examined. In six rats the movement of AChE was found to be reduced 20 per cent ( $p < 0.02$ ) during a 3.5-hour period of hypoglycemia (Schmidt, unpublished).

In the third experiment, pains were taken to avoid the development of hypoglycemia during the actual flow experiment. Therefore, the animals were not treated on the last day of the experiment. As a result the treated animals were severely hyperglycemic at the time of sacrifice (legend, table 2). Again the reduction in accumulation of AChE (21 per cent) and ChAc (40 per cent) activities was observed in the diabetic animals (table 2). Insulin treatment completely reversed the depression for both enzymes.

In both experiments in which ChAc was measured,

the activity of ChAc per unit weight was increased in the nerves of the diabetic animals. However, in both experiments the average weight per unit length of the diabetic nerves was decreased from the control average of 0.275 kg./km. Therefore, in the experiment shown in figure 1, the enzyme activity per unit length was the same in both control and diabetic nerves,  $3.21 \pm 0.08$  and  $3.30 \pm 0.08$  mmoles/km./hr., respectively. In the experiment shown in table 2, the weight loss was somewhat less, and the enzyme activity was actually somewhat higher in diabetic than in control nerves,  $5.04 \pm 0.08$  and  $4.64 \pm 0.14$  mmoles/km./hr., respectively ( $p = 0.02$ ).

#### DISCUSSION

Evidence of a reduction in slow axoplasmic flow in an experimental neuropathy has been obtained by Pleasure et al.<sup>12</sup> using acrylamide-poisoned cats, and by Jablecki and Brimijoin<sup>13</sup> in dystrophic mice. In the present experiments on diabetic animals, a reduction in the accumulation at a tie has been observed for two enzymes: ChAc, which moves by slow flow,<sup>14</sup> and AChE, which moves by fast flow.<sup>15</sup>

In the case of ChAc it seems reasonable to assume that all of the enzyme, being soluble and located in the neurons,<sup>16</sup> is in motion. If so, the rate of slow axoplasmic flow was probably reduced in the motor axons of the diabetic animals. There was no evidence for a loss of cholinergic axons, since the amount of ChAc activity in the diabetic nerves was unchanged or slightly elevated. Jablecki and Brimijoin<sup>13</sup> found a reduction in accumulation of ChAc activity coupled with an increase in total ChAc activity in the nerves of dystrophic mice.

In the case of AChE, the situation is more complicated. The observed reduction in AChE accumulation could have resulted from a reduction in the rate of movement of the AChE-containing particles, which move by fast flow,<sup>9,15,17</sup> or it could have been the consequence of a reduction in the amount of enzyme in motion. Only 5 per cent of the enzyme activity in rat sciatic nerve is in motion toward the periphery (Schmidt, unpublished). A small reduction in the amount of enzyme activity in motion would be difficult or impossible to discern. Such a reduction could have occurred in each AChE-containing axon, but it is unlikely that it was the result of a loss of axons from the diabetic nerve. In rats treated as ours were, and showing reduced conduction velocity, Sharma and Thomas<sup>5</sup> found no evidence for loss of fibers, change in fiber diameter, or change in the myelin-to-axon ratio in tibial or sural nerves.

TABLE 2

Accumulation of acetylcholinesterase and choline acetylase activities twelve hours after a tie in each sciatic nerve of control, diabetic, and insulin-treated diabetic rats\*

	Number of nerves	Acetylcholinesterase§			Choline acetylase		
		Activity in pieces 3 & 4		Accumulation in pieces 1 & 2	Activity in pieces 3 & 4		Accumulation in pieces 1 & 2
		Total	Average		Total	Average	
		<u>nmoles</u> 4 mm. hr.	<u>nmoles</u> kg. hr.	<u>nmoles</u> nerve hr.	<u>nmoles</u> 4 mm. hr.	<u>nmoles</u> kg. hr.	<u>nmoles</u> nerve hr.
Control	12	221 ± 9	205 ± 31	371 ± 17	18.6 ± 0.5	17.2 ± 1.0	6.2 ± 0.7
Diabetic	16	220 ± 10	220 ± 8	292† ± 18	20.2‡ ± 0.3	21.7‡ ± 0.6	3.7† ± 0.5
Diabetic + Insulin	12	234 ± 8	235 ± 8	403 ± 9	18.2 ± 0.8	17.0 ± 0.7	7.0 ± 0.5

\*At the time of sacrifice the body weights and the serum levels for glucose, insulin and glucagon were as follows (mean ± S.E.M.):

	Number of rats	Weights (gm.)	Glucose (mM)	Insulin (μU. IRI/ml.)	Glucagon (pg. IRG/ml.)
Control	(6)	396± 8	8.8±0.3	35.6±3.5	208±26
Diabetic	(8)	233±15	31.9±0.3	14.6±4.1	693±59
Diabetic + Insulin	(6)	339±15	26.8±1.9	23.1±5.8	387±41

†Different from control and insulin-treated,  $p \leq 0.003$ .

‡Different from control and insulin-treated,  $p \leq 0.02$ .

§This assay was done with radioactive acetylcholine.<sup>10</sup> In the other experiments acetylthiocholine was used.<sup>9</sup> The differences in activities between experiments are explicable on the basis of the different assay methods.

A reduction in motor-nerve conduction velocity has been observed in alloxan-<sup>4</sup> and streptozotocin-treated<sup>5,6</sup> rats, and it may be that maintenance of conduction velocity in the axon is dependent on axonal transport. However, there is as yet no evidence linking these two functions. In this regard, it is of interest that some properties of the skeletal muscle membrane, for example the resting membrane potential and extrajunctional sensitivity to acetylcholine, appear to be dependent on axoplasmic flow.<sup>18</sup>

Whether the changes seen in axoplasmic flow are the direct results of insulin lack or are secondary to other changes that occur in diabetes cannot be decided now. Insulin does appear to enhance glucose uptake by nerves<sup>19</sup> and leucine incorporation into the proteins of peripheral nerve myelin.<sup>20</sup> However, some of the biochemical changes observed in brain in experimental diabetes are also seen in severe dehydration.<sup>21</sup> There is evidence that the reduction in motor nerve conduction velocity is secondary to loss of myoinositol from diabetic nerves. Treatment of the diabetic animals with a diet containing supplementary myoinositol returned myoinositol content and conduction velocity to normal, although the severity of

the diabetes was otherwise unaffected.<sup>6</sup>

Whether impairment of axonal flow is in any way causally involved in the development of the peripheral diabetic neuropathy seen in man cannot be answered by using the present system of acute experimental diabetes. Human diabetic neuropathy presents a relatively constant constellation of morphologic changes. But no morphologic changes have been found in experimental diabetes even after one year of illness.<sup>5</sup> Nevertheless, it would seem rewarding to continue the present line of research to assess the possible physiologic functions of insulin, direct or indirect, in maintaining the orderly flow of material up and down peripheral nerves.

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