The Sorbitol Pathway

Enzyme Localization and Content in Normal and Diabetic Nerve and Cord

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SUMMARY

The enzymes of the sorbitol pathway, aldose reductase and sorbitol dehydrogenase, were investigated in sciatic nerve and spinal cord. The substrate specificities of spinal cord aldose reductase indicates that it is a variant of TPN L-hexonate dehydrogenase which possesses poor polyol forming ability. In contrast, the cauda equina and sciatic nerve aldose reductase have a true aldose reductase with considerable polyol forming ability. The distribution of the latter enzyme is associated with the presence of Schwann cells. Wallerian degeneration experiments are compatible with the localization of true aldose reductase in the Schwann cell and sorbitol dehydrogenase in the axon. There was no change in the levels of sorbitol dehydrogenase in diabetic nerves, however, a 30 per cent decrease occurred in the aldose reductase content. This decrease suggests a metabolic abnormality of the Schwann cell, possibly resulting from altered cellular integrity, which may have important implications for the etiology of diabetic neuropathy. DIABETES *17*:239-43, May, 1968.

Since the initial observation by Van Heyningen,[1] studies by Kinoshita and his group[2] demonstrated the accumulation of sorbitol and fructose in diabetic lenses and of dulcitol in the lenses of galactose fed animals by way of the "sorbitol pathway." These accumulations play an etiologic role in the development of galactosemic and diabetic cataracts by inducing osmotic and electrolyte changes culminating in the disruption of the lenticular fibers. Significant accumulations of sorbitol and fructose also occur in the sciatic nerve and spinal cord of diabetic rats.[3,4] Elevation of cerebrospinal fluid fructose levels was recently reported in diabetic patients, with a high correlation between cerebrospinal fluid fructose and glucose levels.[5]

The sorbitol pathway consists of a series of reactions involving two enzymes, aldose reductase and sorbitol dehydrogenase:

$$\underset{\text{Aldose Reductase}}{\text{D-Glucose} + \text{TPNH} \longrightarrow \text{D-Sorbitol} + \text{TPN} + \text{H}^+}$$

$$\underset{\text{Sorbitol Dehydrogenase}}{\text{D-Sorbitol} + \text{DPN} + \text{H}^+ \longrightarrow \text{D-Fructose} + \text{DPNH}}$$

This report describes the properties, levels and tissue location of these two enzymes in normal and diabetic rat spinal cords and sciatic nerves.

EXPERIMENTAL PROCEDURES

Animals: Male rats of the Sprague-Dawley strain weighing 130 to 150 gm. were made diabetic as previously described.[3] A normal control group, a group that received alloxan which did not become diabetic, and an alloxan diabetic group which received no insulin treatment were all sacrificed after four weeks. The animals had free access to food at all times. Constant glycosuria during the period of observation, and blood sugar values at time of sacrifice (normal group, 122 ± 16; normal alloxan group, 133 ± 32; diabetic group, 454 ± 68 mg./100 ml.), established the identity of the three groups. The average body weights at the time of sacrifice were 288 ± 61, 282 ± 53, and 178 ± 36 gm. ± S.D. for the normal, normal alloxan, and diabetic groups respectively.

Wallerian degeneration experiments were carried out on normal albino rabbits of the New Zealand strain weighing 4 to 5 lbs. The sciatic nerve was sectioned on one side only, the other serving as a normal control. With the animal under ether anesthesia, the nerve was exposed behind the greater trochanter, and a ligature was applied just below the bifurcation. A 5-mm. segment of the sciatic nerve was removed and the proximal stump, with the ligature, was placed high behind the trochanteric fossa to prevent reinnervation. The

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animals were sacrificed after ten days, and comparable segments of Wallerian degenerated nerves and control nerves were removed. Comparable normal nerves from unoperated rabbits were also obtained.

Chemicals and materials: TPNH, DPN, DL-glyceraldehyde, and sorbitol were obtained from Sigma Chemical Co.; D-glucuronate, D-glucuronolactone, and D-xylose were obtained from Pfahnstiehl Laboratories, and L-gulonate was prepared from L-gulonolactone by the method of Mano et al.[6] Folin-Ciocalteau reagent for the Lowry protein method[7] was obtained from Fisher Scientific Co.

Tissue for enzyme assays: Rats were killed by decapitation and the sciatic nerves and spinal cords rapidly dissected, weighed, and frozen. Rabbits were killed by air embolism and the sciatic nerves removed and rapidly desheathed. The tissues were homogenized in 10 volumes of cold distilled water in a glass homogenizer and aliquots for protein determinations were obtained when necessary. The homogenates were centrifuged at 46,000 × G for thirty minutes in an IEC Model B-20 centrifuge at 4°.

Assay methods: Sorbitol dehydrogenase was assayed by a modification of the method of Smith.[8] A typical reaction mixture of 1 ml. contained 100 μmoles of glycine buffer (*p*H 9.6); 0.47 μmole DPN; enzyme solution equivalent to 7 to 10 mg. wet weight of sciatic nerve, or 2 to 4 mg. of spinal cord; and was started by the addition of 40 μmoles of sorbitol. The increase in absorbance due to the formation of DPNH was linear for at least ten minutes. A unit of activity was defined as a change of absorbance of 0.001 unit per ten minutes.

Aldose reductase was assayed by a modification of the method of Hayman and Kinoshita.[9] The assay in a 1 ml. final volume contained the following: phosphate buffer 0.067 M (*p*H 6.2); 75 mμmoles TPNH; enzyme solution equivalent to 3 to 5 mg. wet weight of sciatic nerve, or 7 to 10 mg. spinal cord; and 1 μmole DL-glyceraldehyde. Lithium sulfate was omitted from the reaction mixture. The reaction was started by the addition of the glyceraldehyde substrate and was followed at 340 mμ. and 27.5° in a Unicam SP 800A automated double beam spectrophotometer, which automatically compensated for the slight nonspecific TPNH decay in the blank. A unit of activity was defined as a change of absorbance of 0.001 unit per five minutes. The reaction was linear for at least five minutes.

Aliquots were analyzed in duplicate, and those from normal and diabetic tissues were coded with random numbers and were not identified until all the assays were completed.

RESULTS

Sorbitol dehydrogenase: The enzyme activity was directly proportional to the enzyme concentration at lower levels (figure 1). All tissues were assayed in the linear portion of the curve.

Aldose reductase: Under the conditions described above, the aldose reductase activity was proportional to the enzyme concentration for each tissue examined (figure 2). The reaction was TPNH specific in that there was virtually no glyceraldehyde reduction when DPNH was used. The aldose reductase enzyme is capable of reducing a large number of compounds with aldehydic functional groups. Table 1 presents a typical experiment showing the substrate specificities of the aldose reductase enzyme for various segments of rabbit spinal cord and for sciatic nerve. The relative rates of reaction of various substrates (10 mM), as well as the relative rate of oxidation of L-gulonate by TPN, are expressed as percentages of those obtained with DL-glyceraldehyde as a substrate. It can be seen that the aldose reductase of the various segments of the cord, with the exception of the cauda equina, has a poor ability to reduce D-xylose to its polyol, while possessing a considerable ability to oxidize L-gulonate. By contrast, both the cauda equina and sciatic nerve aldose reductase have a considerable polyol forming activity with low L-gulonate oxidation rates. Thus, these substrate specificities would indicate that the cauda equina and sciatic nerve aldose reductase are sufficiently similar to the lenticular enzyme[10] to conclude that these tissues contain the same enzymatic activity. However, the substrate specificities of the cord enzyme would indicate that it is a variant of the enzyme TPN-L-hexonate de-

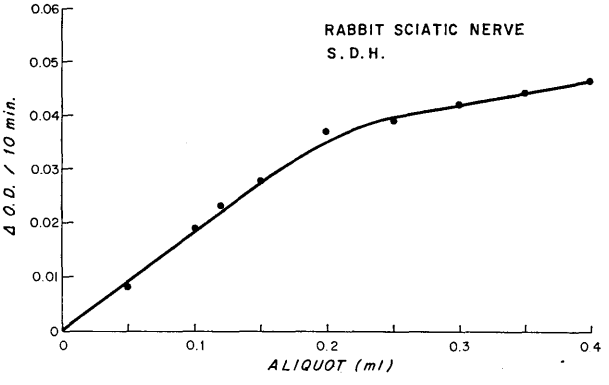

FIGURE 1

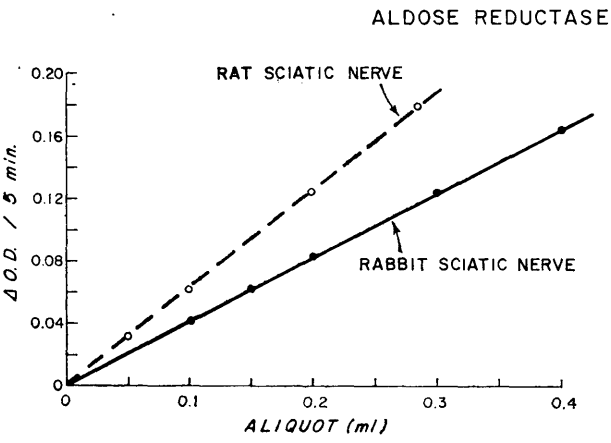

FIGURE 2

TABLE 1

Relative rates of reaction*

Substrate	Rabbit spinal cord				Rabbit sciatic nerve
	Cervical	Thoracic	Lumbar	Cauda equina	
D-glucuronate	123	101	137	68	51
D-glucuronolactone	93	101	96	86	103
D-xylose	16	14	24	55	64
L-gulonate	61	60	66	13	10

*Expressed as percentages of those obtained with DL-glyceraldehyde as a substrate, each point is the mean of four experiments.

hydrogenase found in liver.[6] This latter enzyme, while capable of reducing glyceraldehyde, has a poor ability to reduce hexoses and pentoses to their corresponding alcohols. Since the sciatic nerve and the cauda equina contain a large number of Schwann cells, while the spinal cord is totally lacking in these cells, this suggests an association of polyol forming or true aldose reductase with Schwann cells.

Localization of enzyme activities in sciatic nerve: In Wallerian degeneration, the axons are resorbed and disappear in two to three days. Schwann cells begin to proliferate suddenly on the fourth day following nerve section, and by the tenth day there is a fivefold increase in their number. In table 2 the enzyme activities are expressed on a wet weight basis as well as units per milligram total protein because of a 10 to 15 per cent increase in the nerve water content during Wallerian degeneration.[11]

The results expressed on a wet weight basis show no change in the aldose reductase levels in the control group of nerves when compared to the normal group. However, there is a slight, although nonsignificant elevation in the Wallerian degenerated nerves. When the results are expressed on a total protein basis, a 22 per cent increase of aldose reductase activity in Wallerian nerves becomes apparent with no change in the control levels.

The sorbitol dehydrogenase levels, expressed on either basis, are significantly decreased in the control group and are reduced to less than 10 per cent of normal values in the Wallerian group of nerves.

Enzyme activities in normal and diabetic rats: Table 3 shows the enzymatic activities for spinal cord and sciatic nerve with DL-glyceraldehyde as a substrate. In the normal sciatic nerve, which is a heavily myelinated tissue rich in Schwann cells, the aldose reductase activity exceeds that of spinal cord by approximately threefold. In the spinal cord, which is primarily neuronal tissue with many myelinated tracts but totally lacking in Schwann cells, the sorbitol dehydrogenase activity is three times higher than that of sciatic nerve. From the substrate specificities, the Wallerian degeneration experiments, and these data it would appear that of the two enzymes of the sorbitol pathway, true aldose reductase is predominately present in tissues rich in Schwann cells, and that the sorbitol dehydrogenase is associated with tissues rich in neuronal cells.

In the diabetic group, there was a divergence of effect in the two tissues. In sciatic nerve there was a 30 per cent decrease in true aldose reductase activity with no

TABLE 2

Desheathed rabbit sciatic nerve enzyme levels

	unit/mg. wet weight ± S.D. (n = 6)		unit/mg. total protein ± S.D. (n = 6)	
	Aldose reductase	Sorbitol dehydrogenase	Aldose reductase	Sorbitol dehydrogenase
Normal	3.21±0.44	4.07±0.41	43.38±5.31	52.55±7.97
Control	3.41±0.38 p N.S.	3.02±0.25 $p < 0.001$	44.48±5.93 p N.S.	33.35±4.22 $p < 0.01$
Wallerian	3.77±0.46 p N.S.	0.36±0.23 $p < 0.001$	54.61±9.18 $p < 0.05$	5.21±3.54 $p < 0.001$

TABLE 3

Enzyme levels in rat tissue
(unit*/mg. wet weight ± S.D.)

	Sciatic nerve		Spinal cord	
	Aldose reductase	Sorbitol dehydrogenase	Aldose reductase	Sorbitol dehydrogenase
Normal	10.34±2.35 (n=8)	6.47±2.40 (n=7)	3.68±0.73 (n=8)	18.78±1.44 (n=8)
Normal alloxan	10.65±4.63 (n=4 p N.S.)	6.71±0.89 (n=4 p N.S.)	4.50±1.25 (n=4 p N.S.)	18.22±1.89 (n=4 p N.S.)
Diabetic	7.30±2.82 (n=9 p<0.025)	6.77±1.47 (n=7 p N.S.)	3.87±1.57 (n=10 p N.S.)	24.05±1.36 (n=10 p<0.001)

*1 unit A.R. = △ OD 0.001/5 min.
1 unit S.D.H. = △ OD 0.001/10 min.

significant change in the sorbitol dehydrogenase levels. In the cord there was no change in its aldose reductase levels, while a 30 to 35 per cent increase in the sorbitol dehydrogenase levels was found.

The enzyme levels in the small group of animals that received alloxan but did not become diabetic were not significantly different from the control group, thereby probably eliminating a direct alloxan effect.

DISCUSSION

The ability of aldose reductase to reduce sugars to their corresponding alcohols, and the accumulation of these products play an important role in the formation of various sugar cataracts.[2] The markedly elevated levels of sorbitol and fructose in diabetic sciatic nerves[3] suggested a similar role for these accumulations in diabetic neuropathy. The finding that sciatic nerve aldose reductase has similar substrate specificity to that of the lens enzyme, especially in regard to its ability to form sugar alcohols, is consistent with the noted accumulations. Similarly, the poor ability of the spinal cord aldose reductase to form polyols explains the low levels of fructose accumulation in the spinal cord when compared to sciatic nerve. Presumably the cerebrospinal fluid fructose is to a large extent formed by the spinal nerves of the cauda equina.

The different specificities of aldose reductase in sciatic nerve as opposed to the spinal cord, and the finding of higher levels of sorbitol dehydrogenase in cord, suggested that the two enzymes are located separately in two types of cells. The sciatic nerve is composed of a large number of myelinated axons, with the myelin sheath and the Schwann cell enclosing the axons. These Schwann cells extend along the axon to its entry into the cord, hence the presence of Schwann cells in the spinal nerves composing the cauda equina. Wallerian degeneration experiments in which the axons are resorbed within a few days and the Schwann cells proliferate, provided an opportunity to examine the possible tissue location of the two enzymes. For these enzyme assays, the nerves were desheathed to eliminate any possible contribution from the cellular elements of the sheath itself. The finding of markedly decreased sorbitol dehydrogenase activity in the Wallerian nerves (10 per cent of normal) would almost certainly indicate that this enzyme is an axonal one. The persistence and elevation of the aldose reductase activity in the Wallerian nerves is compatible with its localization in the Schwann cell. However, it should be noted that in addition to the Schwann cell proliferation, there is an invasion of macrophages into the degenerating nerve.[12] It is not known whether these macrophages contribute any aldose reductase activity.

Assuming the validity of the localization of these two enzymes, the finding of a normal sorbitol dehydrogenase level in diabetic nerves would indicate that the axon is not primarily affected. However, the previously described increase in sorbitol levels in total diabetic nerve[3] assumes an even greater significance since this increase would be restricted to the Schwann cell compartment alone rather than to the whole nerve. Our results imply that these localized accumulations are far greater in the Schwann cells than would be apparent from data obtained for the whole nerve. The inability of the trapped sorbitol to penetrate cell membranes may thus cause harmful effects similar to those seen in lenticular fibers,[2] and result in altered Schwann cell integrity and loss. The decreased aldose reductase levels in diabetic nerves would be compatible with this sequence of events. The finding of large accumulations of

sorbitol and fructose in sciatic nerves despite an absolute reduction in the aldose reductase levels would thus be consistent with a segmental demyelinating process resulting in damage and loss to some Schwann cells while others continue to accumulate sorbitol and fructose. This biochemical view of diabetic neuropathy as a metabolic disease affecting Schwann cell integrity is supported by the pathological findings of segmental demyelination in human diabetic neuropathy.[13]

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Arterial Lesions in Wild Monkeys

Many studies on the effect of diet on the development of hypercholesteremia and atherosclerosis in monkeys have been carried out with the hope that these animals, more related to man than the typical laboratory animal, will provide some evidence useful in combating these diseases. Studies on different types of monkeys indicate that addition of cholesterol to a synthetic diet containing various kinds of fat leads to hypercholesteremia and atherosclerosis (*Nutrition Reviews* 20:54, 1962; *21*:151, 1963).

In most studies the *Cebus* monkey has been used, though other studies reporting the effect in *Rhesus* and *Logothrix* monkeys have also been published. Some differences in results have been recorded although, in general, a diet high in a fat containing saturated fatty acids and cholesterol as well leads to hypercholesteremia and atherosclerosis. An interesting study by Pickering and co-workers (*see Nutrition Reviews* 20:81, 1962) reported that in infant *Macaca mulatta* monkeys the development of lesions involving the aorta and muscular arteries (primarily thickening development of fibrous plaques and lipid deposition) was the same with a diet based on cows' milk or one in which corn, olive, and coconut oils had replaced butter fat. Serum cholesterol levels were higher in the infant monkeys fed the milk fat formula, and at one year of age were over twice as high as levels in the other experimental group. However, the report of histological changes indicated that there was no significant difference in the lesions or degree of change in the two groups of animals. It would appear that in this type of monkey development of lesions is less related to diet than in some of the others that have been studied.

The diet of monkeys living in a natural environment is presumed to consist principally of vegetables. J. P. Strong and N. C. Tappen (*Arch. Path.* 70:199, 1965) have recently published a comparison of the aortic lesions found in two species of monkeys living wild in Africa.

The two species studied were *Cercopitheus ascanius* and *Cercocebus albigena.* Forty-nine of the *C. albigena* and sixty-four of the *C. ascanius* were shot during the field investigation in Uganda and the Congo. The animals were classified as to juvenile, sub-adults, and adults on the basis of dentition and skeletal development, and dissected. The hearts and aortas were fixed and brought to New Orleans where staining and evaluation of the

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