Pressure-induced inhibition of fast axonal transport of proteins in the rabbit vagus nerve in galactose neuropathy: prevention by an aldose reductase inhibitor

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Summary. Fast and slow anterograde axonal transport and retrograde axonal transport of proteins were studied in the mainly non-myelinated sensory fibres of the vagus nerve of rabbits fed a diet of 50% galactose over a period of 29 days. Galactose feeding had no effect on the rate or protein composition of slow transport nor on the amount of retrogradely transported proteins. There was a slight retardation of fast transported proteins although their composition was unchanged. The galactose feeding led to a significant increase ($p < 0.005$) in nerve water content and nerve galactitol but no significant change in myo-inositol. When 20 mm Hg pressure was applied locally to the cervical vagus nerve, fast trans-

ported proteins accumulated proximal to the compression zone in the galactose-fed but not in control rabbits. Administration of the aldose reductase inhibitor Statil (ICI 128436) throughout the experiment prevented the increased susceptibility to pressure and the increase in nerve galactitol and water content. The effects of pressure are similar to those found in the streptozotocin-diabetic rat although the underlying mechanisms may differ.

Key words: Axonal transport, nerve compression, neuropathy, galactose, aldose reductase, Statil.

Streptozotocin-induced experimental diabetes in the rat leads to an increase in the inhibition of the fast axonal transport of proteins in the sciatic nerve by local compression [1]. This phenomenon is related to the intraneural accumulation in diabetes of the sugar alcohol sorbitol, since the increased inhibition of transport is prevented by administration of the aldose reductase inhibitor 'Statil' (ICI 128436) [2]. An experimental procedure that has been used to study the consequences of increased sugar alcohol accumulation is galactose feeding, which leads to increased intraneural levels of the polyol galactitol, with consequent osmotic changes leading to increased nerve water content and endoneurial fluid pressure [3, 4]. Similar increases in nerve polyols occur in diabetic neuropathy although the increase in nerve water content is either less pronounced, or absent [5–7].

It was the intention of this study to determine if galactose feeding could lead, like experimental diabetes, to an alteration in susceptibility to pressure and if the effect could also be produced in non-myelinated fibres. The rabbit vagus nerve was chosen since it contains a fairly homogeneous population of non-myelinated fibres in which it is possible to study various phases of axonal transport as well as the effects of applying mild local pressure [8, 9].

Materials and methods

Male albino rabbits (2.6–3.6 kg) were divided into four groups. The first group was fed a standard laboratory diet, while the second received a diet consisting of 50% D-galactose. The galactose diet was manufactured in the laboratory by mixing powdered galactose in a food blender with the standard diet moistened with an equal weight of water. The mixture was formed into bars and baked at 80°C until almost dry; a slightly moist galactose diet was found to be the most acceptable to the rabbits. The galactose-fed rabbits received their diet over a period of 29 days, during which time they had two breaks of 4 days each (days 8–11 and days 18–21) in which they received the standard diet. This was to prevent some slight weight loss observed in preliminary experiments. Both groups of rabbits were given water *ad libitum*. The galactose-fed rabbits showed no signs of neurological defects or any other abnormalities.

A third group of rabbits was fed a galactose-rich diet, as above, and also received a daily dose of 25 mg/kg Statil (ICI 128436; Imperial Chemical Industries plc, Macclesfield, UK) by gastric intubation. The fourth group received the same dose of Statil along with a normal diet.

Rabbits were anaesthetised with Valium (diazepam) 1 mg/kg intramuscularly and Hypnorm (fentanyl 0.2 mg/ml and fluanisone 10 mg/ml) 0.5 ml/kg intramuscularly. The left or right nodose ganglion was exposed and the following procedures carried out on control and galactose-fed rabbits, without administration of Statil.

1. For electrophoretic analysis of fast axonally transported proteins, one nodose ganglion was exposed and 4 injections each of 1–2 μ l totalling 150 μ Ci 35 S-L-methionine (1200 Ci/mmol; Amersham, Amersham UK) were made subepineurally into the ganglion. Animals were killed 5 h later and the vagus nerves and nodose ganglia

removed. For measurement of the rate and amount of fast transport, a total of 60 μCi ^3H -L-leucine (60 Ci/mmol; Amersham) were injected into one nodose ganglion. Animals were killed 3.5 h later and vagus nerves and nodose ganglia removed.

2. For measurement of slow axonal transport, ^{35}S -methionine was injected as in 1) but animals were killed and nerves and ganglia were removed 56 h after radiolabelling.
3. For measurement of retrograde axonal transport, a total of 60 μCi ^3H -L-leucine were injected into one nodose ganglion. The animals were reanaesthetised 16 h later and a pair of ligatures 5 mm apart were applied to the radiolabelled nerve at mid-cervical level. After a further 8 h the animals were killed and nerves and ganglia removed.

The following procedure was carried out on control and galactose-fed rabbits, both with and without administration of Statil.

4. For measurement of the effects of applied pressure: 2 h after radiolabelling with ^3H -leucine as in 3., the cervical vagus nerve was exposed and a small perspex compression chamber applied to the nerve, as described previously [10]. The chamber consisted of two halves to which were attached rubber membranes which could be inflated with nitrogen to a desired pressure – in this case 20 mm Hg. The two halves of the chamber were fastened around the nerve and remained in place for a further 2 h. The animals were then killed and nerves and ganglia removed.
- In 2. and 3. above the animals were placed on their respective diets during the course of the experiment.

Radiolabelled nerves were frozen for storage and thereafter cut into pieces. For nerves radiolabelled with ^3H -leucine, 2.5 mm pieces were washed twice in 10% trichloroacetic acid and then dissolved in Soluene (Packard, Reading, UK) at room temperature prior to liquid scintillation counting of the TCA-insoluble radioactivity. For nerves radiolabelled with ^{35}S -methionine, 5 mm pieces of nerve were homogenised in a buffer containing sodium dodecyl sulphate (SDS), applied consecutively to wells of a gradient SDS-polyacrylamide gel and electrophoresed. The resulting gels were dried, impregnated with fluor and applied to X-ray film to produce fluorographs. The procedure has been described in detail previously [11].

The contralateral non-radiolabelled nerves were cleaned, blotted dry of excess moisture and weighed. They were then freeze-dried to constant weight and reweighed, in order to obtain a measure of nerve water content. In one experiment, the dried nerves were reconstituted in water and processed for measurement of galactitol and myo-inositol by the method of Stribling et al. [12] with the inclusion of galactose and galactitol as standards.

Calculation of results

Fast transport. The apparent rate of fast transport was measured both from the position of the front of the wave of radioactive proteins and from the position of the peak of radioactive proteins in the nerve 3.5 h after radiolabelling, assuming a 'lag-time' between radiolabelling and initiation of fast transport of 30 min [8]. The amount of fast transported radiolabelled proteins in the nerve was expressed as a fraction of that in the nodose ganglion. The main comparison of interest was of the proteins present in the most proximal portions of the nerve, 20–35 mm from the ganglion.

Slow transport. The rate of slow transport was calculated from the position of the fronts of the waves of slowly transported proteins in the nerve 56 h after radiolabelling.

Retrograde transport. The amount of proteins in the nerve immediately proximal to the proximal ligature and distal to the distal ligature were expressed as a fraction of the total amount of radioactive proteins in the nerve between 40 and 50 mm from the ganglion [13]. The latter figure was a measure of retrograde axonal transport while the former was a measure of the amount of fast transported proteins in the nerve once the initial wave of fast radiolabelled proteins had passed that point [11].

Inhibition by compression. The susceptibility of fast transport to compression was calculated as a Transport Block Ratio, i.e. the ratio of radioactive proteins in the two nerve pieces immediately proximal to the compression site plus one nerve piece within it, to the total radioactivity in the nerve between 37.5 and 50 mm from the ganglion [8].

Statistical analysis

A non-parametric statistical test (Mann-Whitney) was used for comparison of axonal transport measurements between control and galactose-fed rabbits. Student's t-test was used for comparison of the direct measurements of nerve galactitol, myo-inositol and water content.

Unless otherwise stated, all chemicals were obtained from Sigma, Poole, UK or British Drug Houses, Poole, UK.

Results

Fast axonal transport

The apparent mean rate of fast transport in control rabbits calculated from the position of the front of the wave of radiolabelled proteins, was 14.8 ± 1.0 mm/h (mean $\pm$ SD; $n = 5$). In the galactose-fed animals the apparent rate was slightly less at 13.0 ± 1.0 mm/h ($n = 5$), as shown in Table 1. The difference was statistically significant at the $p < 0.05$ level. The apparent mean rates, as calculated from the peaks of the waves of radiolabelled proteins were 8.5 ± 0.4 mm/h and 8.3 ± 2.4 mm/h, respectively. The difference was not significant. The amount of fast transported radiolabelled proteins within the nerves expressed in terms of radioactive proteins within the ganglion, was greater in galactose-fed than control animals. The increase was maximal when the proteins present in the nerve 25–30 mm from the ganglia were analysed (Table 1; $p < 0.01$). When the accumulation of delayed fast transported proteins was measured (as outlined above under 'Retrograde transport') there was no significant difference between control and galactose-fed rabbits (Table 1).

Vagus nerve galactitol levels in those animals were below the level of detection of the assay (i.e. < 0.08 $\mu\text{g/g}$ wet weight). The mean galactitol level in the nerves of the rabbits used for the measurement of fast transport rate was 53.0 ± 50.0 $\mu\text{g/g}$. Despite the variation, this was significantly higher than the level in the control rabbits ($p < 0.05$). Myo-inositol levels were not significantly reduced (Table 2).

Fluorograms of fast-transported ^{35}S -radiolabelled proteins failed to show any difference between the control and galactose animals in the nature of the fast transported proteins. In both cases they were similar to previously published work [11] and for that reason are not shown.

Retrograde transport

The amount of radioactive proteins accumulating distal to a ligature on the vagus nerve did not differ be-

Table 1. Axonal transport in control and galactose-fed rabbits

Measurement	Galactose-fed	n	Control	n	p
A. Fast transport rate (mm/h)	13.0 ± 1.0	5	14.8 ± 1.0	5	<0.05
B. Fast transport rate (mm/h)	8.3 ± 2.4	5	8.5 ± 0.4	5	NS
C. Amount of fast transported proteins	0.039 ± 0.014	5	0.017 ± 0.005	5	<0.01
D. Anterograde transport accumulation	4.49 ± 0.51	5	3.43 ± 0.78	4	NS
E. Slow transport rate (mm/day)	23.1 ± 2.1	4	23.8 ± 5.8 ^a	5	NS
F. Retrograde transport accumulation	1.19 ± 0.14	5	1.12 ± 0.34	4	NS

Measurements of axonal transport in rabbit vagus nerve after feeding of 50% galactose for a period of 29 days, mean of *n* values ± SD mean, as calculated from:

- The distance from the nodose ganglion of the front of the wave of ³H-labelled proteins 3.5 h after radiolabelling, assuming a delay for protein synthesis of 30 min.
- The distance from the nodose ganglion of the peak of the wave of ³H-labelled proteins 3.5 h after radiolabelling, assuming a delay for protein synthesis of 30 min.
- The ratio of the radioactive proteins in nerve pieces 20–35 mm from the nodose ganglion to the radioactive proteins in the ganglion 3.5 h after radiolabelling.
- The ratio of the radioactive proteins in the two 2.5 mm nerve pieces immediately proximal to a pair of ligatures applied to the nerve 16 h after radiolabelling to the total radioactivity in the nerve 40–50 mm from the ganglion, 8 h after ligation.
- The distance from the nodose ganglion of the front of the wave of ³⁵S-labelled proteins 56 h after radiolabelling. ^aThe transport rate for the control animals was taken from previously published work [8].
- The ratio of the radioactive proteins in the two 2.5 mm nerve pieces immediately distal to a pair of ligatures applied to the nerve 16 h after radiolabelling to the total radioactivity in the nerve 40–50 mm from the ganglion, 8 h after ligation.

tween control and galactose-fed rabbits (Table 1), despite galactitol and myo-inositol levels similar to those found during measurement of fast transport (Table 2).

Slow transport

The mean rate of slow transport in the galactose-fed rabbits was 23.1 ± 2.1 mm/day (*n* = 4) as calculated from the position of the front of the wave of radiolabelled proteins. This is the same as the normal rate of slow transport in this nerve [8]. Nerve galactitol levels ranged from 90–300 µg/g in the galactose-fed animals. The fluorograms of slowly transported proteins (not shown) from galactose-fed rabbits contained the same slowly transported proteins as those from controls and provided no evidence for qualitative changes in the synthesis or axonal transport of any slowly transported proteins.

Table 2. Galactitol and myo-inositol in vagus nerves of control and galactose fed rabbits

Group (as in Table 1)	Galactitol (µg/g wet weight)	Myo-inositol (µg/g wet weight)
A. Control	nd	872 ± 153
galactose	53.0 ± 50.0 ^a	675 ± 193
C. Control	–	–
galactose	170 ± 101	–
D. Control	nd	805 ± 64
galactose	50 ± 12 ^a	896 ± 166

Galactitol and myo-inositol content of vagus nerves after feeding rabbits 50% galactose for a period of 29 days. The groups correspond to the different experimental groups outlined in Table 1. In most cases levels of galactitol were not detectable (nd) in control rabbits. ^aDifferences between measurements from control and galactose-fed rabbits were statistically significant at the ^a*p* < 0.05 level. A dash (–) indicates that no measurement was made

Table 3. Inhibition of axonal transport by acute compression in control and galactose-fed rabbits

Treatment	Transport block ratio	n	p
Control	0.93 ± 0.61	10	–
Galactose-fed	12.97 ± 20.32	5	<0.02 ^a
Control with statil	1.37 ± 1.31	5	^a NS
Galactose-fed with statil	0.85 ± 0.74	5	^a NS <0.002 ^b

Inhibition of fast axonal transport in rabbit vagus nerve by local compression at 20 mm Hg. Inhibition is expressed as a Transport Block Ratio, which is the ratio of the radioactive proteins in two pieces of nerve immediately proximal and one nerve piece within the compression zone to the total radioactive proteins in the nerve 37.5–50 mm from the ganglion. The effects of feeding 50% galactose for 29 days with and without 25 mg/kg daily Statil are shown as mean values ± SD. Ratios are significantly different from those of ^acontrol or ^bgalactose-only treated rabbits, as indicated

Inhibition of fast transport by compression

The Transport Block Ratio was used as a measurement of the extent of inhibition of fast axonal transport by nerve compression (Table 3). The Transport Block Ratio in control rabbits was 0.93 ± 0.61 (range 0.28–4.23; *n* = 10), while that in galactose-fed rabbits was 12.97 ± 20.32 (range 2.60–49.25; *n* = 5). The difference was significant at the *p* < 0.02 level. The Transport Block Ratio in galactose-fed animals treated with Statil was 0.85 ± 0.74 (range 0.29–2.11; *n* = 5). This was significantly less than the ratio in rabbits fed galactose alone (*p* < 0.002) and was not different from the ratio in control animals. Statil had no significant direct effect on the ratio; in control animals treated with Statil the ratio was 1.37 ± 1.31 (range 0.35–3.05), which was not different from that in control animals without Statil.

Nerve water content in the controls was 2.02 ± 0.52 mg per cm of nerve; after galactose feeding it was 3.18 ± 0.49 mg/cm (Table 4). The difference between controls and galactose-fed animals was significant at

Table 4. Nerve water content in vagus nerve of control and galactose-fed rabbits subjected to nerve compression (as in Table 3)

Treatment	n	Nerve water (mg/cm nerve)	p
Control	10	2.02 ± 0.52	-
Galactose-fed	5	3.18 ± 0.49	< 0.005 ^a
Control with Statil	5	2.32 ± 0.59	^a NS
Galactose with Statil	5	2.47 ± 0.77	^a NS ^b NS

Nerve water content (SD) of vagus nerves after feeding 50% galactose with and without administration of Statil. Values are significantly different from those of ^acontrol only or ^bcontrol rabbits treated with Statil respectively

the $p < 0.005$ level. After treatment with Statil, nerve water was 2.32 ± 0.59 mg/cm of nerve in control rabbits and 2.47 ± 0.77 mg in galactose-fed rabbits. Neither value was significantly different from that in control rabbits without Statil.

In the same animals, galactitol was 22.7 ± 11.9 µg/g in galactose-fed animals and undetectable in controls. Myo-inositol was 152 ± 88 µg/g, compared with 103 ± 39 in controls. The increase in galactitol was significant at the $p < 0.005$ level; the difference in myo-inositol was not significant. In those cases, measurements were made on nerves which had been freeze-dried and reconstituted. Galactitol levels in nerves from control rabbits treated with Statil were undetectable; in galactose-fed rabbits treated with Statil, galactitol was 4.4 ± 9.8 µg/g. This was not significantly different from controls. Myo-inositol in the same two groups was 625 ± 57 µg/g and 548 ± 121 µg/g respectively. The difference was again not significant. In those cases nerves were not freeze-dried before measurement of galactitol or myo-inositol.

Discussion

Sidenius and Jakobsen [14] showed that galactose feeding in the rat produced changes in slow and retrograde axonal transport which were similar to those found in the streptozotocin-diabetic rat. The changes in axonal transport demonstrated in this study are not identical to those observed in the rat, i.e. we were unable to demonstrate any obvious change in slow or retrograde transport but observed a slight retardation and increased amount of fast transport. The only study of axonal transport in the sensory fibres of the rabbit vagus in diabetes [15] led to the conclusion that both fast and slow axonal transport were unaffected.

The slight retardation in fast transport observed in this work may be a consequence of a reduced transport rate or of a delayed outflow of fast transported proteins from the nodose ganglia. A much more severely reduced fast transport rate has previously been seen in this laboratory in the sciatic nerve of the streptozotocin-diabetic rat [16], an effect which has subsequently

been ascribed to reduced nerve temperature [17]. Although body temperature remained constant throughout these experiments, nerve temperature was not measured. The apparent increase in the amount of fast transported proteins after galactose feeding was unexpected. This could not be ascribed to a difference between the two groups in radiolabelling of proteins within the nodose ganglion. However, the possibility of increased diffusion and local incorporation of radioisotope in the galactose-fed rabbits must be considered as a contributory factor, in view of the increased water content.

Whether the differences in these results between rat and rabbit depend on the difference in the species and type of nerve studied (i.e. non-myelinated as distinct from myelinated) or on the regime for administering the galactose is unclear. At least in the streptozotocin-diabetic rat, axonal dwindling is known to be confined to myelinated fibres and to be absent in non-myelinated fibres [18]. The 4-day break employed in this study to prevent weight loss may have been sufficient to restore axonal transport deficits to normal since it is known that galactitol and nerve water content can be restored to normality in the rat as early as two days after cessation of a galactose diet [19]. The higher than average nerve galactitol in the animals used for slow transport measurements in this study may reflect that fact, since those animals were restored to their galactose diet for a full 56 h after surgery. If that were correct, further work with galactose feeding for longer periods may be necessary to detect axonal transport defects in rabbit vagus nerve. However, it does mean that the model is suitable for studying the susceptibility of transport to compression without changes in transport *per se* which could complicate the calculation of the results.

Nerve galactitol levels were markedly elevated in the vagus nerve in all experiments. The values quoted above in the nerves of rabbits used for measurements of fast transport rate were typical of the values found throughout; the fact that galactitol and myo-inositol levels were low in the experiments involving nerve compression in the absence of Statil was almost certainly a consequence of the fact that it was necessary to measure galactitol and myo-inositol in the same nerves which had been freeze-dried for measurement of nerve water content and then re-constituted. This also led to a loss of myo-inositol even in nerves from control animals [20].

The increased susceptibility to compression can therefore be explained in terms of the elevated levels of sugar alcohols. In rat sciatic nerve after galactose feeding, these generate osmotic changes leading to increased water content, and a resulting increase in intercapillary distance [4, 21]. It is this increase in intercapillary distance which is thought to be responsible for reduced endoneurial oxygen tension, and possibly reduced nerve blood flow [22, 23].

While at early stages of galactose neuropathy there is also an increase in endoneurial fluid pressure, it has been argued that that is not in itself sufficient to cause capillary closing and thereby lead to ischaemia [24].

So far no morphological data is available on the changes in rabbit vagus nerve produced by galactose feeding. The elevated nerve water content and the increased galactitol do, however, suggest that the situation is similar to that found in rat sciatic nerve. Further work will be necessary to determine whether the significant influence of the galactose feeding was a compromised nerve blood flow or an elevated endoneurial fluid pressure.

The altered susceptibility of the fast transport process to local mild pressure is consistent with previous work in the streptozotocin-diabetic rat [1, 2]; the results confirm that the effect can be observed in small non-myelinated sensory nerve fibres of the vagus nerve. However, in rat and rabbit sciatic nerve there is conflicting evidence for and against an increased nerve water content [5, 6, 25, 26]. Nor in our own experiments have we been able to observe reduced nerve myo-inositol [2] despite other reports of reductions in myo-inositol in both diabetic and galactosaemic rat nerve [6, 20], and the argument that changes in myo-inositol underlie the development of neuropathy and its prevention by inhibitors of aldose reductase [27] in experimental diabetes. It is likely that reduced nerve blood flow and the ensuing, or associated endoneurial hypoxia in both the galactose-fed animal and the streptozotocin-diabetic rat [21–23] are amplified in the presence of applied mild pressure, which also impairs nerve blood flow, to the extent that the oxygen-dependent fast axonal transport is inhibited [28, 29]. The relationship between elevated nerve polyols and reduced nerve blood flow may, however, be different in the two models.

Inhibition of fast axonal transport is unlikely in itself to be the explanation for longer term changes in nerve function associated with diabetes or galactose feeding [30–32]. In fact recent evidence points to deficiencies in provision of slowly transported neurofilaments [33] in galactose neuropathy. Inhibition of fast axonal transport by pressure as measured in this study is, however, known to be associated with an equivalent inhibition of slow and retrograde transport [13, 34].

Acknowledgements. This work was supported by a grant from the Medical Research Council, UK and Imperial Chemical Industries plc, UK. The author is grateful for the technical assistance of Ms. A. Smith, Mr. N. Hughes and Mr. D. Trafford and to Mr. D. Mirrlees for analysis of polyols.

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Received: 1 February 1988
and in revised form: 28 April 1988

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