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AXOPLASMIC FLOW IN AXONAL NEUROPATHIES

I. Axoplasmic Flow in Cats with Toxic Neuropathies

BY

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Introduction

THE passage of large amounts of protein and other compounds from the nerve cell body down the axon is a widely accepted phenomenon to which the term axoplasmic flow has been applied (*see* Barondes, 1967). Substances may also travel in a retrograde fashion from the periphery of the axon to the neuron (Lubinska, Niemierko and Zelena, 1963; Kerkut, Shapira and Walker, 1967; Watson, 1968; Kristensson, 1970; Kristensson, Olsson and Sjöstrand, 1971; Kristensson and Olsson, 1971), though the amount is probably less than the orthograde flow (Lubinska *et al.*, 1963; Lubinska, 1971; Edström and Mattsson, 1972). Many reports have described material flowing in an orthograde fashion from the neuron to the periphery of the axon at two main rates, the slow at about 1 to 2 mm/day, and the fast at several hundred mm/day. There are, however, reports of many intermediate velocities and this matter has been recently reviewed (Bradley, Murchison and Day, 1971).

The function of the axoplasmic flow material is not known, though at least five possibilities might be mentioned:

(1) The provision of enzymes and precursors for the distal synthesis of transmitter substances. Catecholamine granules (Dahlström and Häggendal, 1966) and choline acetyl-transferase (Frizell, Hasselgren and Sjöstrand, 1970) are both synthesized in the neuron and pass down the axon.

(2) The provision of "trophic factors" which have been supposed to be responsible for the many effects of the presence of an intact nerve supply upon an innervated structure. Proteins released from the neurohypophysis with the octopeptide hormones (neurophysins: Hope and Uttenthal, 1968; Dean and Hope, 1968; Geffen, Livett and Rush, 1969) and from the sympathetic nerves and adrenal medulla with catecholamines (chromogranins and dopamine β-oxidase: Viveros, Arqueros and Kirshner, 1968; De Potter, de Shaepdryver, Moerman and Smith, 1969; Kirshner, 1970), and the trans-synaptic passage of material (Grafstein, 1971) may be examples of this phenomenon.

(3) The transmission of information within the extremely elongate cell which is the neuron (Grafstein, 1969). For instance, a message is required to trigger the degeneration of the distal parts of the axon and the axonal reaction of the nerve cell body following axonal section, and this message may be conveyed by axoplasmic flow.

(4) The transfer of information or trophic factors to Schwann cells. Singer and Salpeter (1966*a* and *b*) have suggested that material is transported from the Schwann cells to the axons. Similarly a reversed passage of trophic influence must be considered to exist if the theory of secondary segmental demyelination advanced by Dyck (1973) were correct.

(5) The provision of nutrients for the maintenance of the distal terminals of nerves.

It has been suggested that axonal neuropathies of the "dying back" type (Cavanagh, 1964) might result from impairment of this fifth possible function of axoplasmic flow, with a decrease of the transport of materials, and in particular of protein from the nerve cell body to the peripheral branches of the axon (*see inter alii* Bradley, Lassman, Pearce and Walton, 1970). Pleasure, Mishler and Engel (1969) reported an experiment to test this hypothesis, studying the movement of radioactive-labelled protein in the dorsal and ventral roots of cats with acrylamide and triorthocresyl phosphate (TOCP) neuropathy. The slower rates of axoplasmic flow only were studied by virtue of the time points chosen for investigation. They found evidence of axoplasmic flow in all situations, except in dorsal roots in acrylamide intoxication, where flow appeared absent. The interpretation was that acrylamide caused axonal degeneration in sensory fibres by damaging the slow rates of axoplasmic flow, and that the mechanism of axoplasmic flow in TOCP neuropathy must be different. Bradley *et al.* (1970) later suggested that the slower components of axoplasmic flow might be impaired in "dying back" neuropathies, and that faster rates of flow might be impaired in neuropathies such as that of vincristine where this morphological distribution was not seen. The present study was undertaken further to investigate axoplasmic flow in toxic neuropathies.

Material and Methods

Young cats (1·5–4·25 kg body weight) of mixed breed were studied. Some animals were treated with vincristine, acrylamide or TOCP to induce a toxic neuropathy of mild to moderate severity. Animals receiving neurotoxins were regularly examined for general well-being, gait, ability to stand from lying, and reaction to pin-prick on the feet. The dosage of drugs was adjusted to induce mild to moderate ataxia of gait and weakness of the hind-limbs in two to six weeks. The dosages used were: vincristine 0·04 mg/kg body weight/twice weekly intramuscularly; acrylamide 20 mg/kg body weight/day five days/week orally; TOCP (Coalite and Chemical Products Ltd., Bolsover, Derbyshire), 0·25 mg/kg body weight every two weeks subcutaneously.

Dorsal root ganglion injection.—Control animals, and those with mild to moderate signs of a neuropathy received an injection of L-leucine-4,5-^{3}H into the seventh lumbar dorsal root ganglion on one side. The method was similar to that of Ochs and Ranish (1969). Cats were anæsthetized with pentobarbitone (30 mg/kg body weight) and halothane in oxygen by face mask. Under sterile conditions, the sixth and seventh lumbar and first sacral vertebræ were exposed, the laminæ on one side removed, revealing the seventh lumbar dorsal root ganglion. Under a dissecting microscope, a glass micropipette of tip external diameter 50–70 μ was introduced through the capsule after puncture with a fine steel needle. The micropipette was lowered to a depth of 0·5 mm (Lasek, 1968) with a micromanipulator. The injection volume was measured with a Hamilton 100 μl syringe (A. V. Howe & Co. Ltd., No. 701) and a volume of 15 μl (equivalent to 15 μCi of L-leucine-4,5-^{3}H; specific activity 17 Ci/mmol, 1 mCi/ml; Radiochemical Centre, Amersham, Bucks) was delivered slowly over three minutes. Any leaking radioactive solution was absorbed by lint. The laminectomy

was covered with sterile gelatine foam, the area sprayed with a polymyxin-bacitracin-neomycin aerosol, and the skin closed with clips. The animals recovered quickly and on the day after operation were able to walk normally. None developed neurological abnormalities as a result of the operation. Animals were killed by an overdose of pentobarbitone six hours, ten days and thirty days after dorsal root ranglion injection. Three animals at each time point from each of the control, vincristine, acrylamide and TOCP groups were studied. In a standard manner the seventh dorsal root was removed *in continuo* with the injected L7 ganglion, the whole of the sciatic nerve and its major branches to the level of the popliteal fossa. The nerve was laid on a metal ruler marked in one mm lengths, frozen with solid carbon dioxide, and cut with a razor blade into 3 mm segments. Each was dissolved in low potassium scintillation vials, and the radioactivity measured as previously described (Bradley, Murchison and Day, 1971). Statistical analysis of groups was made using the Mann-Whitney U Test.

Material taken from the opposite hind-limb was used for histological study. The spinal cord, dorsal and ventral roots, the sciatic nerve in the thigh, the posterior tibial nerve and digital nerves in the foot were examined in paraffin sections stained with hæmatoxylin and eosin, solochrome cyanin, and by the Weigert-Pal and Glees and Marsland methods. The hamstring, gastrocnemius, and plantar muscles were studied in cryostat sections stained for myosin ATPase, NADH diaphorase, and by the method of Namba and Grob; and in paraffin sections stained with hæmatoxylin and eosin, phosphotungstic acid-hæmatoxylin, and the Picro-Mallory method. The histological severity of the neuropathy was graded into mild, moderate and severe. The clinical abnormalities at the time of operation and of sacrifice were similarly graded.

Results

Control Animals

The distribution of radioactivity in the L7 dorsal root and the sciatic nerve of normal animals sacrificed at six hours and thirty days after dorsal root ganglion injection is shown in fig. 1. Each point is the average of three animals. The lines have been arbitrarily fitted to the points by eye, and are simply intended to aid in the interpretation of the graphs. At six hours, the activity in the dorsal root ganglion was much higher than at thirty days. At the earlier time in the sciatic nerve a peak of radioactivity occurred about 80 mm from the ganglion and a front of radioactivity about 120 mm from the ganglion. The peak may therefore be interpreted as being due to material moving with a velocity of 320 mm/day, and the front with a speed of about 480 mm/day. In other animals killed at shorter intervals than six hours, a peak and front advancing at approximately these rates were seen, similar to those described by Ochs and Ranish (1969). At thirty days a similar peak about 45 mm from the ganglion was seen corresponding to material moving with a speed of about 1·5 mm/day. Similar curves were found by Lasek (1968). The sciatic nerve from animals killed ten days after injection showed either no peak or only a very small one. Lasek (1968) similarly found only a vague peak in animals sacrificed ten days after dorsal root ganglion injection. The analysis in this paper is based upon the movement of demonstrable waves of activity, and therefore the animals sacrificed at ten days will not be discussed further.

Assessment of the individual speeds in each control animal and the height of the peak are shown in Table I.

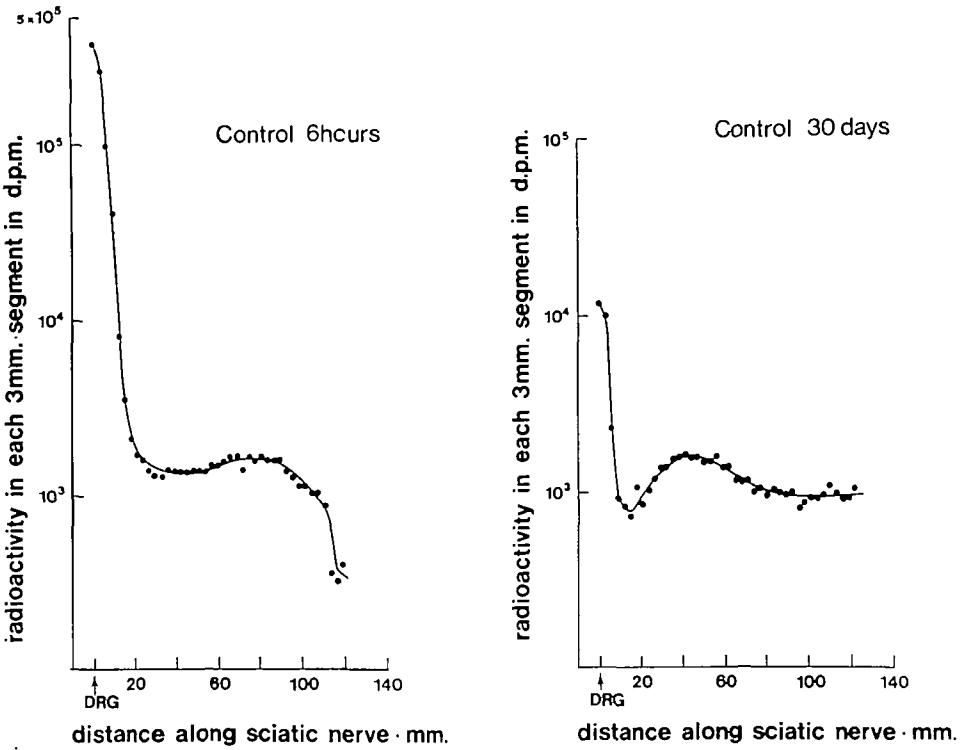

FIG. 1.—Distribution of radioactivity within 3 mm segments of L7 dorsal root ganglia and sciatic nerve of control cats sacrificed six hours and thirty days after injection with ^{3}H-leucine. Each point is the average of values derived from three animals.

TABLE I.—DATA ON AXOPLASMIC FLOW AT SIX HOURS AND THIRTY DAYS AFTER L7 DORSAL ROOT GANGLION INJECTION IN CONTROL ANIMALS AND THOSE WITH TOXIC NEUROPATHIES

Time	*Group*	*Crest dpm*	*Ganglion dpm*	*Crest/ ganglion per cent*	*Average crest/ ganglion per cent*	*Crest rate mm/day*	*Average crest rate mm/day*	*Front rate mm/day*	*Average front rate mm/day*
6 hour	Control	1203	444,800	0·27	0·47	364	385	428	459
		2655	418,700	0·63		432		456	
		1250	240,000	0·52		360		492	
	Vincristine	736	491,600	0·15	0·28	253	276	304	355
		936	559,100	0·17		288		324	
		2078	397,500	0·52		288		436	
	Acrylamide	1350	500,000	0·27	0·75	265	304	324	416
		3800	500,000	0·76		324		504	
		3050	250,000	1·22		324		420	
	TOCP	1600	320,000	0·50	0·87	328	313	444	412
		1675	290,000	0·58		300		372	
		325	214,000	1·52		312		420	
30 day	Control	569	6,700	8·5	12·6	1·1	1·4		
		1925	12,100	15·9		1·4			
		2693	20,000	13·5		1·7			
	Vincristine	165	9,000	1·8	17·5	1·3	1·2		
		3910	10,300	37·9		1·4			
		1246	9,900	12·6		0·8			
	Acrylamide	4622	28,200	16·4	14·3	1·4	1·3		
		2750	18,000	15·3		1·2			
		1800	16,000	11·3		1·2			
	TOCP	2500	13,000	19·3	14·8	0·9	0·9		
		2000	11,200	17·9		0·9			
		2200	30,000	7·3		0·8			

Animals with Toxic Neuropathies

The distribution of radioactivity in the sciatic nerves of these animals are shown in figs. 2, 3 and 4. Again each point corresponds to the average of 3 animals. In acrylamide neuropathy at six hours and in vincristine neuropathy at thirty days, there was a considerable scatter of points which may correspond to a number of minor peaks of axoplasmic flow, or may be due to experimental error or biological variation. Nevertheless, at the two time points in each group, a wave of activity was clearly discernible, indicating that axoplasmic flow occurred in all three groups of animals with toxic neuropathies. The speeds and the crest heights recorded in each individual animal are shown in Table I.

Crest heights.—There is wide variation between animals in all four groups. Thus at six hours in the normal animals, the crest height expressed as a percentage of the dorsal root ganglion activity ranged from 0·27–0·52 per cent, while in animals with vincristine neuropathy studied at this time, one value was comparable to normal, and two values were below the normal levels; similarly at thirty days, the crest height in normal animals varied from 8·5–15·9 per cent of the dorsal root ganglion level, and in the vincristine animals there was one very low value of 1·8 per cent and one very high value of 37·9 per cent. There is clearly no significant difference between the mean crest heights either of the "slow" or "fast" waves in these four groups of animals, though the average height of the "fast" wave crest is somewhat lower in animals treated with vincristine than in the controls.

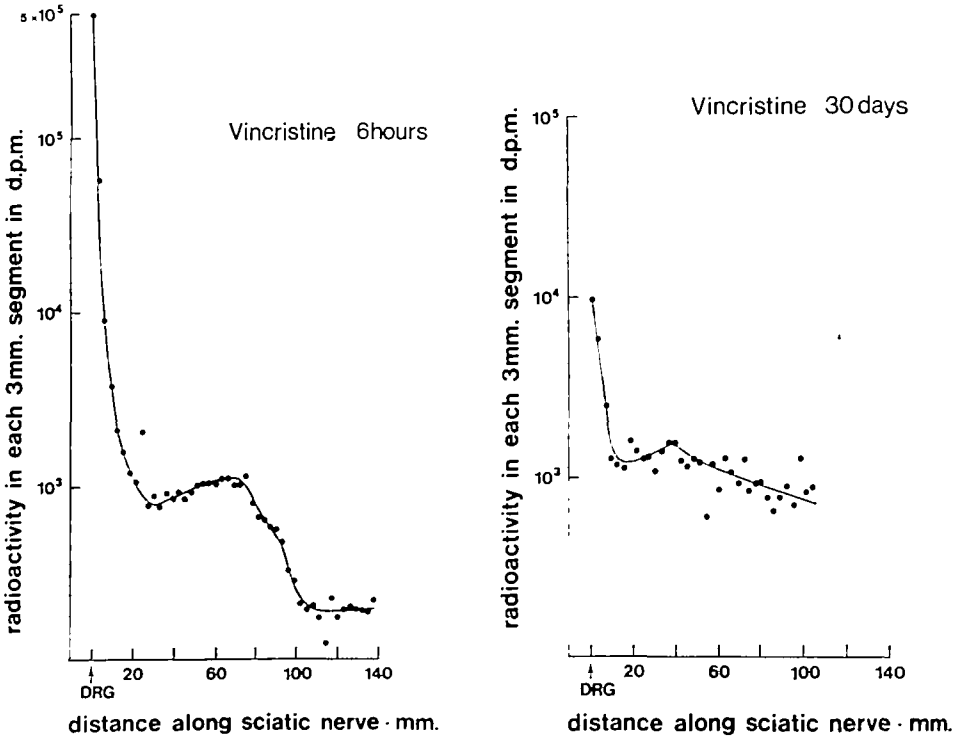

FIG. 2.—As fig. 1 for animals with vincristine neuropathy.

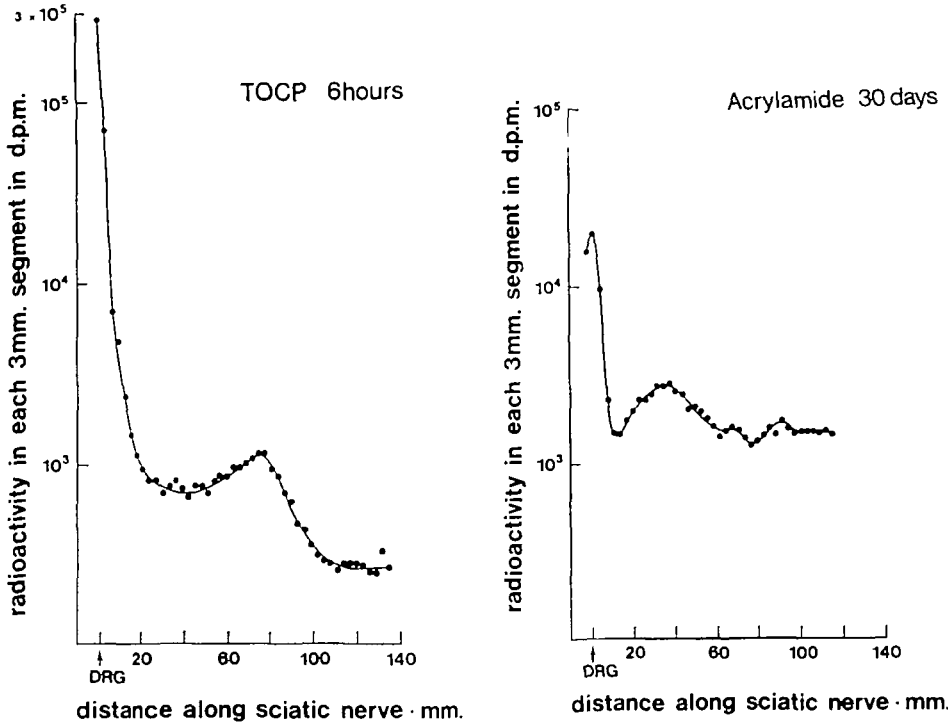

FIG. 3.—As fig. 1 for animals with acrylamide neuropathy.

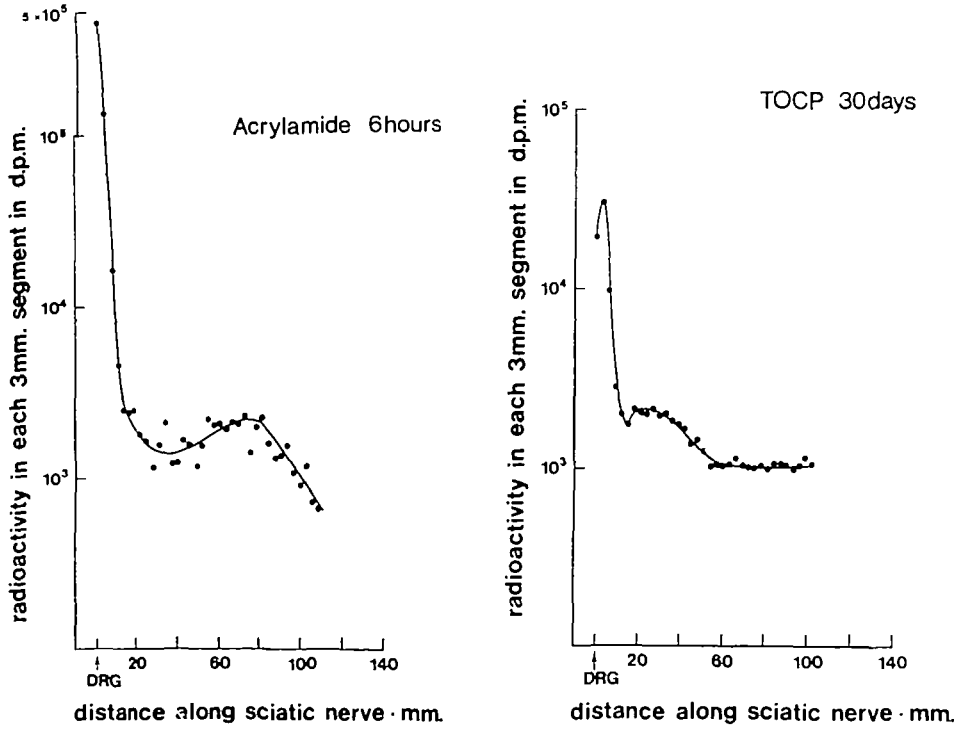

FIG. 4.—As fig. 1 for animals with TOCP neuropathy.

Crest and front velocities.—In this analysis there was less variation between animals of a group. The crest velocity in all the cats with toxic neuropathies sacrificed six hours after ^{3}H-leucine injection, and in all the TOCP-treated cats sacrificed thirty days after injection was significantly below that in the normal animals ($P=0{\cdot}05$). The velocity of the crest of slow axoplasmic flow in vincristine- and acrylamide-treated animals was not different from that in control animals. The velocity of the front of both "fast" and "slow" moving radioactivity did not differ significantly from normal in any group other than in the TOCP-treated animals sacrificed at thirty days after injection of the ganglion ($P=0{\cdot}05$). The reduction of mean velocity for the "fast" wave in each group ranged from 19 per cent in TOCP-treated animals to 28 per cent in vincristine neuropathy. The "slow" wave in the cats with TOCP neuropathy was slowed by 36 per cent compared with normal.

Discussion

The most important conclusion to be drawn from this study is that axoplasmic flow may be shown to occur in a retrograde direction down the sensory nerve fibres of cats with vincristine, acrylamide or TOCP neuropathy. Peaks of radioactivity were seen in the sciatic nerves both at six hours and thirty days in similar positions to those found in normal animals. Even though only three time points were studied, the fact that the peak at six hours after injection of ^{3}H-leucine into the dorsal root ganglion was not seen at ten days, and that a new peak had appeared at thirty days may be taken as clear evidence of the presence of axoplasmic flow (Lasek, 1968; Ochs and Ranish, 1969).

We have therefore been unable to substantiate the finding of Pleasure *et al.* (1969) of no axoplasmic flow in feline sensory roots in acrylamide neuropathy, but to confirm that flow may occur in TOCP neuropathy; Pleasure *et al.* (1969) studied the dorsal root, that is, axoplasmic flow in the orthodromic direction. The findings described in this report relate to the flow along the part of the nerve usually showing the "dying back" phenomenon, that is, the peripheral branches of the sensory fibres. In normal animals, like Lasek (1968), we did not find evidence of moving peaks of radioactivity in the dorsal root. Though Pleasure *et al.* (1969) used autoradiography, they measured the total endoneurial radioactivity, and so like us were unable to define just how much extra-axonal radioactivity was being measured. One refinement of the method would have been to measure autoradiographically only axoplasmic radioactivity as in the study of Bradley and Jaros (1973), but as moving peaks of radioactivity were seen both in the present study and in that of Pleasure *et al.* (1969) it seems likely that the majority of the activity being studied was in fact intra-axonal.

The question which remains therefore is whether there is any quantitative difference between the flow of material in cats with neuropathies and normal cats. Within each toxic group there was marked variation in the height of the waves of axoplasmic flow, and no significant difference from normal was demonstrated between the heights of both the "fast" and "slow" waves. The results therefore give no support

to the hypothesis that a *decrease in the amount* of the axoplasmic flow of protein is the cause of axonal degeneration in toxic neuropathies.

On the other hand, significant differences from normal in the velocities of the crests, but not of the fronts, of these waves, were found. The crests of the "fast" wave in all the neuropathic animals, and of the "slow" wave in the TOCP-treated animals moved more slowly than normal.

The decrease in the rate of delivery of protein at fast rates of flow in vincristine neuropathy is interesting. Vincristine and vinblastine, like colchicine, bind to neurotubular protein and produce neurofibrillary accumulation in the nerve cell (Schochet, Lampert and Earle, 1968; Wisniewski, Terry and Hirano, 1970; Seil and Lampert, 1968; Journey, Burdman and Whaley, 1968; Krishan and Hsu, 1969; Olmsted and Rosenbaum, 1969; Marantz and Shelanski, 1970). Both colchicine and vinblastine impair fast and slow axoplasmic flow (Dahlström, 1968; Fernandez, Huneeus and Davison, 1970; Fernandez, Burton and Samson, 1971) though colchicine affects the fast more than the slow rates of flow (Karlsson and Sjöstrand, 1969; Sjöstrand, Frizell and Hasselgren, 1970). The intraneural injection of colchicine (Angevine, 1957), and the systemic administration of vincristine (Bradley *et al.*, 1970; Bradley, 1970) produce axonal degeneration. It was for these reasons that Bradley *et al.* (1970) suggested that vincristine, which does not produce a "dying back" picture, might cause a decrease in the fast rates of axoplasmic flow, and the "dying back" neuropathies a decrease in the slower rates of flow. The present study supports this suggestion with regard to vincristine, though the situation in the "dying back" neuropathies is more complex. In TOCP neuropathy both "fast" and "slow" crest velocities were reduced, while in acrylamide neuropathy the "fast" but not the "slow" crest was retarded.

There was no correlation between the rates and amounts of axoplasmic flow, and the clinical or histological severity of the neuropathy. It is possible that the clinical signs may in part have been complicated by central nervous degeneration which occurs to some extent in acrylamide and TOCP intoxication (Fenton, 1955; Prineas, 1969*a* and *b*). If, however, the hypothesis that axonal degeneration is due to impairment of axoplasmic flow of protein were correct, the more severely affected animals would have been expected to have the greater impairment of flow, and this was not so.

In this study no attempt was made to measure the specific radioactivity of purified protein from each nerve segment. However as argued elsewhere (Bradley *et al.*, 1971), within three hours of injection the major part of ^{3}H-leucine will have been synthesized into protein. Only a minor part is incorporated into other cell components including lipid. Since very few lipid droplets are to be found within the axoplasm on electronmicroscopy, the major part of axonal lipid is presumably phospholipid. The axonal flow of phospholipid is small in amount, and occurs without evidence of moving peaks of radioactivity (Miani, 1964, 1967). It is therefore unlikely that the presence of components other than protein will have interfered with the analysis made here.

We must now discuss the significance of the demonstrated *decrease in velocity* of *normal amounts* of material with regard to axonal degeneration in toxic neuropathies. If the hypothesis advanced in the introduction were correct, the amount of protein delivered to the distal parts of the nerve might be the significant factor in the maintenance of axonal integrity. It therefore becomes a four-dimensional problem involving both the rate of flow and the amount of material transported. There are insufficient data available from the present study to build up a clear picture of the arrival of material in the nerve terminals in these animals. Nevertheless, at the most the delivery of material was reduced by 36 per cent, and was probably very much less affected than this. It seems unlikely that a biological process would have such a small safety factor that perhaps a 10 per cent reduction would cause structural breakdown. We therefore do not believe that the changes demonstrated here could cause the axonal degeneration demonstrated.

One possibility, which cannot however be excluded, is that axonal integrity depends upon a protein constituting only a minor fraction of axoplasmic flow. Quantitative fractionation of these proteins in disease states would be of value.

The study of axoplasmic flow in toxic neuropathies is by no means as simple as might at first appear. In most neuropathies degeneration and to a greater or lesser extent regeneration occur side by side. A totally degenerated axon will not transport protein. As discussed in the following paper (Bradley and Jaros, 1972), axoplasmic flow may be increased in regenerating fibres. All studies must face this problem, particularly those where a single application of a toxin is used.

Another complexity is that the moving waves used in this study constitute only a minor part of the total axoplasmic flow. As Bradley *et al.* (1971) showed, most of the material moves in a graded manner, the amount of the fast flowing material being much less than that of the slow. A full analysis of axoplasmic flow requires the quantification of the amount of material moving at all velocities, by a method such as that of velocity spectrum analysis described by Bradley *et al.* (1971). A large number of animals are required to allow such an analysis, and to date no such analysis has been attempted.

Summary

A study of waves of "fast" and "slow" axoplasmic flow of radioactivity following the injection with ^{3}H-leucine of the L7 dorsal root ganglion of cats suffering from toxic neuropathies is presented. Comparison was made between normal animals, and those intoxicated with vincristine, acrylamide and triorthocresyl phosphate. The heights of these waves in the intoxicated animals were normal. The rate of movement of the crests of the "fast" waves, but not of the fronts, was reduced in all experimental groups. The velocity of the "slow" wave was reduced only in TOCP neuropathy.

It is argued that this reduction of velocity is unlikely to be the cause of the axonal degeneration. The complexity of investigations of axoplasmic flow in toxic neuropathies is highlighted.

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