SOME QUANTITATIVE ASPECTS OF DIPHTHERITIC NEUROPATHY

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IT has been recognised since the observations on a human case of diphtheritic neuropathy by Meyer (1881) and from a study of experimental animals by Stransky (1903) that the main anatomical abnormality produced in nerves by diphtheria toxin is a degeneration of myelin sheaths. Since this period it has come to be generally recognised that the myelin sheath is in fact an integral part of the cell of Schwann. The juxtanodal region, depicted by Meyer as undergoing the earliest changes, shows a further specialisation of structure. The myelin sheath is here uncompacted and opened out into regular loops which enclose pockets of Schwann cell cytoplasm (Hess and Lansing, 1953 ; Robertson, 1957 ; Luxoro, 1958) and thus may well have a specialised function different from that of the rest of the cell. Recently Waksman, Adams and Mansmann (1957) have confirmed the findings of Meyer and of Stransky and have also drawn attention to differences in localisation of the lesions in the guinea-pig and the rabbit. In the former they find that changes occur along the whole length of the peripheral nerve, while in the latter species they are concentrated in the region of the spinal nerve roots. No account was taken by these last authors of the fact that there are Schwann cells of different sizes and lengths in various parts of the peripheral nervous system, however, and it was not possible, from the technical methods that they used, to gain a clear idea whether the different varieties of Schwann cell were affected in the same manner. If the mechanism of the intoxication of the Schwann cell is to be understood, it would be important to know whether the changes are due to damage to the whole of the cell of Schwann or are selective only to the juxtanodal regions as is suggested by some of Meyer's illustrations ; and also whether long and short internodes are equally affected.

It should be recalled that there is usually a direct relationship in the normal animal between the external diameter of the nerve fibre (including its myelin sheath) and the length of its internodal segments, and that the length of the internodal segments is in large part the result of growth in length of the fibre (Vizoso, 1950). The basic length of a Schwann cell is about 230 μ (Young, 1950), but growth in length of the part may increase this more than four times. Since myelination begins in the various nerve fibre systems at various times during development and growth varies much from one part to another, there will thus be a whole range of internodal lengths present in a mixed peripheral nerve. Fibres subserving the various types of sensory endings will thereby tend to have internodes of different thickness and varying lengths ; the same will also hold for motor fibres to voluntary muscles and to intrafusal spindle muscle fibres.

This study was begun in order to determine whether all Schwann cells were equally affected; whether they were affected contemporaneously during the progress of the disease; or whether any kind of selectivity could be detected in the process, either to cells of different lengths or along the course of the individual internodes. The results reported here show that selectivity does in fact occur in the different fibre sizes and that the whole Schwann cell does not necessarily undergo complete degeneration. We believe that these observations are of significance to the understanding of the mechanism of the Schwann cell lesion and in explaining the manner of the progress of the disease.

MATERIALS AND METHODS

Animals.—Young chickens of White Leghorn strain, usually slightly less than 1000 g. in weight and about 10 weeks old were used. They were housed singly or in pairs in cages and fed on poultry bran.

Toxin and antitoxin.—The toxin and the antitoxin were kindly provided by Dr. Mollie Barr of the Wellcome Research Laboratories, Beckenham, Kent, the toxin (RX 7151) containing one guinea-pig L+ dose in 0·024 ml., and the antitoxin (LX 427) containing 75 units/ml. (By definition an L+ dose of toxin is the least amount of toxin which when combined with one unit of antitoxin will kill a 250 g. guinea-pig in 5 days [Ehrlich, 1906]. Lf dose of toxin is that amount of toxin which will show optimal flocculation when mixed with one unit of antitoxin by the Ramon method [Glenny and Okell, 1924]. Since both toxin and toxoid will combine and flocculate with antitoxin the Lf and L+ quantities of different batches of toxin do not bear a constant relation, and depend on the content of natural toxoid. [Dr. Barr, personal communication.])

The standard mixture used for injection contained 0·48 ml. of toxin and 0·26 ml. of antitoxin made up to 5 ml. with normal saline. One L+ (guinea-pig) dose was thus present in 0·25 ml. and the chickens were injected with fractions of the L+ quantity for each 100 g. weight. Combination of the toxin with antitoxin was found to be fairly rapid and no appreciable difference could be detected in the paralysing effect whether the mixture was given 5 min. after mixing or 1 hr. later. Saline was substituted for the antitoxin when toxin was given alone or separately. All injections were given into the left breast muscle unless otherwise stated.

Assessment of functional disturbance.—The animals were examined daily by 2 independent observers before and after an exercise test. This test consisted in raising and lowering the animal by the retrices (tail feathers) in response to which it normally flaps its wings and makes running movements with its legs. Normal birds of this strain will continue in this manner for 20 or 30 such raising and lowering motions before tiring. Abnormal fatigue and slight degrees of unsteadiness or ataxia are readily shown up by this test. The animal was also shaken to assess the state of muscular tone in the legs and wings, and was carefully watched while walking and running for evidence of unsteadiness.

The functional state assessed in this way was put on to a four-point scale for record purposes: (1) definite tiring on exercise, subdued behaviour, slight hypotonia of the limbs; (2) tiring rapidly on exercise, definite unsteadiness on walking, definite hypotonia of limbs; (3) can stand or walk for short periods but prefers to sit, marked hypotonia, gross ataxia on walking; (4) unable to stand. Two or more of each of these features had to be present before a grade was settled upon. In the early phases of the paresis difficulty was often experienced in categorisation. If the animal had been suspected of being abnormal on one day and showed definitely abnormal features on the next, then it was considered that the first observation had been correct.

Treatment of tissues.—The animals were killed by an intracardiac injection of Nembutal. The nerves were dissected out and laid on to a card after being elongated to their natural length. When a piece of nerve is cut from the body it shortens and shows narrow transverse striations due to a regular folding of the fibres which is probably due to shortening of elastic tissue within the epineurium (Thomas, 1963). The nerves were straightened until these transverse striations disappeared, allowed to dry a little until adherent to the card and then dropped into fixative. The spinal canal was opened and the spinal cord and nerve roots were fixed *in situ*.

Various fixatives were used. Formol-saline (10 per cent) was chosen as the routine fixative since it allowed staining with several methods including osmium tetroxide, and there is a greater elasticity (resistance to deformation) in the nerve fibres and their sheaths after this fixative than after osmium tetroxide. With the latter fixative the process of teasing was found to lead to a considerable amount of distortion both of the nerve fibre at its internodes and at the nodes of Ranvier. Flemming's fluid was used for nerve fibre diameter measurements (Fernand and Young, 1951).

In order to study individual fibres about 1 cm. of nerve was cut out, the epineurium stripped off, and the nerve fibres partly separated. This was then stained either with 1 per cent osmium tetroxide for one hour or with one of the Sudan dyes, such as Sudan black B in 70 per cent alcohol. Subsequent differentiation was in 50 per cent alcohol, and the nuclei were counter-stained with carmalum, or with Meyer's haemalum if a red Sudan dye had been used. The nerves were then stored in 33 per cent glycerine and teased later under the dissecting microscope with sewing needles. The preparation was finally mounted in 33 per cent glycerine under a circular coverslip and sealed with asphalt varnish.

Measurements.—External diameters of the nerve fibres and nodal gaps (the nodal gap was measured as the distance between the rounded ends of the compact myelin) from Sudan preparations were measured with a movable ocular micrometer at an objective magnification of 78 diameters, the grid and the superimposed image being inspected through a $\times 10$ Kellner ocular. Osmium stained fibres were measured at a higher objective magnification ($\times 120$) because of the considerably smaller size of the gaps. The fibre diameter spectrum of the peroneal nerve was obtained by measuring the external diameter of all fibres in a photograph of a transverse section magnified to 1000 diameters in the manner of Fernand and Young (1951).

The differences between the means of each group of measurements were tested for significance with the Student *t*-test.

RESULTS

Paralytic responses and toxicology

Effects of dose and the character of the paresis (*Table I*).—The slightly underneutralised mixture when given in a dose of 0·33 L+ (guinea-pig)/100 g. of bird did not produce any overt effect upon the birds for the first 2 days. On the 3rd day the animal might still be apparently well, but sometimes showed that it was rather more subdued than its colleagues. This particular strain of bird has a tendency to be somewhat aggressive and reactive. On opening the cage it is usual for the normal bird to make active efforts to escape and to fly up into the observer's face. By the 3rd day after inoculation and certainly by the 4th day with this dose, this normal activity was noticeably lessened or absent. On examination the animal was found to be more floppy than usual and it tired on exercise. By the 5th day it tired very easily on exercise and was often reluctant to move at all. When it walked it would show unsteadiness or ataxia, and its wings commonly drooped. By the 6th or 7th day the animal could no longer stand; it was grossly hypotonic; and it had difficulty in breathing and holding up its head. The birds were usually killed when this stage was reached.

With one-tenth of this dose (0·033 L+/100 g.) the delay period before the onset of symptoms was slightly longer (Table I). Following the onset of symptoms the condition advanced over the succeeding days to reach the maximum of dysfunction by about the 15th day. There was some improvement later on but the animals were not specifically studied after 21 days.

With a dose of 0·0033L+/100 g. the delay period was further prolonged to about the 14th day and the degree of paresis reached was considerably less. With intermediate doses there was a corresponding variation in the speed of onset and the ultimate state of paresis.

TABLE I.—*Response to Doses of Toxin Mixed with an Under-Neutralising Quantity of Antitoxin*

Days	Dose in L+/100 g. (Nos. of birds in brackets)					
	0·33(7)	0·033(8)	0·025(2)	0·017(4)	0·0033(2)	0·0017(2)
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0·3*	0	0	0	0	0
4	1·0	0	0	0	0	0
5	1·8	0·1	0	0	0	0
6	3·0	0·7	0·5	0	0	0
7	4·0	1·3	1·5	0·25	0	0
8		1·4	2·0	0·5	0	0
9		1·6	2·5	0·5	0	0
10		1·6	2·5	1·0	0	0·5
11		1·7	2·5	1·0	0	0·5
12		2·1	2·5	1·0	0	0·5
13		2·3	3·5	1·0	0	0·5
14		2·3	3·5	1·0	1·0	0·5
15		2·5		1·25	1·0	0·5
16		2·5		0·6	1·0	0
17		2·5		0·6	1·0	0
18		2·5		0·6	0	0
19		2·5		1·0	0	0
20		2·5		1·0	0	0
21		2·5		0·5	0	0

* See footnote on Table II.

Effects of toxin alone (*Table II*).—When the toxin was given alone either intramuscularly or intravenously in a dose of 0·025L+/100 g. paralysis appeared about the 4th day and reached a (2) or (3) degree by about the 6th day and was thus equivalent to 0·33L+/100 g. of the mixture. One-tenth of this dose led to almost the same degree of paresis by the 7th day which in 2 birds went on to more severe paralysis. One-twentieth of the original dose (0·00125L+/100 g.) produced only minor weakness and increased fatiguability which was inconstant and did not increase over the ensuing days. No substantial difference was found either in the manner of progress of the paresis or in the changes in the nerves whether antitoxin had been administered or not.

TABLE II.—*Response to Doses of Toxin Alone*

Days	Dose in L+/100 g. (Nos. of birds in brackets)				
	0·025(2)	0·0025(5)	0·00125(3)	0·00025(3)	0·000125(3)
1	0	0	0	0	0
2	0	0	0	0	0
3	2·0*	0·6	1·0	0	0
4	1·0	0·8	1·0	0	0
5	1·0	0·8	1·0	0	0
6	3·0	1·2	1·0	0	0
7	4·0	1·8	1·0	0	0
8		2·2	1·0	0	0
9		1·7	1·0	0	0
10		2·0	1·0	0·3	0
11		1·7	1·3	0·3	0
12		1·7	1·0	0	0
13		1·7	1·0	0	0
14		2·0	1·0	0	0

* The degree of paresis following injection of toxin was assessed on a four point scale (see text). The numbers represent the average degree of paresis.

Effects of giving toxin and antitoxin separately (*Table III*).—When the toxin is given alone intravenously in a dose of 0·025L+/100 g. paralysis comes on as stated by the 5th day. If 10 or even 3 times the neutralising dose of antitoxin is given into the opposite wing vein either 5 min. before or after the toxin, almost complete protection is obtained. Slightly increased fatiguability and some subduedness was in fact seen during the second week in 2 birds but no major paresis developed except in one bird. In this animal a large part of the injected antitoxin leaked from the vein and it is believed that its relatively slow absorption from the subcutaneous tissue allowed the toxin time to become fixed to the tissues. The period, indeed, during which the toxin is available for inactivation is a short one, for from 1 hr. onwards injection of antitoxin has no demonstrable effect upon the subsequent progress of the paralysis. It is concluded that the toxin has reached and may be fixed to the Schwann cells within an hour of injection, and the delay period before the onset of lesions and symptoms may thus be an intracellular phenomenon.

TABLE III.—*Effects of Temporal Separation of Toxin and Antitoxin*

	0·025 L+/100 g. toxin given i.v. to all birds							
Time of antitoxin	3 hr. after	3 hr. after	1 hr. after	1 hr. after	5 min. after	5 min. after	5 min. after	5 min. before
Amount of antitoxin*	×3	×10	×3	×10	×1	×3	×10	×10
Nos. of birds	(3)	(3)	(3)	(3)	(2)	(2)	(3)	(3)
Days—								
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	1·0	1·3	0	1·0	1·0	0	0	0
5	3·0	3·0	1·0	3·0	4·0	0	0	0·6†
6	4·0	4·0	3·0	3·0	4·0	0	0·3	1·3†
7			3·0	3·3		0	0·3	0
8			3·0	4·0		0	0·3	0
9			4·0	4·0		0	0·3	0
10			4·0			0	0·3	0
11			4·0			0·5	0	0
12						0·5	0	0
13						0·5	0	0
14						0·5	0	0

* Multiples of amount of antitoxin in 1 L+.
† One bird in which antitoxin leaked from vein during injection.

Qualitative changes in peripheral nerves

Teased preparations were found to be far more informative than preparations by any other method. This is, indeed, the only method by which the Schwann cell can be properly examined. Tibial nerves, sciatic nerves, and lumbar spinal roots were examined at 5, 7, 14 and 21 days following inoculation ; no essential differences were found in the amount or character of the changes occurring in any of these 3 sites.

At 5 days following 0·33L+/100 g. toxin-antitoxin mixture extensive fragmentation of myelin was observed in many small diameter fibres which clearly, by the presence of reactive cell proliferation, had begun at least a day or two before examination, and before the onset of evident paresis (Fig. 2). On the other hand, the larger diameter fibres showed very little alteration in structure

despite severe paresis. From 7 days onwards definitely abnormal changes began to occur in the larger diameter fibres. Most striking was an increase in the width of the nodal gap between the adjacent rounded ends of the myelin internodes (Fig. 3). Occasionally a small nucleus (? macrophage) lay in the gap. The paranodal region was occasionally seen to be slightly swollen and less often at this stage actually hyaline and fragmenting. These alterations of the paranodal region of large fibres became more obvious and frequent in animals examined at 10 and 14 days after inoculation ; at which time little lipid remained in the small diameter fibres that had disintegrated earlier. By 21 days very large gaps (Fig. 4*a* and *b*) appeared in the nodal regions of the larger diameter fibres. At the same time definite evidence of the formation of new myelin began to be found on the small fibres (Fig. 4*c*).

The changes described above were not followed further, since our primary concern has been with the early stages of the lesion. It is apparent, however, that in the first 3 weeks the development of nerve damage follows a regular sequence commencing with the involvement and total breakdown of the whole internode of the smallest fibres, and ultimately partial disintegration of the internodes of larger diameter fibres which commences at and may be confined to the paranodal regions. It is rare for the whole internode of the larger diameter fibres to break down and Wallerian degeneration is conspicuously absent.

Quantitative findings

Effects of fixation and staining upon the size of the nodal gaps.—Fixation in formal-saline and staining with osmium tetroxide (Fig. 1*c*) appeared to give the least amount of artifactual widening of the nodes of Ranvier (Table IV), but there are objections to its use for this study, firstly because of its high cost and its toxicity, and secondly because it is not possible to stain nuclei satisfactorily after its use. Exposing the partially teased nerve fibres to 70 per cent alcohol for the time required for staining with Sudan dyes and then staining with OsO_4 produced a slight increase in the mean width of the nodes of Ranvier, which was statistically significant ($P = <0{\cdot}001$) when compared with osmium alone. Staining with the Sudan dyes produced an even greater width of the nodes that was almost double the gap found after osmium staining (Fig. 1*a* and *b*), and this additional increase was thought probably to be due to failure of the opened out loops of

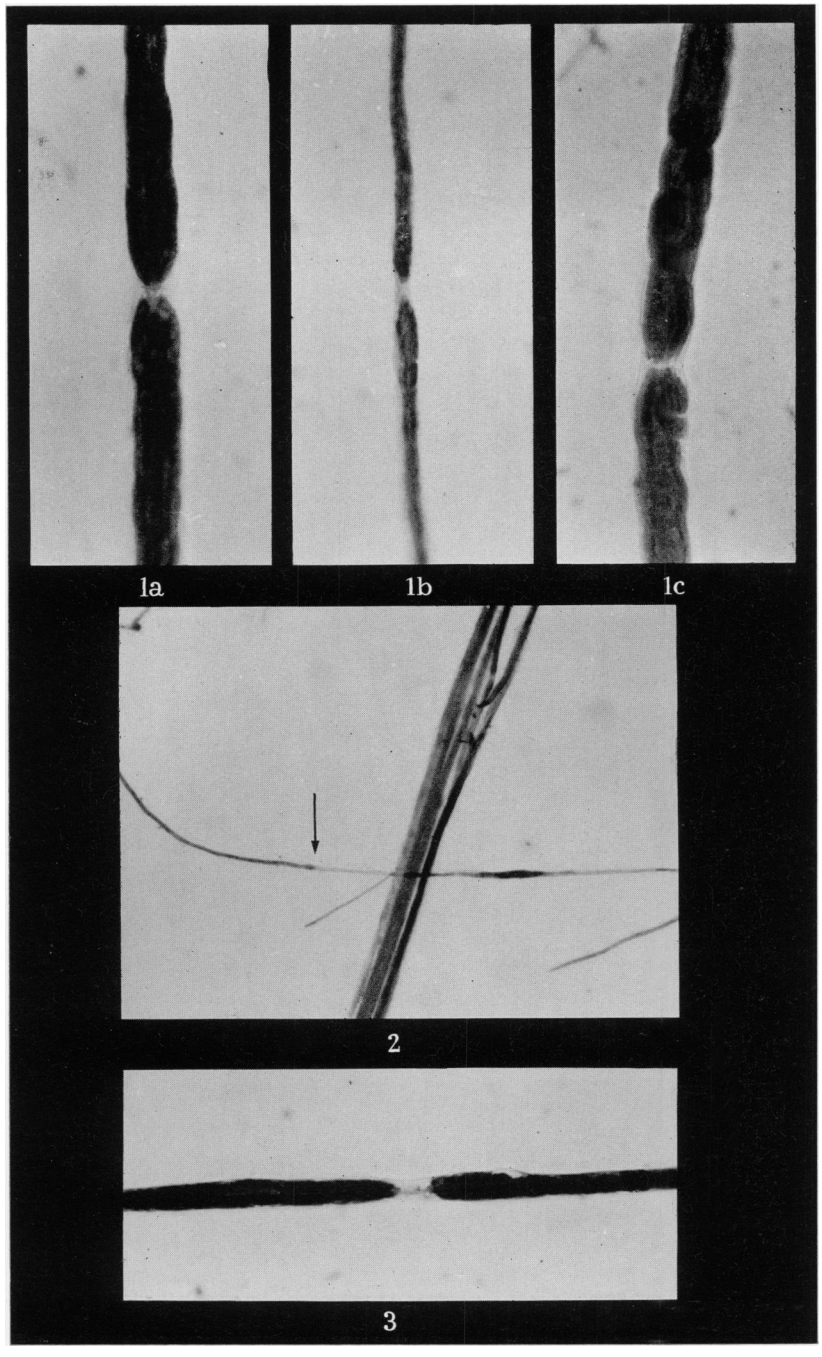
EXPLANATION OF PLATES

FIG. 1.—Normal nodes of Ranvier. ×480.
(*a*) Large diameter fibre. Sudan black B.
(*b*) Small diameter fibre ; the nodal gap is considerably greater than in (*a*) and the edges of the paranodal region are more obtuse. Sudan black B.
(*c*) Large diameter fibre formalin fixed and stained with OsO_4. The nodal gap is about half the width of that in (*a*).

FIG. 2.—Five days after diphtheria toxin. Breakdown of internodal segment in a small diameter fibre. Arrow at node with normal internode to left. Sudan black B. ×120.

FIG. 3.—Seven-day animal. Widening of node of Ranvier without apparent change in adjacent paranodal regions. Sudan black B. ×480.

FIG. 4.—Partial internodal breakdown at 21 days.
(*a*) Gap measuring about 40 micra in width. Sudan black B. ×640.
(*b*) Gap measuring about 250 micra. Sudan black B. ×160.
(*c*) Internodal segment (between arrows) which has broken down and is now undergoing remyelination. Sudan black B. ×160.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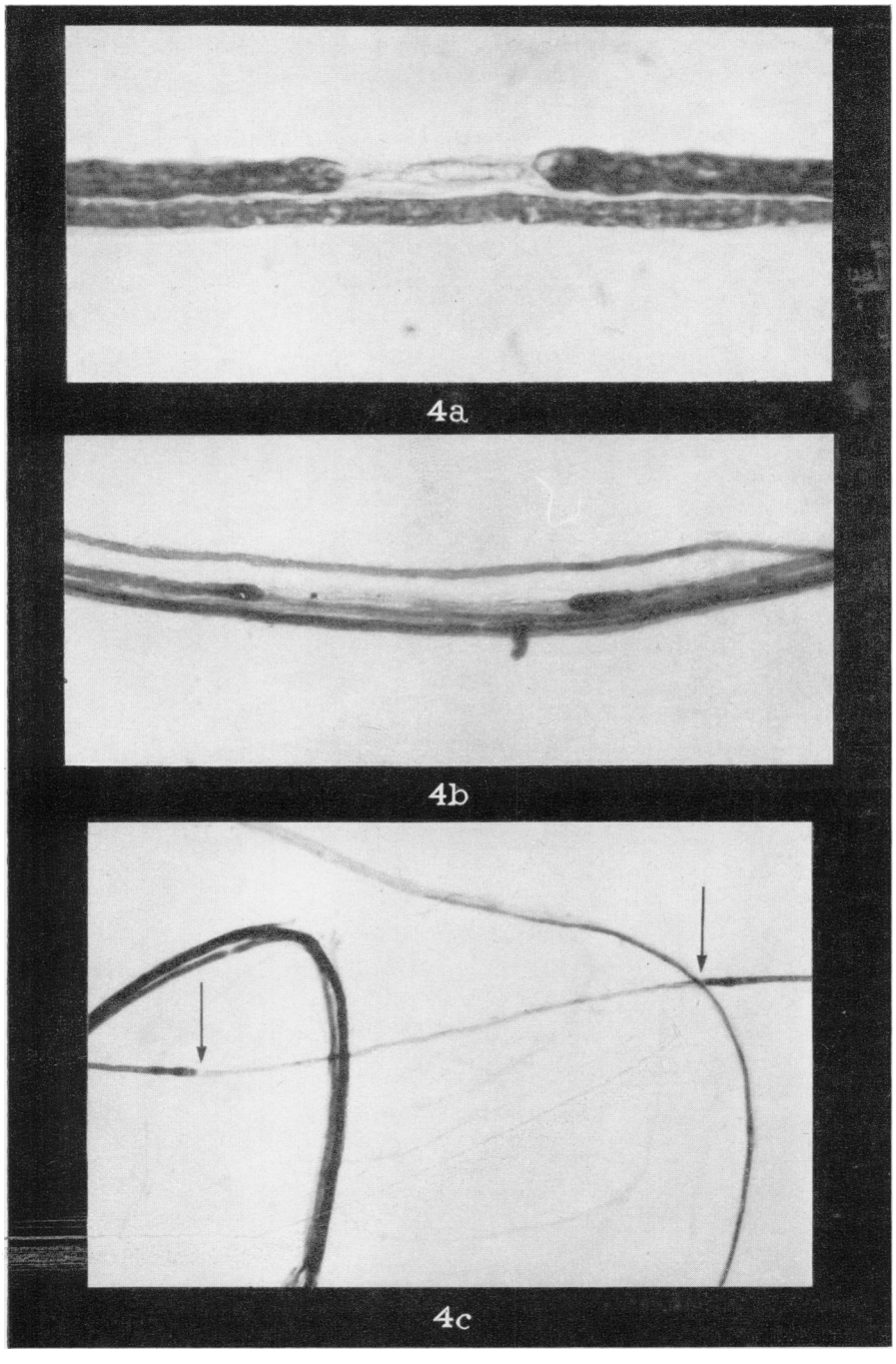

4a

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4c

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myelin in the paranodal regions to take up the dyes, since the shape of the nodes is slightly different in the 2 cases. The fact that the initial width of the nodes of Ranvier were slightly greater when stained with the Sudan dyes conveyed some advantage as is seen 7 days after inoculation in animal H.D.33 (Table V). The mean nodal width in osmium stained preparations in this animal was 3·3 μ as compared with 1·6 μ in the normal by this technique. With Sudan dyes, however, the mean nodal gap was now 6·3 μ as compared with the normal of 2·9 μ. In each case the width has approximately doubled and the increase is statistically significant ($P = <0{\cdot}001$), but the change is considerably more readily appreciated visually with the Sudan dyes than it is with osmium.

TABLE IV.—*Effects of Fixation and Staining on Nodal Gaps*

Animal	State	Fixation and staining methods	Mean nodal gap size (μ)	S.E.M.
H.D.60	Normal	Formalin-OsO_4	1·6	±0·099
H.D.60	,,	Formalin-70 per cent alcohol for 45 min.-OsO_4	2·1	±0·114
H.D.60	,,	Formalin-Sudan	2·8	±0·096
H.D.59	,,	,,	3·0	±0·085
H.D.59	,, (stretched)	,,	3·8	±0·136

TABLE V.—*Effects of Diphtheria Toxin on Nodal Gaps*

Animal	State	Fixation and staining methods	Mean nodal gap size (μ)	S.E.M.
H.D.46	Diphtheria 5 days	Formalin-Sudan	2·8	±0·1885
H.D.79	Diphtheria 5 days	,,	4·7	±0·2872
H.D.33	Diphtheria 7 days	Formalin-OsO_4	3·3	±0·3966
H.D.33	Diphtheria 7 days	Formalin-Sudan	6·3	±0·1977
H.D.38	Diphtheria 7 days	,,	5·6	±0·3895
H.D.41	Diphtheria 14 days	,,	25·4	±9·198
H.D.78	Diphtheria 14 days	,,	27·4	±4·371
H.D.39	Diphtheria 21 days	,,	14·0	±3·147
H.D.77	Diphtheria 21 days	,,	18·0	±4·548

The possible effects of stretching of the nerve and nodes of Ranvier during the process of handling the nerve before fixation and during the actual process of teasing out under the dissecting microscope have also been examined. In one animal pieces of sciatic and tibial nerve were forcibly stretched before they were fixed on to the card and the nodes were then measured after fixing and staining. The nodes in general were not appreciably widened, but a few were considerably increased. The mean was thus slightly increased (Table IV) but the general shape of the histogram of size distribution was not substantially altered. Since the force exerted in stretching these nerves was far in excess of that normally used it was considered that this factor probably would not materially affect the measurements.

Changes in the nodal measurements following inoculation of diphtheria toxin (Table V).—The earliest observations were made in animals 5 days after injection. In one (H.D. 46) the mean nodal width did not differ significantly from the normal.

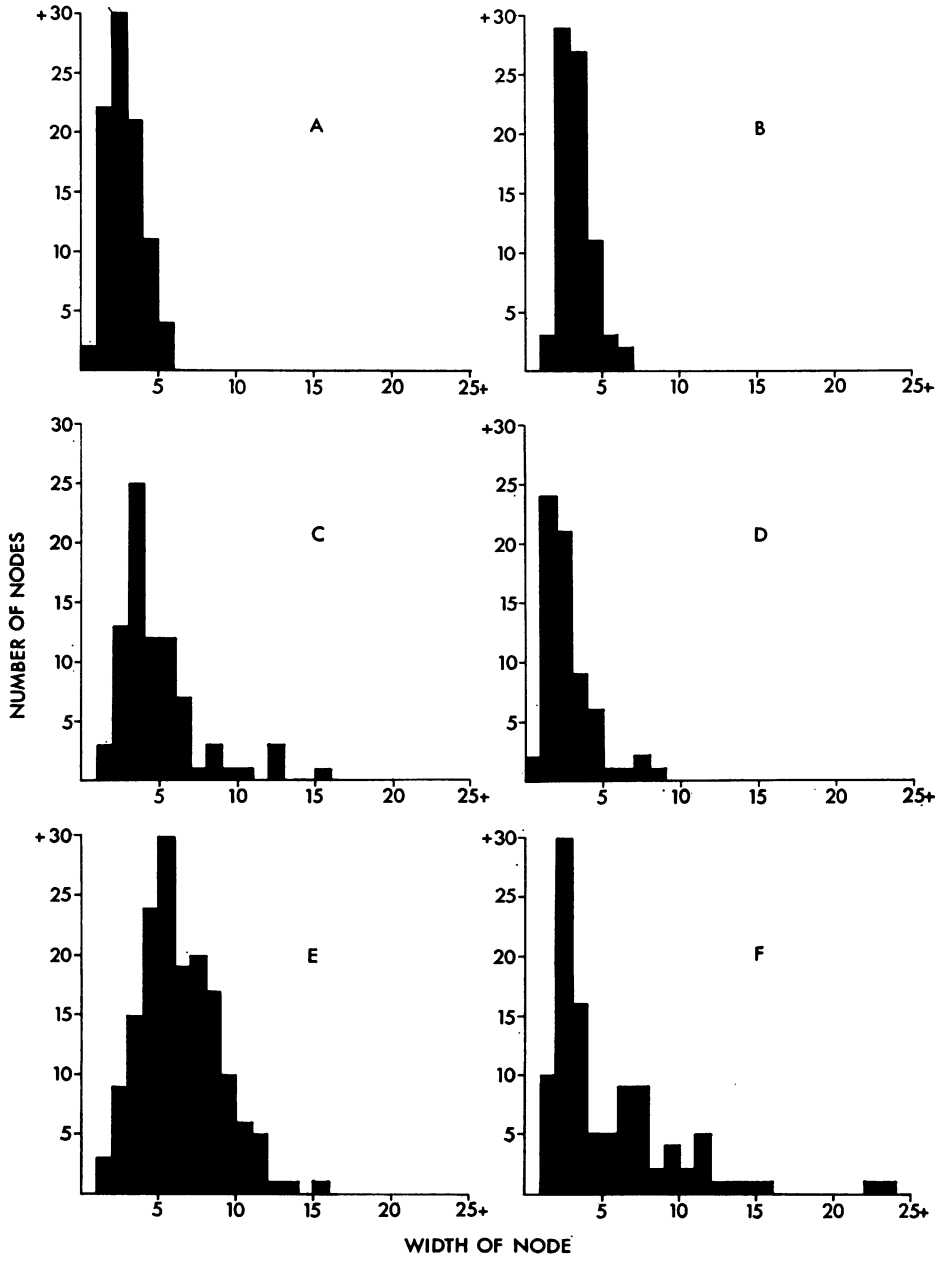
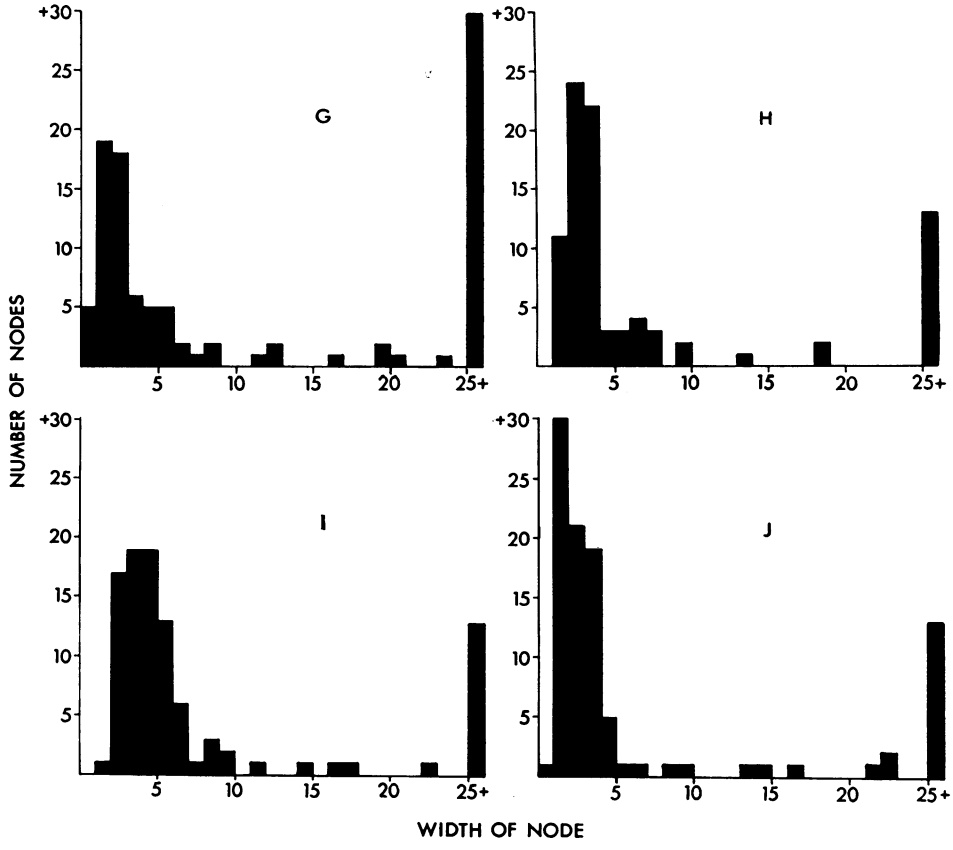


FIG. 5.—Histograms of size frequency distribution of widths of nodes of Ranvier in formalin-fixed-Sudan black B stained nerves.

(*a* and *b*) Two normal birds. After diphtheria toxin at: (*c* and *d*) 5 days; (*e* and *f*) 7 days; (*g* and *h*) 14 days; (*i* and *j*) 21 days.



In the other the mean width was greater than in the controls and the range of nodal size was greatly increased, quite a large proportion being over 5 μ (Fig. 5*c* and *d*). The difference from normal was statistically significant ($P = <0{\cdot}001$). At 7 days there was a general increase in nodal width, the means in the 2 birds being considerably greater than normal. The histograms show a general shift of nodal size to the right (Fig. 5*e* and *f*). When nodal gap size was plotted against external diameter of the internode the increase in nodal gap did not affect any special fibre group in those over 4 μ in diameter. Fibres below 4 μ that were unaffected by whole internodal breakdown were too few for us to be certain whether they took part in the process of nodal widening or not.

At 14 days the mean nodal width in each animal is greater than normal but examination of the histograms shows that these can now be separated into 2 groups. On the one side those in which the gaps are very considerably enlarged, more than 15 μ, and on the other those that now fall into the range of the normal. The same pattern is repeated in the 21 day animals and in both these there is a suggestion that the greatly widened gaps are in the larger diameter fibres, *i.e.* those over 4 μ in internodal width. This is only a suggestion, however, since the numbers of fibres of smaller diameter available for measurement are few.

Degree of involvement of internodes.—Wherever a whole degenerating internode was found the diameter of the next intact internode was measured in more than one place along its length and away from the paranodal region. In Fig. 6 the mean diameter of these adjacent internodes is related to time after inoculation and it will be seen that this increases with time. At 5 days it lies between 2–3 μ, at 7 days it is 3·4 μ, while at 14 and 21 days it has risen to near 5 μ. There is a statistically significant difference between these groups ($P = <0{\cdot}001$) and this confirms the observations commented upon earlier that during the first week there was a striking amount of internodal degeneration in small diameter fibres which appeared to have begun even before the onset of the overt functional disturbances.

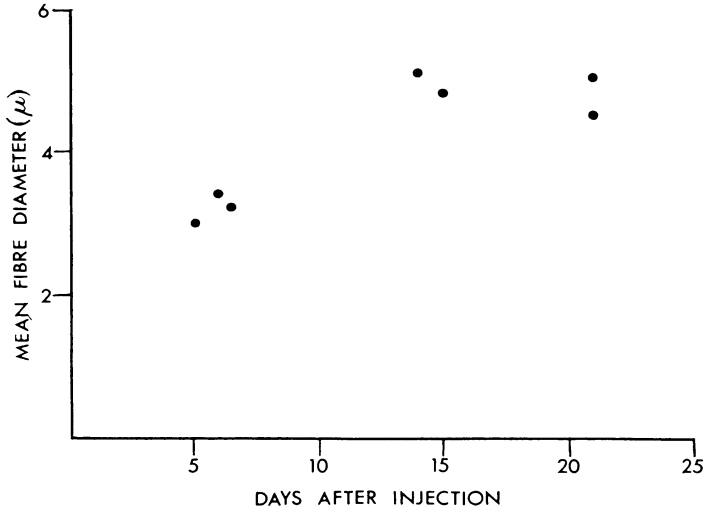


FIG. 6.—Diameters of fibres in which whole internodes are degenerating in relation to time after injection. Each point represents the mean of not less than 25 fibres.

Examination of the lengths of internode undergoing degeneration in the larger diameter fibres during the second week after inoculation shows that very few internodes experience complete disintegration. Measurements of internodal length in the leg nerves in the chicken confirm the finding of Vizoso (1950) that there may be a direct relationship between fibre diameter and length of internode (Fig. 7) and that fibres above 5 μ, in diameter, have internodal lengths of more than 600 μ. The nodal gaps present in these fibres were, however, rarely more than 200 μ in width (Table VI). It is therefore plain that the Schwann cells in the larger diameter fibres tend not to undergo total degeneration, and usually more than two-thirds of the cell remains intact. Selective damage occurs to the complex paranodal region and adjacent parts of the Schwann cell, and this is not a primary demyelinating process in the sense that the myelin as such is specifically affected.

When a nerve is examined by the method of teasing only a very small proportion of fibres is selected. This precludes the possibility of determining the absolute amount of damage to a nerve. An attempt was made therefore, to

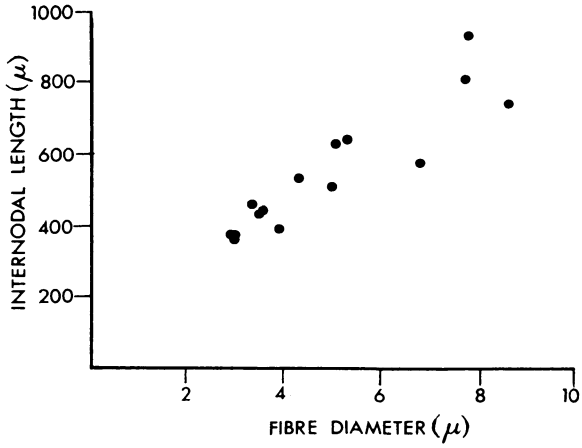


FIG. 7.—Relationship between fibre diameter and internodal length in the tibial nerve in the young chicken.

TABLE VI.—*Nodal Gaps (above 15 μ) in Fibres of Different Diameters from Animals Killed 14 and 21 Days After Injection of Toxin*

Animal	Duration of intoxication (days)	4–5 μ	5–6 μ	6–7 μ	7–8 μ	8–9 μ	9–10 μ
H.D.41	14	17·2	26·0	39·0	45·4	—	18·4
		18·0	37·0	57·0	—	—	—
		25·2	—	—	—	—	—
		71·7	—	—	—	—	—
		97·0	—	—	—	—	—
H.D.78	14	86·0	16·0	19·0	23·0	180·0	—
		—	19·0	25·0	8·70	—	—
		—	22·0	25·0	92·0	—	—
		—	26·0	46·0	180·0	—	—
		—	31·0	46·0	—	—	—
		—	32·0	56·0	—	—	—
		—	48·0	65·0	—	—	—
		—	50·0	88·0	—	—	—
		—	52·0	104·0	—	—	—
		—	53·0	123·0	—	—	—
		—	61·0	160·0	—	—	—
		—	62·0	180·0	—	—	—
		—	70·0	—	—	—	—
		—	97·5	—	—	—	—
		—	120·0	—	—	—	—
		—	130·0	—	—	—	—
H.D.38	21	17·2	33·2	17·0	97·5	—	—
		34·0	36·8	22·6	175·0	—	—
		34·4	39·0	39·0	221·0	—	—
		—	43·0	—	—	—	—
		—	75·5	—	—	—	—
H.D.77	21	16·0	22·0	21·0	—	—	—
		210·0	22·0	59·0	—	—	—
		—	38·0	104·0	—	—	—
		—	42·0	—	—	—	—
		—	52·0	—	—	—	—
		—	78·0	—	—	—	—
		—	155·0	—	—	—	—
		—	180·0	—	—	—	—
		—	220·0	—	—	—	—

assess quantitatively the extent of damage by counting nerve fibres in transverse section using the method of Fernand and Young. Fibre diameter counts from the peroneal nerve from a normal hen, and one showing (4) degrees of paresis on the 14th day after injection, were compared. No significant difference was detectable either in numbers of fibres or in the fibre diameter spectrum. Any quantitative assessment of damage is further complicated by the segmental nature of the demyelination which reduces the probability of cutting through an affected internode.

The absence of any conspicuous difference between the counts from a normal and an affected animal does however indicate that Wallerian degeneration is not taking place. If it were occurring, a cumulative effect from the more proximal nerve fibres affected, would be expected in the distal part of the peroneal nerve which was examined.

DISCUSSION

There are several features of the lesion produced by diphtheria toxin that have been disclosed by this experimental study. The fact that small diameter fibres with short internodes seem to be most susceptible was unsuspected and indeed probably could not be recognised by any other method. In view of the fact that the gamma efferent fibres to muscle spindles would probably be amongst those fibres affected early, there may be here a possible explanation for the pronounced hypotonia that develops early in these animals. Only experiments with electrical methods will establish this. Unfortunately these methods are as yet of little value for revealing selective changes in small diameter fibres. The observations of Kaeser (1962) clearly show that the electrophysiological approach can at present only follow the later and more gross changes in the nerves. His careful analysis of the conduction velocity in the guinea-pig with diphtheritic changes reveals a steady decline in the numbers of fast conducting fibres with time, but the changes are late by comparison with our findings. There is no reason as yet for believing the guinea-pig shows a different kind of lesion from that we have shown in the chicken. If the morphological changes that we have shown at the nodes reflect a functional disturbance it would be predicted that electrical methods probably would not detect slowing of conduction at least until the second week after inoculation by which time both paresis and changes in small diameter fibres are well advanced.

It is not possible to decide from the evidence presented here whether the process of nodal widening is *ab initio* an irreversible process or not. It could conceivably be primarily reversible but in certain fibres it becomes irreversible for secondary reasons later. If so this might depend on the metabolic state of the cell at the time of reception of the toxin. Degeneration of the paranodal and adjacent parts of relatively few Schwann cells may be then only an extreme manifestation of a much more widespread but milder process diffusely affecting the whole Schwann cell system. The lesion could therefore be regarded as the equivalent in the peripheral nerve of cloudy swelling, a classical pathological change which typically occurs in other organs such as liver and kidneys as a characteristic effect of diphtheria toxin. Viewed in this light it may be wondered what in fact is the real significance of the special localisation of the diphtheritic lesion to the nerve roots that has been demonstrated by Fisher and Adams (1956) in man, by Waksman, Adams and Mansmann (1958) in the rabbit, and by

McDonald (1962) in the cat. Waksman (1961) attempted to find evidence for the belief that this localisation was due to a " barrier " phenomenon similar to that which has been postulated for the brain. The evidence for the reality of such a " barrier " is in fact very tenuous (Dobbing, 1961 ; 1963) in either situation, and it is certainly difficult to understand on general grounds how it is that one part of a tissue such as a nerve could have such varying properties at different sites along its length. Clearly a more detailed study of the anatomical changes in these other species is called for particularly in relation to the variation in internodal lengths that there is likely to be in the spinal roots, if this aspect of the problem is going to be better understood.

The localisation of the process to the paranodal region has further points of interest. This region has been known for almost a century to be slightly swollen and to show external grooving of its surface (Ranvier, 1878). The structural significance of this enlargement and folding has recently been explored by Williams and Landon (1963) who have shown that this is a region of the Schwann cell in which there is a large amount of cytoplasm containing numerous mitochondria. There is, indeed, probably a greater amount of cytoplasmic material here than in any other region of the cell, although this may be less true in the immature animal in which myelination is in active progress. It is not, however, clear what is the exact role either of this increased amount of cytoplasmic material or of the complex structural relationship between the two adjacent Schwann cells and the axon in the node itself. The presence of such large numbers of mitochondria suggest the utilisation here of large amounts of energy although to what end is unknown. There is some evidence, however, that diphtheria toxin may interfere with the transference of high energy phosphate bonds particularly for the purpose of protein synthesis (Strauss and Hendee, 1959 ; Kato and Pappenheimer, 1960). The cytological evidence for active protein formation, such as the occurrence of ribosenucleic acid and the electron microscopical demonstration of endoplasmic reticulum, in the nodal or paranodal regions has, however, yet to be found. Our finding (unpublished) that the immature chicken in which myelin is being synthesised is slightly more susceptible to toxin than the mature animal may possibly find an explanation here, but other explanations could also be put forward.

Whatever the metabolic system is which is interfered with by the toxin, its ultimate action upon the Schwann cell is a relatively slow one. Our neutralisation experiments show that even before 1 hr. has elapsed the toxin has become inaccessible to antitoxin. This period is somewhat shorter than that of 10 hr. found by Ramon, Debré and Uhry (1934) in the guinea-pig, but it should be recalled that these authors did not use the intravenous route so that problems of absorption would be expected to prolong the process. Since even 10 times the neutralising dose of antitoxin had no protective effect 1 hr. after injection of toxin it must be assumed that the toxin must already be by that time outside the capillary bed of the nerve and perhaps even at the Schwann cell surface. Strauss and Hendee (1959) found a similarly rapid entrance of diphtheria toxin into HeLa cells in culture and also a distinct delay period, which was dose dependent, before the onset of slowing of protein synthesis. The time scale in their system was of the order of a few hours, however, whereas in the nerve it is of the order of days. What is of particular interest is that the delay seems greatest in the larger fibres which have the longest internodal length. Whether

a transport factor can be brought into the problem should be thus seriously considered. A similar delay period occurs in organophosphorus neurotoxicity but here it is the longest and larger nerve fibres that suffer distal degeneration (Cavanagh, 1954; 1964). In diphtheria the longest internodes show distal changes, and are apparently affected late in the time sequence.

SUMMARY

Young chickens have been injected with diphtheria toxin and it has been shown:

That the neuropathic effects and the delay period are dose dependent.

The toxin becomes unavailable for inactivation by antitoxin within 1 hr.

The earliest changes occur in small diameter fibres which tend to show whole internodal degeneration.

Widening of the gap between the ends of myelin at the nodes of Ranvier occurs as an early feature in many fibres, and partial breakdown of the paranodal and adjacent internode may follow this.

It is uncommon with the doses employed for whole internodes to degenerate in larger diameter fibres.

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