REGENERATION OF NON-MEDULLATED NERVE FIBRES

By D. H. L. EVANS and J. G. MURRAY
Department of Anatomy, University College, London

INTRODUCTION

Return of function following nerve injury involves the downgrowth of axonal sprouts to re-establish contact with the denervated end-organs. The efficiency of the process depends not only on the ability of the axons to grow peripherally but also upon the integrity of the guiding nerve sheaths, which ensure that suitable contacts are made with the effector cells.

Numerous workers experimenting with somatic medullated nerves have shown that the degree of return of function depends, to a large extent, on the nature of the nerve injury (Seddon, 1943; Denny-Brown & Brenner, 1944; Young, 1949). When the nerve is crushed the axons are interrupted, but the connective tissue sheaths may remain intact at the crush site serving as direct pathways along which the axons travel to re-innervate their former end organs (Gutmann, Guttmann, Medawar & Young, 1942; Young, 1949). In these conditions return of function is rapid and to all intents and purposes complete. When a somatic nerve trunk is severed, on the other hand, and especially when a large gap remains between the cut ends, some delay and criss-crossing of axons occurs and many regenerate along sheaths leading to end-organs differing from those with which they were originally connected. The degree of impairment of function largely depends on the proportion of nerve fibres making wrong connexions.

Few studies have been made on the process of regeneration in the non-medullated nerve fibres in the autonomic nervous system. This is partly due to the greater difficulty in staining these fine fibres and to the comparative lack of precision with which return of function can be measured in most of the viscera innervated by autonomic nerves.

Several features in the structure of non-medullated nerve trunks suggest the possibility that the process of regeneration in these may differ appreciably from that in somatic nerves containing medullated fibres. There are marked differences between the sheaths of the two varieties of fibres.

The sheath of a medullated axon consists of three distinct components covering the outer surface of the myelin. Immediately around the myelin is a layer of protoplasm of a satellite cell, the cell of Schwann. This is fully enclosed in a tube of more rigid material whose inner wall is the neurilemma, a thin, smooth membrane, composed of very fine fibres. Outside the neurilemma the wall of the tube is further strengthened by coarser collagen fibres and continued as the endoneurium, by which various fibres are bound together. Each medullated fibre has an individual sheath and each sheath contains a single axon. In contrast, in non-medullated fibres, the sheath forms a continuous network composed of fine trabeculae of varying length and breadth (Nageotte, 1932; Gasser, 1952). The protoplasm of the Schwann cells contained within the trabeculae thus forms a syncytium. Around the trabeculae is

a thin but resistant membrane which corresponds to the neurilemma. The network is enclosed in endoneurium. Each branch of the network is a composite fibre containing a variable number, often large, of axons in the protoplasm. The protoplasm enclosed in the sheath is divided into septa of uniform thickness which separate axon from axon.

In a nerve trunk containing both medullated and non-medullated fibres, the latter come into close association with the sheaths of the former. The medullated fibres frequently pass through the loops formed in the network of the non-medullated fibre sheaths (Nageotte, 1932).

From the above description it might be expected that there is less precise direction of regenerating axons in the diffuse sheath of a non-medullated nerve trunk compared with the situation in a medullated nerve where the axons are guided along individual tubes.

Another striking difference between medullated and non-medullated fibres is seen in the behaviour of their respective Schwann cells following interruption of the axons. In medullated nerves these cells rapidly begin to proliferate, not only close to the lesion but throughout the length of the peripheral stump. Furthermore, in the region of a lesion in medullated fibres, the Schwann cells migrate and elongate into strands, which bridge the gap between the two cut ends (Ingebrigtsen, 1916; Abercrombie & Johnson, 1942). This outgrowth is of great importance in providing conducting pathways along which axons from the central stump reach the peripheral tubes (Young, 1942). The Schwann cell nuclei of non-medullated fibres, in contrast, show little or no proliferation as a result of degeneration of the axons (Tuckett, 1896; Joseph, 1947). As far as can be ascertained, however, no attempts have been made to determine whether the migratory property also is absent in non-medullated nerves.

In the present investigation regeneration in non-medullated fibres of the vagus of the rabbit has been studied. The vagus was selected because of the long lengths over which regeneration can be observed, enabling lesions to be made at different levels in the nerve trunk. The rabbit proved the most convenient species for three reasons. In the first place the vagus nerves at the level of the diaphragm in this animal are fairly constant in arrangement and position, being usually in the form of two discrete nerve trunks in contrast to the nerve plexuses found in the cat and dog. Secondly, the abdominal vagus nerves of the rabbit are composed almost entirely of non-medullated fibres; other species examined contained a larger proportion of medullated fibres. Thirdly, electrical stimulation of the rabbit vagus regularly produces a sharp increase in intragastric pressure, which thus serves as a suitable index of return of function. Similar stimulation in the cat and dog results in more variable effects.

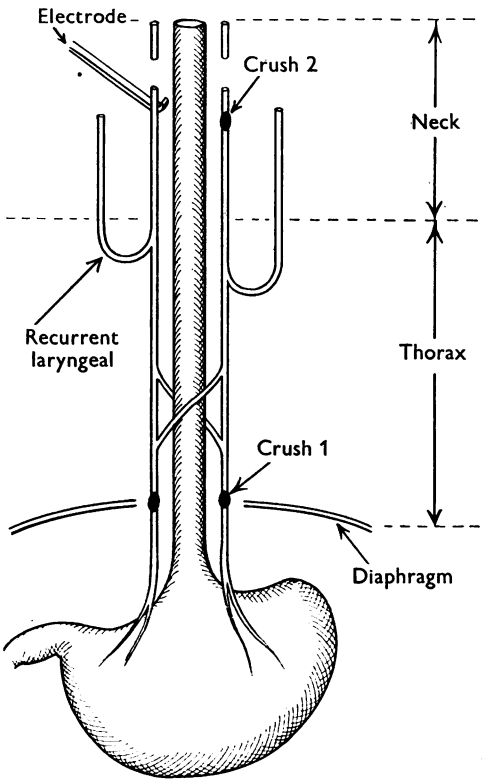
METHODS

Adult rabbits of various breeds were employed throughout this investigation.

Lesions of the vagus nerve

A crush lesion of the vagus nerve was employed as a means of studying regeneration. The nerve was compressed for 10 sec. with a pair of watchmaker's forceps having smooth blades of 1 mm. width. Study of sections of medullated nerves fixed

at various times after such a lesion has shown that axons are interrupted, but the connective tissue sheaths are left intact and the regenerating fibres can advance along their original tubes to regain their old connexions (Gutmann *et al.* 1942; Young, 1949). Crushing a medullated nerve in this way therefore provides a valuable means of producing a standard lesion from which recovery is rapid and complete.



Text-fig. 1. Diagram showing the levels of the lesions: crush 1, at the level of the diaphragm; crush 2, at the level of the lower border of the thyroid cartilage.

The rabbits were anaesthetized with intravenous pentobarbitone sodium (Nembutal) supplemented with ether. Full aseptic precautions were observed. The lesions were standardized as follows:

(*a*) *At the level of the diaphragm* (Text-fig. 1). In a series of rabbits the anterior and posterior abdominal vagi were crushed, great care being taken that any additional branches were dealt with. In another series 15–20 mm. of the anterior and posterior nerves were resected.

(*b*) *At the level of thyroid cartilage* (Text-fig. 1). In a third group either the right or left cervical vagus was crushed opposite the lower border of the thyroid cartilage.

Return of function

At varying intervals after the lesions, the animals were biopsied. Return of function of the vagus nerves was tested and specimens of the nerves removed for histological examination. The methods varied depending on whether the lesions of the nerves were at the level of the diaphragm or in the neck.

(*a*) *After lesions at the level of the diaphragm.* Return of function to the stomach was measured by observing the effect of intragastric pressure of stimulating electrically the right and left vagus nerves in the neck (Text-fig. 1). The animals were anaesthetized with intravenous pentobarbitone sodium and maintained under anaesthesia with ether.

The abdomen was opened by a long mid-line incision, and a gastrotomy opening was made in the greater curvature of the stomach. A balloon, connected by pressure rubber tubing to a Marey's tambour recorder, was introduced into the lumen and the opening in the stomach closed by means of a purse string suture firmly tied around the rubber tube. The system was filled with air and intragastric pressure changes recorded on a revolving drum and calibrated by means of a water manometer. Pressure of from 8 to 12 cm. of water was employed and with the size of the balloon used about 25 ml. of air was required to produce such pressures. In all cases this volume was below that required to take up all the 'slack' in the balloon. The abdomen was widely opened, the abdominal walls being retracted in the manner suggested by Sollmann (1923) so that a trough was formed in which the stomach lay and the whole was kept moist with normal saline at 38° C. The right and left vagus nerves were demonstrated in the neck and divided at the level of the thyroid cartilage. The peripheral cut ends were stimulated in turn and the effect on intragastric pressure recorded. By stimulating only the peripheral cut ends, the complication of central stimulation with reflex effects on the viscera was avoided.

(*b*) *After lesions of the cervical vagus nerves.* The right and left vagus nerves were divided in the neck above the level of the crush and the effect of stimulation of the peripheral cut ends recorded (Text-fig. 1). The normal vagus was stimulated first and this acted as a convenient control. The treated vagus was stimulated proximal to the site of crush. The following observations were made:

(1) Effect on intragastric pressure was recorded by the method described above.

(2) Effect on the vocal cords. An incision through the thyrohyoid membrane allowed direct observations on the movement of the vocal cords. Re-innervation of the laryngeal muscles was assumed when stimulation of the treated vagus produced detectable movement of the vocal cord.

(3) Effect on the heart rate and blood pressure was recorded from the carotid artery.

The nerves were stimulated by rectangular voltage pulses delivered to the nerves through 30 s.w.g. platinum wire electrodes insulated with 'Perspex' from the surrounding tissues. The parameters of stimulation were varied experimentally over a wide range (pulse length 0·1–100 msec., pulse voltage 1–25 V., repetition rate 2–200 per sec.), and maximal responses from the stomach were obtained, in practice, by pulses of 0·1, 1·0 or 10 msec. duration at 10–25 V. amplitude at a repetition rate of 40 per sec. This applied in both normal and regenerating nerves.

HISTOLOGICAL METHODS

The technique used for staining all axons in both medullated and non-medullated was the pyridine-silver method described by Ranson & Davenport (1931). The medullated fibres were demonstrated by the Weigert method which stains the myelin sheaths black. Details of the above methods and of counting and measuring fibres are described by Evans & Murray (1954).

Estimates of the total number of axons present in pyridine-silver preparations were made by a sampling method, the detail being as follows: the image of the cross-section of a nerve was projected at a magnification of 500 on paper of uniform thickness and the outline traced. This area was cut out and weighed and the cross-sectional area of the nerve calculated. The counts were made with a binocular microscope ($\times 12$ ocular, $\times 100$ objective) at a magnification of 1200 diameters A 1 mm.^{2} graticule was placed in one eyepiece and the area of nerve covered by each square calculated. A selection of squares was made from a table of random numbers and the numbers of axons in each square counted. The total area counted was between 17 and 33 % of the total cross-section of each nerve. In the abdominal vagus nerve, where the axons are evenly distributed throughout the section, statistical analysis showed that counting of 17 % of the total area gave a sufficiently consistent and therefore an adequate estimate of the total count. In the case of the regenerating recurrent laryngeal nerve the fibre population was unevenly distributed. The segment containing the large medullated fibres was sampled separately from that containing small fibres. Owing to the unevenness of fibre distribution in both segments as much as 33 % of the total area of each segment was counted.

A special note is required in the case of animals subjected to division of the vagus with excision of a length at the level of the diaphragm. In these, it was necessary to remove the lower oesophagus, upper stomach and the nerves in one block for an examination of the neuroma and the orientation of the regenerating axons in relation to the distal nerve stumps. The complete specimen was lightly stretched on a glass frame and put in Bouin fixative (sat. aq. picric acid 75 ml.; 40 % formaldehyde 25 ml.; glacial acetic acid 5 ml.). The specimen was subsequently embedded in paraffin, cut transversely at 10 μ thickness, and stained by the Bodian method for axons.

RESULTS

For a clear understanding of the results, it is convenient to consider first the fibre content of the normal vagus nerve in the rabbit, then the process of regeneration as measured by return of function and finally the histological findings in regenerating nerves and to correlate these with the functional results.

(1) *Histological appearances of the normal vagus nerve*

An analysis of the fibre composition of the vagus and its branches in the rabbit has been made by Evans & Murray (1954) and the findings relevant to the present study are summarized in Table 1. It will be seen that the cervical vagus is composed of both medullated and non-medullated fibres, whilst the abdominal vagus consists almost entirely of non-medullated and the recurrent laryngeal of medullated fibres (Pl. 1, fig. 1; Pl. 2, figs. 7, 8).

Furthermore, we found that the great majority of the non-medullated fibres of the abdominal vagus nerves are afferent, only about one-tenth of the total being motor to the abdominal viscera. Observations were also made on the number of 'adventitial fibres' present in the abdominal vagus trunks. In three rabbits, 1372, 1721 and 569 axons were present (Pl. 1, fig. 5), and these were presumed to have been added to the vagus nerves in the thorax from communications with the sympathetic trunks.

Table 1. *Number of medullated and non-medullated fibres in the normal vagus and recurrent laryngeal nerves of the rabbit*

(The table shows the mean and standard deviation from the mean and, in brackets, the number of specimens counted.)

Specimens	Medullated fibres	Total number of axons	Non-medullated axons
Cervical vagus	2914 ± 207 (5)	23,132 ± 980 (6)	20,000 (approx.)*
Recurrent laryngeal			
(1) Laryngeal bundle	277 ± 14 (6)	—	(1) } 62 ± 9 (6)
(2) Tracheal and oesophageal bundle	400 – 600†	—	(2) }
Abdominal vagus nerves	65 ± 4 (4)	26,178 ± 1315 (6)	26,000 (approx.)*

* In counts of Ranson preparations of cervical and abdominal vagi no attempt was made to differentiate medullated and non-medullated fibres. The approximate number of non-medullated axons was determined by subtracting the number of medullated axons from the total number of axons.

† No mean value could be determined as the number of fibres present in the individual specimens counted was very variable.

(2) *Functional results*

(*a*) *Normal response of the stomach to stimulation of the vagus nerve*

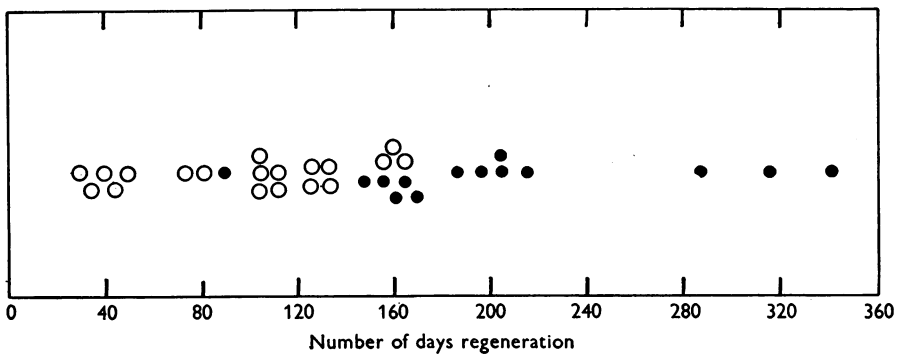
In a series of thirty-six normal rabbits, we obtained unequivocal results on stimulating the peripheral cut ends of the right and left vagus nerves in the neck. In all cases a sharp rise in intragastric pressure was produced, of the order of 15 cm. of water (Text-fig. 5A). The latent period was always brief, in all cases less than 2 sec. In spite of attempts to vary tonus and activity of the stomach by feeding the rabbit within an hour or two before biopsy or withholding solid food for as long as 72 hr., or varying the pressure in the balloon, the response was always similar in character. Stimulation of the right and left vagus nerves in the neck in the majority of cases gave a similar degree of response, but in a few cases the left had a slightly greater effect than the right. The reverse was never observed. This confirms the findings of M'Crea, M'Swiney & Stopford (1925), and emphasizes that the effects on the rabbit's stomach do not depend to the same extent on the degree of tone and peristaltic activity as in the cat and dog.

In all thirty-six control animals, the rise in intragastric pressure on stimulation of the vagus nerves in the neck was abolished by crushing or cutting the nerves at the level of the diaphragm.

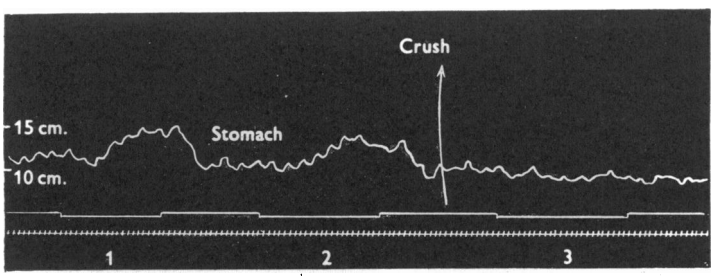
(*b*) *Return of function after crushing the vagus nerves at the level of the diaphragm*

At varying intervals after crushing the vagus nerves at the level of the diaphragm, the animals were brought to biopsy. The results are summarized in Text-fig. 2. Up to 140 days after the lesions, the animals, with one exception (90 days), showed no

rise in intragastric pressure. From 140 to 170 days there was a transition period when the results were mixed, only a proportion showing a rise in intragastric pressure. With post-operative survival periods greater than 170 days, function had returned in all the animals tested. That the rise in intragastric pressure was due to regeneration of the abdominal vagus nerves was shown by the fact that in all cases it was abolished by crushing or cutting the nerves distal to the original crushes



Text-fig. 2. Regeneration following crushing at the level of the diaphragm. ●, rise of intragastric pressure on electrical stimulation; ○, no rise of intragastric pressure on electrical stimulation.



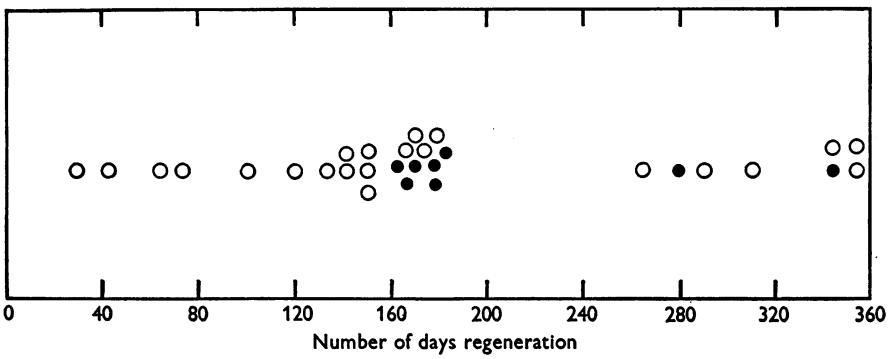
Text-fig. 3. (Rabbit 4.) Intragastric pressure recording showing a positive response after a lapse of 150 days following crushes at the level of the diaphragm. The response was less in amplitude and showed a considerable delay compared with the normal (Text-fig. 5A). The rise in intragastric pressure was abolished by crushing the nerves distal to the original lesions. Time intervals on the tracings equal 1 sec.

(Text-fig. 3). The intragastric pressure response obtained in the early regenerating period (140–220 days) was subnormal in amplitude and showed a prolonged latent period of about 5 sec. However, in the three animals with post-operative survival periods of 285, 315 and 350 days the latent period and amplitude of the response approached normal. Thus we see that there is a period of increasing perfection of function extending over about 100 days.

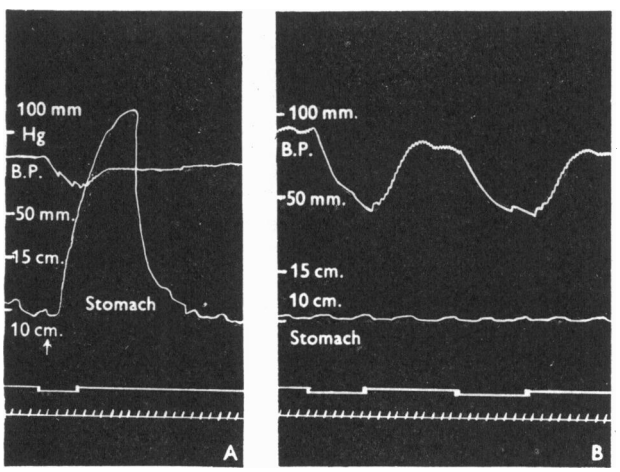
This fact suggests that stimulation of one point in the myenteric plexus by a regenerated vagus fibre is not transmitted by the plexus over a large area of the stomach wall. Presumably the myenteric plexus consists of a series of units, each having an individual vagal nerve supply (White, Smithwick & Simeone, 1952).

(*c*) *Return of function after division of the vagus nerves at the level of the diaphragm*

Text-fig. 4 summarizes the findings obtained in the series in which 15–20 mm. of the abdominal vagus nerves had been resected. As might be expected the return of function is not as sharply defined as in the series of crushed nerves. In each of the eight animals giving a positive response, the increase in intragastric pressure was abolished by cutting the nerves distal to the gaps.



Text-fig. 4. Regeneration following division at the level of the diaphragm. ●, rise of intragastric pressure on electrical stimulation; ○, no rise of intragastic pressure on electrical stimulation.



Text-fig. 5. (Rabbit 19.) Intragastric and blood-pressure records from an animal in which the left cervical vagus had been crushed 670 days previously. A, right side showing normal responses; B, left side showing a return of function to the heart but none to the stomach. Time intervals on the tracings equal 1 sec.

In all cases when function had returned, regeneration was only partial as indicated by a long latent period and a small rise in intragastric pressure. It was observed that after a crush there was a period of increasing perfection of function, but after division this was not the case, at least up to 350 days.

(*d*) *Return of function after crush of the right or left vagus in the neck*

We were unable to demonstrate return of function to the stomach in any of the animals subjected to a crush of the cervical vagus (Text-fig. 5). This was a striking phenomenon and the cause became apparent only after histological investigation.

Table 2. *Regeneration following crushing in the neck at level of thyroid cartilage*

(0 = no reaction on electrical stimulation; + = reaction on electrical stimulation.)

Rabbit number	Right or left vagus	Number of days regeneration	Stomach	Heart	Vocal cords
91	R	42	0	0	0
92	L	51	0	0	0
72	L	61	0	0	0
158	R	70	0	+ slight	+
1	L	75	0	Not tested	+
38	R	84	0	0	+
53	L	110	0	+	+
18	L	135	0	+	Not tested
9	R	164	0	+	+
34	R	168	0	+	+
8	L	183	0	+	+
10	R	187	0	+	+
46	R	200	0	Not tested	Not tested
16	L	205	0	0	+
28	L	307	0	+	+
11	R	311	0	+	+
12	L	436	0	Not tested	Not tested
7	R	670	0	+ slight	+
19	L	670	0	+	+

On the other hand, the return of function to the larynx and heart presented a much more uniform picture. The results are summarized in Table 2. The distances as measured in four animals from the site of crush to the three structures in which function was tested is shown in Table 3.

Table 3. *Distances measured along the nerves from site of the crushes in the neck to the larynx, heart and diaphragm*

(The measurements were made in four rabbits.)

Rabbit number	Weight (kg.)	Larynx		Heart* (cm.)	Diaphragm (cm.)	Stomach† (cm.)
		Right (cm.)	Left (cm.)			
112	2·1	13·5	17	11·5	16·5	8·5
196	2·7	15	18	11	18	10
195	3·0	15	18	11·5	17	10·5
10	3·3	12·5	16	10·5	17·5	10·5

* Measurement to the level of the atria.

† Distance from the level of the diaphagm to the pyloro-duodenal junction as measured along a line mid-way between the greater and lesser curvatures.

(3) *Histological results*

(*a*) *Nerve crush at the level of the diaphragm*

Twenty days after the crush, there were 2709 axons present 20 mm. distal to the crush (Table 4). This indicates a rate of growth of axon tips greater than 1 mm. per day. It was apparent that the regenerating axons were smaller than normal in

diameter (Pl. 1, fig. 2). After a lapse of 40 days following a crush, the axons at a level 20 mm. distally were more numerous than at 20 days, but still appeared smaller than normal. By 70 days the fibre density 20 mm. distal to the crush had returned to normal (Table 4) and the axons appeared to be normal in size.

Table 4. *Non-medullated fibre counts* 10 *mm. proximal and* 20 *mm. distal to a crush of the anterior vagus nerve at the level of the diaphragm*

Rabbit number	Regeneration period in days	Number of axons	
		Proximal	Distal
131	20	8,943	2,709
163	50	18,633	10,518
150	70	7,564	7,770

Before function is restored, it may be presumed that the following processes must occur: (*a*) the regenerating axons must grow across the lesion; (*b*) these axons must grow down to ganglion cells in the myenteric plexus; (*c*) synaptic connexions must be re-established; (*d*) increase in diameter of the regenerating axons may have to occur. Our histological investigations suggest that growth across the crush lesion and rate of growth of axon tips cannot be mainly responsible for the delay. Very little is known about processes (*c*) and (*d*) in non-medullated fibres. We consider that another factor may contribute to the delay in this situation. Owing to the syncytial nature of the nerve sheaths, the regenerating non-medullated fibres have no specific guiding channels along which to reach appropriate end-organs. It is likely, therefore, that many axons fail or take a longer time than anticipated, to make functional connexions. This effect would be accentuated by the dispersed nature of the ganglion cells in the myenteric plexus. Further, the lack of guiding tubes and the widely scattered end-organs may well explain the relatively long period of increasing perfection of function.

(*b*) *Section with excision of* 15–20 *mm. of nerve at the level of the diaphragm*

From transverse and longitudinal sections a picture could be built up of the axons growing out from the proximal cut end. The axons grew out in all directions, some passing cranially along the outside of the nerve trunks and a variable number successfully bridging the gaps and growing into the distal nerve trunks (Pl. 1, fig. 6). In some, large numbers of axons grew into the oesophageal wall. Of the thirty cases which were investigated for return of function, four were rejected because of inadequate staining and three were used for longitudinal section. Of the remaining twenty-three specimens, all showed the presence of axons in the distal nerve stump. In some there were relatively large numbers of axons and in others few. We were unable to observe any correlation between the number of axons present in the distal stumps and the return of function. In the later stages of regeneration, i.e. 260 days and more after the division, there appeared to be no greater number of axons than at the earlier periods. Axons were present in the distal stumps as early as 42 days after the division. A constant and striking finding in transverse sections of the distal stumps was that the axons which successfully bridged the gap were arranged around the periphery of the section, the more central portions being almost devoid of axons.

It is interesting to note that in rabbits with regenerating periods greater than 160 days, only eight of the eighteen animals showed a return of function, although in all cases axons had successfully regenerated down the distal nerve trunks. Several factors could contribute to this state of affairs.

(1) Although in all cases some axons are successful in bridging the gap, in some the proportion is relatively small compared with the normal number.

(2) As we have shown (Evans & Murray, 1954) a large proportion of the non-medullated fibres in the abdominal vagus nerves are afferent, and thus the number of successful efferent fibres may be very small.

(3) It appears that non-medullated fibres do not have specific tubes to guide them back directly to an end organ and thus the likelihood of functional connexion being established is diminished.

(*c*) *Nerve crush at the level of the thyroid cartilage*

After a crush in the neck the medullated fibres destined for the recurrent laryngeal nerve behaved as anticipated. Function returned rapidly, e.g. in 75 days over a distance of 17 cm. (rabbit 1) and there were 220 medullated fibres present in the recurrent laryngeal nerve adjacent to the larynx.

Table 5. *Non-medullated fibre counts in regenerating recurrent laryngeal and abdominal vagus nerves after a crush of the left cervical vagus*

Rabbit number	Regeneration period in days	Recurrent laryngeal	Abdominal vagus nerves
126	280	9,443	2,485
54	550	10,649	5,805
19	670	7,243	2,466

On the other hand, the outstanding finding after crushing the vagus in the neck was that function failed to return to the stomach even after a lapse of 670 days. Histological examination of the abdominal vagus nerves at the level of the diaphragm showed that large segments of the section were almost entirely devoid of axons, even after very prolonged regeneration times (Pl. 1, fig. 4). The appearances were similar to those found in the abdominal vagus nerves 3 weeks after cutting one vagus in the neck (Evans & Murray, 1954). It was thus clear that few axons had succeeded in reaching the abdomen even after very prolonged regeneration times. In order to study this finding more fully, quantitative estimations of the number of regenerating axons reaching the abdomen were made in the following manner: in three of the rabbits with long-term cervical crush, the contralateral cervical vagus was cut and a segment removed. After allowing a period of 3 weeks for fibres to degenerate, the animals were biopsied and the abdominal vagus nerves removed for pyridine-silver staining. The nerves contained remarkably few fibres which were distributed throughout the section (Pl. 1, fig. 3). Table 5 shows the fibre content of the nerves in these three instances. The numbers found in the present series are slightly larger than those of the 'adventitial fibres' discussed previously (Pl. 1, fig. 5). This small increase in number is presumably due to a few vagal fibres having grown successfully from the level in the neck to the abdominal vagus nerves. Some of these

fibres may have been motor, but if so, they were evidently too few to produce contraction of the stomach when stimulated.

'Guidance' of the growing tips of non-medullated fibres by medullated fibre sheaths (Evans & Murray, 1953). The fate of the regenerating non-medullated fibres was elucidated when the recurrent laryngeal, cardiac and bronchial branches of the vagus were examined. These presented a remarkable appearance in that groups of non-medullated fibres were observed forming crescents or rings surrounding the medullated fibres in these branches. This was particularly striking in the recurrent laryngeal nerve, which normally contains very few non-medullated fibres (Pl. 2, fig. 8). In the regenerating nerve, on the other hand, all the medullated fibres were surrounded by non-medullated axons (Pl. 2, fig. 9); in some cases as many as twenty-five to thirty of these were found related to a single medullated fibre (Pl. 2, fig. 10*a*). This appearance was observed in recurrent laryngeal nerves both after long and short regeneration periods. The diverted axons regenerated along the whole length of the nerve, a distance of 14–17 cm. (Table 3) from the site of the crush and entered the laryngeal muscles. Bielschowsky-Gros preparations of these muscles showed numerous fine axons growing alongside the muscle fibres, but making no apparent connexion with the end plates.

Counts of the total number of non-medullated fibres present in the regenerating recurrent laryngeal nerve at the level of the clavicle were made in three rabbits with long survival periods following a cervical vagus crush. Table 5 shows the values obtained and indicates that the number of non-medullated fibres which have been diverted into the recurrent laryngeal nerve accounted for almost half of the total number of non-medullated fibres normally present in the cervical vagus. This is based on the assumption that each regenerating non-medullated axon produced only one sprout. Additional groups of non-medullated fibres were found surrounding the medullated fibres in the cardiac and bronchial branches and these undoubtedly accounted for the majority of the remaining regenerating non-medullated fibres.

Position of the non-medullated axons growing along the sheaths of the medullated fibres. It was not possible to decide the position of the non-medullated axons in relation to the various components of the sheath of the medullated fibres simply from observations made on sections of regenerating nerve stained by the pyridine-silver method. Several methods of counter-staining were tried, the best results being obtained with that described by Foley (1938). Unfortunately, however, it was not possible to distinguish the boundaries between the Schwann cells and the neurilemma and between the neurilemma and the endoneurium (Pl. 2, fig. 11).

More definite, but still indirect, evidence regarding the position of non-medullated axons in the regenerating recurrent laryngeal nerve was obtained when the large medullated fibres in this nerve were caused to degenerate. A two-stage operation was carried out on two rabbits. The first stage consisted of a crush of the vagus trunk in the neck, after which regeneration was permitted in the two animals for 240 and 260 days respectively. Then extracranial section of the nerve between the skull and the upper pole of the nodose ganglion was performed and the animals allowed to survive for a further period of 30 days. In the rabbit such extracranial section of the vagus results in degeneration of all the large medullated fibres present in the laryngeal bundle of the recurrent laryngeal nerve (Evans & Murray, 1954).

These fibres, which constitute the motor innervation of the laryngeal muscles, have their cells of origin in the medulla. On the other hand, the majority (about 75%) of the non-medullated fibres present in the cervical vagus have their cells of origin in the nodose ganglion and these therefore survive supranodose section of the nerve (Evans & Murray, 1954). Examination of pyridine-silver stained sections of the laryngeal bundle of the recurrent laryngeal nerve in these two animals showed the presence of non-medullated fibres in large numbers. This demonstrates that they are not sprouts of regenerating medullated fibres but are, in fact, regenerating non-medullated fibres which have been diverted into the recurrent laryngeal nerve. Most of the fibres were situated within the sheaths of the now degenerated medullated fibres, occupying the centre of the tubes (Pl. 2, fig. 10*c*) in contrast to the more peripheral position they occupy in the presence of medullated fibres. This suggests that these axons were originally placed between the neurilemmal membrane and the outer surface of the protoplasmic layer composed of Schwann cells. When the medullated fibre degenerates, the Schwann cells proliferate and come to occupy the centre of the tube. Evidently most of the non-medullated axons adhere to the surface of the Schwann cells and thus become reorientated.

In the early period following a crush of the cervical vagus the growing tips of the non-medullated axons must presumably penetrate the endoneurium and neurilemma to reach the centre of the tube of the medullated fibre. It was first considered that this penetration would be most likely to occur at the site of the crush. However, this was not the case as in sections taken immediately distal to the crush the non-medullated axons were not orientated in rings around the medullated fibres. This appearance was visible only in sections taken at several millimetres below the crush, and it was evident that more and more axons became added to the rings as the nerve was traced distally.

In the early regenerating period (20 days and less) the outgrowths of the non-medullated axons that have thus penetrated the sheaths grow amongst the thin sprouts of the regenerating medullated fibres. At this stage both types of axons were found distributed amongst the proliferating Schwann cells occupying the centres of the tubes and no clear distinction between the two varieties was possible. Very soon, however, one or more of the thin outgrowths (Pl. 2, fig. 12) within each tube thickens and becomes medullated. The larger fibres originate as outgrowths from a medullated parent fibre and the thickening process begins before the tips of the fibres have made contact with end organs. The growth in diameter which these fibres undergo causes the remaining axons in the tube to be displaced to a more peripheral position (Pl. 2, figs. 10*a*, *b*). A similar displacement occurs during regeneration of a purely medullated somatic nerve. At an early stage following a crush each peripheral tube becomes populated by a number of fine fibres all derived from the one central axon. Later, when one of these fibres reaches a suitable end-organ, it begins to increase in diameter, displacing the remaining axons towards the periphery of the tube and ultimately causing them to disappear (Sanders & Young, 1944; Aitken, Sharman & Young, 1947). From these results it may be seen that, apart from the principal axon, all sprouts arising from the medullated parent fibre are firstly displaced to the periphery of the tube and finally the majority disappear; the non-medullated axons regenerating down such tubes, although similarly displaced, persist, even after full maturation of the medullated axon.

DISCUSSION

The results of this study confirm that the central stumps of non-medullated axons which have been interrupted by crushing or cutting a nerve trunk have a strong regenerative capacity. When the vagus nerve is crushed the axons of non-medullated as well as those of medullated fibres grow directly through the crushed region to invade the distal stump. The subsequent behaviour of the non-medullated axons is profoundly influenced by the presence or absence of medullated fibres. When the abdominal vagus nerve, which consists almost entirely of non-medullated fibres, was crushed the regenerating axons in the peripheral stumps assumed a distribution pattern indistinguishable from the normal. Ultimately they reached end-organs in the stomach wall and function became re-established. Below a lesion of the cervical vagus, on the other hand most of the non-medullated axons became arranged alongside the sheaths of the medullated fibres and were thus diverted from their old pathways. In consequence no functional re-innervation of the stomach occurred.

The part played by the sheaths of medullated fibres in directing the path taken by new axons during regeneration has been studied in detail by Holmes & Young (1942). During regeneration of a nerve composed of myelinated fibres the pattern of the peripheral stump of the nerve is maintained by the neurilemma and endoneurium. These structures persist as a continuous tube leading to the end-organs. The Schwann cells proliferate rapidly soon after the interruption of the axons and they come to occupy the centre of the tube. The nuclei and the protoplasm of the Schwann cells become greatly elongated. During the early stages of regeneration Holmes & Young found that the new axons grew along the interface between the inner wall of the neurilemmal sheath and the surface of the Schwann cells. When a medullated nerve is crushed by the method used in the present study the great majority of the connective tissue sheaths of the medullated fibres are not interrupted and thus most of the regenerating axons grow along their original tubes.

Evidently the sheaths of non-medullated fibres do not provide such efficient pathways for the regenerating axons. This might be expected from the account of the structure of such sheaths given by Nageotte (1932) and Gasser (1952). The syncytial arrangement of the Schwann sheath which they described would provide ample opportunity for regenerating non-medullated axons to wander about within the nerve trunk. Nageotte found that medullated fibres frequently traversed the interstices of this syncytium. Non-medullated axons regenerating along the syncytium thus come in close contact with the endoneural sheaths of medullated fibres, and they grow alongside these fibres in preference to their old pathways.

The experimental findings described above demonstrate marked differences in the process of regeneration of medullated and non-medullated fibres.

SUMMARY

An experimental study has been made of regeneration of non-medullated fibres in the vagus nerve of the rabbit. The vagus nerve in the neck is composed of medullated and non-medullated fibres whereas in the abdomen, the nerves are almost entirely non-medullated. Thus the behaviour of regenerating non-medullated fibres in the presence and absence of medullated fibres can be studied by lesions at these two levels.

1. Following a crush of the abdominal vagi, function returns uniformly to the stomach but is delayed. The delay is possibly due to the lack of specific guiding channels in non-medullated nerves.

2. Following a crush of the cervical vagus, no evidence of functional return to the stomach was found even after survival periods of 600 days. Histological findings showed that few non-medullated axons had succeeded in regenerating to the abdomen.

3. The fate of the regenerating non-medullated fibres was elucidated on examination of the recurrent laryngeal nerve. They had been diverted from their original pathway and 'guided' along the medullated fibres in the recurrent laryngeal nerve.

4. The majority of the regenerating non-medullated fibres were situated within the Schwann tubes of the medullated fibres in the recurrent laryngeal nerve.

The authors wish to thank Prof. J. Z. Young for his advice and encouragement with this work; also, Mr J. Armstrong for the photography and Miss R. Smith for technical assistance.

REFERENCES

ABERCROMBIE, M. & JOHNSON, M. L. (1942). The outwandering of cells in tissue cultures of nerves undergoing Wallerian degeneration. *J. exp. Biol.* **19**, 266–283.

AITKEN, J. T., SHARMAN, M. & YOUNG, J. Z. (1947). Maturation of regenerating nerve fibres with various peripheral connections. *J. Anat., Lond.*, **81**, 1–22.

DENNY-BROWN, D. & BRENNER, C. (1944). Lesion in peripheral nerve resulting from compression by spring clip. *Arch. Neurol. Psychiat.* **52**, 1–19.

EVANS, D. H. L. & MURRAY, J. G. (1953). Orientation of regenerating non-medullated nerves. *J. Physiol.* **120**, 52–53*P*.

EVANS, D. H. L. & MURRAY, J. G. (1954). Histological and functiona lstudies on the fibre composition of the vagus nerve of the rabbit. *J. Anat., Lond.*, **88**, 320–337.

FOLEY, J. O. (1938). A differential counterstain for toned sections of pyridine silver preparations of peripheral nerves. *Anat. Rec.* **71**, 133–139.

GASSER, H. S. (1952). The Neuron. *Cold Spr. Harb. Symp. quant. Biol.* vol. XVII, pp. 32–36. New York: The Biological Laboratory, Long Island Biological Association, Inc.

GUTMANN, E., GUTTMANN, L., MEDAWAR, P. B. & YOUNG, J. Z. (1942). The rate of regeneration of nerve. *J. exp. Biol.* **19**, 14–44.

HOLMES, W. & YOUNG, J. Z. (1942). Nerve regeneration after immediate and delayed suture. *J. Anat., Lond.*, **77**, 63–96.

INGEBRIGTSEN, R. (1916). A contribution to the biology of peripheral nerves in transplantation. II. Life of peripheral nerves of mammals in plasma. *J. exp. Med.* **23**, 251–264.

JOSEPH, J. (1947). Absence of cell multiplication during degeneration of non-myelinated nerves. *J. Anat., Lond.*, **81**, 135–139.

M'CREA, E. D., M'SWINEY, B. A. & STOPFORD, J. S. B. (1925). The effect on the stomach of stimulation of the peripheral end of the vagus nerve. *Quart. J. exp. Physiol.* **15**, 201–233.

NAGEOTTE, J. (1932). Sheaths of the peripheral nerves. In *Cytology and Cellular Pathology of the Nervous System* (edited by W. Penfield), vol. I, pp. 189–239. New York: Hoeber, Inc.

RANSON, S. W. & DAVENPORT, H. K. (1931). Sensory unmyelinated fibers in the spinal nerves. *Amer. J. Anat.* **49**, 331–353.

SANDERS, F. K. & YOUNG, J. Z. (1944). The role of the peripheral stump in the control of fibre diameter in regenerating nerves. *J. Physiol.* **103**, 119–136.

SEDDON, H. J. (1943). Three types of nerve injury. *Brain*, **66**, 237–287.

SOLLMANN, T. (1923). A method of studying peristalsis in situ. *Amer. J. Physiol.* **63**, 395–396.

TUCKETT, L. (1896). On the structure and degeneration of non-medullated nerve fibres. *J. Physiol.* **19**, 267–311.

WHITE, J. C., SMITHWICK, R. H. & SIMEONE, F. A. (1952). *The Autonomic Nervous System.* London: Kimpton.

YOUNG, J. Z. (1942). Functional repair of nervous tissue. *Physiol. Rev.* **22**, 319–347.
YOUNG, J. Z. (1949). Factors influencing the regeneration of nerves. In *Advances in Surgery*, vol. I, pp. 165–220. New York: Interscience Publishers, Inc.

EXPLANATION OF PLATES

With the exception of Pl. 1, fig. 6, all are transverse sections stained by the pyridine-silver method.

PLATE 1

Fig. 1. A segment of a normal posterior abdominal vagus with a high population of non-medullated axons.

Fig. 2. Section of an anterior abdominal vagus trunk 20 mm. distal to a crush. Regeneration period of 20 days. The axons are few and uniformly distributed. They are smaller in diameter than normal.

Fig. 3. A segment of the anterior abdominal vagus showing that few non-medullated fibres have grown back after a crush of the left cervical vagus 670 days previously. The right cervical vagus was divided 21 days before biopsy. On the left side, a group of regenerating non-medullated fibres are arranged around a medullated fibre tube.

Fig. 4. Low-power view of the anterior abdominal vagus, 307 days after a left cervical crush. Few, if any, fibres have regenerated into the segment of nerve occupied by the left vagus (upper segment of section).

Fig. 5. A segment of an anterior abdominal vagus nerve showing a few remaining axons after both cervical vagus nerves have been divided. The right nerve was cut 45 days and the left 21 days before the biopsy.

Fig. 6. Longitudinal section of the lower part of a neuroma showing regenerating non-medullated fibres bridging a gap of 15 mm. to re-innervate the distal stump of the abdominal vagus nerve. A 15 mm. segment of the nerve at the level of the diaphragm had been removed 42 days before biopsy. Bodian stain.

PLATE 2

Fig. 7. A segment of the normal left cervical vagus showing the medullated axons within their tubes and the non-medullated axons in the connective tissue between the tubes.

Fig. 8. Section showing normal left recurrent laryngeal nerve with the medullated axons staining faintly within their tubes and one sharply stained non-medullated axon.

Fig. 9. Section showing regenerating left recurrent laryngeal nerve 280 days after a crush of the left cervical vagus. Non-medullated axons are arranged around the tubes of the medullated fibres. Compare with the normal nerve (Pl. 2, fig. 8).

Fig. 10. Showing orientation of non-medullated fibres in relation to the sheaths of medullated fibres in regenerating recurrent laryngeal nerves.

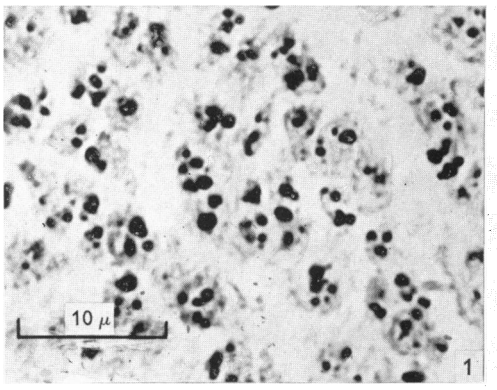
Fig. 10*a*. 550 days after cervical crush. The large central axon is very faintly stained.

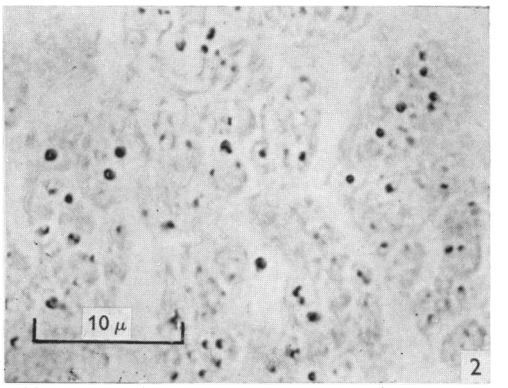
Fig. 10*b*. 670 days after cervical crush showing the medullated axon darkly stained.

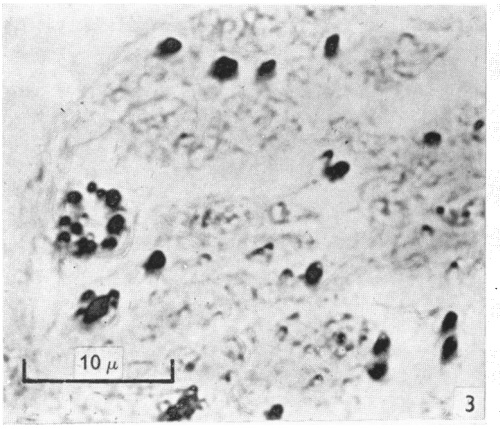
Fig. 10*c*. 240 days regeneration after cervical crush followed by section of the vagus cranial to the nodose ganglion 30 days before biopsy. The medullated fibre has degenerated and the surviving non-medullated axons now occupy the centre of the tube.

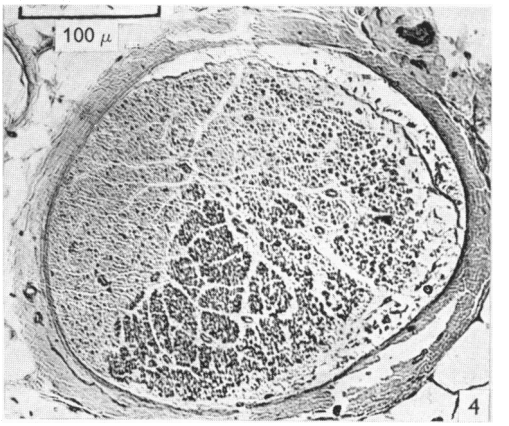
Fig. 11. Regenerating recurrent laryngeal nerve 280 days after a crush of the left cervical vagus showing the orientation of the non-medullated axons (*n.m.a.*) in relation to the medullated axon (*m.a.*). Most of these axons are separated from the myelin (*m.*) by a definite zone of the sheath (*s.*). One tube contains no medullated fibre and in this the non-medullated axons are scattered throughout the centre of the tube. Pyridine-silver, counter-stained by Foley's method.

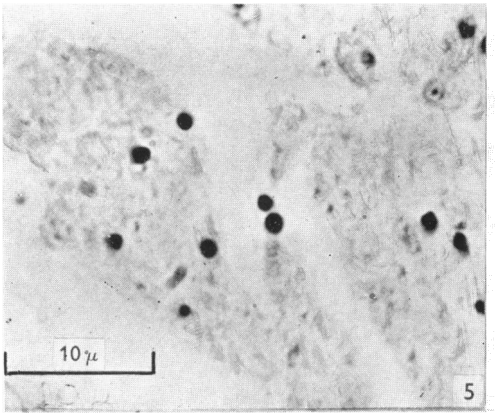
Fig. 12. Cervical vagus 2 cm. below a crush after 28 days' regeneration. At least one axon (*m.a.*) in each tube has already become medullated. Groups of non-medullated axons (*n.m.a.*) are arranged at the periphery and towards the centre of the tubes.

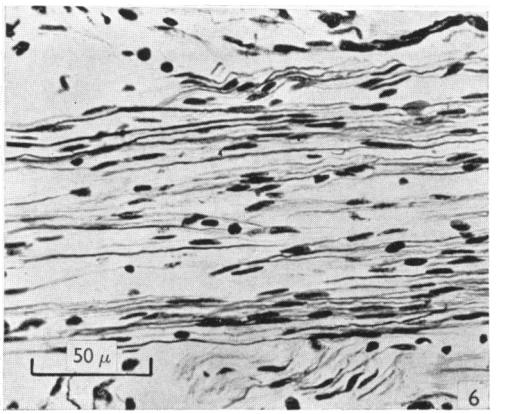

10 μ
1

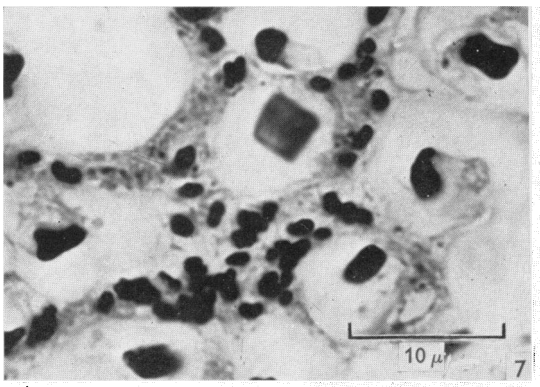

10 μ
2

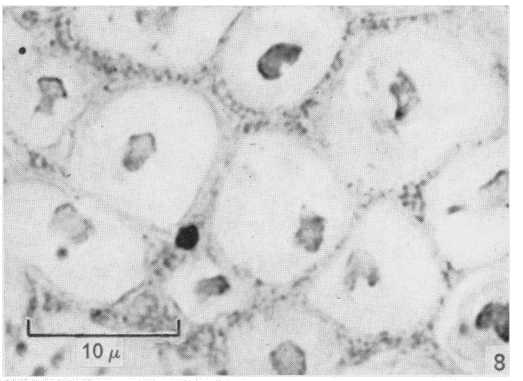

10 μ
3

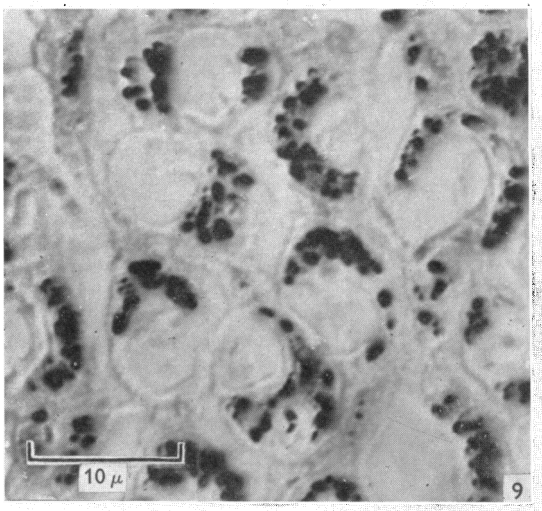

100 μ
4

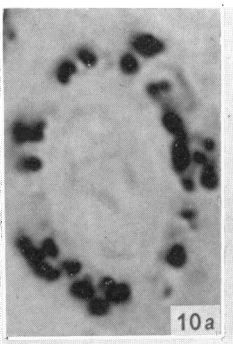

10 μ
5

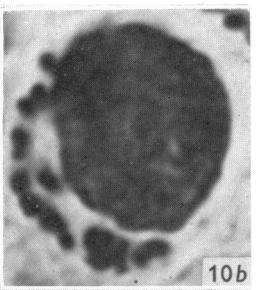

50 μ
6

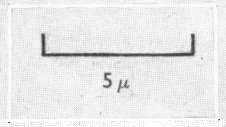


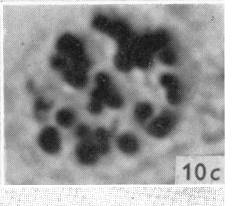


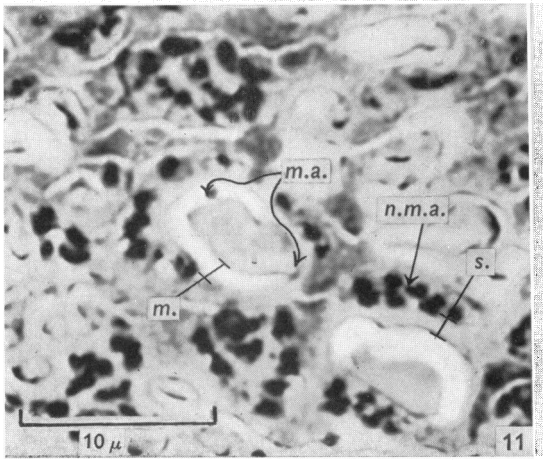


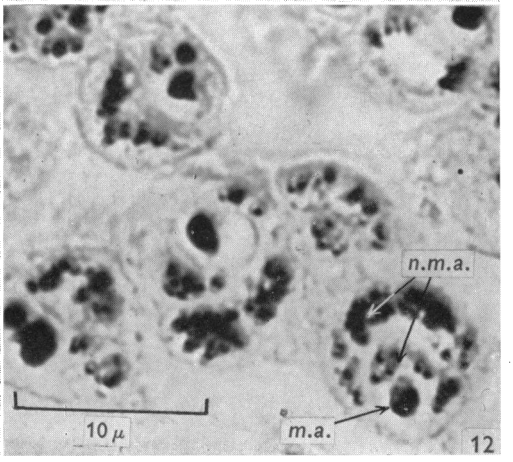












EVANS AND MURRAY—REGENERATION OF NON-MEDULLATED NERVE FIBRE