A BIOCHEMICAL AND MORPHOLOGIC STUDY OF MYELINATION AND DEMYELINATION*

II. Lipogenesis in Vitro by Rat Nerves Following Transection

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Plates 16 to 18

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The morphologic events of Wallerian degeneration have been thoroughly investigated, with the optical microscope (1, 2), with the electron microscope (3), and by histochemical methods (4–7). A considerable body of knowledge has also accumulated on the changes in lipide and enzyme patterns at various stages after cutting or crushing injury, due largely to the work of Rossiter and his collaborators (8–15). Fewer data are available on the metabolic events which take place in a transected nerve (16–21), and attempts to correlate functional and morphologic changes have been focussed essentially on the progressive failure of excitability (22). In the present paper, lipogenetic and respiratory activities of transected nerves have been studied *in vitro* conjointly with changes in their morphology. Considerable emphasis had been placed on the *early* metabolic alterations in the severed nerve; *i.e.*, on the stage at which truly "degenerative" changes are not yet overwhelmed by hypertrophy and hyperplasia of the Schwann cells. Nerves were examined both above and below the level of transection and the fine, newly formed fibers which emerge from a transected stump were also studied. It was thus possible to compare the process of nerve regeneration with previously reported data on normal nerve growth in very young animals (23). The early metabolic changes of Wallerian degeneration were also compared with degenerative changes occurring during incubation *in vitro*.

Material and Methods

Male rats of the Sprague-Dawley and Wistar strains were used. The age was kept as close as possible to 10 to 12 weeks and was identical for all rats within one experiment.

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Wallerian Degeneration.—Under ether anesthesia the sciatic nerve was bilaterally exposed close to its origin, lifted gently on a hook, and sectioned. This operation will be referred to as "high transection." In order to prevent reunion of the severed nerve, the proximal stump was pushed towards its origin with a small plug of cotton which rapidly became anchored in place by a connective tissue reaction. After intervals ranging from 12 hours to 252 days the animals were killed by decapitation, both sciatic nerves were dissected distal to the site of transection, and excised together with the tibial branch; the proximal 8 mm. was discarded. A sample of two pooled nerves weighed 40 to 90 mg. depending on the stage of degeneration.

Experiments with the Proximal Stump.—The three main branches of the sciatic were cut at the level of the popliteal space ("low transection"). Stretching of the main trunk was carefully avoided. No measures were taken to prevent regeneration, and the rats were killed 3 to 32 days later. The proximal stump developed a terminal bulb 3 to 4 mm. in length, which by the 10th day had usually established connection with the peripheral stump of one of the three branches. The portion of the nerve excised for incubation extended from the origin to 5 mm. *above the bulb.* The two pooled segments from one rat weighed 30 to 40 mg. Comparable segments taken from normal animals served as controls.

Experiments with Regenerating Nerves.—This group of experiments was directed toward a study of the new fibres which grow out of the tip of a transected nerve. After "low transection" it was not possible to secure satisfactory specimens of nerve sprouts, because the stream of regenerating fibres would often include islets of adipose cells. Transection was therefore performed at mid-thigh level, where the sciatic runs in a space in which adipose tissue is scanty. The exposed nerve trunk was seized with a small hemostat, and two cuts, above and below the clamped portion, eliminated a segment about 3 mm. long. Spontaneous retraction widened the gap between proximal and distal stump to about 8 mm. When the animals were killed, 10 to 32 days later, the terminal bulb of the proximal stump ordinarily tapered off into a thin, delicate filament attached to the distal stump. This filament was semitransparent and almost gelatinous at 10 days after section, but whiter and similar to a normal myelinated nerve at 32 days (Fig. 1 A). It was dissected free of the surrounding connective tissue, seized at the distal end with Swiss watchmaker's forceps, severed from the terminal bulb of the proximal stump, and briefly stored under the subcutaneous tissue of a freshly killed rat (23). The specimens were weighed in batches of 10 to 12, constituting one sample of 20 to 30 mg.

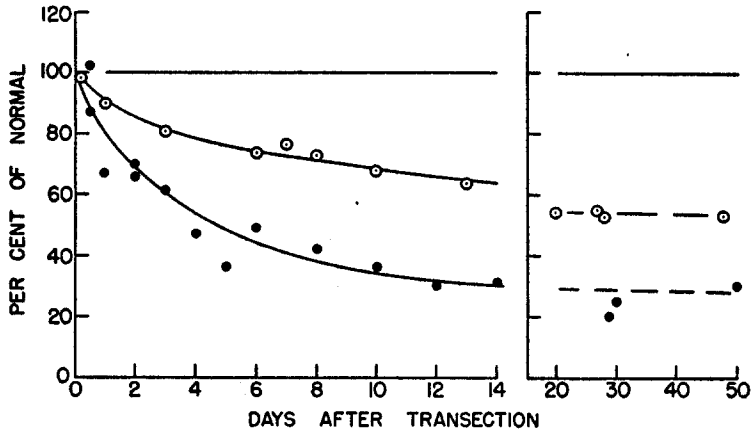
Incubation and Lipide Extraction.—All samples were weighed to the nearest 0.2 mg. on a torsion balance. Incubation was carried out in Warburg flasks, for 4 hours, in Krebs-Ringer-phosphate medium containing glucose as a source of energy (10 μM/ml.), and a labelled lipide precursor. This was either acetate-1-C^{14} at a concentration of 0.66 mg./ml (0.5 to 2.0 $\times$ 10^6 counts per minute per flask) or P^{32}-phosphate (1 to 2 $\times$ 10^6 counts per minute per flask) used at the concentration required for phosphate-buffered Krebs-Ringer medium. After incubation the nerves were rinsed and homogenized, and the total lipides were extracted, plated, and counted as previously described (23). The *total lipide content* was calculated from the amount of lipide which was plated from each preparation. *Dry weights* of tissue were determined as previously described (23). In a typical experiment, three flasks containing normal sciatics were compared with sets of triplicate experimental samples.

Histological Procedures.—Samples of each kind were set aside for histological examination. One set of tissues was fixed in 10 per cent formalin, embedded in paraffin, and stained with hematoxylin and eosin. Another set was fixed in 1 per cent osmic acid, embedded in paraffin, and counterstained with eosin. When appropriate, a protargol method for axis cylinders (24), Peer's modification of the phosphotungstic acid–hematoxylin method (25), and scarlet red–hematoxylin on frozen sections were also used. For the study of the proximal stumps, in which it was expected that histological changes would be very slight, each experimental specimen was coupled with a comparable segment of control nerve, simultaneously fixed, then embedded and cut in the same block.

EXPERIMENTAL PROCEDURE AND RESULTS

Wallerian Degeneration.—

Bilateral high transection was performed on 128 rats. The body weight dropped slightly during the first few days (5 to 7 per cent) and returned to the original value within the 2nd week. By the end of the 1st week, the ankles tended to become edematous and a number of animals developed uni- or bilateral plantar ulcers. As degeneration progressed, the soft, glossy white, ribbon-shaped nerve swelled to a rigid cylinder of characteristic semitransparent appearance, a macroscopic change which reflected an increase in tissue water and a decrease



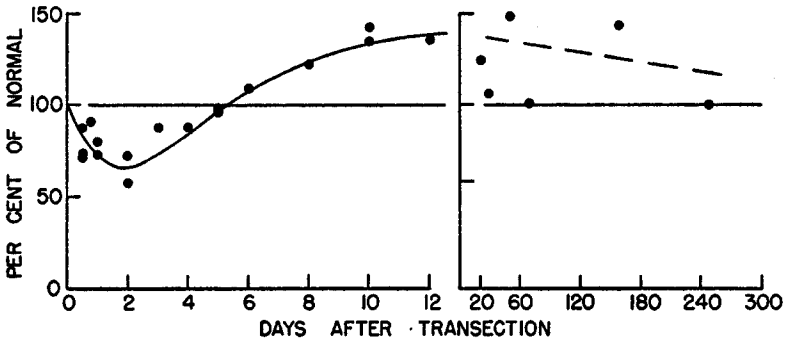
TEXT-FIG. 1. Wallerian degeneration: dry weight (⊙) and lipide content (●) of rat sciatics at various stages after transection, calculated as percentages of fresh weight. The results are expressed as a percentage of values for normal tissue which were set at 100. The histology of these preparations is shown in Figs. 2 and 3.

in lipide content. The animals were killed in groups of 2 to 4—together with controls—at varying intervals after the operation (12, 18, 24 hours, and 2, 3, 4, 5, 6, 8, 10, 12, 14, 29, 50, 72, 158, and 252 days). Control animals were mostly unoperated, except for the study of the earliest stages (see Discussion) in which case they were sham-operated. Thirty-one rats were used for determining the dry weight of the degenerating nerves; there was a progressive fall to 53 per cent of normal, while the lipide content dropped to about 25 per cent of normal (Text-fig. 1) and the total weight of the nerve doubled.

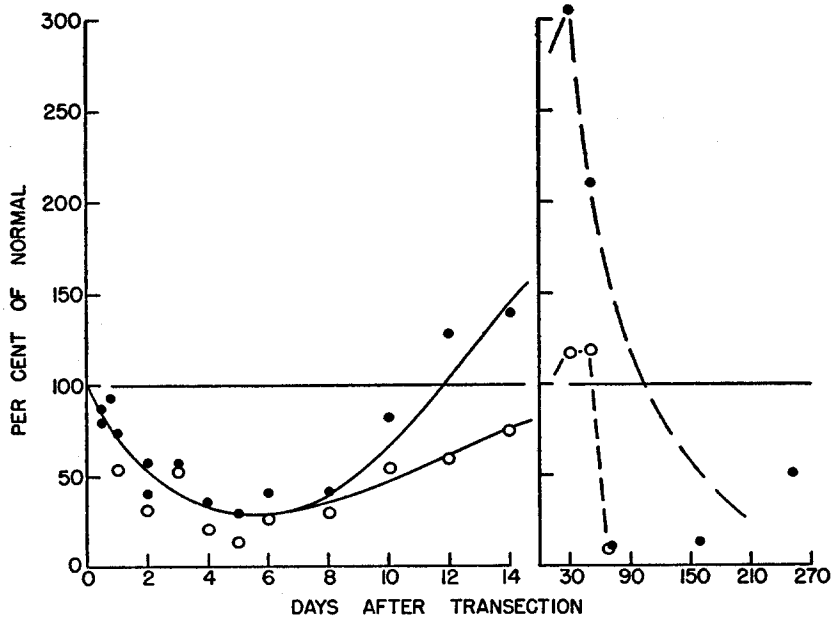
About 20 animals were used for histological study. Though essential as a base for interpreting the biochemical data, the histological material in itself does not contribute new information, and a detailed description will be omitted. Two plates are provided for reference (Figs. 2, 3). Our findings are covered almost exactly by the description given by Noback and Montagna (5).

Sixty-three animals were used for the study of respiration and lipide biosynthesis, using either acetate-1-C^{14} or phosphate-P^{32}. Oxygen uptake, referred to fresh weight, decreased by 10 per cent at 12 to 18 hours, reached a minimum at 48 hours (58 per cent), then rose to a plateau of about 140 per

cent of normal by the 10th day (Text-fig. 2); this plateau is about 210 per cent of normal if respiration is referred to dry weight. The incorporation of acetate into lipide, measured as specific activity, began to drop as early as 12 hours after section, reached a level 30 per cent of normal at 5 days, then

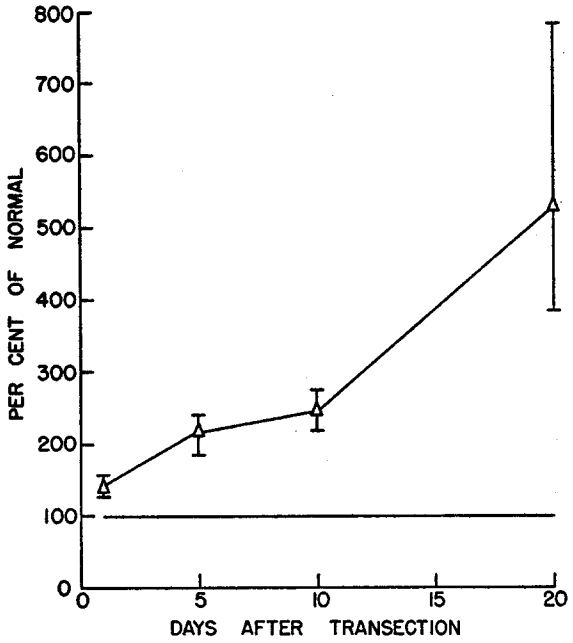


TEXT-FIG. 2. Wallerian degeneration: respiration of rat sciatics at various stages after transection. Results are expressed as a percentage of the values obtained for normal sciatics of rats of the same age (32 μl. O_2 per 100 mg. fresh weight and hour).



TEXT-FIG. 3. Wallerian degeneration: relative incorporation of acetate-1-C^{14} into the lipides of rat sciatics at various stages after transection. Results are expressed as a percentage of the values obtained for normal sciatics: (●) relative specific activity (calculated from measurements of C.P.M./100 mg. lipide) and (○) relative total activity (calculated from measurements of C.P.M. incorporated into lipide by 100 mg. of fresh nerve).

rose steeply to 305 per cent of normal by the 28th day (Text-fig. 3). The incorporation of labelled phosphate was studied 22 hours, 5, 10, and 20 days after transection. It was consistently higher than in the controls even at the earliest stage, while lipogenesis from acetate was dropping (Text-fig. 4). It may be seen that *total* incorporation of activity from acetate drops rapidly for 5 days, and then recovers to attain a constant level, which is 20 per cent above normal, 3 to 4 weeks after transection. In a number of experiments a comparison was made of the conversion of acetate-C^{14} to $C^{14}O_2$ of the center-



TEXT-FIG. 4. Wallerian degeneration: relative incorporation of phosphate-P^{32} into the lipides of rat sciatic at four stages after transection. Results are expressed as a percentage of the specific activity (C.P.M./100 mg. lipide) of the values obtained for normal sciatics.

well or to lipide-C^{14} (23). 2 and 4 weeks after transection only 12 to 19 C.P.M. were incorporated into the lipides for each 100 C.P.M. recovered from the center-well. The normal proportion (23) was 55 to 70 C.P.M. as lipide-C^{14} per 100 C.P.M. $C^{14}O_2$.

Degeneration of Nerves in Vitro.—Normal rat nerves were allowed to "degenerate" in Krebs-Ringer medium with glucose at 37°, and thereafter incubated with radioactive phosphate and compared with normal nerves which had not been pre-incubated.

The experiment was set up as follows: the sciatic preparations and brachial plexuses (23) of 6 rats were distributed among 12 Warburg flasks, containing the usual medium with glu-

cose and 400 units/ml. of both penicillin and streptomycin. Radioactive phosphate was added only to the first three flasks, and incubation was started for all samples simultaneously. At the end of the 2nd hour the first three samples were taken from the bath, homogenized, and their lipides extracted. These preparations were used as controls; *i.e.*, as nerves which had not been pre-incubated. After 4 hours, the nerves contained in the next three flasks were transferred to new flasks containing fresh medium with added radioactive phosphate, and these samples were homogenized at the 6th hour. After 8 hours the next three samples were similarly transferred to new flasks and incubated for 2 additional hours with labelled phosphate. The samples contained in the three remaining flasks were used for histological study only. The duration of the entire experiment was limited to 10 hours, since degenerative changes proceed faster *in vitro* than *in vivo* (18, 22). After the 8th hour an increasing oxygen uptake indicated—despite the antibiotics—an incipient bacterial growth, confirmed by microscopic examination of the medium.

The results are shown in Text-fig. 5. If the incorporation of phosphate during the first 2 hours is considered 100 per cent, during hours 5 and 6 it was reduced to 90 per cent (respiration to 80 per cent) and during hours 9 and 10 it was further reduced to 83 per cent. Histologically there was a progressive retraction of the myelin at the nodes of Ranvier, without interruption of the fibers (Fig. 1 G).

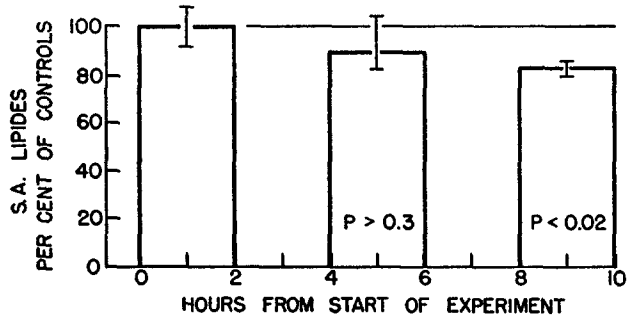
Proximal Stump.—

Seventy rats were submitted to bilateral low transection, then killed between the 3rd and the 32nd day. Proximal to the terminal bulb, for the first 10 or 12 days, the nerve trunk appeared normal in the gross. Thereafter, however, careful comparison with a similar segment of a control rat of the same age showed that the entire nerve trunk was somewhat swollen, its shape having become cylindrical rather than ribbon-shaped. The change extended as far proximally as the nerve could be followed (about 40 mm. above the site of transection).

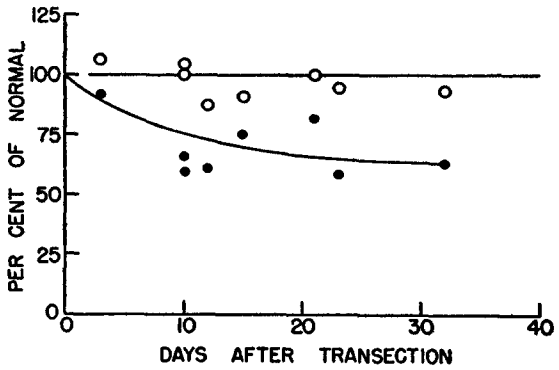
Nine animals were used for the determination of dry weight, 3, 10, and 33 days postoperatively. In this case the sample was a short (*ca.* 10 mm.) segment of the proximal stump from 25 to 35 mm. above the level of transection. The dry weight (compared with that of similar samples from control rats) was 98, 95, and 94 per cent of normal respectively. Thirty rats were killed in groups of three or six (together with unoperated controls) after 3, 10, 12, 15, 21, 23, and 32 days, and the preparations were incubated with acetate-1-C^{14}. Incorporation of the label into the lipides, measured as specific activity, was 60 to 80 per cent of normal from the tenth postoperative day onwards (Text-fig. 6). Two additional groups of 6 animals were killed after 15 and 21 days, respectively, and the proximal stumps were incubated with phosphate-P^{32}. Incorporation into the lipides (specific activity) was increased at both stages, being 185 per cent of normal at 15 days and 118 per cent of normal at 21 days. Oxygen uptake referred to fresh weight showed no significant change, though there was a trend toward a slight depression (Text-fig. 6). The *lipide content* was not significantly different from normal. The salient histological finding is shown in Figs. 1 E and 1 F.

Regenerating Nerves.—

Bilateral transection at midthigh level was performed on 80 rats. After 10 to 11 days, union tissue was found in most cases, but histologically the sprouts did not always reach the distal stump; occasionally a filament visible in the gross emerged from the proximal segment,



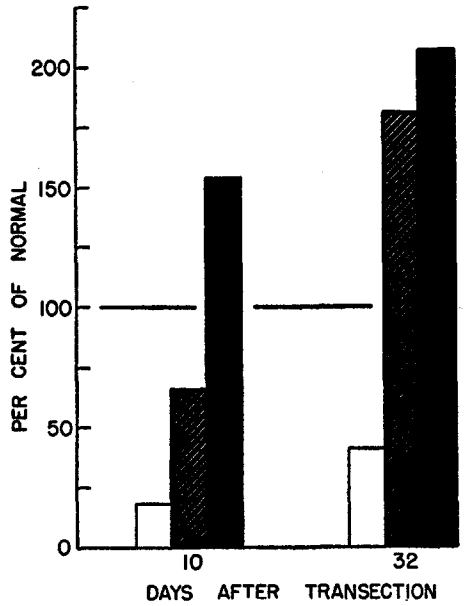
TEXT-FIG. 5. Degeneration of rat nerves *in vitro*: incorporation of phosphate-P^{32} into the lipides during hours 1 and 2, 5 and 6, and 9 and 10 of the incubation. See text for experimental details. Results are expressed as a percentage of the average specific activity (C.P.M./100 mg. lipide) obtained for hours 1 and 2. Histological aspect of the preparations: see Fig. 1 G.



TEXT-FIG. 6. Proximal stumps of transected rat sciatics: (○) oxygen uptake, (as a percentage of normal values) and (●) incorporation of acetate into the lipides (expressed as a percentage of the specific activity values (C.P.M./100 mg. lipide) obtained for normal sciatics). The histology of a 19-day sample is shown in Figs. 1 E, and 1 F.

but had not reached the distal segment; in two or three nerves there was no visible filament at all. A cap of greyish tissue sprouting from the distal stump—Nageotte's "Schwannoma" (26)—was often observed (Fig. 1 A), but it never seemed to reach, in bulk, the size of the regenerating filament. Histologically, a sample of 10 day union tissue consisted mainly of spindle-shaped cells, while the tangled axon sprouts—still unmyelinated—represented only a fraction of the total volume. These relationships are apparent in Figs. 1 C and 1 D. At 32 days, however, the histological appearance was similar to that of a normal nerve, with regularly aligned fibres bearing myelin sheaths.

Batches of 18 rats were killed 10 or 11, and 32 days respectively after the operation, the pooled filaments emerging from the proximal stumps were incubated with radioactive acetate, and compared with whole sciatic preparations of 3 normal rats. The results are shown in Text-fig. 7. Respiration of the regenerating nerves was always higher than normal; lipogenesis from acetate was lower than normal at 10 days, higher at 32 days. The dry weight, deter-



TEXT-FIG. 7. Regenerating sciatics, 10 days (left) and 32 days (right) after transection: open bar, lipide content; Striped bar, incorporation of acetate into the lipides expressed as relative specific activity; Solid bar, oxygen uptake per 100 mg./fresh weight. Results are calculated as a percentage of the values obtained for normal sciatics. The morphology of a preparation obtained 10 days after transection is shown in Figs. 1 A to 1 D.

mined on 14 pooled samples at the 12 day stage, was 19.5 per cent of the fresh weight; *i.e.*, 65 per cent of the normal adult value. This level is similar to the dry weight content of nerves from rats 10 days old (23). A dozen animals were used for histological study.

DISCUSSION

Wallerian Degeneration.—In choosing a mixed nerve such as the sciatic for a biochemical study of Wallerian degeneration, it should be appreciated that the histological sequence of events characteristic of this condition varies with size (22) and type (22, 27) of the fibers. Ideally, bundles of fibers similar in caliber and physiological significance should be used. However, in the rat,

appropriate nerves such as the peroneal or the sural are rather small relative to the techniques here employed. In view of the large amount of data already existing for the sciatic, the latter remains, for the time being, the nerve of choice.

All our test animals were bilaterally operated, and the control nerves were taken from normal animals of the same birth date. This procedure was adopted because there is evidence that the contralateral nerve of a unilaterally operated animal is abnormal histologically (28, 29), chemically (21), and histochemically (7). The trauma of the operation in itself was found capable of causing a transient depression of respiration and lipogenesis from acetate in peripheral nerve. Hence, for the study of early stages of degeneration (24 hours or less) sham-operated animals were used in addition to the normal controls. Particular care was given to using animals of uniform age, in view of the broad changes which occur in growing peripheral nerves (12, 23).

One of the most striking observations on the severed rat sciatic is the rise in water content. Expressing the change as a drop in dry weight (Text-fig. 1) there was a significant 10 per cent decrease at 24 hours, in keeping with histological findings (30) which indicate a swelling of the axon after only 3 hours. At 1 month the nerve has almost doubled its total weight, compared with the normal. In the cat (8) and rabbit (14) the reported rise in water content during Wallerian degeneration is less marked.

It was a surprise to find that the total lipide content of the nerve began to wane within 24 hours (Text-fig. 1). The decrease was evident both when the measurement was based on the dry weight or on the fresh weight of the tissue. A significant drop occurred at 24 hours, and after 5 days the lipide content—referred to dry weight—was only 67 per cent of normal. Data from Rossiter's laboratory show that a significant loss of lipides, in cat (8) and rat (11) sciatics, occurs within 2 to 4 days. Histology is of little help in explaining the disappearance of lipide at 24 hours, when the myelin sheath is still continuous (Fig. 2). By the end of the 3rd day, over ¼ of the total lipide had disappeared; histologically, the axis cylinders were segmented, but large amounts of axoplasm were still present and stainable. Even if the whole axis cylinder disappeared with its entire lipide content, it is unlikely that it could account for the missing lipide. The macrophage reaction was barely under way (Fig. 3). Within this early period it seems that a "non-cellular removal" takes place, as if myelin were diffusely removed, possibly as the result of imbalance between anabolic and catabolic processes. Function is maintained and histochemical changes in the myelin are practically absent for the first 10 days after section (5).

Respiration and lipogenesis from acetate, during the first month, followed a biphasic course. The initial drop in respiration may be related to the rapid disintegration of the axis cylinder with its mitochondria. Concomitantly

there occurs a hypertrophy of the Schwann cells (see below), but the curve swings upwards only when the total number of cells begins to increase. We observed occasional mitoses after 2 days and scattered phagocytes after 3 days (Fig. 3). Crude nuclear counts per microscopic field showed the first increase after 4 days, but if the swelling of the nerve is taken into account the rise would occur at least 1 day earlier, in conformity with previous findings (31, 15). The number of cells per microscopic field by direct count rose to at least three times (32) the normal level after 12 days. If appropriate corrections (32) were made, the number would more properly rise to four- or fivefold the normal value. On the other hand, respiration (referred to dry weight) reached a plateau at only twice the normal value. This indicates that in addition to an increase in the number of cells present, there was also a progressive change in their functional state. In the degenerating frog sciatic the changes in oxygen uptake are slower, but otherwise similar to those here described (18).

The degenerating nerve might be said to contain two categories of lipide: (*a*) lipides of the former myelin sheaths, now degenerating and (*b*) lipides of regenerating Schwann cells. In category (*a*) incorporation of acetate into lipide might drop to very low and possibly to zero values. In category (*b*) acetate incorporation could conceivably be very high. The activity curves of Text-fig. 3 would not be contrary to this suggestion. During the first 5 days incorporation of activity into lipides of category (*a*) probably decreases, and since these constitute the bulk of the lipides, the over-all *specific activity* diminishes. After 5 days, as the Schwann cells proliferate, the activity incorporated into the increasing amounts of lipide of category (*b*) causes the upward swing in specific activity. At about 50 days after section only scattered debris of the myelin sheaths remain (Fig. 2) and lipides of category (*a*) have reached minimal amounts, so that the specific activity measurement reflects essentially that of lipides of regenerating Schwann cells.

It is an intriguing fact that, while respiration and lipogenesis from acetate drop and then rise, the incorporation of phosphate into the total lipides, expressed as specific activity, rises from the start (Text-fig. 4). The earliest period after section examined in this study was 22 hours, when the increase in specific activity was 41 per cent. Similar data have been reported from other laboratories (16, 17, 19, 20). If these specific activity data are correlated with the data of Text-fig. 1, it is apparent that a real increase in phosphate incorporation into nerve-lipide per unit of dry tissue occurs at a time when the myelin shows regressive changes, when lipogenesis from acetate and respiration are depressed, and when cellular multiplication is not yet apparent. There is abundant cytological and biochemical evidence, however, that the Schwann cells—though not yet multiplying—are already undergoing hypertrophy within 24 hours of transection (4, 21). It would seem, then, that the regenerating Schwann cells synthesize some lipide moieties far more rapidly than others.

In the later stages of Wallerian degeneration (72, 158, and 252 days) the sciatic preparations appeared as fine, very cellular threads. Respiration tended to decrease towards normal values, whereas incorporation of acetate was drastically reduced to between 10 and 50 per cent of normal. This situation is a metabolic parallel of the late atrophy so often described when reinnervation is prevented (2, 31, 33, 34).

In a broad sense, then, the biochemical data agree with the histologic findings in indicating that Wallerian degeneration evolves through three stages: (I) a stage of predominantly passive changes (1 to 3 days), (II) a stage of cellular reaction, (4 to 50 days), (III) a stage of atrophy (from 50 days onwards). The duration of each phase, of course, cannot be assigned a fixed value, because of the different time-course of Wallerian degeneration depending on age (23, 12) and species (2).

Degeneration of Nerves in Vitro.—In contrast with the early stages of Wallerian degeneration, in which the incorporation of phosphate into the lipides increased, the incorporation of phosphate dropped when nerves were allowed to degenerate *in vitro* (Text-fig. 5). This finding lends further support to the idea that the increase observed *in vivo* is due to hypertrophy of the Schwann cells. In a medium such as that here employed, which lacks a source of nitrogen for protein synthesis, it is unlikely that even an early hypertrophy could be supported. This difference between degeneration *in vivo* and *in vitro* is also worth noting because the changes of early Wallerian degeneration are often likened to these occurring during incubation (35, 8, 22). Differences between the two processes have been demonstrated by tissue culture methods (34) as well as functionally (18) and chemically (18, 36).

Proximal Stumps.—The changes which take place in the proximal stump are not only in the nature of regeneration. It is now established that there is a slight increase in cellularity (37) while the average caliber of the fibers decreases (38–40), and the thinner fibers are reported to have a thicker myelin sheath (41, *cf.* reference 40). Variations were also reported in conduction velocity (40, 42). Chemical (43) and metabolic (17) data on proximal stumps are very scanty. Bodian and Dziewiatowski (17) injected radioactive phosphate into *rhesus* monkeys at various intervals after transection of the sciatic; 6 to 8 days after the operation, the proximal stumps, excluding the terminal bulb, showed an increased incorporation in all the fractions examined. Several studies of the "proximal stump" actually refer to the region of the terminal bulb, which we eliminated (44).

Histologically, in the present work, a slight interstitial edema was apparent in the proximal stumps between 14 and 32 days (Figs. 1 E, and 1 F), as far up as the sciatic could be followed (30 to 35 mm. from the site of the lesion). This histological change, which is also indicated by a small drop in the percentage dry weight, may represent an accumulation of endoneurial fluid, inhibited in its proximodistal flow by the tissue changes at the level of the

terminal bulb. Weiss has shown that if a nerve is tied, a bulb is formed proximal to the ligature with histological evidence of a "damming up" process (45).

The incorporation of phosphate into the lipides was increased at 15 days (Text-fig. 6), later it dropped. This observation agrees with findings from various sources, which indicate a transient increase in cellular activity in the proximal stump (17, 42).

Lipogenesis from acetate (specific activity) in our material (Text-fig. 6), was reduced by about one-third starting at 10 days, and for the duration of the whole period of observation (32 days). This depression cannot be explained by a "retrograde Wallerian degeneration." Since our samples did not include the terminal 8 mm., degenerating fibers (33, 46) were only an occasional finding, and certainly inadequate for explaining the considerable metabolic change; further, were the changes related to retrograde Wallerian degeneration, the drop should have been followed by a rise, as described for the distal stump. It seems more logical to ascribe the drop in lipogenesis to atrophy of the nerve fibres. This early metabolic sign of atrophy may well be a precursor of the chemical atrophy apparent 3 months later as a decreased phospholipide content (43), and of the morphologic atrophy which has also been described in advanced stages (38–40, 47).

An objection which could be raised to the results reported here on the proximal stump is that the sciatic nerve of the rat is so short that all the metabolic changes found could be related in some way to the proximity of the level of surgical trauma. If this were true, there might be some demonstrable gradient in the abnormalities found. Thus in one experiment the proximal stumps of 6 rats (10 days after low section) were subdivided into two segments of equal length, proximal and distal, which were incubated separately and compared with similar segment from normal rats. Each segment was 10 to 12 mm. long, and the highest level above the lesion was 30 to 35 mm. Respiration and lipogenesis from acetate were the same in both segments. The metabolic disturbance thus presumably extends all the way up to the origin of the axons.

Regenerating Nerves.—In contrast with the numerous histological studies of regenerating peripheral nerves (*cf.* references 48, 49) biochemical data on newly regenerated fibres are very scanty. The information available at present (8, 9) was obtained indirectly, by comparing transected nerves, undergoing Wallerian degeneration, with crushed nerves (9), in which degeneration and reinnervation were superimposed. Since previous work had provided some information on the process of normal growth (23), it seemed of interest to gather comparative data on the process of regenerative growth, uncomplicated by Wallerian degeneration. The sample of choice was therefore the filament which represents a clearly defined anatomical bridge between the stumps

of a transected nerve. It should be emphasized, however, that histogenetically this structure is more complex than a bundle of axon sprouts, as manifested by the non-committal name of "union tissue" (49). During the first few days it consists of spindle-shaped cells (Fig. 1 C) which can be seen to radiate from the *distal* as well as from the proximal stump. Whether these are Schwann cells (26), or rather "endoneurial fibroblasts" (50), is not settled. Along this cord of cells grow tangled bundles of axis cylinders (Fig. 1 D) which acquire the first myelin sheaths after 14 to 21 days (46). For this study, one set of samples was taken before the appearance of myelin sheaths stainable by osmium tetroxide (10 to 11 days), another when myelination was well under way (32 days). As shown in Text-fig. 7, the lipide content is low at first and tends to increase, as would be expected in a nerve undergoing myelination, and respiration is consistently higher than normal, as it is in young nerves compared with adult nerves. Although the specific activity of the lipides at 32 days is higher than normal, as might be expected by analogy with nerves of young animals, it was interesting to find that in the younger sample of union tissue the level of lipogenesis was quite low. This is not the result of contamination with lipides of adipose tissue, since each single filament was carefully examined under a binocular lens and freed of any adherent adipose cells. Lipogenesis, then, is lower in this sample than in normal adult nerves, despite the dense population of cells similar to Schwann cells, and despite the presence of young unmyelinated axons. These facts may be related to other lines of evidence. It is generally agreed that the Schwann cells have no tendency to build myelin unless stimulated to do so by contact with axones. Further, in the peripheral nerves of newborn rats, lipogenesis is lower at birth than it is at 5 days (23).

In summary, then, the basic aspects of normal and regenerative growth were similar. In both cases oxygen uptake was high, and prior to the "wave" of lipogenetic activity which accompanied the advent of stainable myelin sheaths, there was a preliminary period during which lipide biosynthesis was relatively low.

SUMMARY

Bilateral transection was performed on rat sciatics. At varying intervals after the operation, samples of nerve were taken both distal and proximal to the level of transection, as well as from the tissue which bridged the gap between the stumps. These samples were incubated in Warburg flasks, with glucose and a labelled lipide precursor (acetate or phosphate). The total lipides were then extracted and their radioactivity was measured. Normal rat sciatics served as controls, and the biochemical and histological findings were correlated.

In the distal portion undergoing Wallerian degeneration, the lipide content began to fall before any removal of myelin could be detected histologically. It is suggested that there is a period of "non-cellular removal" prior to the

physical breakdown of the myelin. Changes in respiration and in lipogenesis from acetate followed a triphasic course, and agreed with the histological findings in that after a period of predominantly passive changes (approximately 1 to 3 days) there follows a period of cellular reaction (4 to 50 days) and a period of atrophy (from 50 days onward). The incorporation of phosphate into the lipides was increased at all stages examined, even as early as 22 hours after section. This increased P^{32} incorporation could not be reproduced in nerves allowed to degenerate *in vitro*. It is suggested that the hypertrophying Schwann cells synthesize some lipide moieties at a considerably faster rate than others.

Proximal to the level of transection, lipogenesis from acetate was depressed, for as long as 32 days postoperatively. It appears, therefore, that the maintenance of the myelin sheath is impaired also above the level of transection.

In the "union tissue" which developed between the stumps, prior to the appearance of histologically visible myelin, lipogenesis was low; later it rose above levels for normal nerve. This pattern of lipogenesis in regenerating nerve is similar to that found in growing nerves.

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BIBLIOGRAPHY

1. Krücke, W., Erkrankungen des peripheren Nervensystems, *in* Handbuch der speziellen pathologischen Anatomie und Histologie, (O. Lubarsch, F. Henke, and R. Rössle, editors), Berlin, Springer-Verlag, 1955, **13**, 1.
2. Spielmeyer, W., Degeneration und Regeneration am peripheren Nerven, *in* Handbuch der normalen und pathologischen Physiologie, (A. Bethe *et al.*, editors), Berlin, Springer, 1929, **9**, 285.
3. Causey, G., and Hoffman, H., Axon sprouting in partially deneurotized nerves, *Brain*, 1955, **78**, 661.
4. Holmgren, H., and Rexed, B., Metachromatic staining of the Schwann cells in nerve regeneration, *Acta Anat.*, 1946–47, **2**, 287.
5. Noback, C. R., and Montagna, W., Histochemical studies of the myelin sheath and its fragmentation products during Wallerian (secondary) degeneration. I. Lipids, *J. Comp. Neurol.*, 1952, **97**, 211.
6. Noback, C. R., and Reilly, J. A., Myelin sheath during degeneration and regeneration. II. Histochemistry, *J. Comp. Neurol.*, 1956, **105**, 333.
7. Samorajski, T., Changes in phosphatase activity following transection of the sciatic nerve, *J. Histochem. and Cytochem.*, 1957, **5**, 15.
8. Johnson, A. C., McNabb, A. R., and Rossiter, R. J., Chemistry of Wallerian degeneration. A review of recent studies, *Arch. Neurol. and Psychiat.*, 1950, **64**, 105.
9. Burt, N. S., and McNabb, A. R., Chemical studies of peripheral nerve during Wallerian degeneration. 2. Lipids after nerve crush (axonotmesis), *Biochem. J.*, 1950, **47**, 318.

10. Hollinger, D. M., and Rossiter, R. J., Chemical studies of peripheral nerve during Wallerian degeneration. 5. β-Glucuronidase, *Biochem. J.*, 1952, **52,** 659.
11. Mannell, W. A., Wallerian degeneration in the rat: a chemical study, *Canad. J. Med. Sc.*, 1952, **30,** 173.
12. Mannell, W. A., and Rossiter, R. J., Wallerian degeneration in the rat: the effect of age on the concentration of nucleic acid and phospholipid in intact and sectioned nerves, *J. Exp. Biol.*, 1954, **31,** 198.
13. May, R. M., and Thillard, M. J., Etudes microchimiques sur le système nerveux. IX. Chromatographie des acides aminés du nerf sain et dégénéré, *Bull. Soc. chim. biol.*, 1951, **33,** 253.
14. Brante, G., Studies on lipids in the nervous system with special reference to quantitative chemical determination and topical distribution, *Acta Physiol. Scand.*, 1949, **18,** suppl. 63.
15. Cavanagh, J. B., and Webster, G. R., On the changes in ali-esterase and pseudocholinesterase activity of chicken sciatic nerve during Wallerian degeneration and their correlation with cellular proliferation, *Quart. J. Exp. Physiol.*, 1955, **40,** 12.
16. Samuels, A. J., Boyarsky, L. L., Gerard, R. W., Libet, B., and Brust, M., Distribution, exchange and migration of phosphate compounds in the nervous system, *Am. J. Physiol.*, 1951, **164,** 1.
17. Bodian, D., and Dziewiatkowski, D., The disposition of radioactive phosphorus in normal, as compared with regenerating and degenerating nervous tissue, *J. Cell. and Comp. Physiol.*, 1950, **35,** 155.
18. Greengard, P., Brink, F. Jr., and Colowick, S. P., Some relationships between action potential, oxygen consumption and coenzyme content in degenerating peripheral axons, *J. Cell. and Comp. Physiol.*, 1954, **44,** 395.
19. Magee, W. L., and Rossiter, R. J., Chemical studies of peripheral nerve during Wallerian degeneration. 6. Incorporation of radioactive phosphate into pentosenucleic acid and phospholipin *in vitro*, *Biochem. J.*, 1954, **58,** 243.
20. Miani, N., and Marini, I., Aumentata capacità di sintesi fosfolipidica nel moncone periferico del nervo reciso (ricerche *in vitro* con il P^{32}), *Boll. soc. ital. biol. sper.*, 1955, **31,** 29.
21. Causey, G., and Stratmann, C. J., The nucleic acid content of peripheral nerve in degeneration and activity, *Proc. Roy. Soc., London, Series B*, 1956, **144,** 520.
22. Gutmann, E., and Holubář, J., The degeneration of peripheral nerve fibers, *J. Neurol., Neurosurg., and Psychiat.*, 1950, **13,** 89.
23. Majno, G., and Karnovsky, M. L., A biochemical and morphologic study of myelination and demyelination. I. Lipide biosynthesis *in vitro* by normal nervous tissue, *J. Exp. Med.*, 1958, **107,** 475.
24. Adams, T. W., Thomas, R. W., and Davenport, H. A., Staining sections of peripheral nerves for axis cylinders and for myelin sheaths, *Stain Technol.*, 1948, **23,** 191.
25. Peers, J. H., A modification of Mallory's phosphotungstic acid-hematoxylin stain for formaldehyde-fixed tissues, *Arch. Path.*, 1941, **32,** 446.

26. Masson, P., Experimental and spontaneous Schwannomas (peripheral gliomas), *Am. J. Path.*, 1932, **8,** 367.
27. Thomas, G. A., Quantitative histology of Wallerian degeneration. II. Nuclear population in two nerves of different fibre spectrum, *J. Anat.*, 1948, **82,** 135.
28. Nittono, K., On bilateral effects from the unilateral section of branches of the Nervus Trigeminus in the albino rat, *J. Comp. Neurol.*, 1923, **35,** 133.
29. Tamaki, K., Further studies on the effect of the section of one peroneal nerve of the albino rat on the intact nerve of the opposite side, *J. Comp. Neurol.*, 1936, **64,** 437.
30. Setterfield, H. E., and Sutton, T. S., The use of polarized light in the study of myelin degeneration. I. The appearance and progress of degeneration after transection of the sciatic nerve of the white rat, *Anat. Rec.*, 1935, **61,** 397.
31. Abercrombie, M., and Johnson, M. L., Quantitative histology of Wallerian degeneration. I. Nuclear population in rabbit sciatic nerves, *J. Anat.*, 1946, **80,** 37.
32. Abercrombie, M., Estimation of nuclear population from microtome sections, *Anat. Rec.*, 1946, **94,** 239.
33. Young, J. Z., The functional repair of nervous tissue, *Physiol. Rev.*, 1942, **22,** 318.
34. Abercrombie, M., and Johnson, M. L., The outwandering of cells in tissue cultures of nerves undergoing Wallerian degeneration, *J. Exp. Biol.*, 1942, **19,** 266.
35. Gerard, R. W., Nerve metabolism, *Physiol. Rev.*, 1932, **12,** 469.
36. Johnson, A. C., McNabb, A. R., and Rossiter, R. J., Lipids of the nervous system during *in vitro* degeneration, *Canad. J. Research*, 1949, **27,** 63.
37. Logan, J. E., Rossiter, R. J., and Barr, M. L., Cellular proliferation in the proximal segment of a sectioned or crushed peripheral nerve, *J. Anat.*, 1953, **87,** 419.
38. Gutmann, E., and Sanders, F. K., Recovery of fibre numbers and diameters in the regeneration of peripheral nerves, *J. Physiol.*, 1943, **101,** 489.
39. Weiss, P., Edds, M. V. Jr., and Cavanaugh, M., The effect of terminal connections on the caliber of nerve fibers, *Anat. Rec.*, 1945, **92,** 215.
40. Gutmann, E., and Holubář, J., Atrophy of nerve fibres in the central stump following nerve section and the possibilities of its prevention, *Arch. Internaz. Studi Neurol.*, 1951, **1,** 1.
41. Sanders, F. K., The thickness of the myelin sheaths of normal and regenerating peripheral nerve fibres, *Proc. Roy. Soc., London, Series B*, 1948, **135,** 323.
42. Sanders, F. K., and Whitteridge, S., Conduction velocity and myelin thickness in regenerating nerve fibres, *J. Physiol.*, 1946, **105,** 152.
43. Logan, J. E., Protein-bound phosphorus compounds in the proximal stump of peripheral nerve after nerve section or after nerve crush, *Canad. J. Med. Sc.*, 1952, **30,** 457.
44. Greenman, M. J., Studies on the regeneration of the peroneal nerve of the albino rat: number and sectional areas of fibers: area relation of axis to sheath, *J. Comp. Neurol.*, 1913, **23,** 479.
45. Weiss, P., Endoneurial edema in constricted nerve, *Anat. Rec.*, 1943, **86,** 491.
46. Rexed, B., and Swensson, A., Über die Regeneration der Markscheide bei peri-

pheren Nerven nach Kontinuitätstrennungen, *Z. mikroskop.-anat. Forsch.*, 1940–41, 359.

47. Ranson, S. W., Retrograde degeneration in the spinal nerves, *J. Comp. Neurol.*, 1906, **16,** 265.
48. Vizoso, A. D., and Young, J. Z., Internode length and fibre diameter in developing and regenerating nerves, *J. Anat.*, 1948, **82,** 110.
49. Weiss, P., The technology of nerve regeneration: a review. Sutureless tubulation and related methods of nerve repair, *J. Neurosurg.*, 1944, **1,** 400.
50. Denny-Brown, D., Importance of neural fibroblasts in the regeneration of nerve, *Arch. Neurol. and Psychiat.*, 1946, **55,** 171.

EXPLANATION OF PLATES

PLATE 16

FIG. 1. A. Rat sciatic, 11 days after mid-thigh transection. Proximal end to the left. The gap has been bridged by semitransparent "union tissue" (49), which also forms a small cap over the distal stump (peripheral Schwannoma (26)). The dotted line indicates the extent of the specimen excised for incubation as a sample of "nerve sprout". Biochemical data: see Text-fig. 7. Scale on right in millimeters.

B. Specimen similar to A; histological aspect after fixation in 1 per cent osmic acid. Proximal end to the left. Scale: 500 micra.

C. Histological aspect of "union tissue" from an 11 day sample similar to A; hematoxylin and eosin. Scale: 100 micra.

D. Same specimen as C, stained (24) for axis cylinders. Same enlargement as C.

E. Normal rat sciatic, control for F, simultaneously fixed, embedded, cut, and stained with hematoxylin and eosin. Scale: 100 micra, same as F.

F. Proximal stump of rat sciatic, 19 days after transection, approximately 25 mm. above the level of the operation. Slight dissociation of the fibres by increased amount of interstitial fluid. This change could have been considered an artifact unless compared with a control such as E.

G. Rat sciatic, after 10 hours of incubation at 37°C. in Krebs-Ringer-phosphate medium with glucose. Fixation in 1 per cent osmic acid. A non-incubated control sample is shown at the left at the same enlargement. (Biochemical data: see Text-fig. 5).

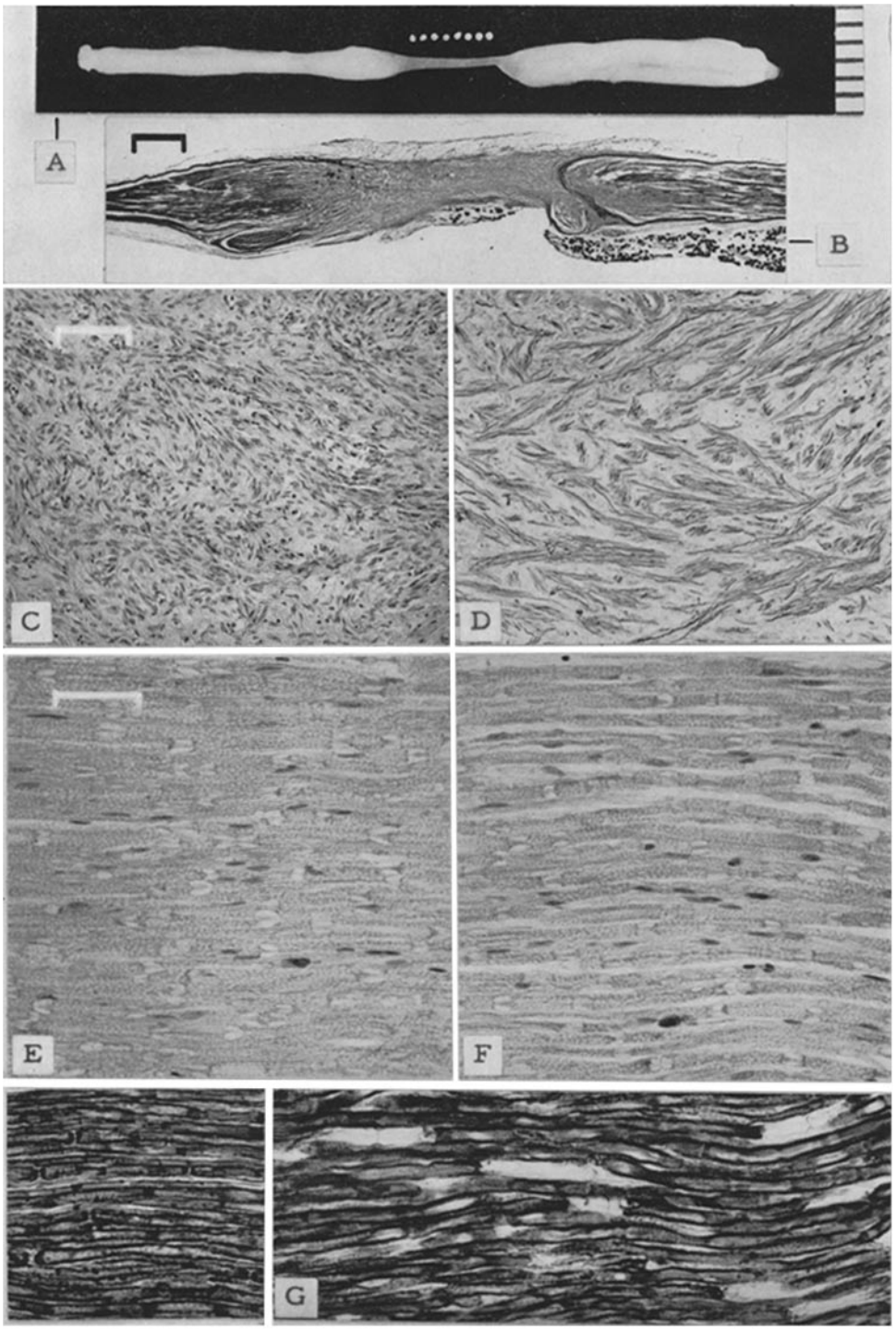

FIG. 1

(Majno and Karnovsky: Lipogenesis in transected nerves)

PLATE 17

FIG. 2. Wallerian degeneration of the rat sciatic. Histological aspect of the progressive changes in the myelin sheaths. Numbers indicate days after transection. Fixed in 1 per cent osmic acid, counterstained with hematoxylin and eosin. Scale: 100 micra.

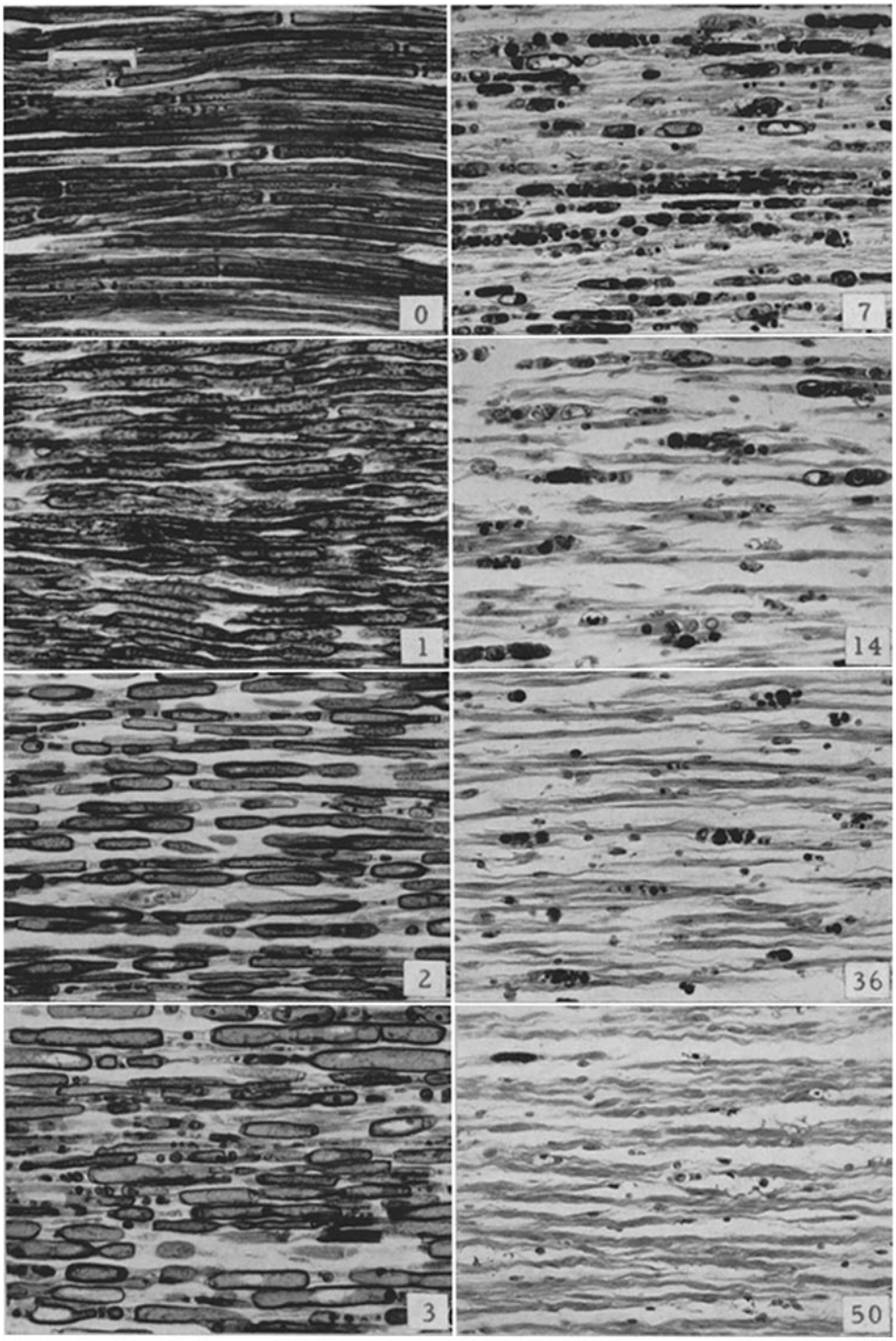

FIG. 2
(Majno and Karnovsky: Lipogenesis in transected nerves)

PLATE 18

FIG. 3. Wallerian degeneration of the rat sciatic. Histological aspect of formol-fixed material stained with hematoxylin and eosin. Numbers indicate days after transection. Scale: 100 micra.

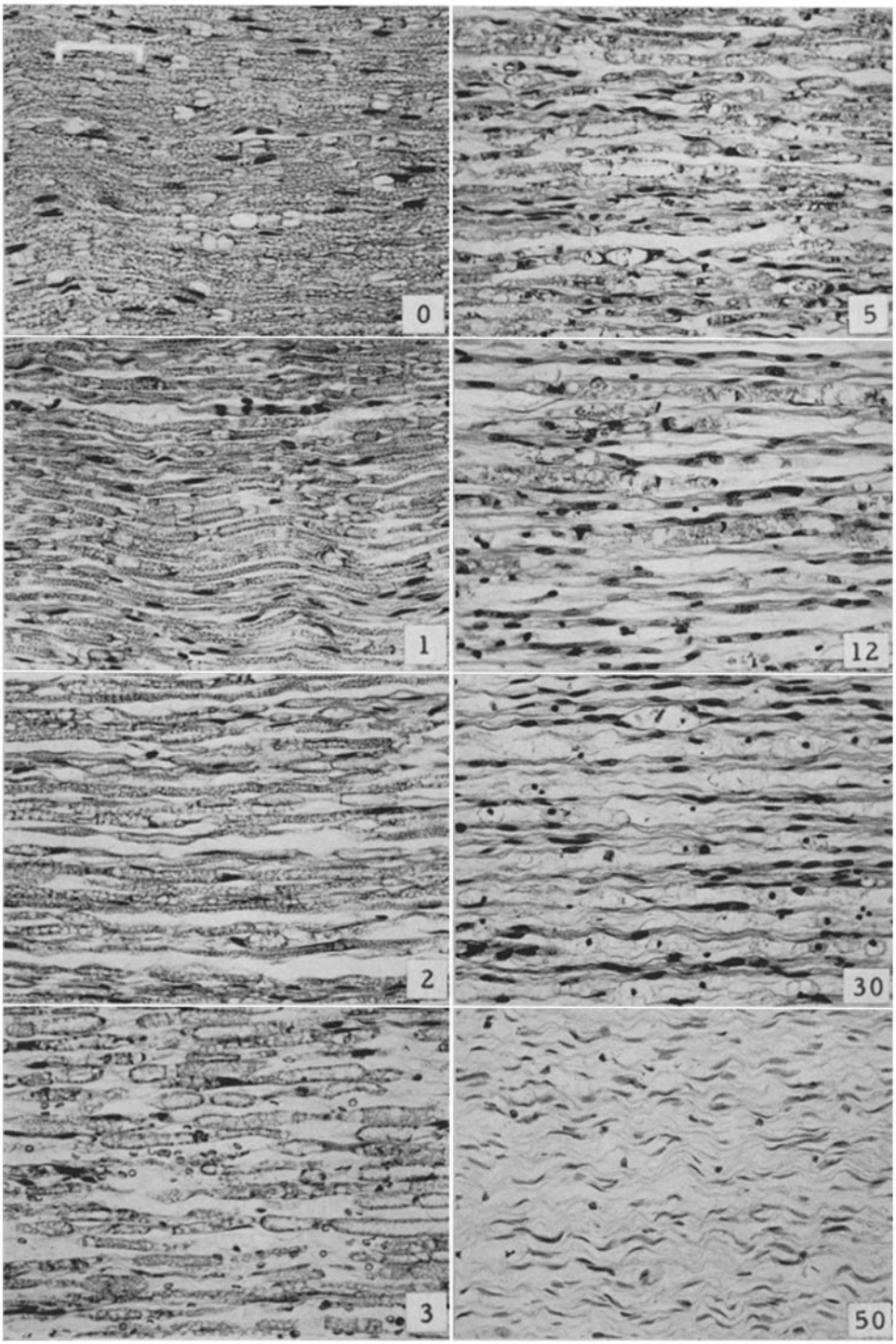

FIG. 3

(Majno and Karnovsky: Lipogenesis in transected nerves)