Pattern of Myelin Breakdown during Sciatic Nerve Wallerian Degeneration: Reversal of the Order of Assembly

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ABSTRACT Myelin sheaths of rapidly growing rats were sequentially labeled with the ^{3}H and ^{14}C isotopes of leucine as precursors of protein synthesis. The two injections were separated by time intervals ranging from 2 to 12 d. Wallerian degeneration was initiated by sciatic nerve neurotomy at 2 or 10 d after the second injection of radioactivity. After 5 d of degeneration, myelin was purified and the ratio of isotopes was determined in the delipidated protein. Regardless of the order in which the two isotopes were administered, the relative recovery of radioactivity resultant from the second injection was greatly reduced in degenerating nerves compared with sham-operated controls. Radioactivity incorporated from the first injection was also reduced, but to a lesser extent. Consequently, the isotope ratio corresponding to the first/second injection was greater in degenerating nerves than in controls, and the ratio increased in proportion to the time interval separating the two injections. The magnitude of the effect of degeneration was only slightly greater when degeneration was initiated 2 d after the second injection than when initiated 10 d after the last injection. Consequently, myelin disintegration rather than diminished incorporation of radioactivity accounts for the losses of radioactivity. Furthermore, the pattern of myelin degeneration preferentially involves the last myelin to be formed.

Peripheral nerve lesions resulting from nerve transection, nerve crush, or severe compression lead to a characteristic and reproducible degeneration of the nerve commonly referred to as Wallerian degeneration (for reviews, see references 21 and 31). These observations include primary effects, such as the accumulation of organelles in paranodal axoplasm (10, 22, 34, 45) and loss of axoplasmic microtubules and neurofilaments (10, 13, 20, 32, 33), and secondary effects involving myelin and Schwann cells. In the latter category, effects appear as early as 12 h, and observations include an increase in the number of Schmidt-Lantermann clefts, a loosening of the myelin lamellae (24, 34), and retraction of myelin loops at the nodes of Ranvier (4, 28). After 3 d of axon degeneration, segmentation of myelin appears paranodally (26) and myelin debris appears in Schwann cells (26, 28, 32). During the 1st wk of degeneration, Schwann cells show increased numbers of cell organelles (24, 26, 32) and an increased amount of cytoplasm (4, 28, 44). Dissolution of the axon and breakdown of myelin are accompanied by a proliferation of Schwann cells, starting at ~4 d in the rat and rabbit (1, 2, 6, 11, 28, 32).

The rapid loss of peripheral nerve myelin as a result of Wallerian degeneration is well known (2, 5, 25, 32, 43), and various studies have demonstrated increased proteinase and lysosome activity in peripheral nerves undergoing Wallerian degeneration (3, 17–19, 29, 37). Because degenerative axonal changes appear to precede myelin breakdown in Wallerian degeneration, Wolman (42) proposed that the increased lysosomal activity in axons was the source of enzymes leading to demyelination. For such cases, he proposed the term "centrifugal demyelination" to denote a process whereby the direction of myelin degeneration is from the axon out through the myelin layers. For neuropathies in which myelin breakdown precedes axonal pathology, Wolman proposed the term centripetal demyelination to denote probable degeneration from the outside inward.

Various morphological and biochemical studies of Schwann

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cells indicate that new (most recently synthesized) myelin layers are laid down upon existing layers (12, 14, 15, 30, 36) to form a growing spiral. Thus myelin growth is highly ordered in the developing rat and involves both the addition of new myelin spirals and an elongation of the internode width as well. Consequently, we alternately tagged myelin growth with ^{3}H and ^{14}C isotopes of leucine to test hypotheses that myelin breakdown during Wallerian degeneration proceeds by pattern.

MATERIALS AND METHODS

Both L-[4,5-^{3}H]leucine, 50 Ci/mmole, and L-[1-^{14}C]leucine, 290 mCi/mmol, were purchased from New England Nuclear (Boston, Mass.). Isotope solutions were evaporated under a stream of nitrogen, redissolved in water, redried through several cycles to remove traces of hydrochloric acid, and reconstituted in phosphate (0.01 M) -buffered physiological saline (pH 7.2). Isotope administration was by intraperitoneal injection. Doses in all cases were 1.0 μCi of [^{14}C]leucine/g body weight, and 3.0 μCi of [^{3}H]leucine/g body weight.

Pregnant rats were obtained from Charles River Co. (Boston, Mass.). The offspring were used to radioactively label myelin synthesis in vivo, starting at 12 d of postnatal age, as growth and sciatic nerve myelination are rapid at this time (40). The ^{3}H and ^{14}C radioactive isotopes of leucine were administered one at a time and separated by intervals ranging from 2 to 12 d, presumably tagging the growing myelin spiral with alternate layers of ^{3}H and ^{14}C radioactivity. The order of myelin loss was determined by measuring ratios of the two isotopes in myelin purified from degenerating nerves. In every experiment (e.g., Fig. 1), controls included the use of animals injected in the reverse order as well as sham-operated rats.

At 2 or 10 d after the administration of second isotope, rats were anesthetized with pentobarbital, and surgical neurotomy was performed on the left sciatic nerve just distal to the sciatic notch of the pelvis. Sham surgery was performed on the right side. An additional control included rats having sham surgery on both sides. Sham surgery consisted of exposing the sciatic nerve as if it were to be cut. Surgical incisions were closed with wound clips. After 5 d of Wallerian degeneration, which we have shown results in a 30–50% decrease in myelin recovery (5), rats were killed by decapitation and myelin was purified from the distal 1.7-cm segments of individual sciatic nerves. At the ages used, a 1.7-cm segment constitutes essentially the full weight and length of the nerve in the lower extremity, and thus various data are reported as disintegrations per minute per nerve myelin. Myelin was purified on discontinuous sucrose gradients by ultracentrifugation, osmotically shocked, and repurified as we have previously described (38–40).

Freeze-dried myelin was delipidated by three cycles of extraction with ether: ethanol (3:2, vol/vol). Polyacrylamide disc gels of myelin protein were prepared as described previously (38, 40). Freeze-dried myelin protein was digested in Protosol (New England Nuclear) and liquid scintillation counting was performed in toluene containing Omnifluor (New England Nuclear) as described previously (39). A Searle Mark III counter (Searle Radiographics Inc., Des Plaines, Ill.), or a Beckman 9000 (Beckman Instruments, Inc., Fullerton, Calif.), were employed with essentially identical results. Tritium spillover into the ^{14}C channel was 1–2%. ^{14}C spillover into the tritium channel was 13–18%. Counts per minute were detected at efficiencies of ~37% for tritium and 74% for ^{14}C; counts per minute were automatically computed as disintegrations per minute. All averaged data show the standard error of the mean. All isotope ratio values are ratios of disintegrations per minute. Isotope ratios are expressed as the ratio of the first administered isotope to the second.

RESULTS

In control studies, the relative incorporation of [^{3}H]leucine and ^{14}C radioactivity into sciatic nerve myelin protein of 12-d old rats was determined by injecting 19 rats intraperitoneally with a 3:1 mixture of [^{3}H]leucine and [^{14}C]leucine. Rats were sacrificed at times ranging from 2 h to 14 d later, and the ratio of $^{14}C/^{3}H$ radioactivity was determined for delipidated myelin protein. The relative incorporation of ^{14}C radioactivity into sciatic nerve delipidated myelin protein exceeded ^{3}H by 27%, resulting in a constant $^{14}C/^{3}H$ ratio of 0.422 ± 0.006 over a period of 2 wk after the injection. However, the accumulation of total radioactivity in myelin increased for up to 10 d after the injection (Fig. 2). The appearance of a decline in radioactivity after 2–3 wk, presumably from turnover, has been substantiated elsewhere.[1]

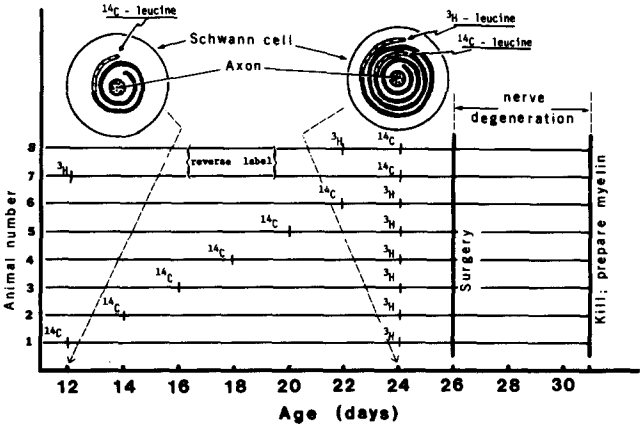

FIGURE 1 The experimental regimen. Rats 1–6 were injected with [1-^{14}C]leucine at an age that varied between the interval of 12–22 d. Each rat was injected with [4,5-^{3}H]leucine on day 24. Rats numbered 7 and 8 are reverse isotope controls. As a result of this labeling regimen, the growing myelin sheath will include an inner zone labeled with the first isotope and outer lamella labeled with the second of the two isotopes. At the age of 26 d (2 d after the 2nd isotope injection), surgery was performed on each rat. Left sciatic nerves were cut to initiate Wallerian degeneration; right sciatic nerves were sham operated. At 5 d after surgery, myelin was prepared from each sciatic nerve. See text for additional details of the experimental design.

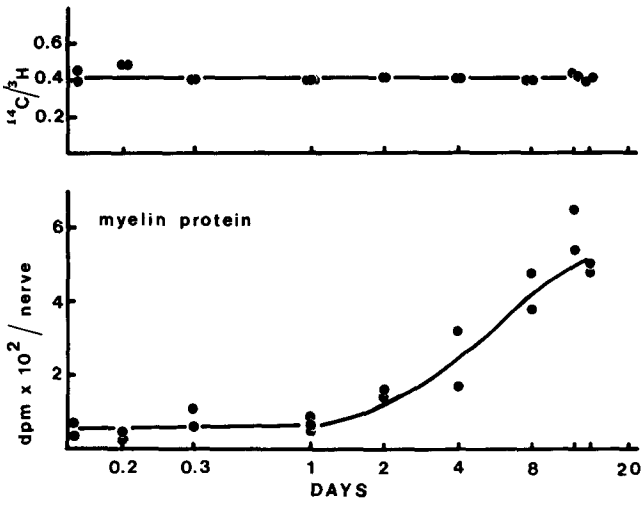

FIGURE 2 The top panel shows the $^{14}C/^{3}H$ ratio of radioactivity in delipidated myelin protein of rat sciatic nerve after the injection of 1:3 mixture of [^{14}C]leucine and [^{3}H]leucine, intraperitoneally, at 12 d of age. The bottom panel shows the increase in radioactivity (using the [^{14}C] data) for up to 2 wk after these injections.

Because the studies of patterned degeneration (to follow) involve sequential administrations of the two isotopes over several days and subsequent surgery, we also examined isotope ratios in control rats injected at different times. Three rats received [^{14}C]leucine at 18 d of age, followed by [^{3}H]leucine at 24 d (in a 1:3 ratio, by body weight). 2 d later, sham surgery was performed on the sciatic nerves of each rat, and rats were killed at 3, 5, and 7 d after the second injection. The $^{14}C/^{3}H$ ratio of sciatic nerve myelin protein in each case was: 0.59, 0.46, and 0.44, respectively. These ratio data are consistent with

[1] Unpublished data from the laboratory of Dr. Wiggins.

the time-course for incorporation (Fig. 2) and show that incorporation of the second injection was almost complete by 7 d, as the ratio at this time is within 5% of the ratio (0.42) obtained by injection of the two isotopes simultaneously (Fig. 2). Furthermore, sham surgery did not alter the incorporation of radioactivity into sciatic nerve myelin protein.

The effect of Wallerian degeneration on the recovery of myelin radioactivity was examined for the case where surgical neurotomy (to initiate degeneration) occurs at 2 d after the second injection (during the period of rapid incorporation of radioactivity into compact myelin) and at 10 d after the second injection (when incorporation of radioactivity should be essentially complete, see Fig. 2). In the first experiment (Fig. 1), the typical order of administration was [^{14}C]leucine followed by [^{3}H]leucine; in the second experiment, the order was [^{3}H]leucine followed by [^{14}C]leucine. As an additional control for isotope effects, the order was also reversed within each set. Equivalent results were obtained in all cases.

In the first experiment, rats were killed for myelin preparation at 17 d after the ^{14}C injection and 7 d after the final ^{3}H injection. In the case of sham-operated nerves, ^{14}C averaged 360 ± 25 dpm/nerve myelin and ^{3}H averaged 850 ± 79 dpm/nerve myelin. The ratio of radioactivity in sham-operated nerves increased slightly from 0.40 to 0.51 (Fig. 3) as the interval between injections increased (see Fig. 1), indicating slightly more complete incorporation of the radioactivity from the first injection relative to the second at the time of sacrifice. The ratio values observed here for the sham-operated nerves are essentially the same as in the preliminary control studies above.

However, for nerves undergoing 5 d of Wallerian degeneration, results are quite different from those observed in the corresponding sham-operated nerves. The recovery of myelin protein labeled by both the first and second injections of radioactive leucine was greatly decreased compared to sham-operated controls, but more importantly, the loss of radioactivity resulting from the second injection (occurring 2 d before surgery) was greater than for myelin labeled by the first injection. Consequently, as the interval separating the two injections increased from 2 to 12 d, the ratio of the first/second injections increased by twofold (Fig. 3). The recovery of radioactivity representing myelin protein synthesis at 14, 10, 6, and 4 d before neurotomy (^{14}C data) was 170, 161, 125, and 89 dpm/nerve. Compared to the incorporation of radioactivity and myelin recovery from the paired sham-operated nerves, these ^{14}C values represent recoveries ranging from 47% for myelin protein synthesized 14 d before neurotomy, to 25% for myelin protein synthesized 4 d before neurotomy. The recovery of ^{3}H-labeled myelin protein, representing synthesis 2 d before neurotomy, was 173 ± 9 dpm/nerve, or 22 ± 1% of the recovery in paired sham-operated nerves. A similar effect was obtained with the reverse isotope controls (Fig. 3). In this experiment, surgery to initiate degeneration occurred 2 d after administration of the second isotope, and consequently it is not clear whether degeneration caused a preferential loss of myelin labeled by the second injection, or reduced incorporation of radioactivity from the second injection. Therefore, a second experiment was designed in which surgery followed the final injection by a period sufficient to afford complete incorporation (see Fig. 2).

In the second experiment, [^{3}H]leucine was administered on day 12, [^{14}C]leucine on day 24, surgery was performed on day 34, and rats were killed on day 39. The order of isotope administration is the same as for the reverse isotope controls (Figs. 1 and 3). The resultant ^{3}H/^{14}C ratio for sham-operated nerves was 2.5 ± 0.2, essentially the same as in Fig. 3. The ^{3}H/^{14}C ratio in paired degenerating nerves was 3.9 ± 0.3, an increase of 1.6-fold over the ratio in paired sham-operated nerves, again as a result of preferential loss of the second label. The radioactivity observed in myelin of sham-operated nerves was 304 ± 38 dpm/nerve for ^{3}H and 123 ± 23 dpm/nerve for ^{14}C. In the paired degenerating nerves, recovery of radioactivity resulting from the first injection, ^{3}H, was 204 ± 52 dpm/nerve, or 71 ± 20%; recovery of radioactivity resulting from the second injection, ^{14}C, was 56 ± 13 dpm/nerve, or 52 ± 12%. Thus, preferential loss of the most recently formed myelin, not a reduction in incorporation, accounts for the altered ratios in myelin protein of degenerating nerves.

The protein composition of myelin recovered during Wallerian degeneration was examined by polyacrylamide gel electrophoresis. Adult rats were used because of the very low recoveries of myelin from degenerating nerves (5), compounded by the small size of young rats (40). Results (Fig. 4), show no marked alteration of the myelin protein composition after 1, 3, or 5 d of Wallerian degeneration. Additional analyses by Bell (5) confirm this conclusion.

DISCUSSION

The present study indicates that the peak of incorporation of leucine radioactivity into sciatic nerve myelin of young rats occurs at 10–12 d after intraperitoneal administration of leucine, and the relative incorporation of ^{14}C radioactivity into normal sciatic nerve myelin protein slightly exceeded (by 27%)

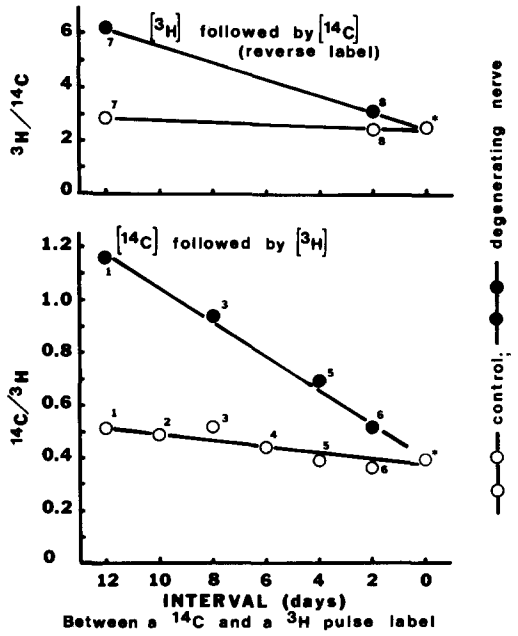

FIGURE 3 The ratio of radioactivities, recovered in myelin, that result from the first and second isotope injections. Animal numbers correspond to the rats described in Fig. 1. For rats 1–6, the ^{14}C/^{3}H ratio is the first (injected) isotope divided by the second; for rats 7 and 8, the ^{3}H/^{14}C ratio is the first divided by the second. The asterisk shows the ratio for normal myelin after the injection of a (3:1) mixture of [^{3}H]leucine and [^{14}C]leucine. The isotope ratio in sham-operated control nerves is similar. For degenerating nerves, the ratio represented by the radioactivity of the first injection/second injection increases as the time between the two injections increases.

the relative incorporation of ^{3}H radioactivity. A similar excess of ^{14}C radioactivity has been previously observed and discussed (39). Because leucine, administered as a protein precursor, is in an active metabolic pool (23) having a half-life measured in minutes, we may conclude that myelin protein synthesized at the time of the injection (and thus radioactively tagged) requires several days to complete its eventual incorporation into compact myelin lamellae. The observations and conclusions appear consistent with autoradiographic studies of glycoprotein incorporation into peripheral nerve myelin (14).

The initiation of Wallerian degeneration in sciatic nerves previously tagged by injections of radioactive leucine reveals two major effects on myelin. First, by comparing equivalent results in sham-operated nerves, the amount of radioactively labeled protein is greatly reduced in degenerating nerves. However, the amount of the reduction depends on the time interval between administration of the radioactive precursor and the onset of degeneration. 5 d of Wallerian degeneration was observed to reduce radioactivity by 80% when the isotope injection preceded surgery by only 2 d and by only 29% when the injection preceded surgery by 22 d. Thus, the total myelin loss was extensive; however, loss of the most recently synthesized myelin was greatest, as evidenced by the increasing myelin ratios representing radioactivity of the first/second injections.

By comparing results of the two degeneration experiments, it is possible to estimate the relative effects of Wallerian degeneration on (*a*) blocking incorporation of radioactively labeled protein into compact myelin and (*b*) actual degradation of radioactively labeled compact myelin. Isolation of these events is permitted by the requirement of a time period of ~10 d for full incorporation of leucine radioactivity into compact myelin. Where surgical neurotomy followed the second injec-

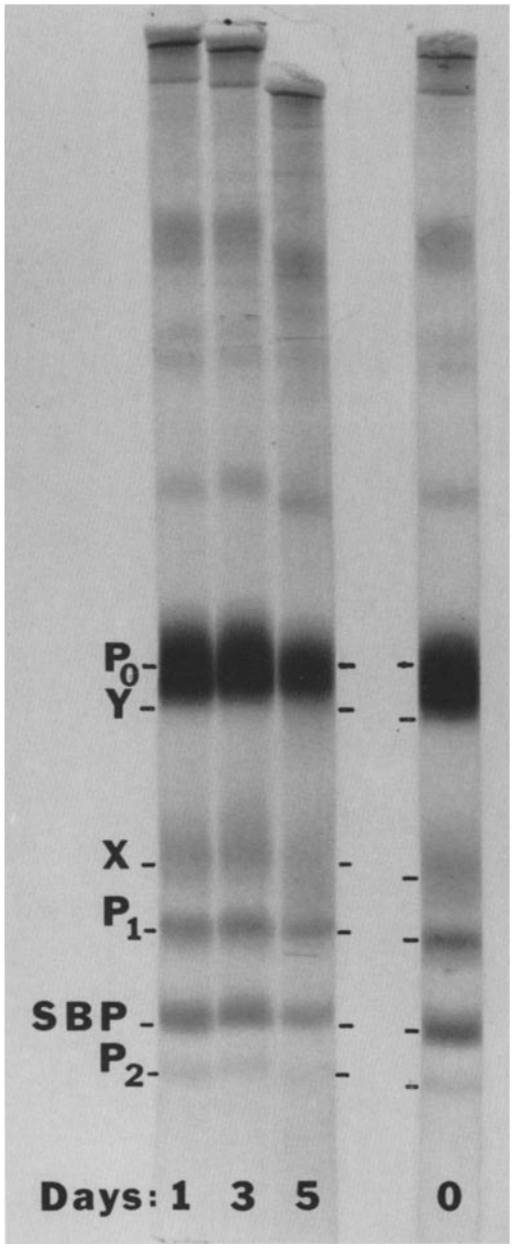

FIGURE 4 Polyacrylamide disc gels showing the protein composition of sciatic nerve myelin at 0 (control), 1, 3, and 5 d after surgical neurotomy to initiate Wallerian degeneration. Each gel is loaded with 50 μg of protein and stained with Fast Green. (See Discussion for a description of the band identification.)

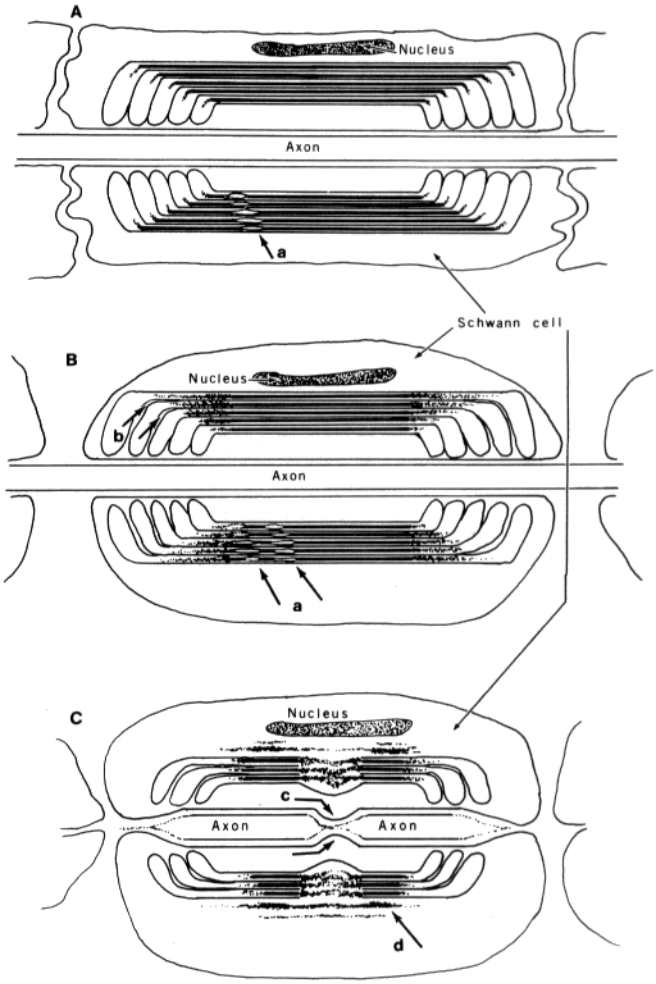

FIGURE 5 A model of sciatic nerve myelin degeneration during Wallerian degeneration. *A* shows a normal Schwann cell, several layers of compacted myelin, and Schmidt-Lantermann clefts (*a*); *B* shows early stages of myelin catabolism, including an increase in Schmidt-Lantermann clefts (*a*), loosening at the interperiod line (*b*), and myelin disintegration; (*C*) progressive degeneration, showing axon segmentation (*c*) and extensive myelin degeneration affecting the outer lamellae (*d*) and all layers in association with segmentation.

tion by only 2 d, and the first by 14 d, the ratio representative of the first/second injections increased by 2-fold over sham controls after 5 d of degeneration. Where neurotomy followed the second injection by 10 d, and the first by 22, the ratio in degenerating nerves increased by 1.5-fold over sham controls. Thus, comparable effects were obtained in each case, and from similarity of the two results we can conclude that disruption of processes leading to myelin assembly cannot account for the altered isotope ratios observed in myelin of degenerating nerves. Instead, the loss of radioactivity is the result of actual myelin disintegration in a pattern that preferentially involves the most recently formed fractions, which includes the outermost lamellae (12, 14, 30), and the lateral loops. Early myelin loss probably occurs in the Schmidt-Lantermann clefts as well. On the basis of these observations, the hypothesis of centrifugal demyelination of Wolman (42) is refuted. Experimental results indicate essentially the reverse, and although the degeneration process preferentially involves the last assembled lamellae, extensive myelin loss occurs in layers representative of synthesis at all ages.

In Fig. 5, we present a model of myelin breakdown during Wallerian degeneration of the sciatic nerve. This interpretation includes features of the histological literature as well as our biochemical conclusions.

Fig. 5 *A* represents an axon segment showing a Schwann cell with several myelin layers. In Fig. 5 *B*, which represents an early stage of Wallerian degeneration: (*a*) myelin lamellae are loosened along the interperiod line (28) and Schmidt-Lantermann clefts are dilated and increased in number (4, 7, 24, 35, 41); (*b*) Schwann cell cytoplasm has retracted from the nodes, leaving small areas of bare axon (9), however, Ballin and Thomas (4) observed no bare axons in their detailed study of the nodal region; and (*c*) myelin disintegration has begun, especially at the paranodal lamellae (4, 28, 41), the outermost (most recently formed) layers, and in the Schmidt-Lantermann clefts.

In Fig. 5 *C*, (*a*) progressive disintegration of the myelin outer lamellae continues; (*b*) axons begin to segment, resulting in a fragmentation and collapse of the myelin sheath parallel to areas of shrinkage of the axon (5, 24, 27, 28). Axonal segmentation and collapse of the adjacent myelin lamellae most likely accounts for much of the myelin loss at inner, as well as outer, layers.

With regard to electrophoretic characterization of myelin recovered during Wallerian degeneration, Fig. 4 shows the persistence of an essentially normal protein composition. Because myelin recovery is operationally defined, one would expect little alteration of composition in the "recovered" fraction. Myelin in various stages of degeneration may contribute to additional subfractions, although no consensus has emerged with regard to the metabolic significance of myelin subfractions. The nomenclature for myelin proteins (Fig. 4) is consistent with our current usage (40) and reflects the recent (16) reevaluation of the small basic protein and P_2. Identification of the Y band is included, although recent evidence indicates that Y is a function of the state of sample reduction (8).

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