

# EVIDENCE THAT CONTACT WITH CONNECTIVE TISSUE MATRIX IS REQUIRED FOR NORMAL INTERACTION BETWEEN SCHWANN CELLS AND NERVE FIBERS

RICHARD P. BUNGE and MARY BARTLETT BUNGE. From the Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, Missouri 63110

## ABSTRACT

Explants of fetal rat sensory ganglia, cultured under conditions allowing axon and Schwann cell outgrowth in the absence of fibroblasts, occasionally develop nerve fascicles that are partially suspended in culture medium above the collagen substrate. In these suspended regions, fascicles are abnormal in that Schwann cells are decreased in number, are confined to occasional clusters along the fascicle, provide ensheathment for only a few axons at the fascicle periphery, and do not form myelin. When these fascicles are presented with a substrate of reconstituted rat-tail collagen, Schwann cell numbers increase, ensheathment of small nerve fibers occurs normally, and larger axons are myelinated. We conclude that, for normal development, Schwann cells require contact with extracellular matrix as well as axons. The Schwann cell abnormalities in suspended fascicles are similar to those observed in nerve roots of dystrophic mice.

**KEY WORDS** Schwann cell proliferation · axon ensheathment · Schwann cell-axonal interaction · myelination · connective tissue matrix · Schwann cell differentiation

The interaction between supporting cells and peripheral axons leading to ensheathment of unmyelinated nerve fibers, or myelin formation along larger axons, has generally been considered to involve only two elements, the Schwann cell and the axon. Our recent observations on myelinating cultures of peripheral nerve tissue indicate that contact with a connective tissue surface is also involved. This report describes the abnormalities of axonal ensheathment seen when developing Schwann cells are not in contact with a substrate, and demonstrates the correction of this abnormality when contact with a collagen surface is provided.

## MATERIALS AND METHODS

The culture system used employs brief initial exposure to antimetabolic agents to obtain sensory ganglion explants that spawn a rich outgrowth of axons and Schwann cells

without fibroblasts or other cell types (16). Explants were prepared from 18- to 21-day fetal rat cervical dorsal root ganglia that were stripped of much of their connective tissue capsules. One or two explants were established in each Aclar plastic minidish (Allied Chemical Corp, Morristown, N. J.) (8) which had been coated with collagen by spreading two drops of dialyzed collagen solution, prepared by acetic acid extraction of rat-tail tendons (3), over the 22-mm (diameter) surface and polymerizing by exposure for 2 min to  $\text{NH}_3$  vapor. Subsequently, the culture dish was rinsed several times with distilled water before culture medium was added. The medium contained 25 vol% human placental serum, 10 vol% 9-day chick embryo extract, 60 vol % Eagle's minimal essential medium with Earle's salts, 0.7 vol % 200 mM 1-glutamine, 3 vol % 20% glucose, and 20 U/ml nerve growth factor (prepared according to the method in reference 2). During the 2nd wk in culture, the explants were cut out and transferred to a new collagen-coated culture dish; this maneuver leaves behind the initial outgrowth which contains considerable debris from supporting cells killed by the antimetabolic agents and also lingering fibroblasts (16). Cultures were evaluated by bright field or phase microscopy in the living state and by electron microscopy. For the latter, cultures were fixed overnight at 4°C in phosphate (0.1

M)-buffered 2% glutaraldehyde (E. M. grade, Electron Microscopy Sciences) to which 0.08 M sucrose was added, rinsed in cold Earle's balanced salt solution (BSS), further fixed in cold 2% OsO<sub>4</sub> in BSS, rinsed again in cold BSS, dehydrated in graded ethanol solutions, and embedded in Epon-Araldite. After sectioning, the preparations were stained with uranyl acetate in absolute methanol and lead citrate (for details, see reference 6).

## RESULTS

After transfer of the explant as described above, a new outgrowth forms. This initially contains axons predominantly, followed within several days by migrating and dividing Schwann cells derived from the explant area; these Schwann cells confine themselves largely to the regions of axonal growth. Over a period of 2 wk, Schwann cells populate the entire axonal outgrowth and, during the 3rd wk, myelin may appear around the larger axons.

Axonal outgrowth occurs by the progression of growth cones across the collagen surface. When the explant is bulbous rather than flattened, some axons must grow over the sides of the explant to reach the collagen surface before progressing into the outgrowth. Although the tips of these axons attach to the substrate, their shafts do not. This phenomenon may lead to a "guy rope" configuration, the axons arising well above the substrate surface and attaching to the substrate 0.2-1 mm from the explant periphery (Fig. 1). This phenomenon, which we have observed intermittently over the last several years, perhaps results from an

inadequate connective tissue framework in this type of fibroblast-free culture. It rarely occurs in cultures in which the collagen surface has been air dried during its preparation to provide a more adhesive surface.

The suspended axons occur in fascicles, are surrounded in their suspended portions by tissue culture medium, and are loose enough to vibrate when the viewing microscope is tapped. That these fascicles lie above the substrate plane is also apparent when their oblique course is traced with the microscope focusing knob or when additional (normally ensheathed) fascicles are present on the substrate beneath them (Fig. 1).

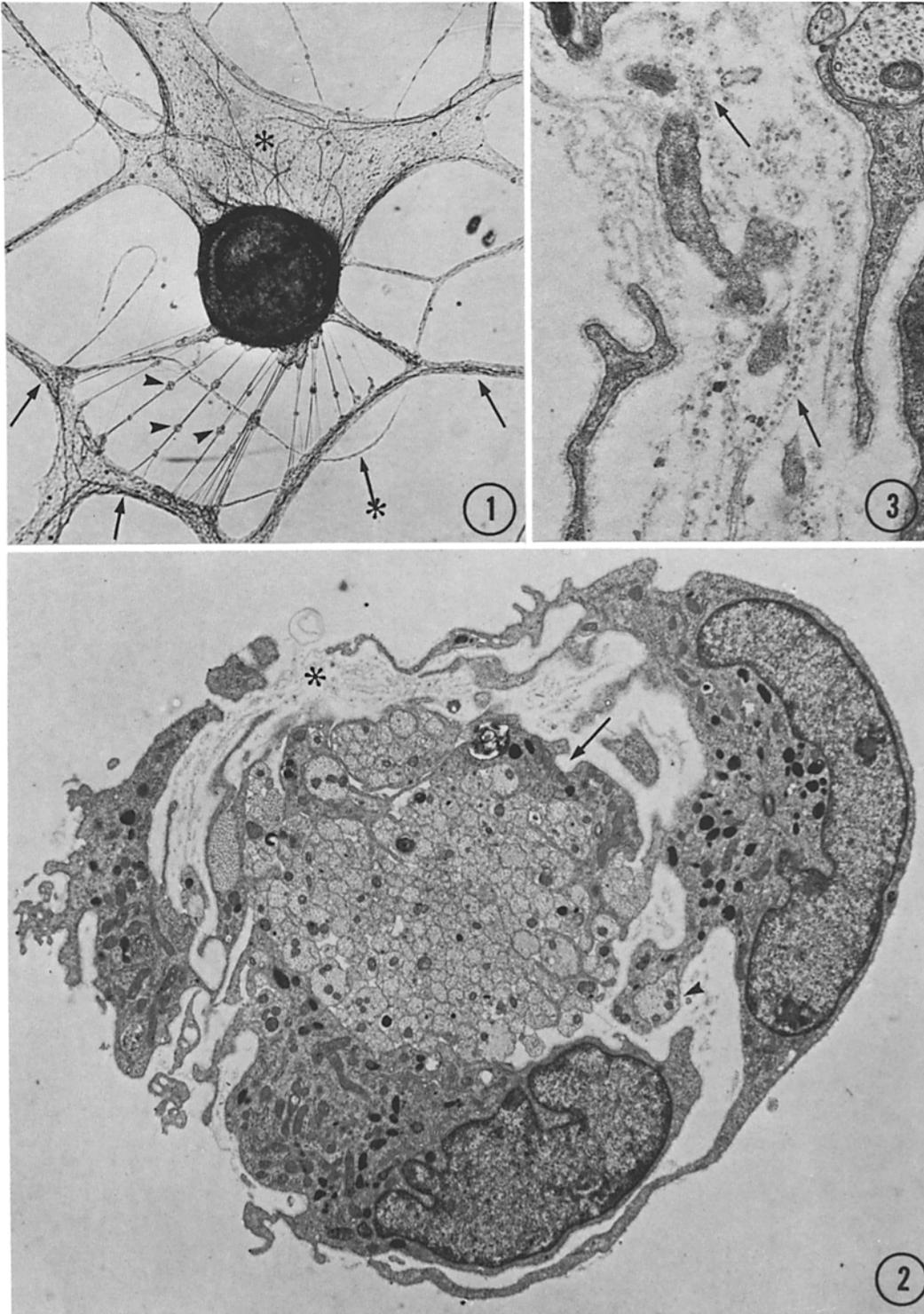
Schwann cell ensheathment of the suspended fascicles is consistently abnormal. Long segments of the fasciculated axons lack ensheathment, and Schwann cells are confined to clusters of varying sizes along these fascicles (Fig. 1) rather than being aligned parallel to the length of associated axons in the normal way. In electron micrographs of these clusters, Schwann cells are observed to occupy a peripheral location in relation to the fascicles of axons (Fig. 2). Their processes usually contact several axons but the ensheathment is incomplete, leaving many axons of both larger and smaller diameters unensheathed. Some Schwann cell extensions meander among axons without encircling them in a typical manner, suggesting that the process of ensheathment has been arrested; this observation is not explained on the basis of insufficient time for ensheathment, because this pattern is maintained for weeks. The Schwann cells exhibit a basal lamina and there is,

---

FIGURE 1 Arising from the central explant (where the sensory neuronal somata are located) are typical outgrowth areas (as at \* and →) composed of nerve fibers and their ensheathing Schwann cells. An occasional neurite is myelinated in these areas, the myelin being manifest as curving dense lines. The outgrowth area emanating from the lower border of the explant is not typical in that the axon bundles are neither attached to the substrate nor ensheathed by Schwann cells; the only Schwann cells present are in small clusters, some of which are designated by arrowheads. A thin, curving fascicle (\*→) on the collagen surface, beneath the suspended fascicles, shows normal Schwann cell ensheathment. Bright field photograph of living culture, 54 days in vitro. × 45.

FIGURE 2 Electron micrograph of an area comparable to those designated by arrowheads in Fig. 1. Four Schwann cells, including a small portion of one at the arrow, surround a fascicle of axons. These Schwann cells actually ensheath only a few of the peripherally located neurites. Although a considerable gap (and therefore an apparent lack of contact) exists between some Schwann cells and the fascicle, study of serial sections has shown that at some point each Schwann cell is in direct contact with an axon (as at arrowhead). The area marked by an asterisk is described in Fig. 3. × 8,900.

FIGURE 3 Another level of the area designated by the asterisk in Fig. 2. Basal lamina coats the Schwann cell processes and extends beyond them as well. Material associated with the basal lamina (as at the arrows) has not yet been identified (see text). × 26,500.



in addition, redundant basal lamina beyond the borders of the Schwann cell processes (Fig. 3). It is known that Schwann cells form basal lamina and associated 20-nm diameter fibrils in the fibroblast-free cultures (7, 15). The material accompanying the basal lamina in the Schwann cell clusters (Fig. 3) appears different from these fibrils and has not yet been identified. When the suspended fascicles are sectioned in areas devoid of Schwann cell clusters, few, if any, Schwann cell processes are observed, axons directly appose one another, and no basal lamina is present (Fig. 4).

Experiments were undertaken to determine whether the juxtaposition of polymerized collagen would correct this abnormal Schwann cell ensheathment. Accordingly, narrow strips of plastic were collagen-coated (as above) and placed on top of suspended fascicles (Fig. 5). The fascicles were visibly stretched under the strip and no longer moved with perturbation. A total of 14 fascicles lie under the strip shown in Fig. 5; suspended fascicles emanating from other areas of the explant served as controls.

Fascicles not covered by the strip continued to exhibit abnormal Schwann cell-axonal relationships (Fig. 6). In the electron microscope, the

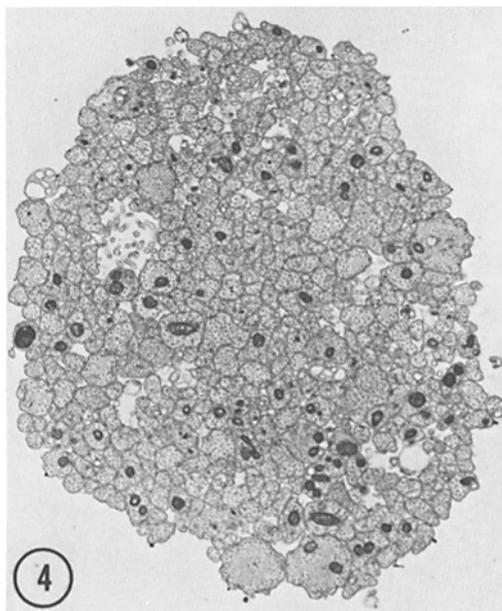


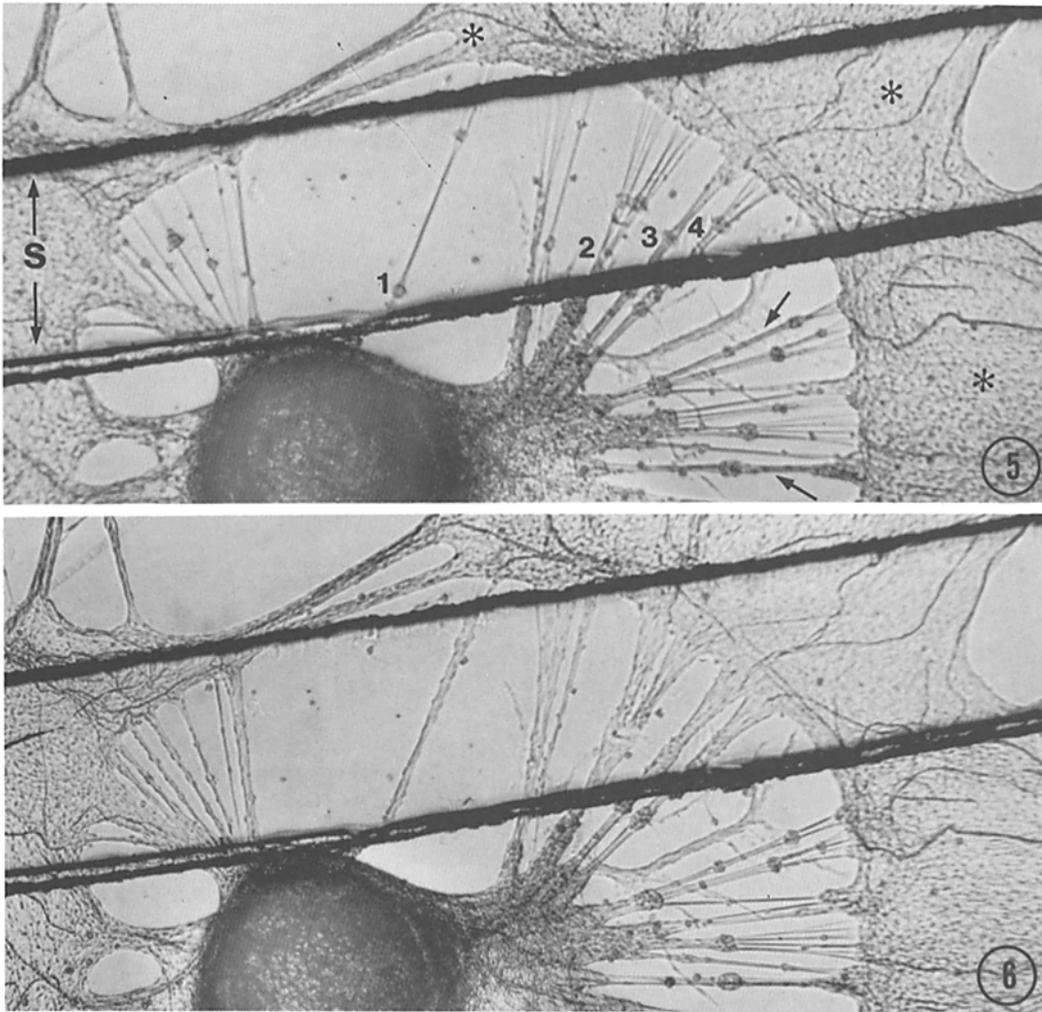
FIGURE 4 Electron micrograph of a suspended fascicle, in a region lacking clustered Schwann cells. There are no ensheathing Schwann cell processes within this fascicle of 393 nerve fibers.  $\times 9,200$ .

abnormal Schwann cell aggregates and intervening axonal fascicles without Schwann cells resembled those areas described above. Suspended fascicles that are only partly covered by the strip are not corrected along the uncovered portions (Fig. 7); the uncovered regions of these fascicles are short and also stretched by the strip and no longer move when the culture dish is perturbed. Because in these regions Schwann cell function remains abnormal, we conclude that vibration of the suspended fascicles does not by itself cause the ensheathment abnormality.

In striking contrast, the abnormal ensheathment in all 14 fascicles beneath the applied collagen-coated strip was corrected within several days (Fig. 6), beginning on the 1st day. The aggregated Schwann cells dispersed and became positioned along unensheathed portions of the fascicles as their numbers increased, and, only 6 days after strip application, myelin appeared in two fascicles. By 10 days, 13 of the 14 fascicles contained one or more segments of myelin (as in Fig. 7). Electron microscope study of these fascicles after 10 days under the strip revealed a remarkably rapid correction of the ensheathment deficit, including well advanced myelination (Fig. 8). Schwann cell processes now surround almost all individual axons whether large or small (Fig. 9). Several larger axons in these fascicles exhibit a substantial degree of myelination (up to 30 lamellae). Thus, the ensheathment of both myelinated and unmyelinated axons now resembles normally developing fascicles of this type of culture. As has been previously observed in normal cultures of this type (7, 15), the corrected fascicles contain basal lamina marking the outward-facing surfaces of the Schwann cell and occasional closely juxtaposed 20-nm fibrils (not shown in Fig. 9); the redundancy of basal lamina noted in the Schwann cell clusters is not generally seen in the corrected areas. If suspended fascicles are provided with collagen, not as a coating on a strip but as a "clot" (formed by adding a drop of dialyzed collagen solution to culture medium) or as filamentous strands (from a clot that was vigorously triturated), the abnormalities are similarly corrected over a period of several days.

#### DISCUSSION

The observations presented indicate that normal differentiation of the Schwann cell requires not only contact with nerve fibers but also contact with a connective tissue matrix (or some material

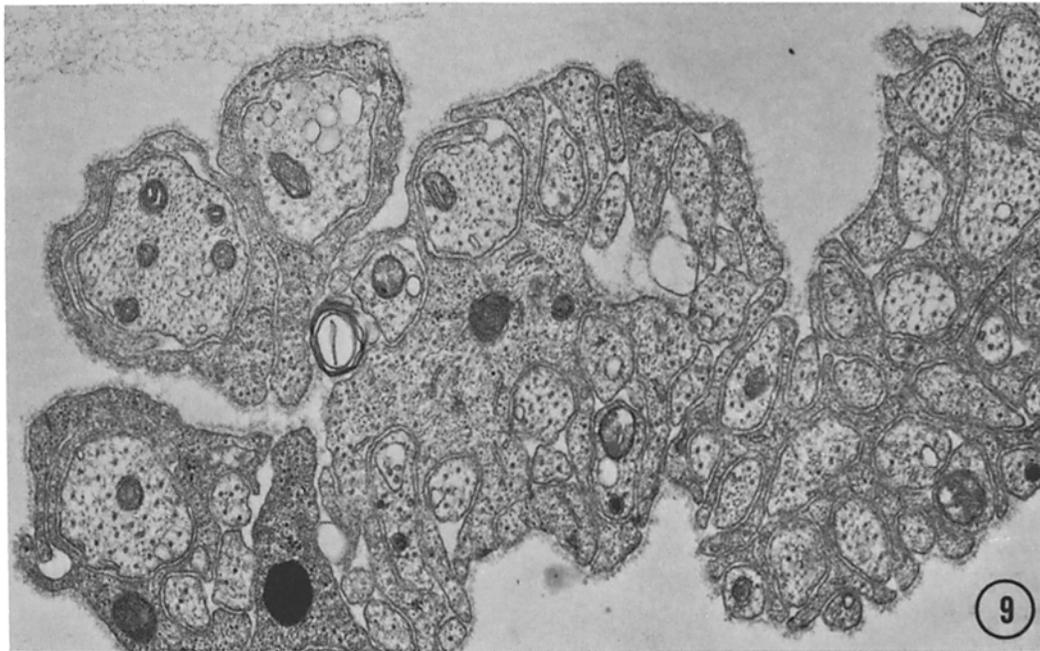
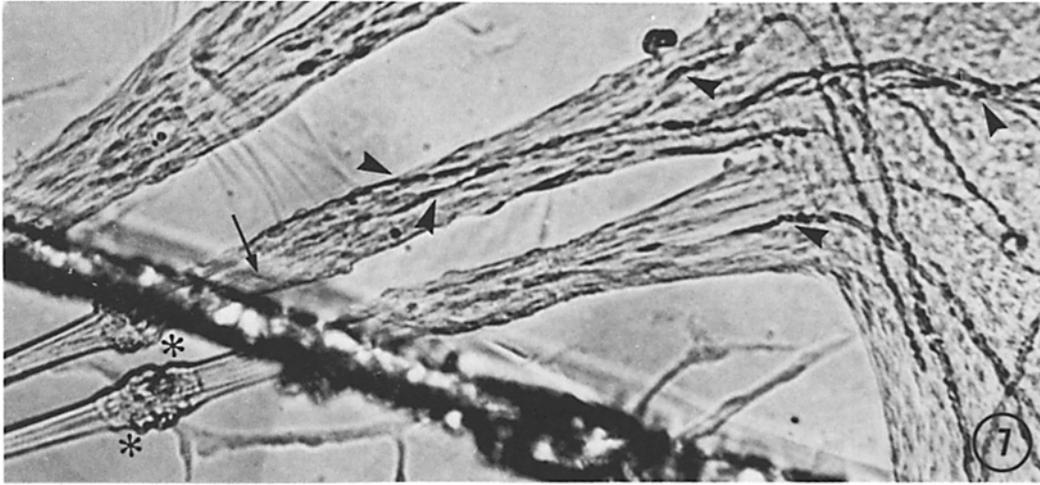


**FIGURES 5 and 6** Bright field photographs of a living culture following placement of a collagen-coated strip (S) on top of suspended fascicles. 60 days in vitro;  $\times 60$ . Fig. 5: Immediately after strip placement. Fascicles under the strip as well as those next to the strip (indicated by arrows) manifest the abnormality illustrated in Fig. 1. The areas marked by asterisks are typical outgrowth regions in which the neurites are attached to the substrate, Schwann cells are distributed along the fibers, and an occasional myelin sheath has been formed. Fig. 6: 3 days after strip placement. In contrast to the atypical fascicles that were not covered by the strip, those beneath the strip have undergone a striking change, namely, Schwann cells have divided and spread out along the fascicles to provide a more typical ensheathment. All the cells along fascicle no. 1 have presumably originated from the two clusters rather than the neighboring explant or outgrowth because the strip edge did not reach either region.

associated with that matrix). Without this additional contact, the Schwann cell appears to be only able to recognize and adhere to the axon and to produce basal lamina. The normal number of Schwann cells is not attained, and the Schwann cells present do not complete ensheathment of unmyelinated nerve fibers and do not myelinate

larger axons. This abnormality of ensheathment is corrected beginning within 1 day after contact with a surface of repolymerized rat-tail collagen. Schwann cell numbers increase, unmyelinated axons are separated and ensheathed, and, within 6 days, myelin formation begins.

These results appear to relate to observations



indicating that during development a variety of cell types requires contact with or close proximity to an extracellular matrix for normal differentiation (for review see reference 10). The requirement for a collagenous substratum in the differentiation of myoblasts to myotubes is perhaps the best known (9). The evidence that a substrate of collagen and glycosaminoglycans (GAG) enhances collagen and GAG production by developing corneal epithelial cells has recently been reviewed by Hay (11). In both these cases, it is known that several types of collagen are able to provide an effective substratum (11, 12).

In both these systems, and in the observations on Schwann cell differentiation described here, the inductive signal provided by extracellular matrix appears to fit the definition of a permissive, or secondary (as opposed to primary), induction as defined by Saxén (14). Secondary induction is considered permissive rather than instructive in that it permits full expression of a cell already showing some differentiated characteristics. Schwann cells on suspended axon fascicles express some ability to recognize and relate to axons but appear unable to fully express ensheathment capacity.

The Schwann cell abnormalities described above are similar to the abnormal axon ensheathment seen in certain spinal and cranial nerve roots of dystrophic mice (1, 4, 5). These abnormalities include failure of ensheathment of both unmyelinated and myelinated nerve fibers in dorsal and ventral nerve roots. Cells believed to be Schwann cells are present in small numbers in these regions but they do not relate normally to the large number of unensheathed nerve fibers. Because these Schwann cells are similar to the cells we have observed relating to nerve fibers in the absence of a connective tissue surface, it seems reasonable to suggest that during nerve root development in the dystrophic mouse a substantial number of Schwann cells fail to receive a neces-

sary inductive signal from connective tissue components in their immediate environment (see also reference 13). They are subsequently separated from the general tissues of the body and are suspended on the root, with no access to the required inductive signal. This would suggest the importance of studies of connective tissue components in the dystrophic mouse.

The authors greatly appreciate the excellent laboratory assistance of Mrs. Ann K. Williams and helpful discussions with Dr. Patrick Wood.

This work was supported by National Institutes of Health grant NS09923.

Received for publication 27 April 1978, and in revised form 6 June 1978.

## REFERENCES

1. BISCOE, T., K. CADDY, D. PALLOT, U. PEHRSON, and C. STIRLING. 1974. The neurological lesion in the dystrophic mouse. *Brain Res.* **76**:534-536.
2. BOCCHINI, V., and P. U. ANGELETTI. 1969. The nerve growth factor: purification as a 30,000-molecular-weight protein. *Proc. Natl. Acad. Sci. U. S. A.* **64**:787-794.
3. BORNSTEIN, M. 1958. Reconstituted rat-tail collagen used as a substrate for tissue cultures on coverslips. *Lab. Invest.* **7**:134-137.
4. BRADLEY, W. G., and M. JENKISON. 1973. Abnormalities of peripheral nerves in murine muscular dystrophy. *J. Neurol. Sci.* **18**:227-247.
5. BRADLEY, W. G., and M. JENKISON. 1975. Neural abnormalities in the dystrophic mouse. *J. Neurol. Sci.* **25**:249-255.
6. BUNGE, M. 1977. Initial endocytosis of peroxidase or ferritin by growth cones of cultured nerve cells. *J. Neurocytol.* **6**:407-439.
7. BUNGE, M., J. JEFFREY, and P. WOOD. 1977. Different contributions of Schwann cells and fibroblasts to the collagenous components of peripheral nerve. *J. Cell Biol.* **75**(2, Pt. 2):161a. (Abstr.).
8. BUNGE, R., and P. WOOD. 1973. Studies on the transplantation of spinal cord tissue in the rat. I.

---

FIGURE 7 Fascicles no. 2-4 located beneath the strip, as shown in Figs. 5 and 6. The configuration shown here (after 10 days under the strip) is a typical one for outgrowth areas in which axons are attached to a substrate. Myelin sheaths are present (arrowheads). Just beyond the actual lower edge of the strip (arrow), the atypical pattern of Schwann cell aggregates (\*) remains.  $\times 230$ .

FIGURES 8 and 9 Electron micrographs of two of the substrip fascicle areas shown in Fig. 7. In Fig. 8, nerve fibers are typically ensheathed with Schwann cell cytoplasm or myelin. The collagen (\*) of the initial substrate and the coated strip sandwiches the fascicle. Fig. 9 depicts the normal appearing ensheathment of unmyelinated axons by Schwann cells and basal lamina.  $\times 7,800$ ;  $\times 22,500$ .

- The development of a culture system for hemisections of embryonic spinal cord. *Brain Res.* **57**:261-276.
9. HAUSCHKA, S. D., and I. R. KONIGSBERG. 1966. The influence of collagen on the development of muscle clones. *Proc. Natl. Acad. Sci. U. S. A.* **55**:119-126.
  10. HAY, E. 1977. Cell-matrix interaction in embryonic induction. In *International Cell Biology. 1976-1977*. B. Brinkley and K. Porter, editors. The Rockefeller University Press, New York. 50-57.
  11. HAY, E. D. 1977. Interaction between the cell surface and extracellular matrix in corneal development. In *Cell and Tissue Interactions*. J. Lash and M. Burger, editors. Raven Press, New York. 115-137.
  12. KETLEY, J. N., R. W. ORKIN, and G. R. MARTIN. 1976. Collagen in developing chick muscle *in vivo* and *in vitro*. *Exp. Cell Res.* **99**:261-268.
  13. MADRID, R. E., E. JAROS, M. CULLEN, and W. BRADLEY. 1975. Genetically determined defect in Schwann cell basement membrane in dystrophic mouse. *Nature (Lond.)*. **257**:319-321.
  14. SAXÉN, L. 1977. Directive versus permissive induction: a working hypothesis. In *Cell and Tissue Interactions*. J. Lash and M. Burger, editors. Raven Press, New York. 1-9.
  15. WILLIAMS, A., P. WOOD, and M. BUNGE. 1976. Evidence that the presence of Schwann cell basal lamina depends on interactions with neurons. *J. Cell Biol.* **70**(2, Pt. 2):138a. (Abstr.).
  16. WOOD, P. 1976. Separation of functional Schwann cells and neurons from normal peripheral nerve tissue. *Brain Res.* **115**:361-375.