BRANCHING PATTERNS OF INDIVIDUAL SYMPATHETIC NEURONS IN CULTURE

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ABSTRACT

The growth of single sympathetic neurons in tissue culture was examined with particular regard to the way in which the patterns of axonal or dendritic processes (here called nerve *fibers*), were formed. The tips of the fibers were seen to advance in straight lines and to grow at rates that did not vary appreciably with time, with their position in the cell outgrowth, or with the fiber diameter. Most of the branch points were formed by the bifurcation of a fiber tip (growth cone), apparently at random, and thereafter remained at about the same distance from the cell body. It seemed that the final shape of a neuron was the result of the reiterated and largely autonomous activities of the growth cones. The other parts of the cell played a supportive role but, apart from this, had no obvious influence on the final pattern of branches formed.

INTRODUCTION

Although it may be that no two neurons have exactly the same dendritic and axonal patterns, it is certain that nerve cells of the same kind tend to have similar, and frequently distinctive, types of outgrowth. Compare, for example, the simple form of many retinal bipolar neurons to the elaborate configuration of the dendritic trees of vertebrate Purkinje or pyramidal cells (1). The development of distinctive form must be largely due to the interactions of the developing cell with its neighbors, but intrinsic features are also known to play a part (2). Properties such as the number, size, and symmetry of the initial outgrowths, their rates of growth, and frequency of branching could be largely independent of external influences and would certainly contribute to the final form of the cell.

For this reason it is of interest to examine the growth of neurons in isolation, to see what properties their outgrowth has in the absence of other cells. Tissue culture conditions are artificial and will impose environmentally determined distortions of shape of their own, but any similarity between the growth patterns in the culture dish and in the animal might reasonably be assigned to growth mechanisms intrinsic to the cell.

Many kinds of nerve cell can produce extensive outgrowths in the absence of other cell types (3–6), but only a few observations have been made of the growth of neurons individually isolated in culture (7, 8). In the present study, sympathetic neurons from day-old rat pups were seeded into culture medium containing nerve growth factor (9). Their production of long branching processes was observed by light microscopy and by time-lapse cinematography, and specimens were fixed in aldehyde for subsequent examination in the electron microscope (see the accompanying paper by M. B. Bunge [10]). In the animal these cells have a single long axon which divides into a number of collaterals within

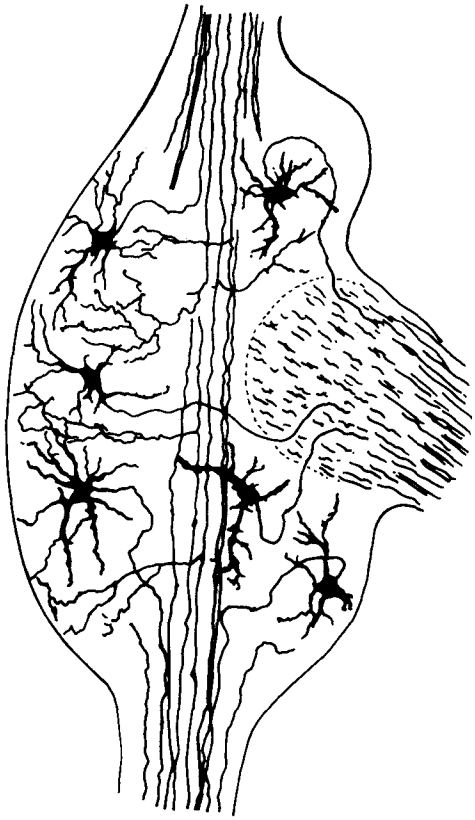


FIGURE 1 The shapes of sympathetic neurons from a 16-day chick embryo. Based on Ramòn y Cajal (1), Fig. 899.

the innervated tissue, and a number of branched dendrites arising from the cell body (Fig. 1.)

METHODS

Details of the culture conditions have been given before (7). Briefly, superior cervical ganglia were dissected from day-old rat pups, dissociated mechanically, and grown on plain glass cover slips set into the bottom of tissue culture dishes. L15 (Leibovitz) medium was used, and was supplemented with serum, glucose, penicillin, streptomycin, 0.6% methylcellulose, and 1 μg/ml of nerve growth factor.

Culture dishes were held on the stage of a Nikon inverted microscope. The temperature was maintained at 37°C by means of a heating coil inserted into the dish, and evaporation was prevented by a thin film of mineral oil floating on the culture surface. Leibovitz's medium maintains a pH of 7.2 in equilibrium with normal atmosphere so that no adjustment of the CO_2 tension was necessary. Pictures were taken with a Nikon automatic camera, and time-lapse movies were taken with a Sage photomicrographic apparatus.

RESULTS

The cells in culture were initially without processes. They were rounded and refractile and possessed an eccentrically placed nucleus. At a variable time after they had settled to the glass surface, usually within 12 h, they began to move across the dish but without flattening onto the surface or putting out ruffling membrane as fibroblasts do. Occasionally, fine spicules were seen to project from beneath the rounded cell and these were followed, as fiber elongation commenced, by the emergence of growth cones.

The way in which the fibers grew out and branched was similar for all cells and will be described in detail for a particular one (cell 1). This neuron was chosen because it had a fairly extensive and widely spaced outgrowth, but was not in any important way atypical. The main variation in the cell outgrowths was the degree of branching which ranged from simple bipolar configurations to those so highly branched that extensive overlapping of their fibers existed (Fig. 2). It is not known whether this variability exists in the animal or whether it is a consequence of explantation.

The Growth of Cell 1

Cell 1 was first observed after it had been in culture for about 18 h (Fig. 3). A time-lapse movie was started at this time and continued for 10 h, with hourly interruptions to take still photographs. Observations were abandoned when it was found that some of the processes had become intermingled with those of a neighboring neuron.

The still photographs of the cell at different times (Figs. 3–10) illustrate the main features of growth seen in the time-lapse movie. By far, the most active parts of the cell were growth cones, and these had two kinds of movement. One appeared in the movie as a flamelike motion involving the projection and retraction of the numerous spicules around the edge of the cone, and was fast enough to be seen by direct observation in the microscope. The other was the slower and more orderly progression of the cone which accompanied the elongation of the fibers. Branches, in almost every case, were generated by the bifurcation of a growth cone, which in-

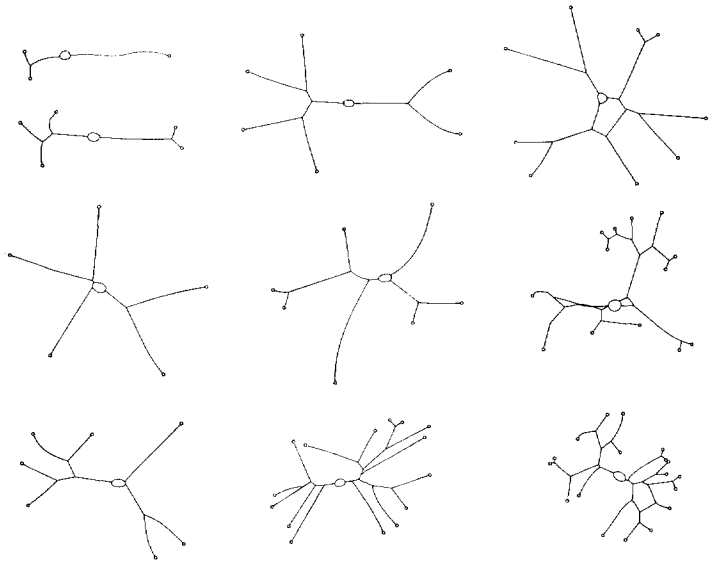


FIGURE 2 Outlines of a number of sympathetic neurons growing singly in culture. Bar, 100 μm.

volved the initial broadening of the cone, its resolution into two or three separate areas of ruffling membrane, and finally, the attenuation of the intermediate areas to give the daughter processes (Figs. 4 and 5). Once they were formed, the branch points remained at about the same distance from the cell body but could undergo small lateral shifts or alterations of the branching angle (Figs. 5 and 6).

The other parts of the cell, the cell body, and the fibers, had little movement of their own but were sometimes passively pulled across the dish during growth. Their behavior suggested that they had only a weak attachment to the culture surface, and this was further explored by displacing them with a microelectrode. Cell bodies and fibers were easily shifted in this way and behaved as though they were held down solely at their tips. Vigorous displacements that broke the fibers would often leave the isolated growth

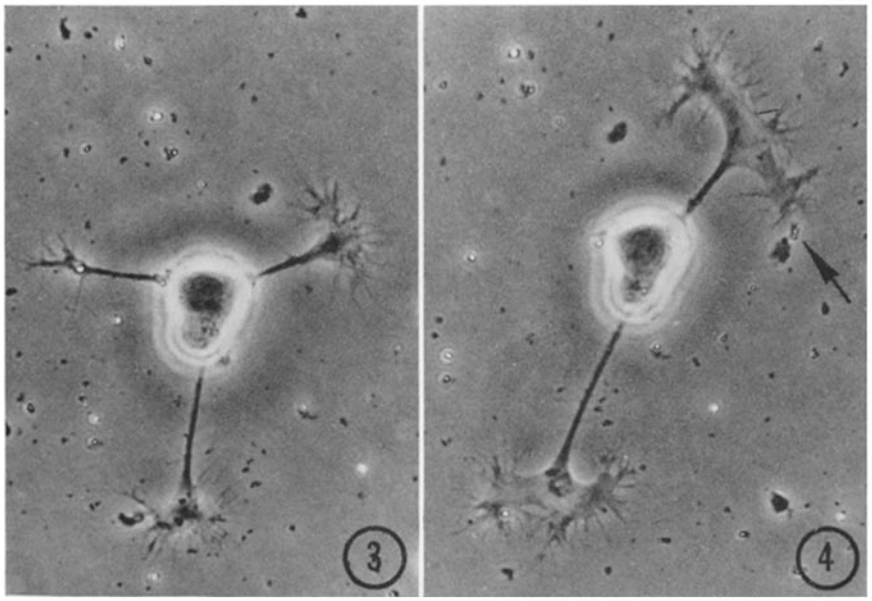
FIGURE 3 Cell 1, 0 min observation, (18 h after explantation). At the start of observations the cell had three short processes tipped with spiky growth cones. One of the cones is smaller than the others, and soon after this picture was taken it was pulled towards the cell body, which itself was displaced slightly (cf. Fig. 4). The cell body is refractile because it has not flattened onto the surface, and acorn-shaped because its nucleus is eccentrically placed. × 580.

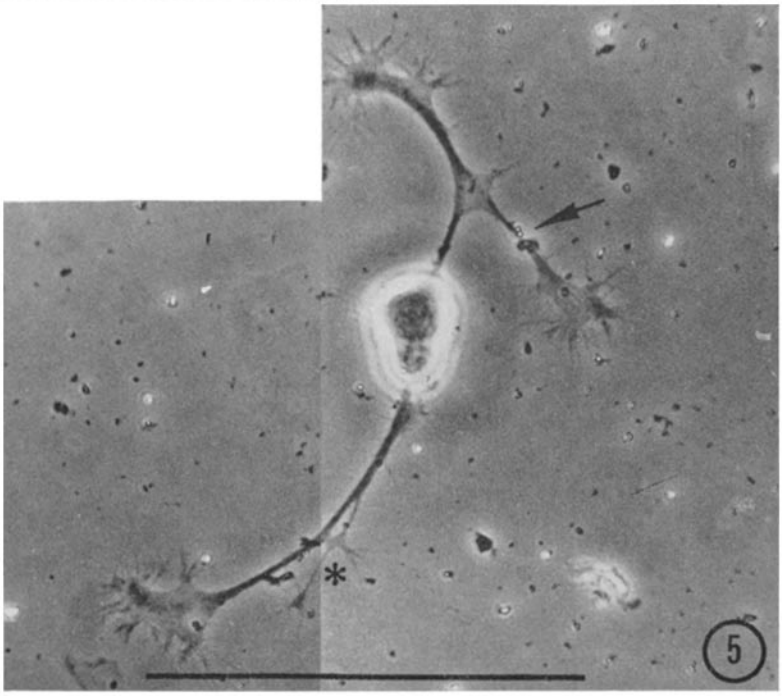
FIGURE 4 Cell 1, 60 min. The retraction of one of the three fibers seen in the previous figure is almost complete and the cell now appears to be bipolar. The numerous fine spicules about the two broad growth cones were continually projected and retracted to give a flickery or flamelike appearance. An apparently related movement is shown by glass particles, such as the double particle above the left-hand cone (indicated by an arrow, Figs. 4 and 5), that are picked up at the periphery of the cone and carried rapidly backwards. This cone was in the process of dividing to form a bifurcation in the fiber (Fig. 5). × 580.

FIGURE 5 Cell 1, 120 min. The pronounced curvature of the cell at this time was a consequence of the retraction of the fiber shown in Fig. 3. This is because the retraction caused the cell body to move without changing the orientation of the two growth cones, so that when the daughter processes were formed they were at an angle to the parent fibers. The angle at the lower right-hand side was so acute that a process climbed back along the fiber, and the residue (*) of this migration is seen alongside the principal fiber. Bar, 100 μm. × 580.

cones attached to the dish. It appeared that the primary attachment of the cell, as well as most of its movement, was at its growth cones, and this was undoubtedly the reason for the geometrical appearance of the outgrowths.

The movements of growth cones described above were repeated at an increasing number of fiber tips throughout the period of observation. Two other kinds of activity were occasionally observed: one was the retraction of a fiber which seemed to occur when a growth cone lost its attachment to the dish (Figs. 3 and 4), and the other was the appearance of a collateral branch from the trunk of an existing fiber. The most





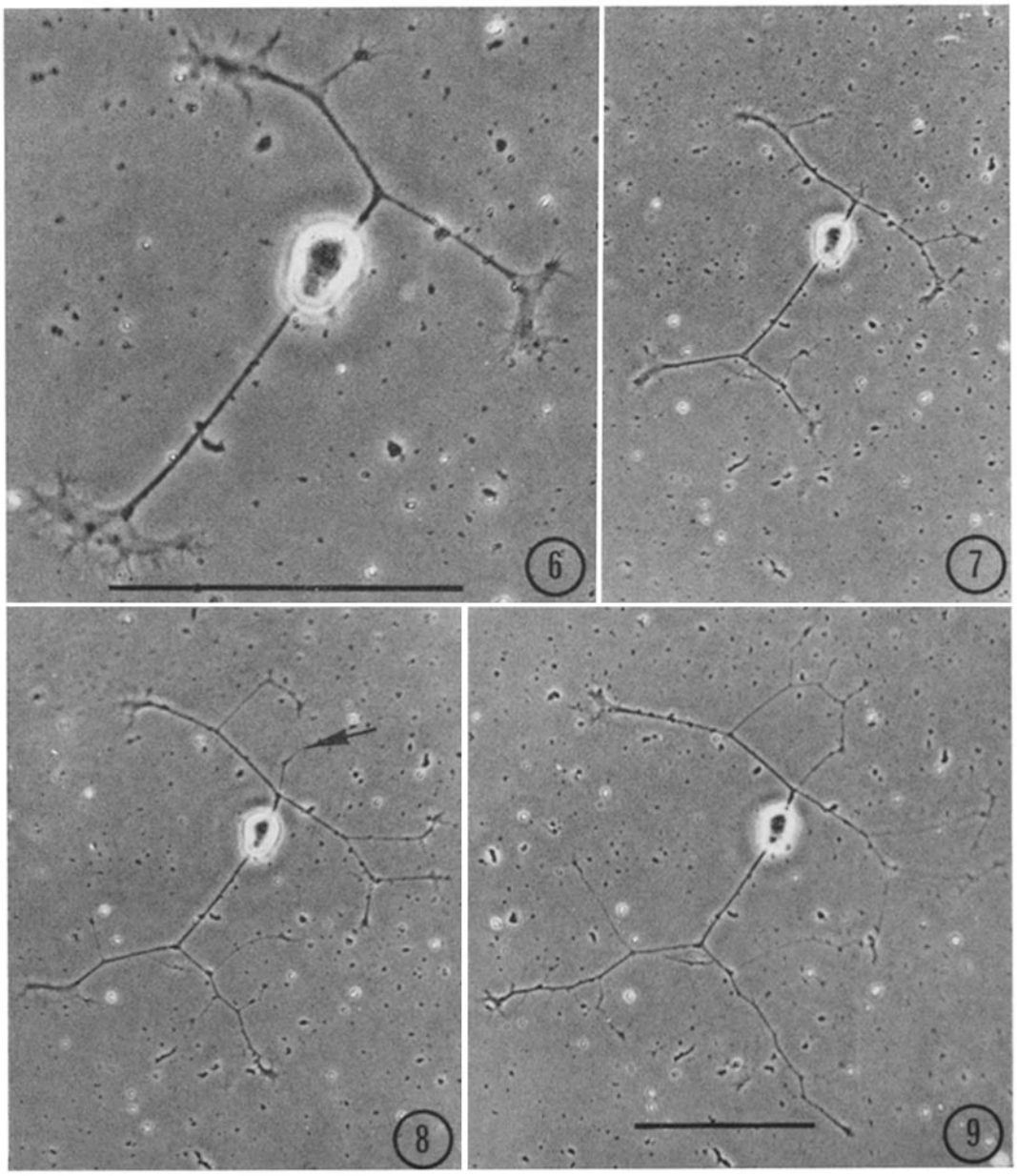


FIGURE 6 Cell 1, 180 min. The cell adopted this more symmetrical shape from that shown in the previous figure by slow movement of the cell body and its processes, apparently caused by the pulling of the growth cones. Bar, 100 μm. × 490.

FIGURE 7 Cell 1, 240 min. × 250.

FIGURE 8 Cell 1, 300 min. Notice the collateral branch (arrow), which has appeared from the principal branch point. × 250.

FIGURE 9 Cell 1, 360 min. Bar, 100 μm. × 250.

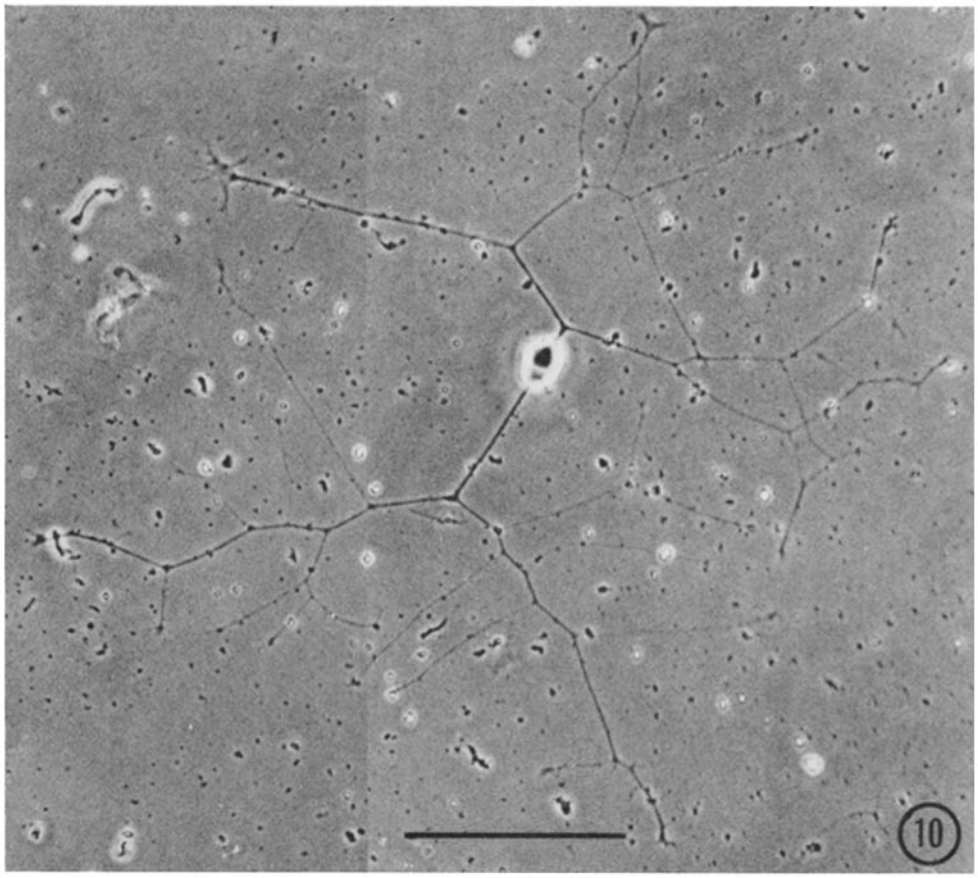


FIGURE 10 Cell 1, 500 min. Bar, 100 μm. × 290.

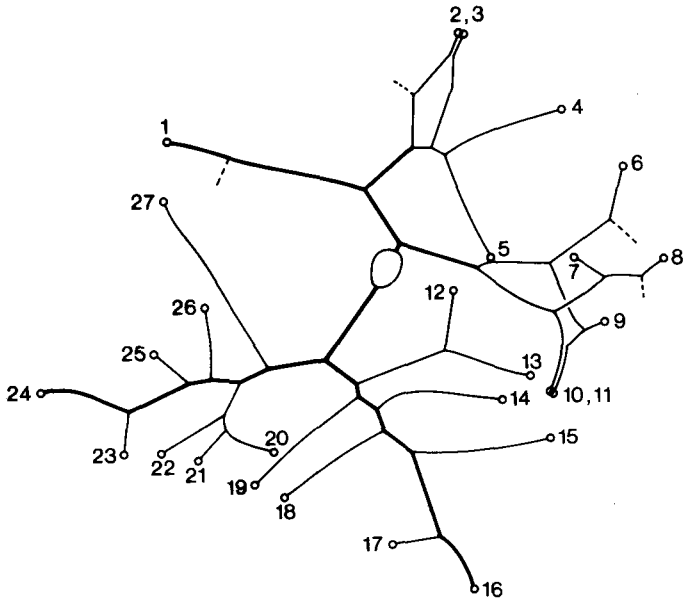


FIGURE 11 The outline of cell 1 at 500 min. The thickest fibers are shown by heavy lines, and those too thin to easily follow by broken lines. In a few regions (see growth cones 2, 3, and 10, 11), overlapping of fibers occurred and in these cases their distribution was assigned on the basis of the previous growth.

clearly seen example of the latter involved an area of ruffling membrane apparently stranded by the division of a growth cone (Fig. 5) which, 2 h later, initiated the growth of a daughter branch (indicated by an arrow in Fig. 8).

Measurements of Cell 1

The simple mechanism of growth of cell 1 suggested that its final form could be accounted for by relatively few parameters, such as the rate of growth of the tips, the frequency with which they bifurcate, and the angles the daughter branches make with each other. A particularly important question is whether there are any regular trends during growth. Are there, for example, areas of the cell or stages in its growth in which fibers grow faster or branch more often than another?

Measurements of the cell were facilitated by the geometrical quality of its outgrowth and were made from outlines prepared as in Fig. 11 at different times of growth. One major limitation was that the diameter of the fibers, which clearly decreased with each bifurcation, was close to the limit of resolution of the microscope so that any regularity in this decrease could not be determined. It is also possible that very thin branches were present but not observed.

TABLE I

Branching Angles of Cell 1

Order	Left	Right	Order	Left	Right
1	90	125	4	100	*157*
1	112	108	4	145	142
2	125	*130*	4	*167*	87
2	*155*	115	4	105	135
2	139	92	4	80	*132*
2	154	167	4	*125*	105
3	120	137	5	*160*	98
3	*137*	125	5	140	120
3	145	147	5	*165*	130
3	*178*	110	5	170	148
3	132	148	5	*133*	104
3	150	117	6	*133*	150
3	125	138	6	114	*132*
			7	*145*	70
			7	*130*	87

The angles in degrees between a parent fiber and the daughter branches to its left and right were measured at each branch point of cell 1. The angles are arranged according to their branching order (number from the cell soma), and those subtended by a branch that is clearly thicker are italicized.

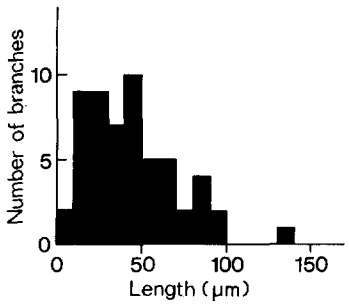


FIGURE 12 Distribution of segment lengths in cell 1. Lengths of fiber were measured (Fig. 11) from the growth cones or the cell body to the nearest branch point, or between adjacent branch points. Intersections with broken lines were included.

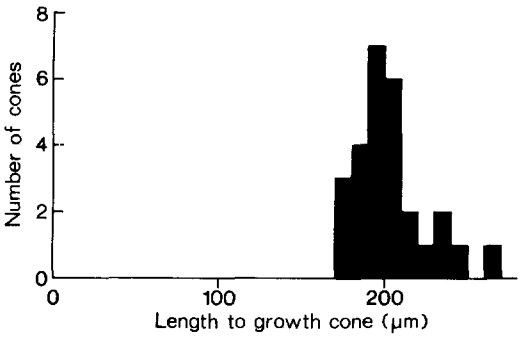


FIGURE 13 Distribution of fiber lengths in cell 1. Lengths were measured from the cell body to the growth cones (Fig. 11).

The branching angles of the final outgrowth, seen in outline in Fig. 11, varied widely but were not obviously correlated with distance from the cell body or with position in the cell outgrowth (Table I). One quite regular feature that appeared was the tendency for thinner branches to subtend a smaller angle than thicker ones. Although accurate measurements of diameter were difficult to make from these pictures, in about half the total number of bifurcations one of the daughter branches had an obviously larger diameter than the other and in every such case this was the branch that formed a larger angle (Table I).

Measurement of the lengths of segments revealed a feature not immediately obvious by in-

spection of Fig. 10. The lengths themselves fell on a broad distribution (Fig. 12) in which short segments (under 10 μm long) were comparatively rare and in which the most frequent length was close to 40 μm. But if the measurements of the most peripheral branches (i.e. those terminating in a growth cone) were separated from those of the inner branches, it was found that the terminal branches were on the average longer (55 μm) than the nonterminal ones (35 μm). This suggests that the branching frequency decreased towards the end of the period of observed growth.

Three kinds of measurement suggested that the growth cones of cell 1 all advanced at about the same rate. The first of these was the measurement, from the cell outline in Fig. 11, of the lengths of fiber from the cell body to each of the 27 growth cones (Fig. 13). The distribution of these lengths was quite sharp and, since growth went on continuously for the 8.5 h of observation, the same was probably true of their rates of growth. In particular, the lengths did not appear to vary with the thickness of the fiber and only slightly with the branching order of the growth cone (Fig. 14). The second measurement was of the increase of length at each growth cone during the last 80 min of observation (Fig. 15), and again these lengths were uniform and such variation as existed was not obviously correlated with any morphological features. Third, and most direct, the growth rates were measured from the time-lapse movie of the cell. The time course of the elongation of one of the major processes of the cell and of three of its daughter processes is shown in Fig. 16. Clearly, the tips grew at a fairly constant rate, close to 40 μm/h, and this was maintained through two divisions. In contrast, the branch points formed with each daughter process did not travel away from the cell body and remained at about the same length from the cell body.

DISCUSSION

It is very important to the interpretation of these results that the cells observed were in fact single neurons. To be sure, they appeared to be solitary and had only one visible nucleus. They did not divide and very often the fibers that they extended were over 0.5 mm in length. But it could still be said that glial cells could wrap around neurons so tightly as to become invisible, and that cells other than nerve cells can produce long thin processes. Both of these possibilities are made less likely by the results of the examination of these cells in the electron microscope (10, 11), which shows them to have many of the ultrastructural characteristics expected of developing neurons, and to be free of associated glia. Their identity as nerve cells is also indicated by the fact that they did not survive if nerve growth factor was omitted from the culture medium (9).

The outstanding impression gained from this work was of the important part played by the growth cones. These were seen to dominate the movements of the cells, to provide the major points of attachment to the culture surface, and to form most of the new branches by their bifurcation. Evidence was presented previously that growth cones are the sites at which new surface is accumulated (7), and much of their behavior

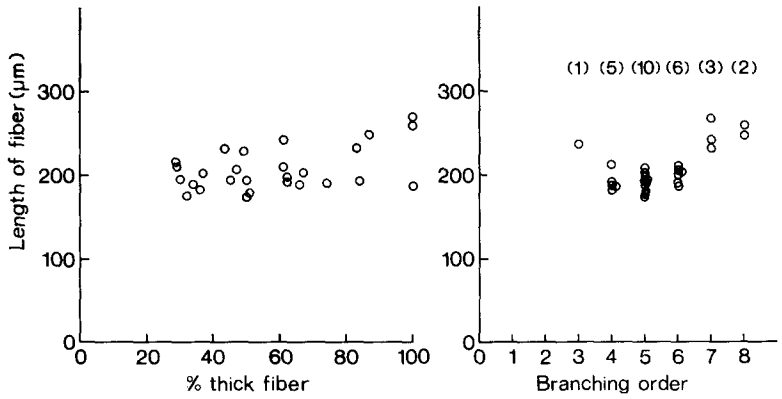


FIGURE 14 The fiber lengths of cell 1 plotted against the percentage of thick fiber (Fig. 11), and the branching order of the growth cones. The branching order is defined as the number of intersections between a growth cone and the cell body, the numbers of tips of each order are given in parentheses.

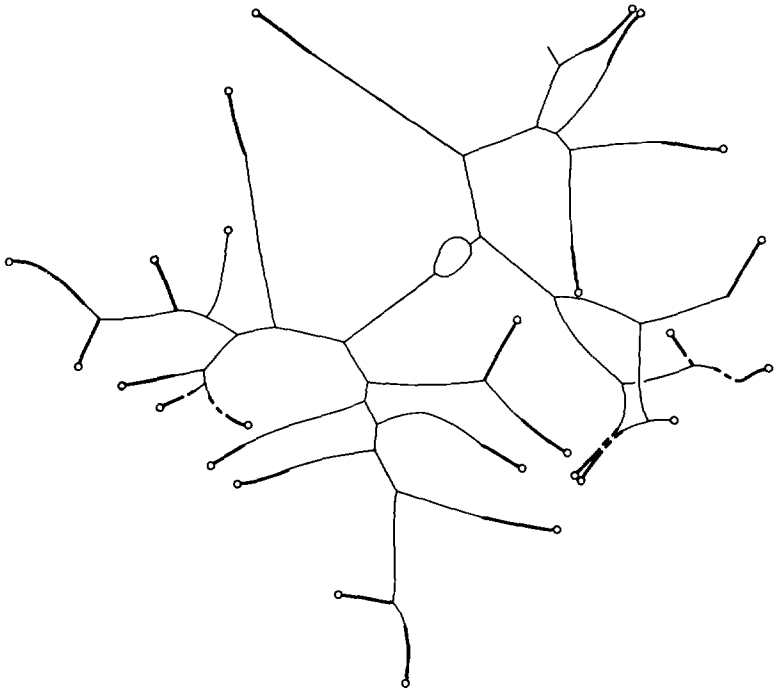


FIGURE 15 The outline of cell 1 showing the fiber increase in length during the last 80 min of growth. Fiber added during this time is shown by heavy lines; broken heavy lines indicate uncertainty of measurement.

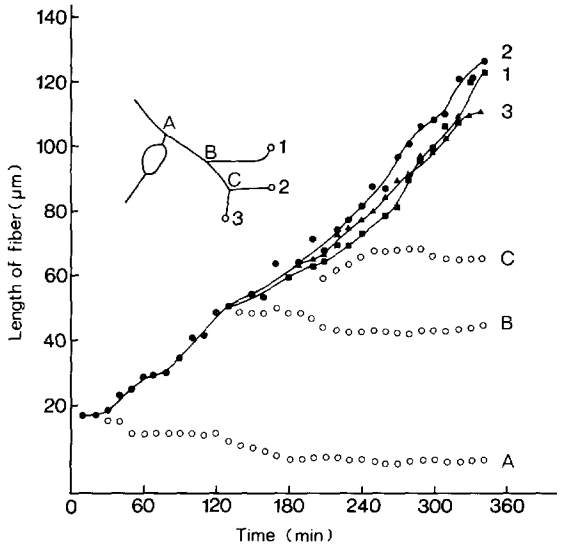


FIGURE 16 Measurements from the time-lapse movie of cell 1. Lengths of fiber were measured from the cell body to growth cones and branch points in the field. The inset diagram shows the points measured and corresponds to the cell at 300 min (Fig. 8).

could be understood in these terms. In particular, the finding that branch points, once formed, did not move away from the cell body while the tips advanced hundreds of microns shows that at least part of the fiber is deposited by the tip. The constant rate of advance of growth cones implies that the accretion of new surface is carefully regulated. But whether this regulation

occurs in the supply of new materials to the tips or in the actual assembly process cannot be said.

The behavior of the growth cones was also the main factor in the determination of the final form of the cell outgrowth. The rectilinear appearance of the outgrowths was due to growth cones progressing in straight lines and pulling on the trailing fibers. The sharpness of the branching angles, and the finding that thinner fibers usually subtended small angles, could be due to tension exerted from the tips. The branch points were formed by growth cones in an apparently random way, and there was no evidence of any predetermined plan. The only regularities that were observed were that branches were slightly less frequent at later stages of growth, and that branch points did not occur very close together (Fig. 12). With these small exceptions all of the cell shapes seen could have been formed by a simple mechanism in which growth cones proceeded in straight lines at a constant rate and had, with every unit of length advanced, a fixed probability of forming a branch.

The main advantage of the study of isolated neurons is that all parts of the cell may be seen at every stage of growth, but this is unfortunately not possible with cells in more normal environments. Developing neurites in embryos (12, 13), or even in the more usual kinds of tissue culture (14), show a strong tendency to fasciculate so that what appears as a single process may contain the outgrowth from dozens of cells, and what appears as a bifurcation may be a splitting of a large bundle into two smaller ones. Histological methods are handicapped in a different way because, although they can give a detailed picture of an entire cell, they can do this only for one stage of growth.

Comparison of the outgrowths of the cells in this study with those of sympathetic neurons of about the same stage of development in the animal (Fig. 1) shows one outstanding difference. Although the outgrowths in culture often bear an ostensible resemblance to the arrangement of dendrites in vivo, there is never, as there always is in the animal, a single long unbranched axon-like process. There are many possible reasons for this. The growth of axons may require a longer period to develop than the 4–5 days for which the cultured cells were observed, or may need specific extracellular cues not present in the culture. It is also possible that the axons of sympathetic neurons are normally produced at an earlier stage of development and by a mechanism that is essentially different from that which produces dendrites. Rakic (13) has described how cerebellar granule cells and late-forming neurons in fetal neocortex appear to elaborate their axons as they migrate to their positions in the cortex, and only later produce dendrites.

A recent attempt to reconstruct the growth of dendrites from their patterns in silver-impregnated preparations has shown agreement with the results presented here. In an analysis of the branching of pyramidal and Purkinje cell dendrites from rat, Hollingworth et al.[1] have found that their lengths, measured from tip to perikaryon, are uniform, and that their patterns appear to be formed by random branching in the terminal dendritic segments. These similarities in the structures of normal dendrites to the fiber outgrowths of isolated neurons in culture raise the possibility that the patterns in vivo may also be determined by the activities of the growth cones. Grosser features of the cellular architecture, such as the course of the axon or the disposition of axonic and dendritic stems on the cell soma, may be established in other ways (2, 13). However, the exploration of surrounding tissue by growth cones that are free to respond to local variations in environment could easily account for the detailed structure of dendrites, and perhaps of axons at their terminal ramifications.

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[1] T. Hollingworth, M. Berry, E. M. Anderson, and R. Flinn. Network Analysis of Dendritic Fields. Manuscript in preparation.

REFERENCES

1. RAMON Y CAJAL, S. 1911. Histologie du Système Nerveaux de l'Homme et des Vertébrés. Maloine, Paris. **2**.
2. VAN DER LOOS, H. 1965. The "improperly" oriented pyramidal cell in the cerebral cortex and its possible bearing on problems of neuronal growth and cell orientation. *Bull. Johns Hopkins Hosp.* **117**:228.
3. LEVI-MONTALCINI, R., and P. U. ANGELETTI. 1963. Essential role of the nerve-growth factor in the survival and maintenance of dissociated sensory and sympathetic nerve cells *in vitro*. *Dev. Biol.* **7**:653.
4. VARON, S., and C. W. RAIBORN. 1969. Dissociation, fractionation and culture of embryonic brain cells. *Brain Res.* **12**:180.
5. AUGUSTI-TOCCO, G., and G. SATO. 1969. Establishment of functional lines of neurons from mouse neuroblastoma. *Proc. Natl. Acad. Sci. U. S. A.* **64**:311.
6. CHEN, J. S., and R. LEVI-MONTALCINI. 1970. Axonal growth from insect neurons in glia-free cultures. *Proc. Natl. Acad. Sci. U. S. A.* **66**:32.
7. BRAY, D. 1970. Surface movements during the growth of single explanted neurons. *Proc. Natl. Acad. Sci. U. S. A.* **65**:905.
8. NAKAI, J. 1956. Dissociated dorsal root ganglia in tissue culture. *Am. J. Anat.* **99**:81.
9. LEVI-MONTALCINI, R., and P. U. ANGELETTI. 1968. Nerve growth factor. *Physiol. Rev.* **48**:534.
10. BUNGE, M. B. 1973. Fine structure of nerve fibers and growth cones of isolated sympathetic neurons in culture. *J. Cell Biol.* **56**:713.
11. BUNGE, M. B., and D. BRAY. 1970. Fine structure of growth cones from cultured sympathetic neurons. *J. Cell Biol.* **47**(2, Pt. 2): 241a(Abstr.)
12. TENNYSON, V. M. 1970. The fine structure of the axon and growth cone of the dorsal root neuroblast of the rabbit embryo. *J. Cell Biol.* **44**:62.
13. RAKIC, P. 1971. Guidance of neurons migrating to the fetal monkey neocortex. *Brain Res.* **33**: 671.
14. GRAINGER, F., D. W. JAMES, and R. L. TRESMANN. 1968. An electron-microscopic study of the early outgrowth from chick spinal cord *in vitro*. *Z. Zellforsch. Mikrosk. Anat.* **90**:53.