*IN VITRO DIFFERENTIATION OF A MOUSE NEUROBLASTOMA**

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Communicated by Clifford Grobstein, July 7, 1969

Abstract.—Mouse tumor C1300 has been established in tissue culture. The cells have a round cell morphology in both the subcutaneous tumor and in suspension culture. However, when given a surface on which to attach, they send out processes up to 3 mm in length and assume the morphology of mature neurons. The attached cells are stained by the Bodian silver procedure for neurons, whereas the cells grown in suspension are not. Electron microscopy reveals that the attached cells contain neurofilaments, neurotubules, and dense-core vesicles indicative of nerve fibers. Both free-floating and attached cells have tyrosine hydroxylase activity characteristic of sympathetic nervous tissue. Apparently cell attachment can induce morphological differentiation from an anaplastic round cell to a cell which has many properties of a mature neuron.

The analysis of differentiated function is hampered by the fact that the end cell usually does not divide. In the case of extremely heterogeneous cell populations such as those found in the endocrine, nervous, immune, and hepatic tissues, further advances require a way of establishing clones of end cells which express their differentiated phenotype in tissue culture.[1, 2] Such cells are most easily derived from neoplasms and have been used successfully in the study of endocrine,[3] hepatic,[4] and endoreticular[1] cell function. This communication extends these studies to the nervous system by describing a cloned, tissue-culture adapted, mouse cell line which can be induced to differentiate to a cell which has some properties of a mature neuron.

Materials and Methods.—Tumor lines: Mouse tumor C1300 was obtained from Jackson Laboratories. This cell line has been previously described as a round cell tumor, possibly a neuroblastoma.[5, 6] The spontaneous neoplasm originated in the body cavity in mouse strain A/J in 1940 and has been maintained by subcutaneous transfer in A/J mice. The solid tumor was adapted to tissue culture conditions by dispersing the cells in modified Eagle's medium[7] containing 20% fetal calf serum. The cell cultures were maintained at 37°C in an 85% air, 15% CO_2 incubator. When grown in plastic Petri dishes, the established cell line had a doubling time of 17 hr. Cells were cloned twice by spreading dilute cell suspensions on solid agar (0.5% agar, 4.5 mgm/ml trypticase soy broth in Eagle's modified medium plus 20% fetal calf serum) and picking visible colonies with a platinum loop after a 2-week incubation.[8] Clones had doubling times comparable to the initial explant, and were homogeneous with respect to cell morphology, staining characteristics, and karyotype.[9]

Staining and electron microscopy: For light microscopy, cells were grown in Petri dishes with glass cover slips. After 2 to 4 weeks, the cover slips were washed several times in Eagle's medium and the attached cells stained according to the Giemsa,[10] and Bodian[10, 11] procedures. Cells growing in suspension were centrifuged, washed twice in modified Eagle's medium, spread on a slide, and stained as described above.

For electron microscopy, free-floating cells were pelleted, washed three times with Eagle's medium, and fixed. Attached cells were washed, fixed, and embedded in the plastic dishes on which they were grown. Cells were fixed in phosphate-buffered (pH 7.6) glutaraldehyde, washed, and postfixed in OsO_4. Dehydration through ethanol and propylene oxide was followed by embedding in epon. The propylene oxide was omitted for attached cells.

After hardening of the epon, the culture plate embeddings were examined under the light microscope and selected areas marked. The epon was then cleaved from the plate by applying Dry Ice. The selected areas were cut out, glued to specimen blocks, and cut parallel or perpendicularly to the substrate on which the cells were grown. Sections were examined after uranyl acetate and lead citrate staining.

Catecholamine assay: Attached cells grown in tissue culture dishes were washed twice *in situ* with Eagle's medium minus tyrosine; cells grown in suspension in Petri dishes were centrifuged and washed twice in the same medium. Cells were incubated in Eagle's medium minus tyrosine with 10 μc/ml of ^{3}H-tyrosine (spec. act. 40 c/mmole) for 1 hr. The cells were centrifuged, resuspended in 0.4 *M* $HClO_4$, and sonicated. Debris was removed by centrifugation at 30,000 $\times$ *g* for 15 min, and the catecholamines absorbed to alumina.[12] After elution with 0.5 *M* acetic acid, catecholamines were assayed by thin-layer chromatography.[13] As a control, mouse myeloma tissue culture line MOPC21 was labeled with tyrosine and extracted by the identical technique.

Results.—Mouse tumor C1300 was initially adapted for tissue culture in bacteriological plastic Petri dishes. The cells divided rapidly in suspension and retained the round-cell morphology of the *in vivo* tumor line. But when cells were transferred to a surface to which they could attach, such as glass, collagen, or commercially treated tissue culture dishes, most cells attached to the surface within 24 hours (Fig. 1) and within three days sent out processes from the cell body, some extending several millimeters (Fig. 2). Individual cells possessed from one to four such processes. After two weeks, the surface of the plate contained a network of these processes (Fig. 3).

First, the *in vivo* tumor morphology will be described, and then the round cells growing in suspension will be compared to the highly morphologically polarized attached cells.

In vivo characteristics: The morphology of the solid subcutaneous tumor was similar to that previously described.[5, 6] The tumor was soft, partially encap-

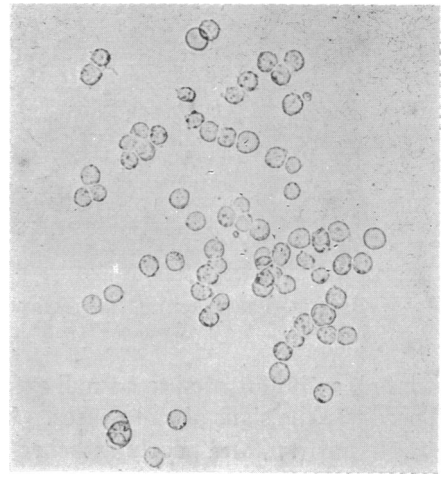

FIG. 1.—Round cell population 24 hr after transfer from a Petri dish to a tissue culture dish.

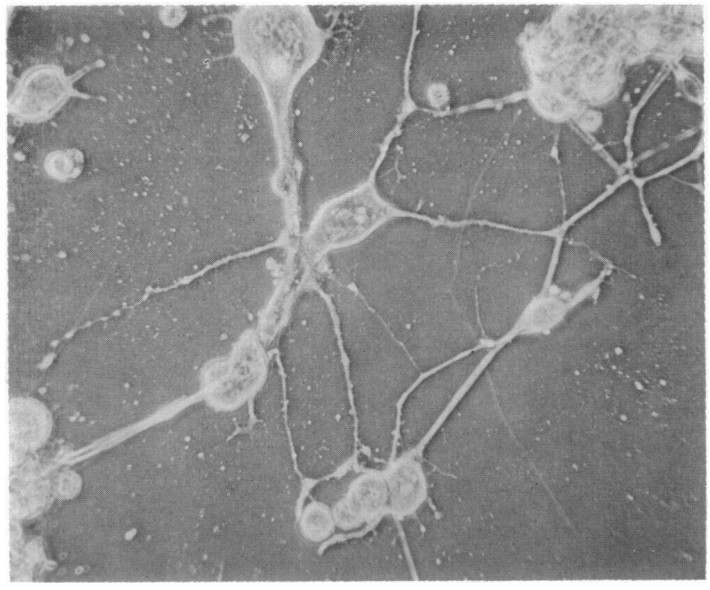

FIG. 2.—Phase-contrast micrograph of attached cells 8 days after transfer from a Petri dish to a tissue culture dish.

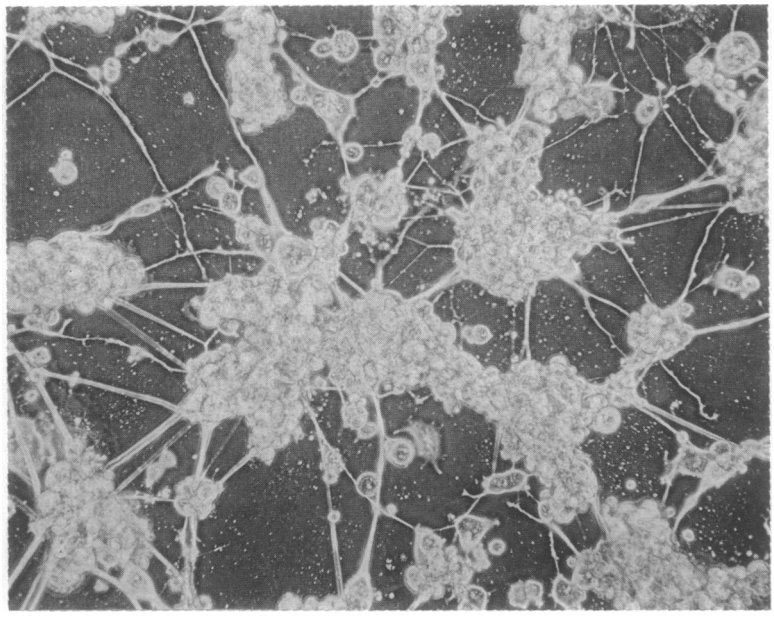

FIG. 3.—Phase-contrast micrograph of attached cells 14 days after transfer from a Petri dish to a tissue culture dish.

sulated, and infiltrated to a small extent the surrounding fibrous-adipose tissues. There were no signs of metastases. The cells were round or ovoid, arranged in a diffuse pattern, and poorly attached to each other. They had a high nuclear:

cytoplasmic ratio, and multinucleate cells were rare. No cells were impregnable with the Bodian silver neuron stain.

In vitro characteristics: Giemsa stains of the cloned cells showed that the round cells growing in suspension were like the *in vivo* tumor cells. They were 40 μ in diameter and contain one to three nucleoli. The cell body of the attached cells growing on glass varied from 40 to 150 μ in diameter and contained one to eight nucleoi; some of their processes extended two to three millimeters from the perikaryon. The processes occasionally dilated along their length and at the end of each process a faintly stained growth cone was usually present. Cells grown on glass showed a tendency to aggregate; these aggregates were usually loosely associated with a number of attached cells which had previously sent out processes. Occasionally giant multinucleate cells were observed whose perikaryon was greater than 150 μ in diameter. Such cells were seen in both the primary culture and the clones. While the round cells in suspension culture are mostly tetraploid, about 50 per cent of the attached cells are octaploid or greater.

Since the cellular morphology of C1300 attached to a surface closely resembles that of nervous tissue, a neuron-specific staining procedure was used to identify these cells. The Bodian stain positively stained the attached, highly branched cells. Cells grown in suspension, like the *in vivo* tumor cells, did not stain.

Electron microscopy: Cells grown in suspension: Suspension cultures contain an apparently homogeneous population of round cells having many unspecialized small interdigitating processes less than a micron in diameter (Fig. 4). Cellular organization is dominated by a diffuse system of endoplasmic reticulum and associated virus particles. The virus appears to form on the outer surface of the endoplasmic reticulum and the maturing particles bud into the cisternae in a manner analogous to other murine neoplasms.[14] Few viral particles are found free in the cytoplasm. There are two populations of mitochondria; one population is normal or slightly shrunken, while the other appears swollen. A similarly mixed mitochondrial population has been observed in virally infected cells[14] and cultured rat nerves.[15] In addition to multivesicular and lysosomal bodies, dense-core vesicles with homogeneous contents slightly withdrawn from the limiting membrane and a maximum diameter of 0.4 μ are found in the cytoplasm.

Attached cells: The majority of the attached cells are bipolar, tapering into long processes on either side of the cell body. Their nuclei are flattened and deeply indented, similar to nuclei in cultured rat nerves.[16, 17] Most cells are mononucleate; a few are clearly in mitosis. Nuclei of nonmitotic cells always have some marginated chromatin and many nuclear pores; only the latter is characteristic of normal nerve cells. In contrast to that of round cells, attached cell endoplasmic reticulum is compact and not dominated by developing virus. Some mature and defective viruses are found free in the perikaryon cytoplasm. Initial stages of virus formation have not been observed in attached cells.

Processes extending from the bipolar cells frequently have bulbous dilations and usually have widened tips; they frequently bifurcate. These extremely long processes may be classified as containing components which are mostly neurofibrillar, mostly neurotubular, or essentially perikaryon cytoplasm. The

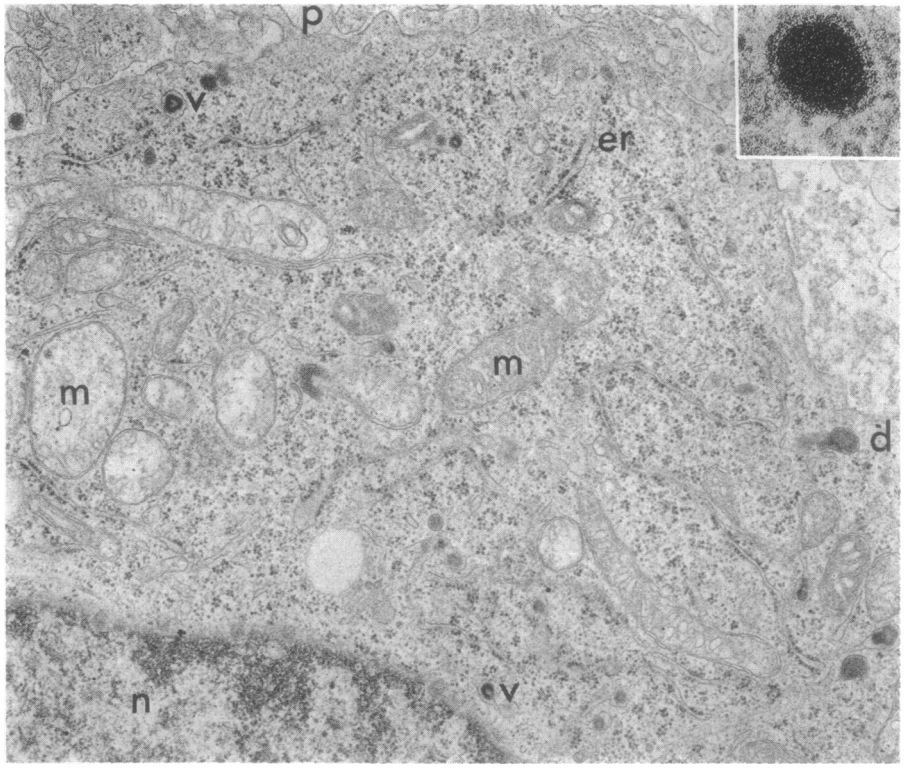

FIG. 4.—Round cells growing in suspension culture. Nucleus (*n*), two populations of mitochondria (*m*), small cell processes (*p*), and viral particles (*v*), which develop in association with the endoplasmic reticulum (*er*). 20,700× magnification. Insert: dense-core vesicle, 85,000× magnification.

latter two are the most frequently observed, and the neurofibrillar type becomes more prevalent in older cell populations. Mitochondria, microtubules, glycogen, and dense-core vesicles are always found in all three types of processes. Dense-core vesicles are found in much greater abundance than in the perikaryon. They are always included in a section of the end of a process if that end is, as frequently observed, in contact with another cell (Figs. 5 and 6). The dilations along the processes often include mitochondria and dense-core vesicles.

Catecholamine assay: Since the C1300 neoplasm originated outside the spinal cord, the tissue of origin might be sympathetic nerve. The synthesis of catecholamines from tyrosine is a property of sympathetic nervous tissue[18] and a sufficient criterion for the identification of sympathetic cells. Table 1 shows that the tissue culture line of C1300 accumulates dopamine derived from tyrosine. Thus these cells have tyrosine hydroxylase, the initial enzyme in the pathway of catecholamine synthesis.[18] Norepinephrine has also been observed, but the amount is small with respect to dopamine accumulated after a one-hour incubation with ^{3}H-tyrosine.

Discussion.—The following evidence suggests that the cloned tissue culture line of mouse tumor C1300 is a neuroblastoma which can be induced to differentiate into neurons. (1) Cells grown in suspension culture are highly ana-

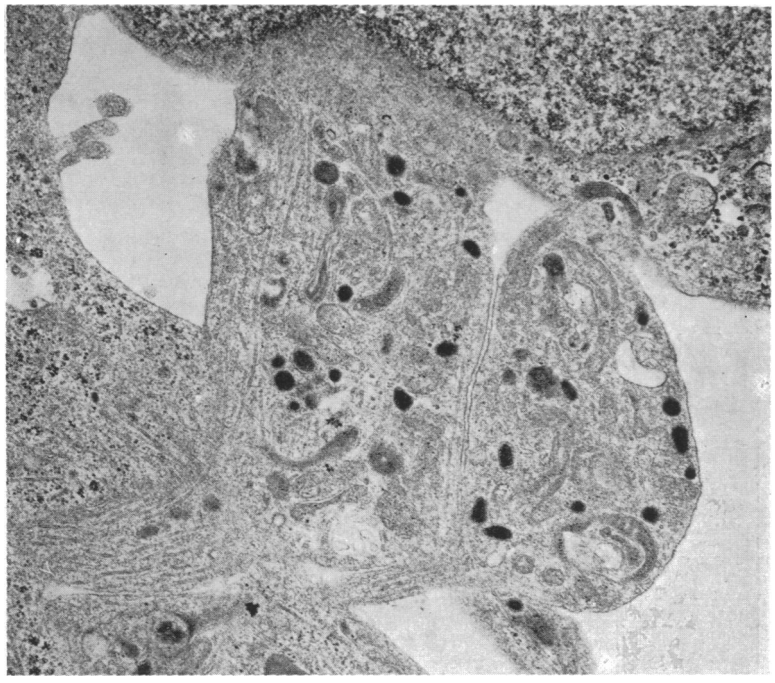

FIG. 5.—Attached cells. Endings of two processes pass under a cell to indent into the soma of another cell. Junctional membranes are sectioned obliquely and thus do not appear sharp. 13,000× magnification.

plastic; they have a high nuclear:cytoplasmic ratio and do not stain by Bodian procedure. (2) When these round cells are presented with a surface to which they can attach, they adhere and send out long processes. These cells are impregnable with Bodian stain and contain an extensive network of neurotubules which is not observed in the round cells. (3) Both round and differentiated cells accumulate dopamine derived from labeled tyrosine, indicating the presence of tyrosine hydroxylase, an enzyme confined to neurons and specialized tissues that contain norepinephrine.[18] Dense-core vesicles are correlated to the putative catecholamine neurotransmitter.

Although correlated with catecholamines, the content and function of the dense-core vesicles are unknown. They are larger than those thought to contain catecholamines and the clear area between the vesicle membrane and core is smaller.[19] The dense-core vesicles observed in C1300 are similar to some large neural vesicles of unknown content,[19] neurosecretory particles,[20] and vesicles in the epithelial side of the epithelial-neural junction in regenerating newt limb.[21] It is difficult to compare structures which have been prepared for microscopy in different ways. Furthermore, tissue-culture conditions can modify the size of the vesicles. For example, some dense-core vesicles in cultured satellite cells are enlarged to about twice their *in vivo* size.[16]

Evidence for synaptic contacts in this cell line is only suggestive. Cell contacts between processes are frequently observed by light microscopy, and the

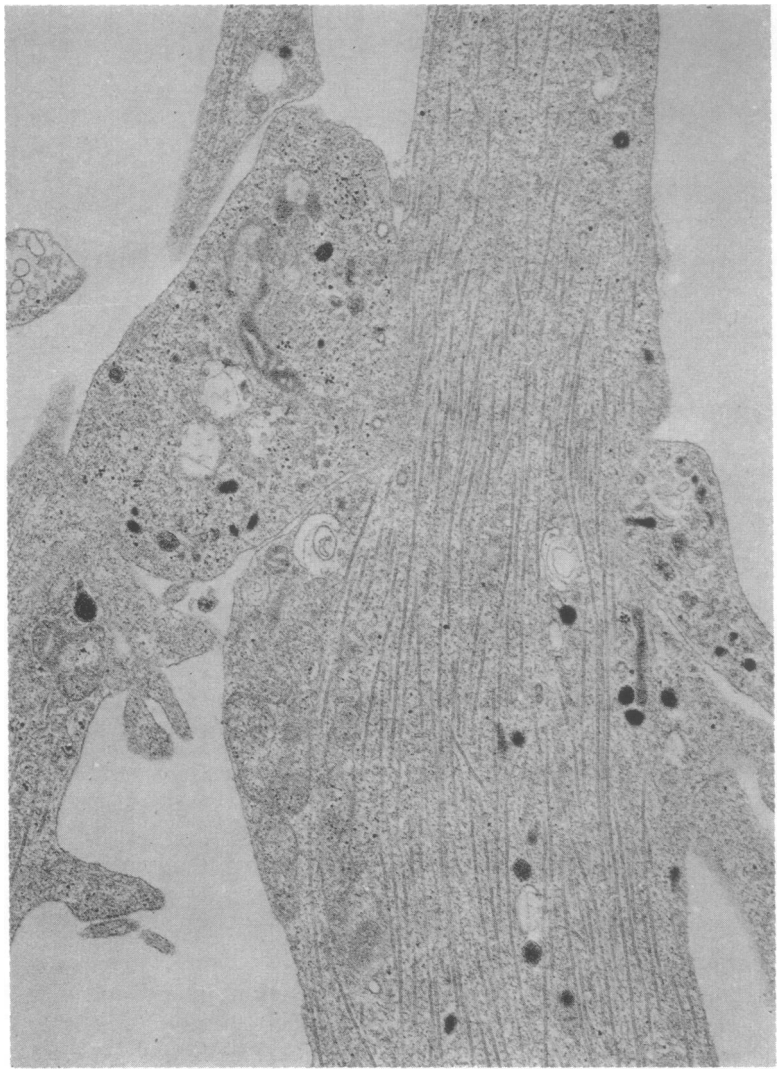

FIG. 6.—Attached cells. Endings of several processes terminate in indentations of a long process with many neurotubules. 13,800× magnification.

electron microscope reveals process endings in indentations of adjacent soma (Fig. 5) and other processes (Fig. 6). These areas of contact frequently contain a unilateral concentration of dense-core vesicles and mitochondria, but membrane specializations have not been demonstrated, possibly because of the difficulty in finding them in sections. These results do, however, indicate that the attached cells of C1300 may be able to form functional synapses under the proper culture conditions (see ref. 22).

Although the majority of the round cells can be induced to differentiate, the generic relationship between this rapidly dividing precursor cell and the infrequently mitotic end cell remains to be defined. The question of critical importance is, however, whether all of the end cells are identical. If the round cell

TABLE 1. *Cathecholamine assay of mouse tumor C1300 tissue culture lines.*

Source	Cpm recovered			
	Dopa	Dopamine	Norepinephrine	Origin
Round cells	226	5048	250	325
Attached cells	245	8530	350	265
Tyrosine standard	20	36	120	50
Dopa standard	4800	56	58	234
Dopamine standard	116	5240	253	141
Norepinephrine standard	424	311	3532	120
MOPC-21 cells	15	25	64	136

Approximately 10^6 cells of C1300 attached and round cells grown in suspension culture were labeled with ^{3}H-tyrosine for 1 hr. Mouse myeloma MOPC21 was labeled under the same conditions as a control. Cells were extracted and assayed for catecholamines as described in *Materials and Methods*. Tyrosine, dopa, dopamine, and norepinephrine were added to lysed unlabeled myeloma cells and carried through the same procedure as standards. The above data represent the radioactivity recovered from the thin layer plate.

gives rise to attached cells of different neurological specificities, presumably defined in terms of their synaptic membrane proteins,[23] an enormous amount of information could be obtained about the development of neuronal specification.

Note added in proof: It was shown by intracellular recording that the differentiated cells of this tissue culture line can be stimulated electrically or by the iontophoretic application of acetylcholine to generate action potentials of up to 85 mv. Although connections morphologically similar to chemical synapses appear to be present, no chemical transmission was observed. Some of the differentiated cell were, however, electrically coupled by way of their axons. (Drs. A. J. Harris and M. J. Dennis, manuscript in preparation.)

* Supported by a National Institutes of Health grant A105875, a training grant 5213 to M. C., and National Science Foundation grant G. B. 5462 to Dr. Tom Humphreys.

† Supported by U.S. Public Health Service fellowship no. 6-78110-24202-3.

[1] Cohn, M., in *Cold Spring Harbor Symposium on Quantitative Biology*, vol. 31 (1967), p. 211.

[2] Cohn, M., in *Differentiation and Immunology*, ed. K. B. Warren (New York: Academic Press, 1968), p. 1.

[3] Sato, G. H., and Y. Yasumura, *Trans. N.Y. Acad. Sci.*, **28**, 1063 (1966).

[4] Thompson, E. B., G. M. Tomkins, and J. F. Curran, these PROCEEDINGS, **56**, 296 (1966).

[5] Dunham, L. C., and H. L. Stewart, *J. Natl. Cancer Inst.*, **13**, 1299 (1953).

[6] Stewart, H. L., K. C. Snell, and L. C. Dunham, in *Atlas of Tumor Pathology* (Armed Forces Institute of Pathology, 1959), sect. XII, fascicle 40.

[7] Vogt, M., and R. Dulbecco, these PROCEEDINGS, **49**, 171 (1963).

[8] Sarkar, S., unpublished technique.

[9] Bunker, M. C., *Can. J. Genet. Cytol.*, **7**, 78 (1965).

[10] Humason, G. L., in *Animal Tissue Techniques* (San Francisco: W. H. Freedman and Co., 1967), p. 212.

[11] Williams, T., *Quart. J. Microscop. Sci.*, **103**, 155 (1962).

[12] Anton, A. H., and D. F. Sayre, *J. Pharmacol. Exptl. Therap.*, **138**, 360 (1962).

[13] Johnson, G. A., and S. J. Boukma, *Anal. Biochem.*, **18**, 143 (1967).

[14] Bernhard, W., "Cellular Injury," in *Ciba Foundation Symposium*, eds. de Reuck and Knightied (Boston: Little Brown and Co., 1964), p. 209.

[15] Bunge, R., M. Bunge, E. Peterson, and M. Murray, *J. Cell Biol.*, **32**, 439 (1967).

[16] Bunge, R., M. Bunge, and E. Peterson, *J. Cell Biol.*, **24**, 163 (1965).

[17] Lumsden, C., in *Structure and Function of Nervous Tissue*, ed. Bourne (New York: Academic Press, 1968), p. 67.

[18] Undenfriend, S., *Pharmacol. Rev.*, **18**, 43 (1966).

[19] Hokfelt, T., *Z. Zellforsch.*, **91**, 1 (1968).

[20] Bargmann, W., *Intern. Rev. Cytol.*, **19**, 183 (1966).

[21] Salpeter, M., *J. Morph.*, **117**, 201 (1965).

[22] Stephanelli, A., "Growth of the Nervous Tissue," in *Ciba Foundation Symposum*, eds. Wolstenholme and O'Conner (Boston: Little, Brown and Co., 1968), p. 31.

[23] Cohn, M., in *Ciba Foundation Symposium*, in press.