DISTINCTIVE CHARACTERISTICS OF THE SYMPATHICOBLASTOMA CULTIVATED IN VITRO

A Method for Prompt Diagnosis *

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The tumor here designated *sympathicoblastoma,* sometimes called *neuroblastoma,* is a highly malignant neoplasm composed of small sympathicoblasts which are often undifferentiated but sometimes arrange themselves in the form of rosettes or pseudorosettes. It is generally regarded as being derived from sympathetic nervous tissue, usually, although not always, arising in connection with a sympathetic ganglion in the mediastinal or retroperitoneal regions, or in the suprarenal medulla. Its chief incidence is among infants and young children, although it may be found in adults of various ages.

The observations here reported were gleaned in the course of a long-term study of the form and behavior of human tumors *in vitro,* during which eight sympathicoblastomas were investigated. These eight tumors behaved in a uniformly distinctive manner *in vitro;* their differences from other tumors with which they are likely to be confused clinically or histologically were so marked that we were led to use tissue culture as a diagnostic method for this tumor. By this means the diagnosis can now be made with a high degree of certainty in 24 hours or less.

Case Histories

Case 1

W. H. (S.P. 67384), an American boy, 3 years old, suffered for 3 months with symptoms due to metastases in the skull and elsewhere. When examined there were retroperitoneal masses and evidences of metastases throughout the skeleton, in the orbit, the scalp, and the abdominal wall. He died 6 weeks after admission to the hospital. Specimen taken for biopsy of one of the tumors in the scalp showed masses of undifferentiated sympathicoblasts with much hemorrhage and necrosis and no evidence of rosette or pseudorosette formation.

Case 2

Baby M (S.P. 73120), a male child of German-American parentage, was born with a large globular mass in the outer part of the left leg near the knee. After biopsy a mid-thigh amputation was done at the age of 1 month. Eighteen months later a retroperitoneal mass near the right kidney was explored and examined by biopsy. Roentgenotherapy was used over a period of 2 months but did not prevent the appearance of other metastases. When the child died at the age of 3⅓ years, autopsy showed metastases in the liver, right kidney, lungs, pleura, mesentery,

* Received for publication, June 13, 1946.

vertebrae, skull and humerus, from the retroperitoneal tumor. Microscopically, the tumor consisted of undifferentiated sympathicoblasts packed together tightly without the formation of either true or pseudorosettes.

Case 3

R. D. (S.P. 85876), an American male child, 12 months old, was found to have fluctuant swellings in both temporal regions and in the right thigh 1 week before admission. Tissue was taken for biopsy from the mass in the thigh and the child died 3 months later with extensive bone metastases and nodules in the abdominal cavity, pleurae, left lung, liver, and right testis. There was no autopsy but the primary site was believed to be retroperitoneal. Microscopically, the tumor was made up of sympathicoblasts which in some areas surrounded irregular spaces partly filled with tangled neurites. This represented partial differentiation. No perfectly rounded pseudorosettes were found.

Case 4

C. C. (S.P. 88130), a colored man, 33 years old, had noted a progressively increasing solitary swelling in the left groin. The mass was discrete, measured 8 by 5 cm., and was examined by biopsy. He received roentgenotherapy over a 1-month period. The field was 15 by 15 cm., later reduced to 10 by 10 cm. The factors were 190 kv., 10 ma., target skin distance 50, filter 1 mm. Cu, and a total dose of 3000 r. was given. As a result the tumor disappeared entirely and he was well 18 months after biopsy. Microscopically, the tumor was composed of solid masses of sympathicoblasts with extensive necrosis. Occasional pseudorosettes were present.

Case 5

H. F. (S.P. 88959), a boy, 6 years old, of Hungarian Jewish descent, complained of pain in the thoracic vertebrae for 6 months. Roentgenograms showed a mediastinal mass which at operation proved to be a lobulated, smooth, rubbery tumor, projecting into the right pleural cavity and pushing the trachea forward and the arch of the azygos vein downwards. It was impossible to remove the neoplasm completely and the child died 3½ months later. Microscopically, the tumor was made up of sympathicoblasts which formed a considerable number of pseudorosettes, and was extensively necrotic. It was found in a mediastinal node and involved a partly calcified sympathetic ganglion.

Case 6

R. A. (S.P. 90971), an American, 59 years old, developed nocturia and dysuria which persisted 6 months after prostatectomy elsewhere. It was reported that the prostate showed no tumor. At exploration a pelvic retroperitoneal mass was found. He died 3 days later, and at autopsy the tumor involved both seminal vesicles, the bladder wall, the prostatic bed, and had metastasized to the pelvic and retroperitoneal nodes. Microscopically, the biopsy section showed a tumor composed of solid masses and strands of rather well preserved, rounded, and sometimes slightly elongated hyperchromatic cells which showed no definite differentiation. The autopsy material showed the occasional formation of pseudorosettes and was very suggestive of sympathicoblastoma.

Case 7

D. F. (S.P. 93335), was an American male child, 16 months old. During a routine physical examination hard masses were felt in the umbilical region and the left flank. At exploration there was a retroperitoneal mass which extended from behind the cecum upward, pushing the stomach forward and the transverse colon downward. He was failing rapidly when he left the hospital 18 days later. Sections

taken for biopsy showed a tumor of sympathicoblasts, most of which were necrotic. In a few places pseudorosettes were present.

Case 8

A. A. (S.P. 97260), was an Italian-American female child, 13 months old. Following an attack of pneumonia, she was brought to the hospital with signs of fluid in the chest. When this was not confirmed an exploratory operation was performed and tissue was taken for biopsy from an inoperable mediastinal growth. She received some roentgenotherapy but the tumor did not regress and she was transferred to another hospital at the parents' request. Microscopically, the tumor was made up of sympathicoblasts which occasionally formed pseudorosettes and even immature ganglion cells. A Cajal impregnation showed many delicate neurites among the tumor cells. Occasionally one appeared to originate in a tumor cell.

Material and Methods

The material for explantation was obtained in the course of biopsy of either primary or metastatic sites. Both are equally satisfactory, so long as necrotic areas are avoided. Even the existence of inflammatory tissue associated with necrosis and hemorrhage does not vitiate the results if a fair number of viable tumor cells remain in the specimen used for explantation.

The tissue cultures were handled by the Maximow lying-drop method, modified slightly for our purposes (Murray and Stout, 1942). In the tumor the sympathicoblasts were grouped in solid masses with little supporting framework, thus forming a very friable tissue which broke up readily when cut or otherwise mechanically disturbed. Consequently, in setting out the cultures many fragments of various sizes were scattered through the medium. These small aggregates of pure tumor cells, uncomplicated by the presence of fibrous tissue, have been found to be more satisfactory objects for observation than the main explant.

Diagnosis can be made from the living cultures, but for records and for more detailed study permanent preparations are desirable. The reduced silver method of Bodian (1936), adapted to the cultures as whole mounts, is simple and reliable and furnishes brilliant contrast between neurites and background. For this it is best to fix the cultures in Bouin's fluid for ½ to 1 hour, and ripen in 80 per cent alcohol, with several changes, for at least 2 weeks before treating with silver. The following steps must be controlled with the microscope, but the approximate timing is as follows: protargol solution, 20 to 24 hours; hydroquinone, etc., 5 to 10 minutes; gold chloride, 1 minute; oxalic acid, 1 minute; sodium thiosulfate, ½ minute. Silver impregnation is the only histologic method in our experience which does justice to the finer structures of these sympathicoblasts (see Figures 3 to 5 and 7 to 11), but fairly satisfactory results can be obtained with phospho-

tungstic acid hematoxylin following Zenker fixation and omitting the Mallory bleach.

Characteristics of the Tumor in Vitro

The sympathicoblasts do not migrate to any significant extent, but within 24 hours some of the small round or oval cells cohering in the clumps scattered about the clotted plasma have produced neurites of varying lengths, easily recognizable, and distinct from any form of outgrowth evolved *in vitro* by nonnervous tissues (Fig. 2). These neuroblasts, remaining *in situ,* project filamentous processes which are sometimes beaded, and which grow in the manner of an axone, with pseudopodial ends. The cells are usually monopolar, although sometimes bipolar and very occasionally multipolar. Within 48 hours the neurites have become longer and more numerous, and sometimes have begun to branch (Figs. 1 and 11). In favorable material this branching may become very elaborate as time goes on, so that after a fortnight's cultivation an isolated clump of cells may produce structures resembling a plexus (Figs. 8 and 10).

However, the sympathicoblasts as well as their newly grown neurites are very fragile, and overly responsive to handling; consequently it is best to confine the washing time to a maximum of 5 or 10 minutes, 2 or 3 times per week, and to keep the pH of the washing saline solution below 7.4. The viability of these cells *in vitro* is very variable and is probably connected with the original location of the explant within the tumor, whether it comes from a poorly nourished or moribund area or from a rapidly growing margin.

Necrosis among the cells composing the outer rim of a clump is so common as to be characteristic of this neoplasm *in vitro* (Figs. 4, 6, 8, and 11). This is not at all the case in clumps of cells derived from lymphosarcomas cultivated under the same conditions. Surprisingly, it reverses the pattern of necrosis which is commonly observed in sections of the sympathicoblastoma *in vivo,* in which the best preserved areas tend to lie close to the blood vessels and the pyknotic and necrotic regions are farther from the surfaces at which food, oxygen, and wastes may be exchanged. Such a pattern of necrosis as we observe *in vitro* leads to the inference that our tissue culture medium is not ideal for this material.

The cell body of the sympathicoblast is often about the size of a lymphocyte, though size may vary within a single tumor. The tumor cells may be larger, however, as in our case 7, in which they had two to three times the diameter of a lymphocyte. In this instance there was

considerable variation in size, and some rather large multipolar cells resembling more mature ganglion cells were present. Tumors have been reported (Stout, 1947) which combine ganglioneuromatous areas with others characteristic of the sympathicoblastoma, but we have not obtained one of these for cultivation. The sympathicoblastoma *in vitro* is entirely different from the benign ganglioneuroma, the behavior of which is essentially similar to that of nonneoplastic adult sympathetic ganglion cells (a description of which will be published shortly). These are large multipolar cells, which do not form tissues but remain isolated one from another and are relatively slow to grow and migrate.

Although the production of neurites is the most conspicuous trait of sympathicoblasts *in vitro,* these tumor cells are also prone to adopt epithelial formations. After 4 to 5 days *in vitro,* tongues or cords of cells appear, usually ending in a filamentous process (Figs 6 and 8). Cell boundaries in such an outgrowth are often indistinct, giving the whole mass the appearance of a syncytium. Nuclei are sometimes lobate or kidney-shaped, and since mitotic figures are only very rarely observed among them *in vitro* it is assumed that these nuclei may multiply by direct division. Within a week or more, flat membranes may be seen in favorable cultures; these differ from typical epithelial membranes in that they frequently develop dendritic outgrowths (Fig. 7). The sympathicoblastoma thus appears to be related to the neuro-epithelioma, one example of which we have been able to study in tissue culture (Stout and Murray, 1942). This exceedingly undifferentiated tumor, arising in the radial nerve, produced membranous sheets of epithelium when cultivated, but no neurites. The neuro-epithelioma might possibly be compared to the neural plate stage in nervous development. It seems probable that the sympathicoblastoma typifies a state of differentiation similar to that found in the neural tube stage of the embryo, since the tumor cells are small, capable of forming membranes *in vitro* (as does columnar epithelium from other germ layers), and are unaccompanied by satellite cells of any description. The rosette formations found in sections of neuroblastomas in general have been thought to represent cross sections of neural tubes formed in these neoplasms.

By the fourth day *in vitro,* fibroblasts are fairly numerous. At the end of 1 week, neurites and other evidences of the sympathicoblasts in the explant are usually obscured by the fibroblastic growth, but the islands of pure neuroblasts scattered throughout the clot may remain distinct for at least 1 month (Fig. 5), the duration of our observations.

Explants of these tumors frequently contain considerable numbers

of lymphocytes and other blood elements, which migrate out into the clot. Differences in form are usually sufficient to distinguish these from the neoplastic cells, but the distinction may be heightened by vital staining with basic dyes. The living sympathicoblasts stain quickly with neutral red applied supravitally in a dilution of 1:10,000. The stain may appear in the cell as a compact group of small granules occupying a juxtanuclear position, or as a few scattered granules in a diffusely pink cytoplasm. In either case the sympathicoblast can be distinguished readily from the normal or neoplastic lymphocyte, which, although it may aggregate in clumps, does not take up neutral red at all. The addition of a dilute solution of Janus green B to the neutral red solution serves to emphasize the distinction further, since the sympathicoblasts contain few or no Janus green staining particles, while the lymphocytes take up the dye readily.

Discussion and Summary

The cultivation of the sympathicoblastoma *in vitro* provides a more rapid as well as a more certain means of identifying this tumor than the customary histologic section methods. Frozen sections are often unsatisfactory; they are particularly unreliable in distinguishing the sympathicoblastoma from members of the lymphosarcoma group or from the small-celled carcinomas.

Small fragments of the tumor, isolated in the medium of clotted plasma, can be counted on to produce neurites within 24 hours. This faculty, coupled with the tendency to marginal necrosis and the affinity of the tumor cells for neutral red applied supravitally, provides a very satisfactory means of differential diagnosis between this tumor of nervous origin and the lymphosarcomas and Ewing's tumor with which it is sometimes confused clinically. As cultivation is continued, from 2 to 5 days, the whole picture becomes increasingly clear and distinctive. There is great advantage in being able to see the whole cell with all its processes and extensions, as well as the characteristic patterns in which the cells group themselves. When this can be done, the sympathicoblasts present a very different appearance from lymphocytes or lymphoblasts or from small carcinoma cells.

Of the eight tumors which we have studied *in vitro,* two have occurred in adults, aged 33 (case 4) and 59 (case 6) years, respectively. Because of their equivocal histologic features these could not be accepted as unquestionable sympathicoblastomas without the examination *in vitro*.

We are indebted to Mrs. I. A. Pogogeff for technical assistance in the handling of the cultures and to Mr. W. I. O'Neill for the photographs.

BIBLIOGRAPHY

Bodian, D. A new method for staining nerve fibers and nerve endings in mounted paraffin sections. *Anat. Rec.*, 1936, **65**, 89–97.

Murray, M. R., and Stout, A. P. Demonstration of the formation of reticulin by Schwannian tumor cells *in vitro*. *Am. J. Path.*, 1942, **18**, 585–593.

Stout, A. P. Ganglioneuroma of the sympathetic nervous system. *Surg., Gynec. and Obst.*, 1947, **84**, 101–110.

Stout, A. P., and Murray, M. R. Neuroepithelioma of the radial nerve, with a study of its behaviour *in vitro*. *Rev. canad. de biol.*, 1942, **1**, 651–659.

[*Illustrations follow*]

DESCRIPTION OF PLATES

PLATE 72

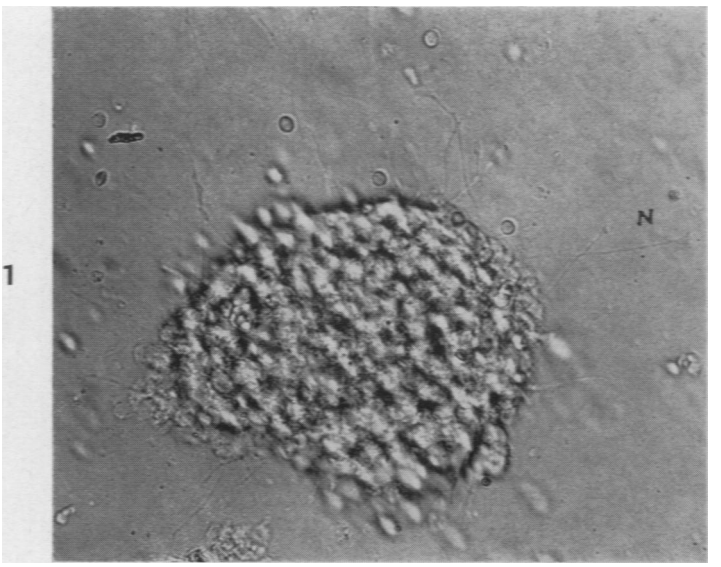
FIG. 1. Case 8. Living isolated clump of sympathicoblasts, with neurites (N) having pseudopodial ends; 48 hours *in vitro*. × 290.

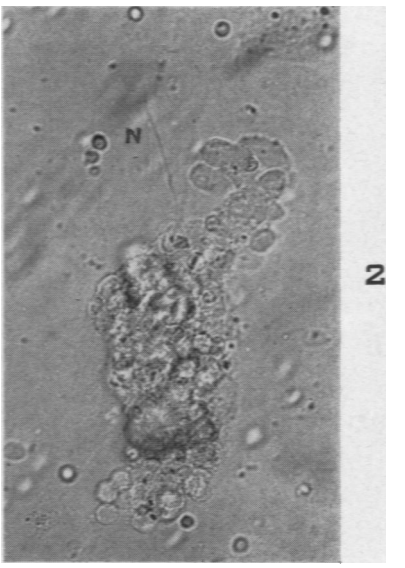
FIG. 2. Case 8. Living clump of sympathicoblasts with neurites (N); 24 hours *in vitro*. × 290.

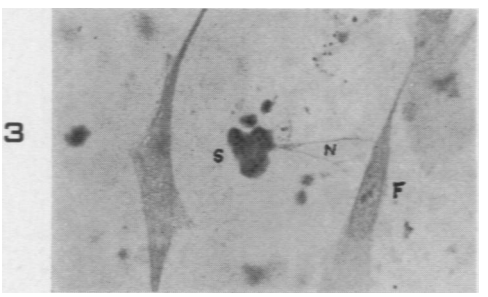
FIG. 3. Case 4. Small clump of sympathicoblasts (S) with neurites (N); F indicates a fibroblast; 10 days *in vitro*. Bodian method. × 380.

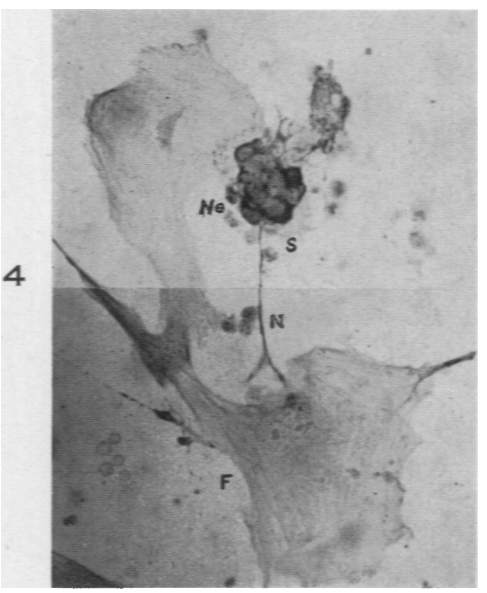
FIG. 4. Case 4. Clump of sympathicoblasts (S) with branching neurite (N), and marginal necrotic area (Ne). Flattened fibroblasts (F) are shown for comparison of size; 10 days *in vitro*. Bodian method. × 380.

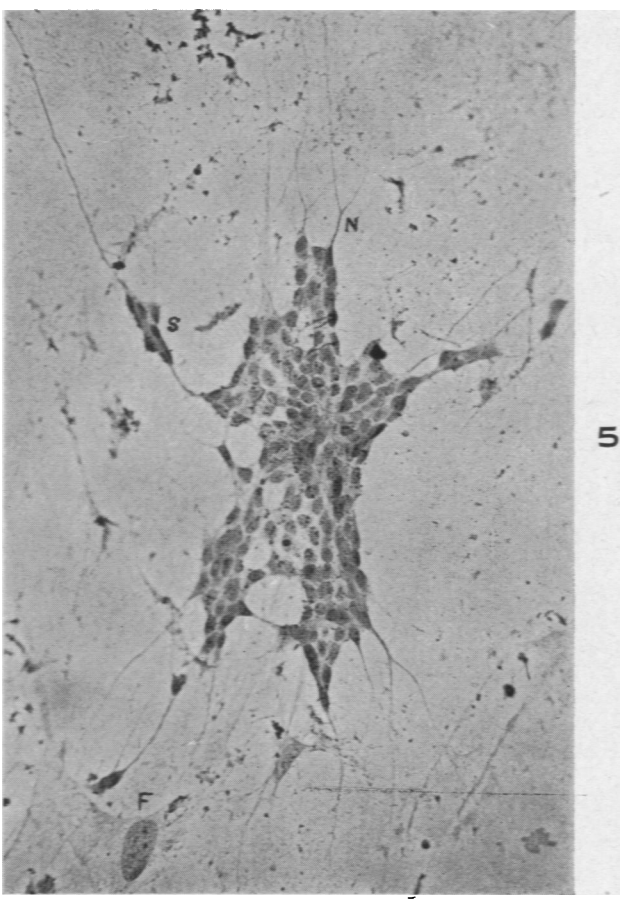
FIG. 5. Case 5. Isolated clump of sympathicoblasts (S) with neurites (N) surviving 30 days. F is a fibroblast. Bodian method. × 255.











Murray and Stout

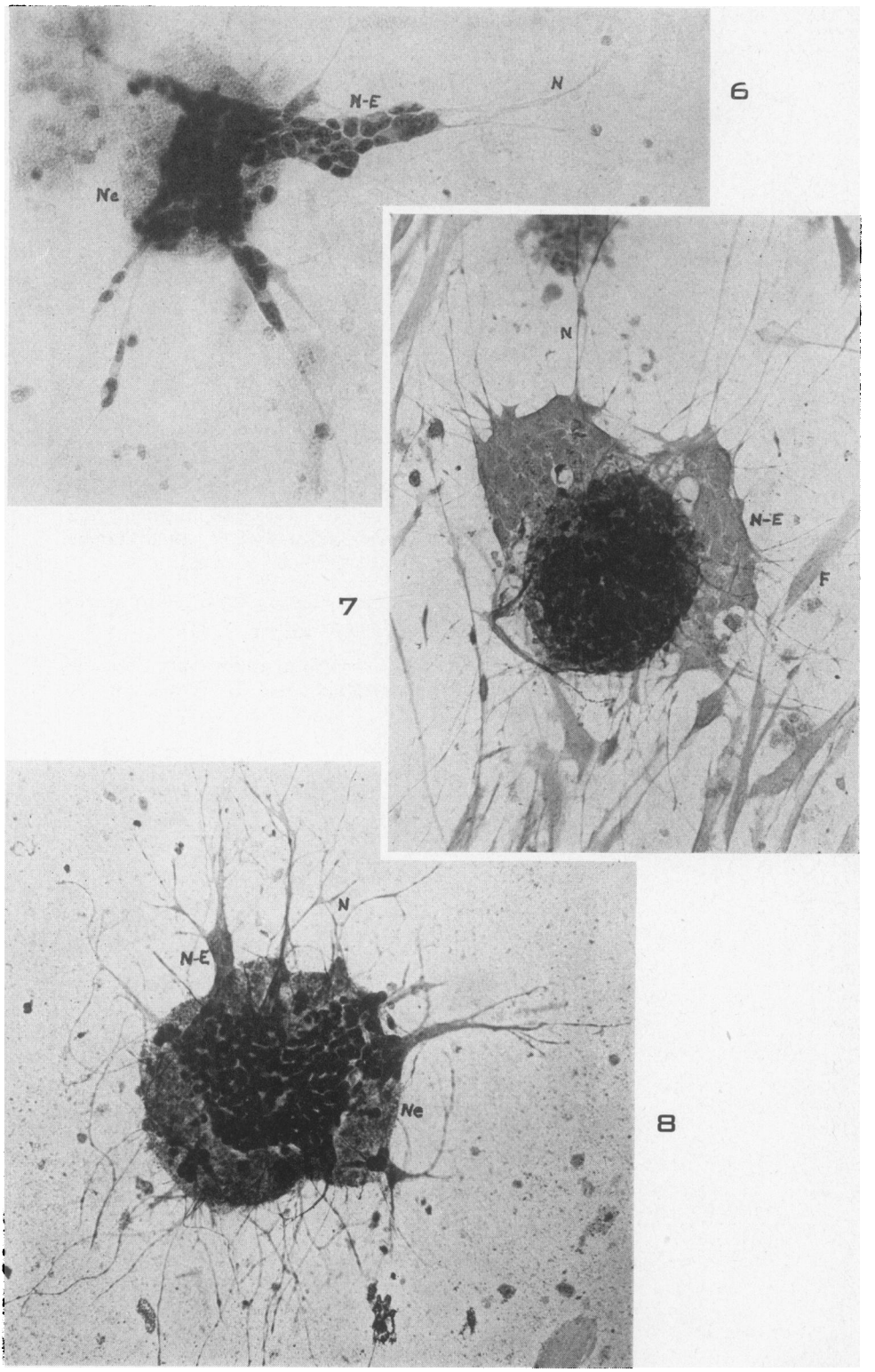
Sympathicoblastoma Cultivated *in Vitro*

PLATE 73

FIG. 6. Case 3. Isolated clump with projecting tongues of neuro-epithelium (N-E) ending in neurites (N), and marginal necrosis (Ne); 5 days *in vitro*. Phosphotungstic acid-hematoxylin stain. × 305.

FIG. 7. Case 3. Sympathicoblasts growing as a flat epithelium (N-E) with neurites (N). Fibroblasts (F). 17 days *in vitro*. Bodian method. × 245.

FIG. 8. Case 3. Clump of sympathicoblasts with pyknotic center, necrotic margin (Ne), epithelial tongues (N-E), and many branched and beaded neurites (N); 5 days *in vitro*. Bodian method. × 220.



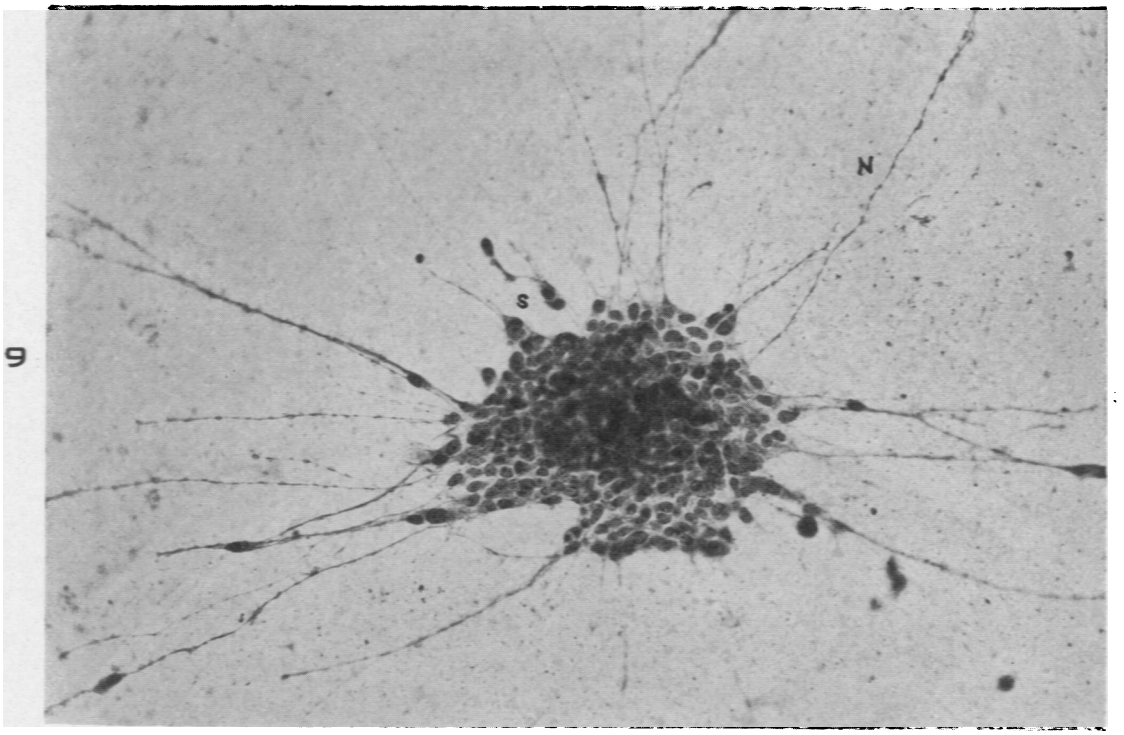
Murray and Stout Sympathicoblastoma Cultivated *in Vitro*

PLATE 74

FIG. 9. Case 5. Membranous clump of sympathicoblasts (S) with very long, beaded neurites (N); 7 days *in vitro*. Bodian method. × 245.

FIG. 10. Case 3. Neurites (N) tending toward plexus formation; necrotic material (N-E); young fibroblasts (F); 17 days *in vitro*. Bodian method. × 140.

FIG. 11. Case 6. Clumps of sympathicoblasts (S), with necrotic margins (Ne) and neurites (N) at 48 hours. Bodian method. × 360.



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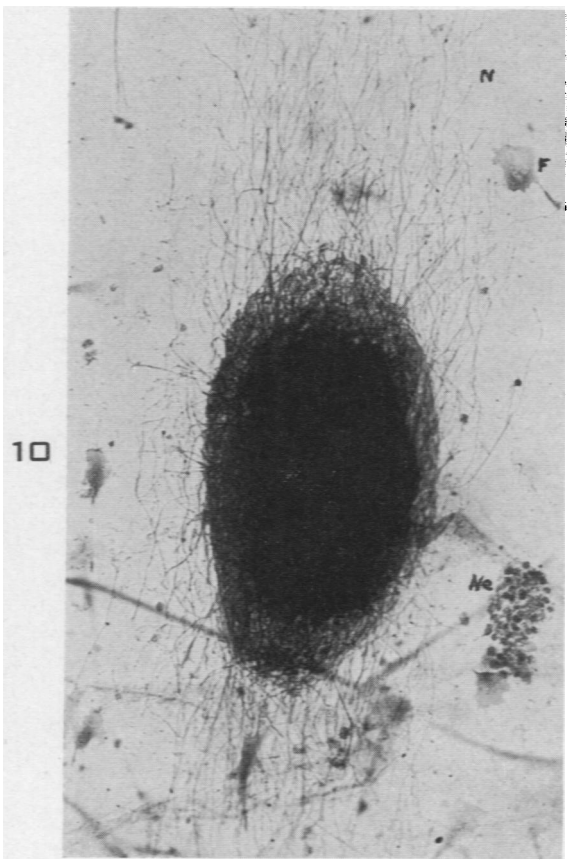

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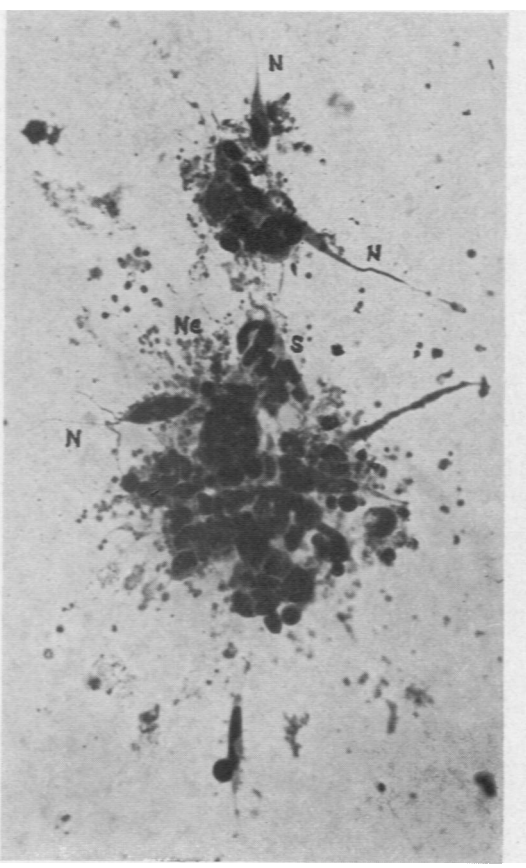

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