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17. After the cover slip was removed with liquid nitrogen, slides were passed through absolute alcohol, ether-alcohol, and chloroform-methanol (for removal of lipids), and then hydrated to saline. Slides were then placed in 0.2*N* HCl (20 minutes, cold) for histone removal, in 10 percent TCA (90°C, 20 minutes), dipped in 70 percent alcohol and 5 percent TCA, and rinsed under running tap water. Slides were fixed for 5 minutes in 10 percent neutral formalin, rinsed in saline, and processed for autoradiography as follows: slides were dipped individually in 2 to 5 dilution of Ilford L-4 nuclear track emulsion, dried overnight, and stored in refrigerator. Slides were developed in D-19 (5 minutes), rinsed, and fixed in hypo. Photomicrographs were taken on the Leitz Ortho-Lux microscope with the Ortho-Mat camera, using Adox KB-14 film.
18. Supported by the Alma Toorock Fund. W.B.B. is the recipient of a CDA grant No. CA 4-2461-01. We thank Dr. A. Gellhorn for suggestions and encouragement, Dr. A. Deitch for assistance with the photomicroscope, Mrs. J. Spivack for photographic processing, and Miss G. Nette and Mrs. S. Beck for technical assistance.

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Axonal Outgrowth and Cell Migration in vitro from Nervous System of Cockroach Embryos

Abstract. *Brain and ganglia of embryonic* Periplaneta americana *were grown for 2 to 3 weeks in a chemically defined medium. Nerve fibers sprouted vigorously from intact brains and ganglia, interconnecting nerve cultures, and adjacent cultures of the digestive tract. Glia, tracheal, and nerve cells were present in large numbers in the migratory zone surrounding the explants.*

We report the successful in vitro culture of the brain, the ganglia, and the gut of embryonic cockroaches. Success in this case hinged on the discovery of a suitable culture medium consisting of five parts of Schneider insect solution (Grand Island Biological Company) to which was added four parts of Eagle basal medium (Microbiological Associates). The two solutions were mixed just prior to use.

Roach embryos were obtained from oöthecae that were collected daily and incubated at 29°C to obtain embryos that were 16 to 18 days old. Brains, as well as subesophageal and thoracic ganglia and the entire digestive tract, were surgically removed under aseptic conditions and gently pressed onto a small cover slip. The latter was placed in a culture chamber consisting of glass vessels (internal diameter, 13 mm; height, 7 mm) containing 0.18 ml of the above-mentioned medium. Five to six culture chambers were placed in each petri dish and stored in a desiccator containing 95 percent air and 5 percent carbon dioxide. Incubation was at 29°C in a water-saturated atmosphere, attained by placing wet cotton in the desiccator and in the petri dish. The cultures were examined daily with an inverted microscope. After 1 or 2 weeks, they were studied and photographed by Zeiss-Nomarski optics and then stained with toluidin blue or by a modified Cajal technique (*1*).

Up to 95 percent of the explants showed substantial development, including the outgrowth of axons and the centrifugal migration of other cell types. The following description is based on a study of 500 cultures, each containing 5 to 6 explants.

By the end of the 2nd day of culture, the brains or ganglia showed axonal

Fig. 1. Photomicrograph of living culture taken with the interference differential (Zeiss-Nomarski) microscope, showing nerve cells in the migratory zone around brain explant from a 16-day embryo 12 days in vitro. Large dilatations are evident on the axon of two nerve fibers. Arrow points to junction of the axon of one nerve cell with another nerve fiber; *n*, nerve cells (× 300). Fig. 2. Silver-stained (Cajal-DeCastro) preparation of the migratory area around brain explant from an 18-day embryo 9 days in vitro. Nerve fibers are encrusted on the surface of a large glial cell. To the left, arrows point to a fragment of glial cytoplasm adhering to nerve fibers (× 640). Fig. 3. Photomicrograph of living culture taken with the interference differential (Zeiss-Normarski) microscope, showing migratory area around brain explant of a 16-day embryo 15 days in vitro; *n*, nerve cells (× 256).

outgrowth that continued during the life of the culture. Some axons consisted of tiny filaments that joined with others to form large fiber bundles which, in turn, often split again into thin filaments. Other outgrowths consisted of large fiber bundles that separated into thin fibers whose growing tips spread fanlike along the glass surface (see cover). The outgrowing axons branched repeatedly and formed connections with each other and with the outgrowth of other explants. The ending of an axon on another axon was characterized by a small enlargement reminiscent of a synapse. This interpretation is supported by preliminary microscopic studies.

The size of the individual axons varied enormously. It was possible to see, in the same outgrowing zone, barely visible filaments alongside large axons that, after fixation, showed intense Cajal staining. Round or fusiform dilatations were seen in most of the large fibers (Fig. 1). The beaded character of these insect axons is in accord with the histological studies reported by Wigglesworth (*2*).

In addition to axons, the outgrowth from ganglia included three other recognizable cell types: glia, tracheoblasts, and neurons. The glial cells were most numerous and consisted of several different types. Particularly noteworthy were giant flattened cells that formed a carpetlike layer around the explants and showed remarkable affinity for the axons (Fig. 2). Smaller glial cells enveloped the axons in the manner of the Schwann cells of vertebrates. Another type of glial cell was characterized by intracellular vacuoles and glycogen inclusions.

The outgrowing zone also contained a second distinct cell type consisting of round cells that showed a large vacuole that often displaced the surrounding cytoplasm into a thin periodic acid–Schiff positive ring. We believe these cells to be tracheoblasts—an interpretation supported by the large number of these cells surrounding gut explants.

The third cell type consisted of neurons (Figs. 1 and 3). In living cultures they were easily distinguished by their oval cell bodies, their prominent nucleolus, and the presence of an axon that could often be followed for a considerable distance to its termination on other axons (Fig.1). The identity of these cells as neurons has been confirmed by electron microscopic studies which demonstrated the presence of neurofilaments. The exit of neurons from the ganglia raises the question whether the nerve cells of invertebrates, unlike those of vertebrates, may be endowed with the ability to move. Our studies suggest an alternative interpretation: namely, that, as the glial cells migrate from the explants, the tightly adhering neurons are passively pulled out.

As illustrated in Fig. 4, explants separated by 1 or 1.5 mm developed rich interconnections, both cellular and axonal. This seems to be the direct result of the centrifugal migration of cells and the outgrowth of axons. A similar thigmotropic effect was described by Marks and co-workers between combined transplants of leg regenerates and thoracic ganglia (*3*). It has not yet been determined whether directed outgrowth can be provoked by explants of tissues other than ganglia and gut.

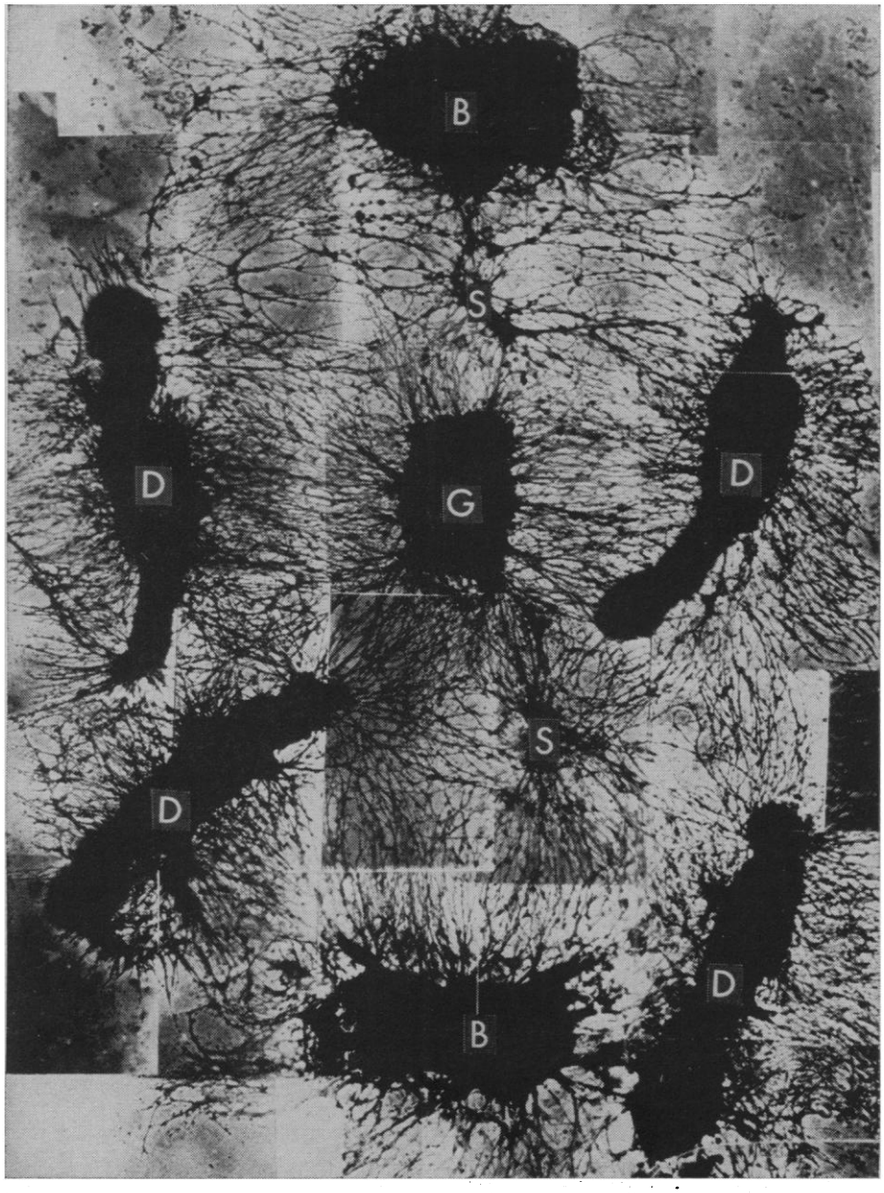

Fig. 4. Array of explants of intact brains (*B*), three fused thoracic ganglia (*G*), hemisection subesophageal ganglia (*S*), and intact digestive tracts (*D*) from a 17-day embryo. The explants were fixed after 9 days of culture and stained with the silver Cajal-DeCastro technique. Fibrillar and cellular bridges interconnect facing explants (× 47).

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