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TISSUE CULTURE OF THE NERVOUS SYSTEM

Edited by
GORDON SATO

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Preface

The impetus for compiling this book was the recent development of culture strains of neuroblastoma and glial cells and the immediate and enthusiastic way they have been taken up as model systems. After the first sudden rush of activity, it seems appropriate to pause, to assess progress, and to contemplate the future contributions that may be possible using these culture techniques.

Long before the advent of established strains, cultures of nervous tissue had already contributed to neurobiology. Ross Harrison, in 1906, in a single experimental series, established tissue culture as a promising new technique in cell biology and settled the Golgi–Cajal controversy as to whether axonic processes originated as outgrowths from the cell body or were formed first in the intercellular spaces and were later connected to the cell body. Harrison observed process growth from nerve cells in cultures, thus settling the matter in favor of Cajal. Of great importance to neurobiology is the discovery by Rita Levi-Montalcini of nerve growth factor. Cultures of spinal ganglia played a major role in the discovery, isolation, and characterization of the factor (Levi-Montalcini *et al.*, 1954). In my opinion, this discovery, although very well known, has not yet been adequately recognized for its germinal influence on neurobiology and embryology.

Progress since the advent of clonal cultures has been more modest. I would like to cite two pieces of work which emphasize the technical advantages of these cultures. The culture techniques have separated the neural and glial elements of nervous tissue, have freed the cells from restrictions found *in vivo*, and have enormously increased the ease with which these cells can be manipulated. To illustrate the first point, Jean de Vellis and coworkers demonstrated the stimulation of glycerol phosphate dehydrogenase in rat brain by adrenocortical steroids. It was not known whether this reaction was taking place in neurons or glia or both. Culture experiments have shown that glia respond and neurons or neuroblastoma cells do not (de Vellis and Inglish, 1969). The second point is illustrated with work on vitamin B_{12}

deficiency. Nervous tissue along with hemopoetic tissue is adversly affected by vitamin B_{12} deficiency, and the biochemical nature of the lesion has escaped elucidation. If neuroblastoma or glioma cultures are made vitamin B_{12} deficient, they can accumulate large quantities of odd chain fatty acids in their plasma membranes. Cultures of nervous tissue origin are more prone to accumulate odd chain fatty acids than cultures from other tissues. This phenomenon was more readily discovered in culture than in the whole animal because the whole animal probably cannot tolerate this substitution to any great extent and would be dead before levels of odd chain fatty acids became easily detectable. Cells in culture, freed from the responsibility of performing sensitive functions vital to life, can tolerate substitution to a much greater extent (Barley *et al.*, 1972). This example illustrates to an extreme degree the ease of manipulation in culture. Here membrane constituents can be varied at will and correlated with physiological function.

Given the technical advantages of these cultures, what can we expect in the future? Are problems such as the regulation of synapse formation, the mechanism of neurotransmitter substances, the biochemistry of action-potential generation, and the function of glia amenable to culture experimentation? What about higher cognitive processes? Are these even in the realm of biochemistry and cell biology? These questions are the ultimate concern of our contributors. Although all share in common the *in vitro* culture methods, their individual approaches and concerns are, appropriately for this stage, diverse.

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Chapter 1

Long-Term Cultures of Embryonic and Mature Insect Nervous and Neuroendocrine Systems

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I. INTRODUCTION

More than a half century of extensive work on the vertebrate nervous system cultured *in vitro* under different experimental conditions has brought to light the merits and the limitations of these techniques. While a considerable amount of information has been gathered on growth and differentiation of nerve cells, on axonal growth, on the relationship between glial and nerve cells, and, recently, also on bioelectrical properties of neuronal circuits *in vitro* (Crain and Peterson, 1964, 1967; Crain *et al.*, 1970), little has been learned concerning the organization of nerve cells at the supracellular level and no attempts have been made to explore, with the aid of these techniques, the problem of neuronal specificity and the building of wiring circuits between nerve cell populations and between nerve cells and their end organs. The reasons which suggested restriction of the study to only a few neurobiological

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problems are numerous. To mention only some of the limiting factors, we remind the reader that this system in vertebrates is from its very inception a highly organized system and cannot operate when submitted to dissociation into small fragments, with each one cultured alone or in proximity to other parts of the same system or of nonnervous structures. Disruption of the continuity of the neuraxis and destruction of the blood capillary network, which permeates the entire system and provides the nutrition and blood supply of individual nerve units, are most damaging factors which cannot possibly be mitigated by any technical skill or ingenious device. In order to permit survival if not proper function of the nerve cell populations, which must rely on diffusion rather than on blood vascular channels, the explants must be reduced to what has been defined as "the critical cubic millimeter" (Lumsden, 1968). The fragments of the nervous system undergo, as a rule, flattening and thinning in long-term cultures. This condition favors exchanges with the medium, and nerve cells located at the periphery of the explants survive reasonably well, but cell-to-cell interconnections through nerve circuits are grossly altered and nothing can be learned about their normal function, leaving aside the more complex problem of the operation of neuronal circuits between distant nuclei and between these and their end organs.

In the hope of finding a more suitable object for the *in vitro* study of the organization and functional properties of the nervous system, we started 4 years ago a research project on the insect nervous system. For reasons to be mentioned in the following section, the object of choice was the cockroach, *Periplaneta americana*. A synthetic medium devised in our laboratories proved to support not only long-term survival of explants of brain, ganglia, and the neuroendocrine system of this insect but also nerve cell migration and vigorous axonal growth from nerve cells located in intact explants or migrated out into the medium. Ganglia dissected out from cockroach embryos became interconnected by cable-like fiber bundles similar to the connectives which form in ontogenesis between these ganglia; nerve fibers emerging from thoracic ganglia assembled in nerves and innervated limb primordia positioned at some distance from them.

The results to be reported below should be considered as preliminary excursions in a field which is opening for the first time to exploration. Although cells and organs from a variety of insects are now routinely cultured in many laboratories with a success which stands comparison with that scored long before with vertebrate cells and organs (Silvana, 1971; Echalier and Ohanessian, 1970), *in vitro* studies on the insect nervous and neuroendocrine systems have not until now been the object of specific investigations. While in fact Marks and coworkers have since 1965 presented evidence for nerve fiber outgrowth from regenerating leg tissues dissected out from cockroach

nymphs and cultured *in vitro* under different experimental conditions, this and subsequent papers by the same authors focused mainly on the effects of hormones and of humoral substances released by explants on nerve fiber outgrowth from the cut stump of the leg regenerates (Marks and Reinecke, 1965; Marks *et al.*, 1968).

In contrast to these and other investigations whose main object was the *in vitro* study of hormonal action on various insect tissues (Marks, 1970; Schneider, 1967; Williams and Kambysellis, 1969) inclusive of nerve tissue, our research program has centered entirely on problems which are unique to the nervous system and apply to that of insects as well as of lower and higher vertebrates. Among the advantages presented by the insect nervous system is the fact that it consists of fairly small ganglionic masses the whole of which can be explanted *in vitro*, thus avoiding disruption of nerve tissue and injury to cell populations and neuronal circuits. Lack of a closed circulatory system in arthropods has, according to Treherne (1968), favored the evolution of rapid diffusion processes of ions and molecules within the solid nerve structures. Metabolites and gas exchanges in brain and ganglia explanted *in vitro* from insect embryos or nymphal forms do not therefore differ from those in the living organisms to the extent that they differ in vertebrate nerve tissues. An additional reason which may account for the long-term survival *in vitro* of intact insect brain and ganglionic nerve cells is their location in a cortical rind: thus nerve cells are far more accessible to metabolites and oxygen than the vertebrate nerve cells embedded in the dense matrix of the neural tube.

In this chapter, we shall consider the results of some of the investigations performed on embryonic and mature insect nervous and neuroendocrine systems. Authors of these studies, partly in association with the writers, are J. S. Chen, R. S. Chen, and P. Amaldi.

II. THE OBJECT OF CHOICE: *Periplaneta americana*

Practical considerations as well as the availability of an extensive literature on *Periplanata americana* suggested its selection as the test object of these studies.

This insect is widespread throughout the world in temperate and tropical climates, it survives and breeds remarkably well under laboratory conditions, and large colonies can be maintained with no specially trained technical help. The insect, which is 30–35 mm long, has a life span of 12–14 months and does not face the cataclysm of metamorphosis but goes through a slow and continuous developmental process which transforms the embryo into the adult without substantial changes in the structural organization of its nerve structures. The nervous system of the embryo (which spends 29–45 days in the

ootheca depending on the outside temperature: at 29°C the embryos hatch at the end of the first month, whereas it takes $1\frac{1}{2}$ months to complete the development at 15°C) is a miniature but otherwise faithful copy of the nervous system of the mature insect. Molting, which occurs first in embryonic life and then repeatedly during the 12 subsequent months in the nymph, makes possible the stepwise size increase of the immature insect, which emerges from the last molt with a well-developed, although rather inefficient flight apparatus but is similar in other respects to the tiny embryo.

Another advantage, which became apparent as we learned more about this insect from early embryonic life up to maturity, is its remarkable resistance to environmental changes and to infectious and other noxious agents. The embryos can be extracted from the oothecae, where they are aligned in two tightly packed parallel rows, and can be reared from the second week to hatching in a CO_2-conditioned humidified incubator at 29°C. Throughout this time, they can be manipulated, submitted to surgical intervention and microinjections of various drugs, and examined for hours under the stereomicroscope without, in most instances, lethal consequences. Mortality is in fact very low, even when limbs or antennae are amputated or additional organs implanted at the beginning of the third week of incubation. The fact that each ootheca contains 14–16 embryos, available for *in vivo* and *in vitro* experiments, gives this insect a remarkable advantage over other blattoids or orthopterans, which are either viviparous or hatch from individual eggs.

A considerable amount of work has been invested in the study of the nervous system of this cockroach, which is in fact better known at the structural, ultrastructural, biochemical, and electrophysiological levels than most other insects. Here are listed only some of the treatises and articles which are pertinent to the objects of our studies: Beattie (1971), Callec *et al.* (1971), Cornwell (1968), Farley and Milburn (1969), Frontali and Mancini (1970), Hyde (1972), Pearson (1972), Pearson and Iles (1970), Pichon and Callec (1970).

Closely related to the nervous system from a structural and functional viewpoint is the neuroendocrine or retrocerebral complex, which plays a key role in the life of insects, comparable in many respects to that of the vertebrate hypophyseal system. The similarity between the vertebrate and invertebrate neuroendocrine systems extends also to their relationship with brain structures and to the segregation in both phyla of this organ complex in a neural and a nonneural derivative. In insects, the paired corpora cardiaca have a neural origin and share some aspects in common with the neurohypophysis, while the corpora allata, small paired glands, possess important morphogenetic and nonmorphogenetic hormonal functions. "While the structural analogy between parts of the endocrine systems of insects and vertebrates is an

interesting one," writes Smith, "it should not be pressed too far; for, unlike the corpus cardiacum, the neurohypophysis possesses no intrinsic secretory cell bodies and, of course, the hormones associated with the two complexes are entirely different in their effects" (Smith, 1968*a*, p. 96).

III. MATERIAL AND TECHNIQUES

Oothecae protruding from the ovipositor are detached from the insect body, cleaned, and stored in a humidified incubator at 29°C. At the moment of use, the oothecae are rinsed in iodine, alcohol, and distilled water; the seams of the dorsal cristae of the oothecae are carefully split with sterilized forceps and the embryos are collected in Schneider's insect solution For the present experimental studies on the nervous system and nonnervous tissues (alimentary canal and limb and antenna primordia), 16-day-old embryos were used, while the neuroendocrine complex was isolated from 12- and 20-day embryos.

Whole brains and subesophageal, thoracic, and abdominal ganglia are dissected out under the stereomicroscope. Together, in some instances, with the brain is explanted also the rostral part of the alimentary canal, the pharynx, which closely adheres to the posterior brain vesicle known as the tritocerebrum. Other segments of the alimentary canal, the esophagus, the gizzard, the midgut, and the posterior gut, are also dissected out and stored until the moment of use. All the embryonic explants are of a size not exceeding 1–1.5 mm. The neuroendocrine complex, consisting of corpora cardiaca and allata from nymphal and adult specimens, is dissected out under rigorously sterile conditions from nymphal and adult forms and cultured in the same medium used for embryonic tissues. This consists of 5 parts of Schneider's insect solution and 4 parts of Eagle's basal medium, mixed at the moment of use. Tissues dissected out from embryos or post-hatching forms are cultured in small cylindrical glass vessels 13 mm in diameter and 7 mm high, half filled with culture medium. The tissues are pressed on a coverslip laid on the bottom of the culture dish until they firmly adhere to its surface. Five to six culture vessels are then arranged in petri dishes 64 mm in diameter, and cotton soaked with sterile water is placed inside the dishes between each vessel to maintain a vapor-saturated atmosphere; the dishes are then stored in a desiccator filled with 95% air and 5% CO_2 and placed in an incubator at 29°C. The cultures are examined daily at the inverted microscope and then at higher magnification, still in living condition, with the Nomarski interference microscope. The same cultures are studied again upon fixation and staining with routine histological techniques or, more frequently, with the silver Cajal-DeCastro technique. Many cultures are also fixed and used for electron