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CULTURE OF NEURAL TISSUE AND CELLS
IN
RESEARCH METHODS IN NEUROCHEMISTRY, VOL. II.
(R. Rodnight and N. Marks, Eds) Plenum Press

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I. INTRODUCTION

The extensive history of tissue and cell culture of neural preparations contains a wealth of experimental contributions. Because of the large bibliography on this subject I shall not attempt to provide a comprehensive discussion of either the methods employed for such cultures or the information available in the literature on their biochemical properties. I shall, instead, describe the major experimental approaches used and cite representative physiological and biochemical observations which illustrate the unique contributions these in vitro techniques can make to neurobiology.

Culture of tissue falls into two broad areas; the use of (1) explant cultures of small fragments, or (2) single cell preparations from tissue dissociated by mechanical or chemical procedures. Primary culture of neural tissue explants will be described initially, as befits its historical role. Dissociation and culture of cells from neural tissue, a much more recent technique, will be discussed subsequently. Advantages and drawbacks of the alternative methods of primary culture will be compared and contrasted, and their utility for the investigation of specific problems in neurochemistry will be presented.

Primary explant or cell cultures of normal tissue do not remain viable indefinitely. Subculture generally yields a non-functional population of cells, due either to selection or dedifferentiation (Yasumura, 1968). One alternative to this problem has been the culture of functionally differentiated tumors. In many instances culture of appropriate neoplastic tissue has lead to the isolation of clonal functional cell lines. Such populations, derived from a single cell, exhibit biochemical characteristics unique to the tissue of origin. They can be serially propagated, apparently indefinitely, without losing function. Techniques for the induction, adaptation to culture, cloning

and propagation of functional cell lines from tumors of neural origin will be described. Functional clonal lines derived from tumors of the nervous system are a recent development in neurobiology. A comprehensive discussion of the observations on clonal lines appears practical and will be attempted. The chapter will conclude with a discussion of prospects for the future use of cultured neural tissue.

II. HISTORICAL BACKGROUND

The basic dictum, rationale and defense of culture studies centers on experimental attempts to remove tissue from the influences of the whole organism, and place it instead in a more defined environment, where external factors can be eliminated or modified by design. The use of culture is a clear example of the reductionist approach to the study of biology, whether the discipline in question is embryology, endocrinology, or neurobiology. An entertaining and informative discussion of the general history of tissue culture with particular reference to the study of viruses and tumor biology may be found in Willmer's introduction to Cells and Tissues in Culture. Willmer's three volume work will be invaluable to the tissue culture neophyte.

It is generally conceded that the field of tissue culture was initiated with the studies of Ross Harrison in 1907. It is appropriate that a description of Harrison's experiments should mark the beginning of this chapter, since his research served as a decisive factor in a classic disagreement between giants in the field of neurobiology. Ramon y Cajal and His contended that nerve fibers originate at and grow away from a single cell; i.e. that the nervous system is composed of independent cellular units. Held and his supporters contended that the nervous system is syncytial in nature, with the neuronal processes serving as cytoplasmic bridges between portions of the structure. Harrison placed fragments of embryonic tadpole tissue into a

culture made with clotted lymph from an adult frog. His cultures remained viable for several weeks; long enough to observe the outgrowth of neuronal fibers from the bodies of individual cells, and thus confirm the neuronal theory.

Harrison and his associates pioneered the "hanging drop" culture method for explanted fragments. In this procedure the tissue is immobilized in a plasma clot on a coverslip, which is then inverted, placed on a depression slide, and sealed with paraffin. This early work is reviewed by W. H. and M. R. Lewis (1924). Significant limitations exist with hanging drop cultures, however, because of the limited medium available for explants. The use of a double coverslip assembly has helped to overcome this problem (Maximow, 1925). The tissue explant is placed on a small coverslip, which is held by capillarity in the center of a larger coverslip. The larger coverslip is covered with an inverted depression slide, and the unit is sealed with paraffin. Such cultures can be washed and refed by transferring the smaller coverslip to a second unit. Murray and Stout (1947) were the first to use the Maximow assembly for neural tissue. This technique has been exploited most successfully by Murray and her co-workers, and has been reviewed in detail by her (1965, 1971).

While the double-coverslip method has proven valuable for many studies, the optical limitations of the depression slide for microscopy has lead to the development of several types of slide chambers which have optical surfaces above and below the living culture. The most commonly used is the Rose chamber (Rose, 1954), which utilizes two coverslips separated by a rubber gasket, and held together by a set of steel plates. The use of Rose chamber cultures for neural tissue has been primarily confined to microcinographic studies of growth and movement of cells, and has been reviewed by Kasten (1968).

The preparation and culture of single cell suspensions has a somewhat

sporadic history prior to the work of Moscona and Moscona (1952). These workers developed enzymic procedures for the dissociation of embryonic tissue into single cell suspensions which remained viable and divided in cell culture. Moscona, Trowell and Willmer (1965) have discussed the advantages and disadvantages of chemical and enzymatic dissociation methods, particularly as they relate to embryonic tissue.

Dissociation of neural tissue, fractionation of neural cells, and characterization of the cell types are problems of enough significance that the field has become an independent sub-discipline and is discussed in Chapter _____ of this series. The morphological, physiological and cytochemical contrasts between neurons and glia suggest vastly different functional and biochemical properties. Most workers concede that culture of dissociated central nervous system tissue without prior separation of cell types would not provide much additional information beyond that gained from explant studies. Cavanaugh (1955) described probably the first attempt to dissociate and culture CNS tissue in his study of chick embryo spinal cord preparations. The separation and culture of different cell types from embryonic CNS has begun only in recent years (Varon and Raiborn, 1969; Varon, 1970) and is still in its infancy.

Many biochemists have avoided the CNS because of its complexity and/concentrated instead on studies of the periphery. Autonomic and sensory ganglia have been extensively used for monolayer cell-culture studies. The earliest descriptions of dissociated embryo ganglia (Nakai, 1956; Nakai and Kawasaki, 1959; Nakai, 1960) were concerned primarily with morphological studies of cellular differentiation. When the response of sensory and sympathetic ganglia to Nerve Growth Factor (NGF) was observed (reviewed by Levi-Montalcini and Angeletti, 1968) the use of explant and dissociated cultures for the study of this phenomenon at a cellular level was initiated (Levi-Montalcini and Angeletti,

1963), and has been intensively pursued. The environmental and nutritional requirements for the morphological, biochemical, and electrophysiological maturation of these cells in culture has been energetically investigated.

A culture system lying between the use of tissue fragments and dissociated monolayer cultures, known as "reaggregation culture" was initially introduced by Moscona (1961). In this procedure dissociated embryonic cells are placed in rotating cultures and reaggregation is allowed to proceed under controlled conditions. Histotypic aggregation often occurs, and organotypic structures may be recognized even in mixed cultures of various tissues. By using various combinations of species, organs, and developmental stages, the biochemistry of morphogenesis and cellular adhesiveness can be explored (Moscona, 1965). Although experiments on retinal reaggregation (Stefanelli, Zacchei, Caravita, Cataldi, and Ieradi, 1967; Lillian, 1968) suggested that maturation and synaptic formation in reaggregation culture occurs, the techniques have only recently been applied to CNS tissue. The initial morphological (DeLong, 1970; DeLong and Sidman, 1970) and biochemical (Seeds, 1971; Seeds and Gilman, 1971) studies of reaggregates of normal and mutant mouse brain suggest this system has great potential for the investigation of neural maturation in vitro. These preparations will be described in detail, since they appear to have great promise for the controlled study of the biochemistry of morphogenesis and cellular interaction in the nervous system.

The culture techniques described above utilize heterogeneous populations of cells. Biochemical analysis of such preparations (other than single cell analysis) suffers from the same criticisms often leveled at biochemical studies of whole brain. The question of the relevance of a biochemical measurement on a section of brain to the situation in a single cell is recognized by all biochemists studying the nervous system and rationalized in a

variety of ways. Many of us have either secretly or openly longed for a homogeneous preparation of functional neural tissue in which the parameter of choice may be examined without regard to cellular heterogeneity, despite the fact that many aspects of neural function and cellular homogeneity are incompatible.

Close on the heels of the development of methods for dispersing cells by enzymatic techniques Puck and his co-workers (Puck and Marcus, 1955; Puck, Marcus and Ciecura, 1956; Puck, Ciecura, and Fisher, 1957) developed methods for isolating clonal populations. Studies of growth, generalized macromolecular synthesis, nutritional requirements, genetics, radiation biology, etc. have benefited considerably from such preparations. The use of clonal cultures for the study of differentiated functions has not proceeded as rapidly as might be expected however. Tissues are not composed of one cell type; otherwise there would not be a necessity for cloning procedures. All too frequently a monolayer population from an organ does not possess the characteristic function of that organ. The reasons for such "dedifferentiation" of growing monolayer cultures have been hotly contested. Two opposing views on this subject have emerged. The first position has suggested that phenotypic alteration to a dedifferentiated state is a necessary concomitant of culture. The second viewpoint has proposed that overgrowth by non-functional cells is responsible. These are not mutually exclusive possibilities, and both phenomena have been described. As a general rule it has been observed that, in attempts to establish functional cell lines, overgrowth by connective tissue cells which appear to have a selective advantage under culture conditions has proved to be a formidable or insurmountable barrier to the establishment of functional lines. Neural tissue, in which cell division is a limited phenomenon would potentially be among the most difficult of tissues from which to derive clonal

lines of "normal" cells. In many cases the cell biologist has circumvented this problem by utilizing functionally differentiated tumors. Tumors are by definition independent of the growth control mechanisms of their parent organs, and frequently grow well in culture. The use of such tumors to establish clonal functional endocrine cell lines, first described in 1962, (Buonassisi, Sato and Cohen) has been exploited to produce functional clonal lines of adrenal, Leydig, pituitary, (Yasamura, Tashjian, and Sato, 1966) and liver (Richardson, Tashjian, and Levine, 1969) cells. Recently clonal populations of cells which continue to carry out brain specific functions in culture have been isolated from functional astrocytomas (Benda, Lightbody, Sato, Levine, and Sweet, 1968; Lightbody, Pfeiffer, Kornblith, and Herschman, 1970). Concomitantly the isolation of clonal lines from tumors of the sympathetic nervous system which maintain some of the differentiated functions of mature neurons has been described (Augusti-Tocco and Sato, 1969; Schubert, Humphreys, Baroni, and Cohen, 1969). While they are a far cry from an intact nervous system, as are explant, dissociated, or reaggregation cultures, the potential utility of clonal cell lines for the investigation of the regulation of specific function is clear, and experimental exploitation has been extensive during their relatively brief existence.

III. METHODS OF PRIMARY CULTURE

A. GENERAL

By the term "primary culture" I refer to all those forms of explant or cell culture in which neural tissue from embryonic, young or adult organisms is used in culture without a sub-passage. This includes explant culture of tissue fragments, dissociated monolayer cultures, and reaggregation cultures. The manipulation and utility of these culture techniques are clearly different, and will be dealt with separately.

Explant cultures have typically been optimized for two major criteria, the maintenance of the organotypic interaction of neural cells and their visualization in the light microscope for experimental analysis. The objective in the bulk of the early explant studies has been the development and maintenance of organotypic structures, i.e., cultures which closely resemble the analogous in situ tissue architecturally and histologically. Neural preparations, like other tissues, show a migration of cells at the periphery of the explant, however. A flattening of the explant occurs, and the cell layer frequently becomes thin enough so that sectioning is not required, and the living culture can be examined directly by microscopy.

The major objectives of workers using dissociated monolayer cultures have been directed toward determining what environmental factors influence neural cells when isolated from their normal structural matrix. The studies performed on dissociated cells of autonomic ganglia, sensory ganglia, or central nervous tissue have emphasized culture conditions which have enhanced (1) the greatest number of cells surviving and (2) the attainment of a differentiated state, as determined morphologically, physiologically, ultrastructurally, or biochemically. Emphasis has been on the single cell, rather than on complex system of cells (i.e., a histotypic structure).

Reaggregation cultures have been utilized to study the transition from a unicellular to a multicellular condition. They provide a means to analyze the genetic, developmental, organotypic and environmental factors which affect the self-assembly phenomenon and the subsequent formation of histotypic differentiated structures. Consequently the basic culture condition sought is the optimization of the ability of cells to aggregate in a three dimensional culture resembling the tissue/origin.

Each of these general methods has been reviewed individually, often in

chapters as long as this one. A comprehensive description of the history, methods, and results of each is not possible or desirable here. What I shall do instead is to refer the reader to suitable reviews at appropriate times, describe the currently used methods for each culture preparation, and compare some, but by no means all, of the basic results from these approaches to the primary culture of neural tissue. For detailed discussion of the general laboratory methods of cell culture such as glassware preparation, sterilization techniques, media preparation, culture incubation, etc. the reader is referred to the texts on this subject by Willmer (1965), Paul (1970), and Parker (1961).

B. EXPLANT CULTURES OF NEURAL TISSUE

The use of explanted neural cultures dates from Harrison's previously cited work. In their Bibliography of Research in Tissue Culture, which covers the years between 1884 and 1950, Murray and Kopeck (1953) cite over 800 references to studies with cultured neural tissue. The bulk of these reports deal with explant cultures. Studies on peripheral tissue from a variety of amphibia, as well as cat, chick, dog, guinea pig, horse, human, mouse, pig, rabbit, and rat are cited. The bulk of the entries, listed under "NERVE, Brain" include studies with cat, cow, chick, dog, fish, guinea pig, human, monkey, mouse, rabbit and rat. Cited among the various regions of the brain cultured in explant are the cerebellum, cerebrum, medulla, mesencephalon, optic lobe, prosencephalon, rhombencephalon and telencephalon.

While the period between 1950 and the present has seen neither the evolution of a new species for study nor the appearance of a new anatomical region of the brain, it has witnessed the spawning of a large number of practicing biologists with an interest in the nervous system. The variations in methodology and the amount of data on explant cultures of the nervous system are staggering. Several reviews of the subject have been published, including

those of Thomas (1956), Geiger (1957), and Pomerat (1958). Probably the most comprehensive description of the literature, however, is by Murray (1965, 1971), one of the leading contributors to the field. I shall concentrate on the techniques of explant culture of (1) autonomic and sensory ganglia from chick and rodent, (2) spinal cord cultures of chick, rat, and mouse, and (3) cerebellar and cerebral cultures of rodents and kittens.

1. Preparation of Coverslips

The original explant preparations utilized plasma clots for immobilization of the fragment. The clotted preparation was then inverted onto a depression slide and sealed with paraffin. Short term cultures of this type were used extensively for the examination of fiber outgrowth. Attempts at long term culture were, however, not generally possible since the cultures could be refed only with difficulty. In the 1940's Murray and Stout adapted the double coverslip assembly to the culture of nervous tissue (1942, 1947). The use of a double coverslip allows convenient washing of waste material from the explant, as well as the opportunity to refeed by simply placing a drop of medium over the clot. Growing cultures frequently produce enzymes which digest the fibrin, however, resulting in liquification of the plasma clot. A method of "patching" the fibrin clot in order to provide a continuous support (Peterson and Murray, 1955) was subsequently developed for neural tissue. This procedure permitted maintenance of explants for several months in culture. The problem proved to be a rather vexing one, however, and has subsequently been eliminated by the use of collagen-coated coverslips. Erhman and Gay (1956) demonstrated that reconstituted rat-tail collagen provided a suitable surface for the attachment and growth of a variety of cells. Bornstein (1958) adapted this procedure to the preparation of Maximow assembly cultures of neural explants. The use of collagen tends to encourage the growth and spread of

cells to two dimensions rather than three, as often occurs with plasma clots. This makes both observation and photography much more convenient.

Round 22 mm coverslips are prepared for collagen-coating/^{by}immersing them in a boiling 7X solution for 10-15 minutes, then rinsing in 5-10 washes of double-distilled and millipore-filtered water. The coverslips are wiped dry individually with a lint-free cloth, placed in 60 mm glass petri plates (3 coverslips per plate) and sterilized in a drying oven. A drop of dialized collagen (see Appendix A) is placed on each coverslip and spread with a glass stirring rod. The collagen is set by placing the open 60 mm petri plate inside a larger inverted plate, along with several cotton swabs dipped in ammonia. The coverslips are exposed to ammonia vapor for four minutes, then to air for five minutes. The ammonia vapor is removed with five distilled water rinses (five ml for five minutes each). The collagen surface is then conditioned by putting five ml of Basic Salts Solution (BSS, see Appendix B) in each plate and allowing the coverslips to sit for 24 hours before use.

Murray (1965) has pointed out the "meticulous attention to purity" required to maintain neural explants in culture for long periods of time. Glassware should be scrupulously cleaned, and great care should be taken with the water used for washing and preparation of solutions. (Many workers use double distilled and millipore filtered water.) In explant studies with chick spinal ganglia Peterson, Deitch, and Murray (1959) observed toxic factors in glass coverslips from various suppliers, and reported a comparative study of several brands of cover glasses. Although the toxic factors were not clearly identified, the data suggested that small amounts of lead or arsenic present in some glass coverslips might be responsible for the toxicity. In the most frequently cited reference (Bornstein and Murray, 1958) Gold Seal #1 22 mm round coverslips were used.

Coverslips containing explant cultures are handled frequently during long term culture and are often broken, even under optimal conditions. In addition, with the advent of electron microscopy, the removal of the cells from glass after embedment for sectioning has become a significant problem (Firkett, 1966). To circumvent these problems Masurowsky and Bunge (1968)

investigated the suitability of collagen-coated fluoroplastic coverslips for long-term neural explants. These workers reported that cultures grown on Aclar 33C were essentially indistinguishable from parallel cultures prepared on glass, and could be most easily processed for electron microscopy.

2. Preparation of Explants

The origin of explanted tissue will depend, of course, on the orientation and interests of the investigator. A detailed description of cultured explants from the list of species and regions of the nervous system/^{previously cited} is clearly beyond either the capabilities of the author or the interest of the reader. Similarly, a description of the embryonic anatomy and dissections required for each preparation is not possible in the space available in this chapter. There are some important generalizations to be made, however, and some of the experimental techniques employed will now be described in the context of specific preparations.

a. Dorsal Root Ganglia (Bunge, Bunge, Peterson, and Murray, 1967)

Cervical or lumbar dorsal root ganglia are removed from 16-1/2 to 19 day old mouse embryos or 8-14 day old chick embryos under sterile conditions, using a binocular dissecting microscope. During the explant procedure the central and peripheral branches of the dorsal root are cut close to the ganglion. The tissues may be washed with BSS in a depression slide. Alternatively they may be placed directly onto collagen-coated coverslips which have been fixed by capillarity to a 40 mm square coverslip. When the experimenter

Fig. 1

is satisfied that the tissue is adhering to the small collagen-coated coverslip a drop or two of medium is placed on the explant. The Maximow assembly is completed by touching a small dot of petroleum jelly to either side of the larger coverslip, inverting a sterile depression slide and placing it over the larger coverslip, as shown in Fig. 1. The chamber is then inverted and made air-tight by brushing hot paraffin around the edge of the square cover-glass. The paraffin should be thinned with about 25-30% petroleum jelly. During culture, fiber outgrowth can occur from the cut root or from any or all parts of the explant.

b. Newborn Rodent and Feline Cerebellum (Bornstein and Murray, 1958; Allerand and Murray, 1968; Silberberg and Schutta, 1967)

Cerebella are removed from newborn mice or rats after cold anesthesia (-20°C for 5 minutes) and placed on a moistened sterile glass plate. The tissue is kept moist with BSS. The meninges and choroid plexus are removed under sterile conditions. The tissue is cut into fragments of from 0.5-1.5 mm^2 . The tissue may, if necessary, be washed with BSS by irrigation on the plate. Most workers do not describe their methods of cutting tissue. Parker (1961) recommends the use of cataract knives, while others use Bard-Parker Blades (No. 11). The cuts should be made cleanly, with a minimum of tearing and gouging. This type of tissue damage, either during cutting or transferring of the tissue will result in an unsatisfactory culture. Two to three explants are placed on a collagen-coated coverslip and fed with a drop or two (0.05-0.1 ml) of nutrient medium. The small coverslip is placed in a Maximow assembly and put in the incubator at 35° .

Recently Allerand and Murray (1968) have demonstrated that cerebellar cultures myelinate more synchronously (i.e., the onset of myelination occurs around a single discrete time point in culture) if topographically paired

contiguous explants are used. In this report the cerebellum was parasagittally sectioned into eight explants, and contiguous and non-contiguous pairs were cultured either widely separated (6-7 mm) or paired with their edges touching. Myelinogenesis also appeared to proceed to a greater degree with contiguously, as opposed to randomly, paired explants (Fig. 2). For those studies in which the synchronous onset and extent of myelination of cerebellar fragments is an important objective or criterion the pairing of contiguous parasagittal sections appears to be of great benefit.

Fig. 2

Kitten cerebellum has been a favorite material for explant culture. Bornstein and Murray (1958) reported excellent development of myelin in these cultures, using precisely the same technique described for rodent cerebellum. Hild and his co-workers have described kitten (and rat) cerebellar cultures, as well as other brain regions, grown as "flying coverslips" in roller cultures. In this case the coverslip bearing the explanted cerebellar tissue is placed in a test tube (16 x 150 mm) with about 2 ml of medium. The test tubes are incubated in roller drums rotating approximately 8-10 revolutions per hour. Roller tube cultures of neural tissue need to be fed only every 7-10 days, simply by aspiration and repipetting, and thus require less attention than Maximow assemblies. They are more difficult to observe, however, since the coverslip must be withdrawn, examined, and returned to the roller tube aseptically. In contrast, a Maximow assembly culture can be viewed continuously. The principal advantage of flying coverslip cultures lies in the relative ease with which large numbers of cultures can be prepared and fed. Myelination and electrophysiological activity in flying coverslip cultures proceed quite satisfactorily (Hild, 1957, 1966; Hild and Tasaki, 1962).

c. Spinal Cord (Peterson, Crain, and Murray, 1965).

Spinal cord of fetal rat between the ages of 11-20 days in utero may be

successfully cultured in the basic Maximow assembly described above. After removal of the vertebrate arches the exposed cord can be dissected from the animal. The cord may be removed either with or without attached ganglia. (In the case of younger animals Peterson et al., reported that the removal of the cord is facilitated if the ganglia are retained.) The meninges may either be retained or stripped from the cord prior to sectioning. The dissected cord is then cut into either whole or half cross-sections of 0.5-1.0 mm thickness. Sectioning may easily be done with cataract knives or with Bard-Parker holders fitted with No. 11 blades. The fragments can then be explanted (1 to 2 per coverslip) in the Maximow double coverslip culture assembly. Peterson et al. reported that chick embryo spinal cord from animals 6-9 days in ova and human spinal cord from six week old embryos will also grow well in culture. Human embryo spinal cord from older fetuses (12-16 weeks), however, did not culture successfully.

d. Autonomic Ganglia

Benitz and Murray (1965) have reported that the sympathetic autonomic ganglia of 5-19 day old chick embryo can be cultured for long periods in the Maximow assembly. In their brief initial report they also stated they were able to culture the superior cervical and stellate ganglia from human, rat, and mouse embryo, as well as the sympathetic chain and isolated ganglia from rat, mouse, and kitten. These cultures could be kept in vitro for up to four months in the Maximow assembly. Crain and Benitez (1964) reported that the addition of 400 BU/ml of Nerve Growth Factor to the standard media used by Murray and her collaborators resulted in healthier mouse and chick sympathetic ganglia. Many more ganglion cells survived than in control cultures, and appeared to be able to propagate electrical impulses over longer distances. This work, of course, extends the earlier short term culture observations of Levi-Montalcini and her

associates (reviewed by Levi-Montalcini and Angeletti, 1968).

e. Mixed Explant Cultures

Study of the biochemical and electrophysiological maturation of various areas of the nervous system in isolation has, of course, been one of the primary objectives of the preparations described above. The in vitro effect of one tissue type on the maturation and function of another has also been one of the most active areas of investigation (Szepsenwol, 1946, 1947) with particular reference to studies of neuromuscular interactions. The use of culture techniques has also permitted questions of species barriers to be examined. As an example, the functional innervation of fetal rodent skeletal muscle by heterologous spinal cord explants has been investigated (Peterson and Crain, 1970; Crain, 1968, 1970). The question of the potential for interaction between parts of the nervous system which normally do not make direct functional connections can also be investigated in this manner. In the neuromuscular preparations, spinal cord with dorsal root ganglia still attached is removed from 12-16 day old rodent embryo and explanted in whole cross section in the standard Maximow assembly. Small muscle fragments from the hindlimbs of 18 to 12 day old rat or mouse embryos are subsequently placed at a distance of approximately 1 mm from the ventral edge of the spinal cord explant. The cultures have then been monitored morphologically, ultrastructurally and electrophysiologically for interaction of nerve and muscle. Crain et al. (1970) have also utilized human embryonic muscle successfully in this culture system.

3. Media for Explant Cultures

Media for primary explants varies greatly from laboratory to laboratory. The particular media suitable for a given investigation is usually a function of the intended duration of the experiments in question and the prior experience

of the investigator. The medium generally contains a balanced salt solution (BSS, Appendix B) supplemented with glucose to 300 to 600 mg percent (found to increase the viability of neural cultures), serum, and various embryonic extracts. The medium described by Bornstein and Murray (1958) for culture of cerebellum of rat and kitten contained equal parts of glucose-supplemented Simms BSS, human placental cord serum, bovine serum ultrafiltrate and extract from nine day old chick embryo. This medium was supplemented to 600 mg percent glucose and 200 units per ml of penicillin for the long-term culture of rat dorsal root ganglia (Bunge et al., 1967). In later cerebellar cultures of mouse (Allerand and Murray, 1968) the glucose level was raised to 600 mg percent, no antibiotics were used, and Eagle's minimal medium (Appendix C) was substituted for Simms' BSS.

Myelinating cultures have been prepared from rat cerebellum using a medium containing 60% Gey's BBS and 40% fetal calf serum, supplemented with
and
650 mg percent glucose, /0.3% thiamine HCl (Silberberg and Schutta, 1967). Hild and his co-workers have routinely used a medium consisting of 45% calf-serum, 5% chick embryo extract and 50% Gey's BSS for their cultures. In their recent studies with mixed explant cultures Peterson and Crain used a medium containing 33% human placental serum, 53% Eagle's minimal essential medium with glutamine, 10% rat embryo extract, 600 mg percent glucose, and 1.2 µg/ml achromycin. It appears that the complex media can vary over quite a wide range and still produce satisfactory cultures. For initial investigations the medium described by Bornstein and Murray (1958) seems to be acceptable as a starting point. In general, one is advised to consult the literature on a specific explant preparation. Peterson and Murray (1960) have described alterations in the nutritional and physical environment of explant cultures which are able to suppress various aspects of the developmental

process of dorsal root ganglia explants, while promoting others.

4. Feeding Maximow Cultures

Because of the relatively small volume of medium used, Maximow cultures generally require feeding every two to three days. To perform this operation four cultures at a time are placed in the sterile hood. The outer coverslip is freed from the depression slide by running a flamed single edge razor blade through the paraffin. The outer coverslip is removed from the depression slide with a forceps and inverted. The smaller coverslip, bearing the culture, is carefully removed with a forceps and a pointed Bard-Parker knife (No. 11). If the explant culture is quite small and no washing is required, the residual media on the culture may be drained by holding the collagen-coated coverslip in a vertical position and touching its edge to a sterile surface. To wash waste products from cultures the coverslips are placed in Coplin jars (Columbia staining dishes) containing either a balanced salt solution or culture medium. Each Coplin jar has vertical grooves which hold four coverslips. If more extensive washing is desired the cultures can subsequently be transferred to a second Coplin jar. The collagen-coated culture-bearing coverslip is placed on a new sterile #3 40 mm coverslip, where it is held in place by capillarity. Fresh medium is placed on each culture and the Maximow assembly is completed by sealing the culture in a new depression slide, using the methods described previously. The cultures are generally incubated in the "lying drop" position. Special incubation racks for this purpose have been designed (Parker, 1961).

C. DISSOCIATED CELL CULTURES OF NEURAL TISSUE

Explant culture can effectively isolate the experimental fragment from various internal and environmental fluxes, and can provide a preparation whose milieu can be manipulated at the discretion of the investigator. The cells

migrate from the explant, but they maintain extensive histotypic organization; indeed this is often one of the basic objectives of explant experiments. Neuronal cell bodies do not move extensively. The nerve fiber and some perikarya, while visible and to some degree accessible, are generally in intimate contact with other cells. It has frequently been suggested that the development and integrity of nerve cells are dependent on an undisturbed intercellular contact. The role of tissue integrity for the maintenance of differentiated function in the nervous system is often stressed (Hyden and Lange, 1962; Galambos, 1961). This concept is not limited to the nervous system, and has been mentioned before in the context of a "dedifferentiating culture". Dissociated neural tissue preparations have been used by a variety of investigators to assess the ability of isolated embryonic neurons of the peripheral and central nervous systems to mature cytologically, biochemically and electrophysiologically. The most commonly used procedure for the dissociation of embryonic tissue, first described by Moscona (1952) uses Ca^{++} Mg^{++} -free trypsin. Discussions of the use of trypsin and other enzymes for tissue dissociation are found in a number of general texts on methods of cell culture (Parker, 1961; Moscona et al., 1965; Paul, 1970).

1. Chick Spinal Ganglia Dissociation

a. Preparation of Cultures

Like so many other areas of neurobiology, the study of dissociated chick sensory ganglia began as a casual observation of Rita Levi-Montalcini and her associates. Nakai, who published the first report of dissociated spinal ganglia cultures (1956), mentions that Levi-Montalcini demonstrated the procedure earlier. These preparations have been used most extensively in the laboratories of Scott and Varon. Their methods of culture and their results are quite similar.

The dorsal root ganglia are dissected from 10 to 11 day old chick embryos. The eggs are opened at the blunt end and the embryo is removed. The embryo is placed on its back on a frosted glass slide and rinsed with sterile BSS. The limbs are stretched out and held in place by surface tension. The embryo is decapitated. The exposed ventral body wall is cut completely away with iridectomy scissors (staying just inside the limbs), the embryo is eviscerated, and the kidneys are removed. The vertebrate column is removed by making lateral cuts down each side and peeling it away with a forceps. The spinal cord is grasped at the caudal end and lifted upward. This shears the dorsal roots connecting the cord to the ganglia. The successive ganglia can then be removed with a jeweler's forceps and transferred to collecting medium (BSS) in a depression slide. Between 20-30 ganglia can be collected from one embryo. Both Scott, Engelbert, and Fisher (1969) and Varon and Raiborn (1971) start with approximately two hundred ganglia. The ganglia are placed in 10 ml of Ca^{++} Mg^{++} -free BSS (CMF, see Appendix B) containing 0.25% crystalline trypsin, and incubated at 37° for 45 minutes. The trypsinized ganglia are washed gently three times with successive 10 ml portions of medium 1415 (Appendix C), resuspended in culture medium, and subsequently dissociated by repeatedly pipetting the solution through a Pasteur pipette. The dissociation should be monitored microscopically, since once a single cell suspension is achieved excess pipetting will damage the cells. The cell suspension is diluted to approximately 25,000 cells per ml (about a tenfold dilution) with complete medium. Two ml aliquots of the cell suspension are added to disposable culture dishes (35 mm diam., Falcon Plastics) containing collagen-coated coverslips. The dishes are incubated at 37° in a 5% CO_2 -95% air atmosphere with medium changes on alternate days.

b. Media for Dissociated Spinal Ganglia

Both Varon and Scott have used Medium 1415 supplemented with fetal calf serum for relatively long-term cultures of dissociated spinal ganglia cells (4-7 weeks). These investigators apparently have obtained similar preparations; a confluent monolayer of fibroblasts with either single nerve cells or nerve cells in small groups connected by fascicles growing on the surface of the monolayer. The cell bodies of the neurons in these cultures are approximately 30-35 μ across.

Varon and Raiborn also cultivated some of their cultures in the presence of 100 BU/ml 7S Nerve Growth Factor (Varon, Nomura, and Shooter, 1968). There appeared to be more nerve cells in NGF-supplemented cultures. There were approximately equal numbers of the 30 μ neurons in NGF-free and NGF-supplemented cultures, but an increase occurred in smaller unipolar neurons of approximately 10-15 μ in diameter. The increase in the number of surviving neurons in these dissociated embryonic ganglia in the presence of NGF is not surprising, since it has been known for a long time that (a) two neuronal populations with differing size, function, and location exist in these ganglia (Levi-Montalcini and Angeletti, 1968) and (b) one of these populations (the smaller, medio-dorsally located neurons) responds to NGF in vivo. Levi-Montalcini and Angeletti (1963) demonstrated that both sympathetic and sensory nerve cells would not remain viable in Eagle's medium alone, but would apparently establish contact with the culture surface, put out processes, and remain morphologically intact for periods of up to 7 days in Eagle's medium supplemented with NGF (0.05 μ g/ml). Cohen, Nicol, and Richter (1964) also reported a requirement for NGF for the growth and development of embryonic sensory and sympathetic neurons in culture. They also found that a high glucose concentration and the presence of insulin enhanced the morphological maturation of these cultures. These results strongly suggest that NGF should be used in cultures of spinal ganglia to optimize

neuronal survival in vitro. The role of insulin and glucose in such cultures should be pursued.

Scott and Fisher have systematically examined the effects of several small molecules on the survival of neurons in these preparations. They have demonstrated that (a) approximately 10 times as many chick embryo spinal ganglion neurons survive in culture at 40.8 mM K⁺ / ^{then survive} at the physiological level of 6.8 mM K⁺ (Fig. 3); (b) the optimal K⁺ concentration for survival of human spinal ganglion neurons is 20.8 mM (Scott, 1971); and (c) high levels of choline can also enhance the number of surviving neurons (Scott and Fisher, 1971). All of these experiments were done with medium 1415 supplemented with fetal calf serum, but no NGF. It is not possible from the existing data to tell if there is a differential survival of the existing classes of neurons in the spinal ganglia in Scott's experiments. However, the increased concentration of K⁺ clearly enhances neuronal survival.

In a recent set of experiments, Varon (1971) reported on the effects of the presence of 7S NGF protein during the isolation and trypsinization of 8 day old chick embryo spinal ganglia. NGF appeared to increase the plating efficiency of dissociated ganglia if present during the washing and aspiration of the ganglion cells following enzymic treatment. The NGF requirement for this effect could be replaced by the α subunit of the 7S protein. Almost a two-fold increase in plating efficiency could be achieved by this method. The specificity of this phenomena has not yet been examined, however. It still remains to be demonstrated whether the effect of 7S NGF or its components on plating efficiency is (a) confined to spinal ganglion cells, (b) generalized to other cell types, or (c) can be replaced by other proteins.

Although these data have been gathered in several different laboratories it appears that Medium 1415, supplemented with fetal calf serum, high potassium,

increased glucose, and NGF would offer the best growth potential for dissociated spinal ganglia. Several important questions remain to be answered before the appropriate culture medium can be settled on, including (a) whether NGF and K⁺ promote the survival of the same cells and are thus redundant, (b) whether NGF and K⁺ alone, without fetal calf serum, can sustain the neuronal population, while eliminating the non-neuronal population due to lack of serum components, (c) whether selective plating techniques can be used to enrich the population of neuroblasts, (d) whether the dividing non-neuronal cells can be selected against with agents which inhibit cell division but will not damage the neuronal fraction and (e) whether other species besides chick and the less studied human spinal ganglia are subject to these in vitro effects in dissociated cell culture. Like so many other aspects of these methods, the establishment of culture conditions and growth media are major areas of current investigation.

2. Dissociated Central Nervous System Cultures

Despite obvious potential interest in the characteristics of dissociated CNS tissue, relatively little work has been reported in this area. Cavanaugh (1955) applied Moscona's dissociation technique to embryonic chick spinal cord tissue, and observed both extensive reaggregation and outgrowth of processes in plasma clot Maximow assembly cultures. Meller et al. (1969) have made similar preparations, and reported the presence of axo-dendritic, axosomatic, and axo-axonal synapses in twelve day old cultures examined by electron microscopy.

The culture of dissociated cortical tissue has only recently been investigated. Sensenbrenner and her colleagues (1971) have been primarily interested in characterizing the nutritional requirements and the supporting matrix necessary for morphological differentiation of chick embryo cerebral cultures.

Cells from tissue dissociated by mechanical sieving were plated on collagen coated coverslips, placed in Rose chambers, and fed with several different media. Varon and Raiborn (1969) have reported a systematic study of the dissociation, fractionation, and culture of eleven day old chick cerebral hemispheres. Mechanical dissociation followed by differential centrifugation, trypsin dissociation, and differential attachment to glass preceded culture in these experiments. Using these procedures, Varon and Raiborn fractionated embryonic chick cerebral hemisphere cell suspensions into three preparations, each enriched for what morphologically appeared to be distinct cell types upon subsequent culture. One cell type, appears to be a unipolar neuron approximately 15 μ in diameter. A second class of cells which are primarily bipolar and somewhat smaller, are also thought by Varon and Raiborn to be neuronal. The final cell type in these fractions is definitely not neuronal. These cells are probably derived either from the ependymal epithelium, or from pieces of meninges not removed during the initial dissection.

To date the analysis of dissociated cerebral cultures has been almost exclusively morphological. Emphasis has been placed on characterization of the supporting matrix, the dissociation and fractionation procedures and the media necessary to produce cultures which are enriched for cells which have a neuronal appearance. Little or no biochemical or electrophysiological characterization of dissociated cerebral cultures exists as yet. At the present time the methods themselves for preparing such cultures are the basis for most of the literature in the field. Optimal conditions for preparing large scale cultures of cerebral cells enriched in one population or another are not yet clearly defined. Consequently, it would be inappropriate at this time to report in detail a single procedure which could be used as a starting preparation for the specific biochemical analysis of dissociated cerebral tissue in culture.

Readers who are interested in such a preparation are referred to the papers of Varon and Sensenbrenner, with the warning that the establishment of appropriate culture conditions will undoubtedly be a major portion of the investigation under consideration.

3. Neuromuscular Preparations from Dissociated Cells (Fischbach, 1970, 1972)

Mixed explants which form de novo neuromuscular synapses have been described above. The previous comments concerning the relevance of histotypic structure for neural maturation are also applicable to the neuromuscular junction. Can an individual neuron, dissociated from its organotypic matrix, form a physiologically competent neuromuscular junction in vitro, or is this developmental process one which is dependent on more extensive cellular organization? This question has been answered by two laboratories, using essentially identical culture preparations. The maturation of functional neuromuscular junctions between embryonic chick spinal cord and muscle cells has been demonstrated cytologically, ultrastructurally, and electrophysiologically, and has provided a potentially invaluable tool for the microchemistry of this phenomenon, in which the entire neuron is identifiable and accessible.

Pectoral thigh muscles of 11 to 12 day old chick embryos are removed under sterile conditions, with the aid of a dissecting microscope. The dissected muscle tissue, kept moist with BSS, is cut into 1 mm^3 fragments and transferred to a sterile test tube. A solution of 0.25% trypsin prepared in Ca^{++} Mg^{++} -free BSS is added. The tissue is incubated at 37° under a 5% CO_2 -95% air atmosphere for 30 minutes. The culture is gently centrifuged (1000 rpm) and the trypsin is removed. The pelleted tissue is resuspended in complete medium and dissociated with a Pasteur pipette whose tip has been narrowed by flaming. Undissociated tissue fragments, blood vessels and other debris are removed from the

dissociated cells by filtering the suspension through a double layer of lens paper held in a swinnex filter apparatus. The concentration of single cells which pass through the filter is determined by hemocytometer count.

Single cell suspensions prepared from muscle tissue by this procedure also contain a significant number of fibroblasts, which will proliferate and eventually overgrow the cultures. Fischbach (1972) has described a combination of several techniques to reduce fibroblast contamination prior to the addition of neurons. Approximately 10^6 muscle cells, plated in a 60 mm Falcon tissue culture plate, are kept in a CO_2 -air incubator at 37° for 30-45 minutes. The media and cells which have not plated are removed and transferred to a second tissue culture dish previously coated with collagen. 3×10^5 cells are plated in a total of 3 ml of medium. The myoblasts are permitted to grow for 2-3 days. D-arabinofuranosyl-cytosine (10^{-5} M) is then added to the cultures for 24-48 hours. After the drug is removed the cultures are washed with BSS, and "stepped down" to a medium containing only 2% chick embryo extract. These cells, which are now ready to receive the suspended spinal cord cells, are fed every 2-3 days.

The spinal cord from the brachial enlargement and cervical and thoracic segments of a 7 day old chick embryo is removed and stripped free of spinal ganglia and connective tissue. The spinal cord cells are dissociated in the same manner as the muscle cells. To each culture of muscle cells, prepared as described previously, is added $1-2 \times 10^5$ spinal cord cells suspended in 3 ml of medium supplemented with 2% chick embryo extract. The spinal cord cells are added 1-2 days after removal of the D-arabinofuranosyl cytosine (preferably after one day). Cultures are maintained at 37° in 5% CO_2 -95% air, with a medium change every 2-3 days. Such cultures are able to form cytologically, ultrastructurally and electrophysiologically identifiable neuromuscular

junctions.

The initial plating medium for muscle cultures consists of 80% Eagle's Minimal Essential Medium in Earle's Salt solution, 10% horse serum, 10% chick embryo extract, 2 mM glutamine, 50 u/cc penicillin and 50 µg/cc streptomycin. The "step down" medium is the same basic medium as above, but supplemented only with 2% chick embryo extract, glutamine, and antibiotics.

D. REAGGREGATION CULTURES OF NEURAL TISSUE

Cells which have grown out from tissue fragments, under some conditions, frequently become organized into histotypically distinguishable structures (Ebeling and Fischer, 1922; Drew, 1923). The history of this phenomena has been reviewed by Moscona (1965) who pioneered the study of controlled reaggregation of dispersed cells, in order to study the properties which lead to histotypic association of cell populations. Prior to his studies the observations on cell aggregations were limited to descriptions of self-aggregating systems which proceeded primarily on the surface of the culture vessel. Such cultures are generally not reproducible, and are subject to a variety of complex interactions among cells, culture vessel surface, and media. In an attempt to provide a more controlled environment Moscona introduced the use of rotating culture vessels. Moscona and his associates have utilized this system to study the relationship of embryonic age, developmental state, environmental factors, and specific tissue components on the formation of such reaggregating complexes (Orr, 1968; Lilien, 1968; Garber, 1967). Recently several laboratories have applied the rotation culture technique to questions of biochemical and histological maturation of mammalian brain.

1. Preparation and Incubation of Reaggregation Cultures

In the experiments of DeLong and Sidman (1970) 17-18 day old mouse fetuses are removed aseptically. The brain is removed from the fetus and

the appropriate region is isolated with the aid of a dissecting microscope. The preparation is kept moist in calcium-magnesium-free basic salts solution (CMF, see Appendix B). The meninges are removed in the appropriate cases. If several animals are being prepared the isolated tissue is kept in CMF during the dissecting period. Cells are dispersed by incubating the tissue in a 1% trypsin solution prepared in CMF. Incubation with trypsin is carried out at 37° in a 5% CO₂-95% air atmosphere for 10-15 minutes (optimal time of trypsinization must be determined experimentally). The tissue fragments are allowed to settle and the trypsin solution is aspirated away. The fragments are washed with three changes of CMF, by simply allowing the fragments to settle to the bottom of the tube. One ml of culture medium is added, and the brain tissue is dissociated by repeatedly drawing the fragments up and down in a capillary pipette. Cell dispersion is monitored microscopically. Isocortical cells are transferred to a 25 ml Erlenmeyer flask and brought to a total volume of 3 ml with medium. Hippocampal cells are transferred to a 10 ml flask and brought to a total volume of 1.3 ml. The dissociated cultures are gassed with a mixture of 5% CO₂ and 95% air, sealed with rubber flask caps, and placed on a rotating shaker (9/16 inch rotation diameter, 70 rpm) at 36-37°. The cultures are fed every 3-4 days by adding 0.5 ml of medium to the flask (0.2 ml in the 10 ml flasks). Optimal histotypic aggregation occurs under these conditions in 7-8 days. Cultures often deteriorate after this period of time.

Seeds (1971) uses whole mouse brain to prepare reaggregating cultures. The brains are removed aseptically from 18 day old mice fetuses and minced into small fragments. The tissue is dissociated in 0.25% trypsin for 20 minutes at 37°. The preparation is pipetted several times and returned to the incubator for an additional 15 minutes. The cell suspension is resuspended by

pipetting again and filtered through a nylon screen to remove cell clumps. Fetal calf serum is added to a final concentration of 10%, and the cells are pelleted by gentle centrifugation. Approximately $1-2 \times 10^7$ cells are placed into 25 ml Erlenmeyer flasks, brought to 3.5 ml by additional medium, gassed with a mixture of 5% CO₂-95% air, capped and incubated at 37° with constant rotation at 70 rpm. After 2 days the cultures are transferred to 50 ml flasks and 5 ml of fresh medium is added. Every two days 5 ml of medium is removed and replaced with fresh medium. In this system cultures have been maintained for up to 20 days in vitro.

2. Media for Reaggregation Cultures

DeLong employed Moscona's basic medium: basal Eagle's medium with 0.1% glutamine, 10% horse serum, 2% chick embryo extract and 600 mg % glucose. Seeds also used basal Eagle's medium but supplemented it with 10% fetal calf serum and 400 mg % glucose.

IV. METHODS FOR CLONAL CULTURES OF DIFFERENTIATED TUMORS OF THE NERVOUS SYSTEM

The primary culture preparations previously described are subject to several obvious limitations which are particularly troublesome to the biochemist. The amount of material available for analysis is often quite small in the case of explant cultures or cultures of dissociated ganglia (either because of the limitations imposed by the culture conditions or because of the laborious isolation of embryonic tissue). The initial cell populations are quite heterogeneous, making biochemical analysis at any level other than that of the single cell subject to obvious qualifications and criticism. Different classes of cells may have selective advantages in plating efficiency and/or cell division when put into culture, thus magnifying the initial problems of heterogeneity and limitation of raw material. The latter problem is particularly significant for the neurochemist since neurons, which are end-cells and do not divide, are

generally diluted in culture. Finally, in order to do successive studies on the same subject experimenters must return time and again to a laboratory stock of animals in order to derive their cultured material. They are thus subject to all the genetic, environmental, experimental and practical problems of obtaining and/or maintaining animal colonies which can provide reproducible tissue for culture.

To circumvent these problems several groups of investigators have attempted to establish cell populations which can be sub-cultured from primary cultures (and therefore provide more material for study) and which stem from a single cell (and are thus a homogeneous cell population). Since many functions of the nervous system result directly from the interactions of heterologous cells, the limitations of such clonal populations are apparent. For many fundamental biochemical studies, however, these clones offer obvious advantages.

At the present time it is not generally possible to establish clonal cell lines from normal tissue. Enough exceptions to this rule exist to remove it from the category of an axiom, but its applicability to neuronal populations is certainly valid to date. To circumvent this general problem several groups of investigators have utilized cultures of differentiated neural tumors to establish clonal functional cell lines. (If a dispersed culture of primary cells can be sub-cultured successfully and will continue to proliferate, the new population of cells is referred to as a cell strain. In many cases the investigator is able to isolate a single cell from a cell strain and maintain a proliferating population. The cloned cells resulting from this procedure are termed a cell line.) Tumors which maintain glial specific functions such as the production of S-100 protein and cortisol-inducible glycerol-phosphate dehydrogenase, and mouse neuroblastoma tumors which retain the capabilities for transmitter biosynthesis and bioelectric activity have been successfully cultured

and cloned. The induction, adaptation to culture, and cloning of neural tumors will be described in this section.

A. INDUCTION OF NEURAL TUMORS

The most thorough studies of carcinogens which have "organotropic" effects on neural tissue have been described by Druckrey and his associates (Druckrey, Ivankovic, and Preussmann, 1965; Ivankovic and Druckrey, 1968; Druckrey, Preussmann, and Ivankovic, 1969). When adult rats are given repeated injections of N-methyl-N-nitrosourea they routinely develop tumors of the brain and spinal cord. Weekly intravenous administration of 5-10 mg/kg body weight leads to a wide variety of nervous system tumors, including oligodendrogliomas, ependymomas, glioblastomas, neurinomas and spongioblastomas. Thirty to forty weekly injections are necessary to induce tumors of the nervous system in adult animals. Using a similar protocol Benda et al. (1968) were able to induce glial tumors which were successfully adapted to culture and proved to be functional nervous system tumors. More recently Ivankovic and Druckrey (1968) have shown that a single dose of ethyl-N-nitrosourea given to pregnant rats on the 15th day of gestation induces malignant tumors of the central and peripheral nervous system in nearly all the offspring. Tumors can be induced with doses ranging from 5-80 mg/kg (2-33% of the LD₅₀). The latent period prior to the presence of observable tumors is an inverse logarithmic function of the dose, ranging between 175 and 500 days. The drug can be administered to pregnant animals either intravenously (by tail-vein injection) or by stomach tube. This extremely simple procedure, which does not require the extensive handling necessary for tumor induction in adult animals, produces tumors in the offspring which are similar to those described for adult animals. Although the adaptation to cell culture of functional tumors derived by in utero administration of ethyl nitrosourea has not yet been described, several laboratories are

actively engaged in projects with this ultimate objective.

The bulk of the tumors induced by these procedures are not neuronal. Similarly, spontaneous tumors of the central nervous system are only very rarely thought to be of neuronal origin. The frequency with which tumors of neuronal origin and function will occur as a result of such tumor induction techniques has yet to be determined. In contrast, neuronal tumors of the sympathetic nervous system (neuroblastoma) are a relatively common clinical phenomenon. Although the spontaneous occurrence of such tumors in various species has not been systematically examined, one transplantable mouse neuroblastoma from an inbred strain has been continuously propagated since its discovery in 1940. Recently several groups have independently adapted this tumor to cell culture and demonstrated its potential for differentiation under appropriate conditions (Augusti-Tocco and Sato, 1969; Schubert et al., 1969; Klebe and Ruddle, 1969).

A large body of literature suggests that a "two-stage" theory of carcinogenesis is responsible for the origin of tumors of liver, lung, bladder, gastrointestinal tract, reticuloendothelial system, subcutaneous tissue, skeletal muscle, thyroid, mammary gland, and skin (Berenblum, 1969). The common features of this theory are the requirements for (a) a carcinogen and (b) a co-carcinogen, i.e., a proliferation and/or differentiation inducing molecule for the tissue in question. The unique and profound response of sympathetic ganglia to NGF suggests that aberrant interactions of this factor, environmental carcinogens, and target cells may be responsible for the relatively high incidence of sympathetic nervous system tumors. This hypothesis is currently being tested in our laboratory, with the ultimate aim of inducing neuroblastomas for adaptation to cell culture.

B. PRIMARY CULTURE OF DIFFERENTIATED NEURAL TUMORS

The induction of functional tumors is generally done in inbred animal strains, to permit the transplantation of tumors to recipient hosts without immunological rejection. Either primary or secondary (i.e., passaged) functional tumors may be used for adaptation to cell culture. Non-human neural tumors which have been successfully cultured to date have come from secondary subcutaneous tumors. Several laboratories are currently attempting to culture primary ethyl-nitrosourea induced tumors, however.

An animal carrying a palpable tumor of one to two grams is selected for culture. The animal is killed by cervical dislocation. The skin covering the tumor is washed thoroughly with alcohol, to disinfect the area. The skin around the tumor is carefully removed, using sterile forceps and scapel, or dissection scissors. The tumor is lifted free and placed in a sterile petri plate where all connective tissue, skin, etc. is carefully trimmed away and discarded. (In the case of primary neural tumors, the brain or peripheral nervous tumor is aseptically exposed, excised, and placed in a sterile petri dish.) The tumor is washed with one or two portions of sterile saline, to remove any blood, etc. The saline is removed by aspiration. The washed tumor mass is minced with fine dissecting scissors. With some soft tumors, the mechanical dissociation from this procedure will be sufficient to permit preparation of a cell suspension. In this case, culture medium is added to the minced tumor, and the preparation is dissociated by pipetting until a homogeneous cell suspension is prepared. Aliquots of this preparation are dispensed into culture flasks and/or petri dishes and placed in the incubator.

If the tumor is not easily dissociated by pipetting, enzymatic release of single cells will be required. In this case the minced fragments are again washed with sterile saline, then suspended in either a 0.25% trypsin solution or 0.5% viokase, (see Appendix B) and transferred to a sterile Erlenmeyer flask

containing a magnetic stirring bar. A gauze-wrapped cotton plug is used to prevent contamination. The cell suspension is gently stirred in a 37° incubator for ten minutes. Care should be taken to insure that the magnetic stirrer does not over-heat the cell suspension. After enzymatic treatment the suspension is dissociated by pipetting until a single cell suspension is achieved. The cells are then pelleted by low-speed centrifugation in a clinical centrifuge. Sterile disposable screw-cap plastic tubes useful for this procedure are commercially available. The cell pellet is resuspended in culture medium and aliquoted into culture flasks. Attachment of cells to the plastic surface of the culture dish will occur over the next 48 hours. At this point the culture medium should be aspirated off and replaced with fresh medium. Primary cultures are fed every two to three days and monitored microscopically. In many cases the cells will adapt readily to culture, and can be subcultured from the primary plate. In other cases, however, the growth of the neural tumor cells will appear to be quite slow, and the experimenter may wish to try the alternate culture and animal passage technique.

C. ANIMAL PASSAGE OF PRIMARY CULTURE

One or several of the primary cultures are washed several times with BSS. The cells are scraped from the plate in buffered saline with a rubber policeman and centrifuged in the clinical centrifuge. The cell pellet is resuspended in a small volume (0.5-2.0 ml) of BSS. Portions of the cell suspension are injected subcutaneously on the back or flank of several animals of the appropriate strain. When a subcutaneous tumor develops the procedure for primary culture is repeated. With each culture passage cells better able to adapt to the restricted conditions of cell culture are selected. Animal passage permits repopulation by tumor cells exclusively, since other cell types will not participate in tumor formation. Eventually tumor cells

that will adapt to culture conditions and compete effectively in cell culture with fibroblasts can be selected.

D. SUBCULTURE OF CULTURED CELLS

Monolayer cells are subcultured by removing them from the culture surface with trypsin, diluting in growth medium and plating in fresh culture flasks or petri plates. Culture medium is removed from the flask to be subcultured by aspiration. The plate is quickly washed with two ml of a sterile trypsin solution (0.1%). The trypsin wash is also removed by aspiration. After addition of one to two ml of trypsin, the plate is incubated at 37° until the cells lift off (determined microscopically). Cell lines vary in the length of time required for trypsinization. In general, the period of exposure to trypsin should be kept to the minimum time commensurate with detachment. The cells are pipetted repeatedly to break up clumps. Many investigators spin the cells out of the trypsin solution and resuspend them in fresh medium. Most cell culture media, however, contain sufficient serum to provide enough trypsin inhibitor upon dilution prior to plating to inactivate the enzyme. In my own laboratory we dilute the trypsinized cells in fresh growth medium and dispense this cell suspension. Cell numbers are determined by haemocytometer count, and appropriate dilutions are made for plating. While established cell strains and lines may have high plating efficiencies, and can be plated at quite low cell numbers, this is usually not the case with primary cultures, even of tumor origin.

E. CLONING OF CULTURED CELLS

Once a cell strain has been established by successfully subculturing the primary culture of a differentiated tumor, it is advantageous to clone the cells, and derive a cell line. This will help to insure that all cells in the subsequent studies will have similar genotypes, and will hopefully lead to populations which (1) are biochemically identical or (2) which have differences

which reflect instability of genetic expression in a genetically homogeneous population. By using clonal cell lines ambiguities which arise from a mixed cell population in the primary culture or cell strains can be eliminated. Sub-cloning is also used to isolate variant or mutant cell lines from a parental population, and for the isolation of cell hybrids.

The cell strain to be cloned is plated in serial tenfold dilutions, from 10^4 to 10^1 cells per plate. After approximately 10-20 days (depending on the doubling time of the cells in question) visible colonies will be present on the plates. An appropriate dilution plate, containing about ten colonies should be selected. The number of cells initially plated to attain this number of colonies will vary from strain to strain, and will be a function of the plating efficiency of the cell strain in question.

Cloning rings of either stainless steel or glass (6 mm diam., 12 mm high, of 1 mm wide) are placed in a glass petri plate containing a layer/silicon grease. The petri plate and its contents are sterilized in the autoclave. The colony(s) to be cloned is outlined with a marking pen on the bottom of the culture plate. The culture medium is removed by aspiration. The plate is quickly washed with sterile 0.1% trypsin in Saline A (see Appendix B). A sterile cloning ring is removed from the plate with a flamed forceps and pressed firmly over the colony to be cloned. Several drops of trypsin solution are placed inside the ring, using a sterile syringe. The plate is incubated at 37° until the cells detach from the plate. Microscopic observation of detachment is advisable. When the cells have detached, the needle of a sterile one ml syringe is imbedded in the plastic surface of the dish, within the cloning ring. This will prevent the experimenter from accidentally dislodging the cloning ring while removing the cells. The trypsin suspension should be pumped up and down in the syringe several times in the cloning ring prior to

transfer, to dislodge attached cells. The trypsinized cells are removed from the cloning ring with the syringe, and transferred to another plate. Finally, the cloning well is washed with a small amount of medium which is also transferred to the plate. Additional medium is added to the plate and it is placed in the incubator.

Some cell strains are unable to grow when plated at such low densities. In order to clone such cells, they may be plated on a "feeder layer" (an irradiated monolayer of another cell line). The "feeder layer" is no longer viable, and will not give rise to colonies upon replating, but will be able to support the growth of many cell strains at low density, and thus facilitate their clonal isolation (Puck et al., 1956). Those neural cell lines which have been cloned to date, however, have not required the use of "feeder layers". A detailed discussion of cloning theory and practice has recently been presented by Ham (1972).

F. CULTURE MEDIA

A variety of culture media have been used for the growth of established cell lines of neural origin. Our own laboratory has used Nutrient Mixture F10 supplemented with either 15% horse and 2.5% fetal calf serum or with 10% fetal calf serum alone, as well as Dulbecco's Modified Eagle's medium supplemented with 10% fetal calf serum. Nutrient Mixture F12 supplemented with 10% fetal calf serum has been used for the growth of clonal schwannoma cells (Pfeiffer, personal communication). In general the literature should be consulted for optimal growth medium for each cell line. Clonal cells of this type are generally not nearly so sensitive to variation in media as primary explants or dissociated cultures.

G. HYBRIDIZATION OF CULTURED CELLS

Somatic cell hybridization has been used to investigate the maintenance

and control of differentiated function in cultured cells. The technique can be used to study questions of genetic dominance, complementation, co-ordinate expression, and segregation of specific genes. The use of cell hybrids for the study of the regulation of nervous system specific functions has been applied to both functional glial and neuroblastoma cell lines.

1. Biochemical Basis for Hybrid Selection

Mammalian cells contain "salvage pathways" which permit the reincorporation of purines and pyrimidines into DNA. Normally these pathways are not the major route of biosynthesis of nucleotides, and cells are able to survive without these enzymes. When nucleotide biosynthesis is blocked, however, the salvage enzymes become essential. Two salvage enzymes, thymidine kinase (TK, converts thymidine to thymidine monophosphate) and hypoxanthine-guanine phosphoribosyl-transferase (HGPRT; converts guanine to guanylic acid and hypoxanthine to inosinic acid) have been utilized to develop a selective technique for isolating hybrids of cell lines.

Cells containing both of these enzymes can grow in the presence of aminopterin (an inhibitor of nucleotide synthesis) if the medium is supplemented with thymidine and hypoxanthine (Hypoxanthine, Aminopterin, and Thymidine, or HAT medium). Cells which are TK^- cannot grow in HAT medium, since they are unable to make TMP. Similarly, cells which are $HGPRT^-$ are unable to grow in HAT medium, since they cannot synthesize either GMP or IMP. Thus HAT medium is a selective medium for hybrids of TK^- and $HGPRT^-$ parents, since the hybrid cell will have both TK and HGPRT (Littlefield, 1964). The problem thus becomes one of isolating TK^- and/or $HGPRT^-$ parental cells strains which retain function. TK^- mutants may be selected for by isolating cells which are resistant to bromodeoxyuridine (BUDR). This thymidine analogue is phosphorylated by thymidine kinase prior to incorporation into DNA. Thus a TK^- mutant will be viable in

the presence of this drug, while a TK^+ cell will not survive. $HGPRT^-$ cells may similarly be selected for by growth in the presence of either azaguanine (AG) or thioguanine (TG). These characteristics are summarized in Table I.

TABLE I

<u>GENOTYPE</u>	<u>GROWTH IN BUdR</u>	<u>GROWTH IN AG OR TG</u>	<u>GROWTH IN HAT</u>
TK^+ , $HGPRT^+$	-	-	+
TK^- , $HGPRT^+$	+	-	-
TK^+ , $HGPRT^-$	-	+	-

In practice it is frequently difficult if not impossible to select drug-resistant mutants of functional cells which maintain their biochemically differentiated characteristics. To circumvent this problem a modification, termed the "half-system" of Littlefield's HAT system has been employed (Davidson and Ephrussi, 1965). In this case an undifferentiated BUdR-resistant cell line is fused with a much smaller number of differentiated cells not bearing a selective marker. The cells are grown on HAT medium to select against the TK^- parent. Colonies of hybrid cells are distinguished from parental colonies of the unselected type by morphology. The clonal isolates of such hybrids are analyzed biochemically (e.g. isozyme patterns) and karyologically to verify that the presumptive hybrids are correctly identified.

Spontaneous hybridization of cells is a relatively rare event, occurring once for perhaps every 1000 to 10,000 minority parent. Okada (1962) and Harris and Watkins (1965) have described a method employing inactivated Sendai virus which promotes increased frequencies of cell fusion. This procedure was adapted to the isolation of viable hybrids by Yerganian and Nell (1966). Coon and Weiss (1969) subsequently combined the Sendai virus fusion procedure with Littlefield's HAT selective technique in order to (1) produce relatively high frequencies of hybridization (100 times greater than spontaneous hybrids) and

(2) at the same time select against the parental cell lines.

Hybrids of functional neuroblastoma and glial clones with mouse fibroblasts have been selected. Mouse neuroblastoma cell lines resistant to thioguanine have been fused with BUdR resistant L cells with the aid of inactivated Sendai virus. Hybrid colonies were subsequently selected for in HAT medium (Minna, Nelson, Peacock, Glazer, and Nirenberg, 1971; Minna, Glazer, and Nirenberg, 1972), and confirmed both biochemically (by glucose phosphate isomerase isoenzyme analysis) and karyologically. A functional rat glial cell line (C₆) has been hybridized with two BUdR resistant mouse fibroblast lines, using the "half-system" selection against the parental fibroblasts. Presumptive hybrids were cloned and verified karyologically (Benda and Davidson, 1971).

2. Selection of Drug Resistant Neuroblastoma Cells

Minna et al. (1971) describe the following method for isolating HGPRT⁻ neuroblastoma cells. Clonal cells were treated with 5×10^{-3} M ethyl methane sulfonate (EMS) for two hours. This concentration of EMS will kill 60% of the neuroblastoma cells. The EMS-treated cells were grown in standard culture medium for five generations. The plates were subsequently shifted to 10^{-6} M 6-thioguanine (TG) to select for resistant mutants. Survivor frequency was approximately 2×10^{-5} . Surviving cells were pooled, subcultured, grown in standard media, and exposed to TG at a concentration of 4×10^{-6} M. Surviving cells were again pooled, then cloned in 10^{-4} M TG. A single clone (N4TG1) was selected for hybridization. This clone was maintained in 10^{-4} M TG. After growth for several weeks in the absence of TG, progeny retained resistance to the drug. Reversion frequency for this TG resistant line was on the order of 10^{-6} - 10^{-7} . Several other TG-resistant clones have subsequently been isolated from the same spontaneously occurring mouse neuroblastoma by these workers

(Minna et al., 1972). These are the only studies in which drug-resistant clonal neural cell lines have been isolated.

3. Hybridization Techniques

Minna and his associates and Benda and Davidson, the two groups which have hybridized functional neural cells, used inactivated Sendai virus to enhance fusion. The techniques for growing the virus and reducing its infectivity, either with β -propiolactone or UV-irradiation, are outside the scope of this chapter. A description of the preparation and inactivation of the virus may be found in the work of Coon and Weiss (1969). Inactivated virus is commercially available from Connaught Medical Research Laboratories, Willowdale, Ontario.

Minna et al. grew their parental strains in Dulbecco's modified Eagle's media containing 10% fetal calf serum. They hybridized N4TG1 (the TG resistant neuroblastoma line) to mouse L cell line B82, which lacks TK. Cells were hybridized at a final concentration of 2×10^6 cells per plate, in 5 ml of standard growth medium. Parental ratios varied between 1:0.7 and 1:200 (N4TG1: B82). For hybridization, parental cells were mixed in desired ratios and plated in 60 mm plates in 5 ml of growth medium. After 18-20 hours at 37° plates were washed with cold serum-free medium. Cells were then incubated with 200-500 hemagglutinating units/inactivated Sendai virus in serum-free medium at 0° for 10 minutes. The plates were rinsed twice with serum-free medium to remove unadsorbed virus, then incubated for ten minutes at 37° in serum-free medium. Plates were then incubated in HAT medium (DME with 10% fetal calf serum supplemented with 1×10^{-4} M hypoxanthine, 1×10^{-5} M aminopterin and 1.6×10^{-5} M thymidine). The procedure for Sendai virus promoted fusion is described in detail by Davidson (1969). Colonies were counted after 15-20 days to determine hybridization frequencies, and clonal isolates were picked.

In the hybridization studies of Davidson and Benda, the rat glial cell line RG6A, a sub-clone of C6 was hybridized to 1D, (a TK⁻ clone of mouse L cells) and 3T3-4E (a TK⁻ clone of 3T3 cells). In this case the glial cells did not carry a drug resistant marker. 1.5×10^6 mouse TK⁻ fibroblasts were mixed with 10^3 glial cells and plated in Nutrient Mixture F-10 supplemented with 10% fetal calf serum. After twenty-four hours the cells were treated with 100 hemagglutinating units of UV-inactivated virus, as described by Davidson (1969). The cells were then exposed to HAT medium prepared in an F10 base. After twenty-four hours the cells were removed with trypsin and plated into 40 6 cm culture dishes, in HAT medium. Colonies of RG6A x 1D hybrids and RG6A (the only two cell types surviving in HAT) were distinguishable by cell size and by morphology. Cells were picked, cloned and characterized karyologically. In this case one of every 25 parental glial cells formed a viable hybrid.

The studies of Minna et al., and Davidson and Benda are the only experiments using hybrids of cloned neural cells. These valuable techniques, which aid in the analysis of regulatory mechanisms will undoubtedly be used extensively in the future by cell biologists interested in the expression of neural function.

V. STUDIES WITH PRIMARY CULTURES

I will describe examples of neurochemical studies which are essentially unapproachable by any methodology other than culture. Apologies are offered to those investigators whose work has been omitted, either by choice or oversight, from this representative discussion.

A. PRIMARY EXPLANTS

1. Myelination

The bulk of these studies is primarily histochemical or cytological. Tissue as diverse as spinal ganglia and cerebellar explants myelinate extensively

in vitro, and can easily be monitored microscopically in the living state. Although many histochemical and ultrastructural reports exist concerning myelination by explants (see Murray, 1971), relatively few biochemical studies have been carried out because of the limited amount of material present. Recently Silberberg et al. (1972) have attempted to correlate the incorporation of radioactive sulfate into sulfatide (a major component of myelin lipid) with in vitro myelinogenesis. An increase in sulfatide labeling coincided with morphological myelination. There also appeared to be a correlation between the amount of sulfatide labeling and the density of myelination in well and poorly myelinated cultures. Studies of the synthesis of the specific protein components of myelin have not yet been reported. The technical requirements can be met by current procedures, however, and these studies should be feasible.

2. Inborn Errors of Metabolism

Although demyelination is not necessarily one of the initial events associated with congenital retardates, it has been noted in a number of such cases. Deficient myelination has been observed in phenylketonuria (Malamud, 1966) and maple syrup urine disease (branched chain ketonuria), two of the most studied aminoacidurias. Silberberg (1967) used an in vitro cerebellar myelinating system in an attempt to determine which of the accumulated metabolites in these pathological states are responsible for the observed demyelination. Phenylalanine produced no visible effects on cerebellar cultures at concentrations up to 9×10^{-3} M. This concentration is 4-5 times greater than blood levels. In contrast several indole-acids including indole acetate, indole-pyruvate, and indole-lactate were toxic at concentrations from 10 to 50 fold lower than this value. Normal development proceeded for 10 to 14 days in vitro, but was followed by progressive degeneration and vacuolation. Silberberg suggests that this indole-acid induced toxicity in cultures "may be relevant

to the pathogenesis of the mental defect and seizures" in phenylketonuria.

Patients with maple syrup urine disease present elevated levels of the branched chain amino acids leucine, isoleucine and valine, as well as their derivative branched chain keto acids α -keto-isocaproate, α -keto- β -methylvalerate, and α -keto-isovalerate. The biochemical block responsible for this elevation is apparently in the oxidative decarboxylation of the branched-chain keto-acids (Dancis, Hutzler, and Levitz, 1963). The neurological deterioration present in this disease can be prevented, and patients will show normal physical and mental development, if put on a diet low in branched chain amino acids soon after birth. Physiological (i.e. patient serum levels) concentrations of α -keto-isocaproate were able to prevent myelination in cerebellar explants, while other metabolites studied had no effect on myelination even at concentrations 6 times greater than α -keto-isocaproate (Silberberg, 1969). Silberberg concludes the report with the comment that "These results in cultures suggest that α -keto-isocaproic acid is directly responsible for at least some of the damage produced by maple syrup urine disease".

3. Neurological Mutants

Jimpy is a sex-linked recessive lethal mutation in the mouse characterized by a tremor, first apparent at ten to twelve days of age, of the rear legs and spine. The Jimpy brain shows a marked deficiency in myelin. The mutation is commonly said to closely resemble sex-linked sudanophilic leukodystrophy in humans. Wolf and Holden (1969) reasoned that myelination of Jimpy cerebellar explants would suggest either protection from some myelinotoxic agent produced in vivo, or the presence in the culture medium of a trophic factor found in normal animals but missing in the mutant. Failure of explants to myelinate would suggest that this inability was intrinsic to Jimpy CNS tissue. Only two of 23 presumptive Jimpy explants myelinated adequately. Jimpy and normal ex-

plants, when grown on the same coverslip, did not influence one another's myelination. The Jimpy mutation must therefore be intrinsic to CNS tissue, and apparently does not produce a diffusible myelinotoxic factor. The direct effect of the Jimpy mutation on CNS tissue could only be demonstrated in this fashion.

4. Hormonal Influences

Hamburgh (1966) has used cerebellar explants to investigate the effect of thyroid hormone on the maturation of neural tissue. Thyroxine treated rat and mouse cerebellar cultures displayed an accelerated myelinogenesis when compared with unsupplemented controls. At the concentration used in these experiments (1.5-3 $\mu\text{g/ml}$) toxic effects could be seen on glial cells in the outgrowth zone. The myelin-stimulating effect of thyroxine was enhanced if cultures were maintained at 32° rather than 37°. Cultures did not myelinate at the lower temperature unless thyroxine was present. These studies demonstrated a direct effect of thyroxine in vitro on neural tissue which mimicked the in vivo effect of the hormone, suggesting that the in vivo stimulation of maturation was the result of a direct interaction of neural cells with thyroxine. Additionally, since cultures at 32° did not myelinate in the absence of thyroxine, while those supplemented with this hormone did so, this system offers the potential for initiating myelination experimentally. Studies of this nature are invaluable in determining whether hormonal influence on neural processes occur by direct action, or are mediated by other organs.

5. Demyelinating Disorders

Experimental allergic encephalomyelitis (EAE) and experimental allergic nephritis (EAN), auto-immune diseases initiated by injecting central and peripheral nervous tissue respectively in Freund's adjuvant, produce demyelination in vivo. Multiple sclerosis (MS) and Guillain-Barré syndrome, two naturally

occurring diseases, also demonstrate extensive demyelination in vivo. Remarkable histologic similarities exist between EAE and EAN induced lesions and those observed in patients with these demyelinating diseases. The use of neural explants allows the investigator to ask about the immediate effects on neural tissue of cells, extracts, sera, etc. from EAE or EAN animals or patients with demyelinating diseases. Penetration of the blood-brain barrier, protective host reactions, and mediated rather than direct reactions are eliminated. Concentration and duration of exposure can be precisely controlled.

The earliest reported studies of EAE sera employed fully myelinated cerebellar cultures from neonatal rat. Sera from EAE rabbits caused initial swelling of neuroglia and myelin sheaths, followed by fragmentation and eventually dissolution of myelin, over a period of six to ninety-six hours. Normal rabbit serum or serum from rabbits inoculated with kidney tissue had no effect. EAE serum was unable to produce any observable morphological changes in cultured liver, muscle or fibroblasts (Bornstein and Appel, 1961). Only one study, using parabiotic animals, had demonstrated the passive transfer of a demyelinating agent (Lipton and Freund, 1953) at that time.

The specificity of sera causing demyelination in culture is quite extensive, and reflects the in vivo result. Sera from EAN animals can demyelinate peripheral nerve. The myelinotoxic agent can be absorbed by peripheral nerve, but not by CNS or non-neuronal tissue (Yonezawa, Ishihara, and Matsuyama, 1968). The specificity of EAE serum for CNS myelin can be demonstrated in cultures of combined spinal cord and dorsal root ganglia, which contain both central and peripheral myelin (Raine and Bornstein, 1970).

The demyelinating agent from EAE sera was shown to be complement-dependent and present in the gamma 2 globulin fraction, suggesting it was a 7S immunoglobulin(s) (Appel and Bornstein, 1964). Yonezawa et al. (1968) reported that

the IgM fraction of EAE and EAN serum possessed demyelinating activity. Dowling et al. (1968) demonstrated complement-dependent demyelinating factors in the 7S and 19S serum fractions from MS patients. The active 7S fraction was characterized as IgG by chromatography and immunoelectrophoresis. These authors conclude that MS sera has both IgM and IgG demyelinating antibodies.

EAE can also occur in the absence of circulating humoral antibody. The present consensus favors a cellular rather than humoral immunological basis for the clinical and histological reactions. Lymph node cells taken from EAE animals cause demyelination of CNS neural explants (Bornstein and Iwanami, 1971). Lymphoid cells from EAN animals are able to demyelinate peripheral, but not CNS cultures (Winkler, 1965; Arnason, Winkler, and Hadler, 1969).

Remyelination will occur when myelinotoxic sera from EAE or MS serum is removed from demyelinated CNS cultures (Bornstein and Appel, 1961). Cultures can, in fact, be subjected to repeated demyelination and remyelination. These observations were subsequently confirmed in observations of intact animals (Bubis and Luse, 1964; Lampert, 1965; Prineas, Raine, and Wisniewski, 1970) and MS patients (Feigin and Popoff, 1966; Suzuki, Andrews, Waltz, and Terry, 1969). EAE serum causes a disappearance of myelinating oligodendroglia and an increase in fibrillary astroglia in explant cultures (Raine and Bornstein, 1970). Remyelination and the simultaneous reappearance of oligodendroglia occur one to two weeks after removal of EAE serum.

At appropriate EAE antiserum concentrations initial myelination of the CNS portion of combined spinal cord-dorsal root explants is totally inhibited, while myelination of the peripheral nerve proceeds normally (Bornstein and Raine, 1970). Much lower concentrations of EAE antisera are required to prevent the initial myelination than are necessary for demyelination of mature cultures. Cultures can be maintained free of myelin for two months. Myelin

appears within three to five days when the EAE sera is removed. In the absence of myelin neurons and their synapses appear grossly normal with no apparent decrease in the number of synapses. Nonmyelinated cultures do not have recognizable differentiated oligodendroglia or astroglia, however. This system thus appears able to temporally dissociate neuronal maturation from glial maturation and myelin production in cultured explants.

6. Synaptogenesis and Onset of Bioelectrical Activity

The bioelectric maturation of explanted central and peripheral neural tissue has been reviewed several times (Crain, 1966; Crain, Peterson, and Bornstein, 1968; Murray, 1971). The reader is referred to these sources for extensive and expert analysis of this work. Maturation from simple axonal spike conduction to complex bioelectric activity indicative of the formation of synaptic networks appears to proceed subsequent to explantation of embryonic neural tissue. Initially absent axosomatic and axodendritic synapses appear in spinal cord (Bunge, Bunge, and Peterson, 1967) and cerebellar cultures. Spontaneous complex discharges can also be seen in cord cultures and in cerebellar explants (Crain, 1966). Embryonic cord with attached myotomes exhibits ultrastructural and electrophysiological in vitro maturation of neuromuscular junctions (Crain, 1964; Bornstein, Iwanami, Lehrer, and Breitbart, 1968).

Several experiments will be cited to demonstrate the unique contributions which cultured explants have made to our understanding of the electrophysiological development of neural tissue. Crain and his co-workers have investigated the establishment of functional neuromuscular junctions between heterologous explants in culture. Functional neuromuscular junctions can form between explanted rat muscle and mouse cord and vice versa (Peterson and Crain, 1970). Histochemical, ultrastructural, and electrophysiological studies have demonstrated that these heterologous neuromuscular junctions form functional

connections between rat muscle and mouse nerve. Fetal rodent spinal cord explants can also innervate either adult rodent muscle or adult human skeletal muscle and form functional neuromuscular junctions (Crain, Alfei, and Peterson, 1970). These studies, which permit the analysis of neurotrophic effects of embryonic neurons and facilitate the investigation of the phylogeny of neuromuscular interaction, represent significant and unique contributions of tissue culture to neurobiology.

The question of the necessity of spontaneous electrical activity for progressive maturation of complex synaptic systems has occupied neurobiologists for a number of years (Carmichael, 1926; Matthews and Detwiler, 1926). Experiments on sensory deprivation in amphibian embryos (Hamburger, Wenger, and Oppenheim, 1966) while instructive, have been unable to resolve this question. There is no apparent interference with morphological or ultrastructural maturation when explants of 17 day fetal mouse cerebral cortex or 14 day fetal rat cord (prior to appearance of synaptic function) are cultured in the presence of either xylocaine (50 $\mu\text{g/ml}$) or a high Mg^{++} concentration (Model, Bornstein, Crain, and Pappas, 1971). Either treatment blocks complex bioelectric activities. After removal of these agents, cultures which have been kept under conditions in which no spontaneous electrical activity occurred are able to respond to the first electrical stimulus with a complex bioelectrical discharge, indicative of multisynaptic activity identical to that of sister cultures grown in conventional medium (Crain, Bornstein, and Peterson, 1968). These results suggest that the development of functional complex interneuronal networks in the central nervous system is indeed able to proceed in the complete absence of any previous electrical activity in the component cells.

7. Nerve Growth Factor (NGF)

Nerve growth factor causes an extensive outgrowth of neurites from

explanted chick embryo sensory ganglia (Levi-Montalcini, Meyer, and Hamburger, 1954). This "halo effect" in culture has become the standard method of detecting and quantitating nerve growth factor in biological fluids. The characteristics and quantitation of this response are described in the excellent review of Levi-Montalcini and Angeletti (1968). Numerous in vitro biochemical studies of the effects of nerve growth factor on explanted ganglia have been described. Levi-Montalcini and Angeletti reviewed studies on glucose metabolism, lipid metabolism, protein and RNA synthesis published prior to 1968. In general NGF appears to stimulate and maintain the bulk of biosynthetic processes in these explanted ganglia. Selective synthesis of acidic proteins by ganglia cultured in the presence of NGF was indicated by column chromatography or acrylamide gel electrophoresis of soluble protein fractions (Gandini-Attardi, Calissano, and Angeletti, 1967). Double labeling experiments indicated an increased polysomal population in NGF treated cultures, as expected from the observed increase in RNA and protein synthesis. Qualitative differences in size of the polysomes or the distribution of free and bound polysomes were not observed, however (Amaldi, 1971).

B. DISSOCIATED CELLS IN CULTURE

The primary objective of this type of study has been to determine whether neuronal precursors can mature into functional neurons when isolated from other cells, or whether cellular interaction is required for the development of neuronal characteristics. Cultures have been studied primarily by light and electron microscopy and electrophysiology. Little biochemistry has been done on such preparations, because of this focus on the maturation of individual cells. Great emphasis has been placed on the development of media and supplements which enhance the plating efficiency and morphological maturation of neurons in culture.

Electrophysiological maturation in culture of neurons from chick spinal sensory ganglia has been demonstrated. A greater number of cells achieved morphological and electrophysiological neuronal characteristics in the presence of NGF. Intracellular recordings demonstrated that these neurons were not spontaneously active, but were able to generate action potentials when subjected to a cathodal stimulation. No electrophysiological evidence for synaptic potentials could be observed (Varon and Raiborn, 1971). The observation that NGF appeared to promote the survival and maturation of sensory neurons confirmed the earlier report of Angeletti and Levi-Montalcini (1963). However, over 60% of selected neurons from chick sensory ganglia cultured in the absence of NGF are reported to respond to electrical stimulation and produce action potentials (Scott et al., 1969). The role of NGF on electrophysiological maturation is difficult to assess in these experiments, since (1) Varon and Raiborn do not report the relative frequency of electrically excitable cells in NGF and NGF-free cultures and (2) Scott et al. analyze cells in culture from 33 to 50 days, while Varon and Raiborn observe much younger cultures. The inherent biases in the selection of cells for electrophysiological observation also make a quantitative assessment of this question difficult. Despite the lack of electrophysiological evidence for synapses in dissociated sensory ganglia cultures, electron microscopic evidence of synaptic structures has been reported (Miller, Varon, Kruger, Coates, and Orkand, 1971).

Although individual neurons could be observed ultrastructurally and electrophysiologically in the experiments of Varon and Scott, these cells grow on a monolayer of fibroblasts and glial cells derived from the cultured ganglia. Cell separations and growth media selective for the neuronal populations need to be devised to permit isolation of neuroblasts free from

supportive cells, in order to investigate completely the question of neuronal autonomy. Preliminary experiments with spinal ganglia suggest that neuroblasts isolated on density gradients are viable (diZerega, Johnson, Morrow, and Kasten, 1970). Cultures enriched in neurons may also be prepared by preferentially adsorbing non-neuronal cells from dorsal root ganglia on glass beads (Okun, Ontkian, and Thomas, 1972). Neuronal populations from chick embryonic cerebrum can be isolated relatively free of glial elements and cultured successfully (Varon and Raiborn, 1969). Biochemical and electrophysiological studies of such preparations have not yet been described, however.

Mitochondrial enzymes (i.e., succinic dehydrogenase, monoamine oxidase and glutamic dehydrogenase) and cholinesterase are reduced to histochemically undetectable levels in two days when sensory and sympathetic neurons from 7-12 day old chick embryos are cultured in total embryo extract. Neurons cultured in the presence of NGF demonstrated both mitochondrial enzymes and cholinesterase activity after two weeks in culture (Ciesielski-Treska, Hermetet, and Mandel, 1970; Hermetet, Treska, and Mandel, 1969). Less than 3% of dissociated 12 day chick embryo spinal ganglia neurons incorporate tritiated thymidine, while 27% of the non-neuronal cells do. Labeled uridine is initially incorporated into nuclei, and then distributed over the perikaryon. No label is found in the fibers. Tritiated amino acid incorporation occurs exclusively in the perikaria. Label appears to flow down the fibers at a rate of 10 mm per day (Utakoji and Hsu, 1965). Sensenbrenner, Treska, Lodin, and Mandel (1970) confirmed the tritiated uridine observations and extended them to older cultures maintained in the presence of NGF.

Morphological and ultrastructural evidence for synaptic contacts between dissociated embryonic muscle cells and dissociated spinal cord neurons has

been reported (Shimada, Fischman, and Moscona, 1969a, 1969b). A low-density culture system in which neuromuscular synaptogenesis could be demonstrated electrophysiologically has been described (Fischbach, 1970, 1972). Non-myogenic and non-neuronal elements may be selectively eliminated prior to the establishment of functional cholinergic neuromuscular synapses (see Sec. III, C4, and also Fischbach, 1972). These cultures provide a system in which the neurochemist may study chemical influences on trophic phenomena and synaptogenesis at the single cell level.

C. REAGGREGATION CULTURES

The first report of reagggregates of neural tissue described the histotypic reaggregation of embryonic chick cerebral hemispheres, mid-brain, and neural retinal cells, dissociated and cultured by the methods described by Moscona. Aggregation was age dependent, and was able to sort-out in commingled cultures of neural retina and cerebrum (Ishii, 1966). In another early report four day old chick embryo eye cups, when dissociated by trypsin and subsequently allowed to reaggregate in rolling tubes for 25 days, formed ultrastructurally identifiable synaptic regions (Stefanelli et al., 1967).

DeLong (1970) dissociated hippocampal and pallial isocortex structures from near-term fetal mice using Moscona's methods. Optimal histological reaggregation was observed after seven days of rotation culture. A characteristic "early organization" containing two distinguishable cell populations could be observed when hippocampal cultures were prepared from fetuses prior to 18.5 days of gestation. Cultures derived from hippocampal preparations of fetuses of 18.5 days gestation showed a series of stages of progressive histological complexity, culminating in the formation of a layer of pyramidal cells which ultimately had the histological characteristics of the Ammon's horn structure. Hippocampal structures were not observed in the relatively non-specific and randomly

organized aggregates derived from fetuses of 19 days gestational age or greater. Development of the Ammon's horn structure in aggregates requires parallel alignment, uniform orientation, and formation of a structure of characteristic curvature by a relatively homogeneous population of pyramidal cells, in addition to their simple sorting out. This cellular interaction, more complicated than the histotypic sorting out of cells from different organs, requires self-organization among a population of structurally homogeneous cells. The ability to form this complex histotypic structure must be a property of these cells themselves, since they assemble in isolation. The factor(s) responsible must also be able to survive dissociation or be produced subsequently by the cells in culture. Optimal histological organization of reaggregated isocortex was also dependent on gestational age. The aggregates from dissociated isocortex of 17.5 to 18.5 days gestation were compact, spherical and composed of three histologically distinct concentric layers resembling the molecular layer, the cortical plate (in which cells were in parallel alignment) and a third inner layer of fibers and cells with no characteristic organization.

DeLong and Sidman (1970) used the same culture system to characterize the defect in the autosomal recessive Reeler mutation (Falconer, 1951) which affects the structure of the cerebellum and cerebrum (Hamburgh, 1960, 1963). The isocortex is similar to the wild-type mouse in size and cell number, but is abnormal in cellular arrangement. Both neuronal layering and orientation are defective (Fig. 4). DeLong and Sidman suggest defective control of cellular migration, alignment, and orientation is responsible for the Reeler phenotype. Fetal 18.5 day-old Reeler isocortex formed spherical aggregates in which there was only poor cell sorting, and no cellular alignment. These observations are summarized in Fig. 5. Similarly, Reeler cerebellar aggregates

Fig. 4

Fig. 5

2

did not form the characteristic histotypic patterns present in aggregates of wild-type cerebellum. The Reeler mutation therefore operates directly on the cortical cells, and is not mediated by some other system.

Reaggregation cultures provide a useful system for studying the effects of a variety of compounds and conditions on biochemical maturation of neural tissue. In the first report of biochemical studies on aggregates, the specific activities of choline acetyltransferase, acetylcholinesterase and glutamate decarboxylase of whole mouse brain aggregates were compared with those activities in vivo during development (Seeds, 1971). All three enzymes reached their peak specific activity after about 17 to 20 days in culture. The activities rose twenty fold, ten fold and five fold, and attained levels of 70%, 40%, and 30% of their respective specific activities in adult brain. In contrast, the specific activities of these enzymes decreased in cells grown in mouse brain monolayer cultures. Seeds and Gilman (1971) have demonstrated a norepinephrine-induced increase in cyclic AMP levels in neural aggregates which parallels the response seen in vivo. Neither fetal tissue or early aggregates (i.e., 15 hours) responded to norepinephrine or isoproterenol. After nine days in culture the aggregates showed a four to six fold increase in cyclic AMP in response to either drug, a stimulation similar to that of adult mouse brain.

Dissociated cells from various regions of embryonic chick and mouse brain exhibit characteristic age profiles for homologous aggregation (Garber and Moscona, 1972a,b). Co-aggregation and internal sorting are related to the degree of homology between co-cultivated brain regions of the two species. Cerebral 14 day fetal mouse cells cultured as monolayers produce a soluble factor which specifically promotes the aggregation of mouse and chick cerebral cells. The factor does not extensively promote aggregation of corpora

quadrigemina, cerebellum, medulla or liver cells of fetal mouse, or optic tectum, cerebellum, neural retina, kidney or liver cells of chick embryo. Production of this factor in monolayer culture is dependent on the embryonic age of the cerebral cells. Responsiveness of cerebral aggregating cultures is also age-dependent. The cerebral aggregation factor promoted a 350-fold increase in aggregate volume when prepared from monolayers of 14 day old fetal cerebral cultures and titrated on 14 day old fetal cerebral aggregating cultures.

VI. STUDIES WITH CLONAL CELLS

A. GLIAL CELLS

Several groups have described the culture of human "glia-like", glioma, or astrocytoma cells, and the establishment of cell strains (Manuelidis, 1965; Pontén and Macintyre, 1968; Westermarck, Pontén, and Hugosson, in press). Studies of proliferation, movement, and density dependent inhibition of division of "glia-like" human cells have been reported (Westermarck, 1971; Pontén, Westermarck, and Hugosson, 1969). Similarly, human fetal brain cell strains characterized as astrocytic or spongioblastic have been reported (Shein, 1965). Strains of cells from hamster brain which have some morphological and classical staining characteristics of astrocytes have also been established (Shein, Britva, Hess, and Selkoe, 1970). These established strains of fetal human and hamster cells have been employed primarily to study the susceptibility of brain cells to transformation by the oncogenic DNA viruses SV40 and polyoma (Shein, 1967, 1970).

Many of the studies on cultured glial cells are primarily concerned with morphological, ultrastructural, and histochemical characterization of cells from normal brain or tumors. Morphological and histochemical characteristics are based on observations of tissue in situ, however. Their extension to

dispersed cells in culture may well be somewhat tenuous. Cultured cells which do not possess the appropriate morphology, but which synthesize specific cell products can be cited. Alterations in cellular morphology due to variations in culture conditions can be demonstrated. A more reliable criterion of organ-specific cell lines in culture would be the production of an organ-specific compound or the expression of a unique physiological function. Several cogent suggestions for neuron-specific products can be put forth, based on the production or destruction of neurotransmitters. There is little information on glial function which points to a chemical or macromolecule specific to these cells, however, with the singular exception of myelin (or its components) in oligodendroglia or Schwann cells.

Brain-specific function has been approached by attempting to detect and purify proteins unique to the nervous system (Moore and McGregor, 1965). Moore (1965) purified a highly acidic protein, S-100, from beef brain. S-100 is confined to the nervous system, is found in a wide variety of vertebrates, and is serologically quite similar among various species (Kessler, Levine, and Fasman, 1968). Several chemically induced tumors of rat CNS with histological characteristics of astrocytomas produce S-100 protein. Alternate culture and passage of one of these tumors was used to establish a cell line (termed C₆) which produces S-100 (Benda, et al., 1968). Subsequently a second cell strain, CHB, was established which also produces this protein (Lightbody, Pfeiffer, Kornblith, and Herschman, 1970). This second strain, originally reported to be of human origin, has been recently shown by both karyological and isoenzyme analysis to be derived from rat tissue (de Vellis, Stoolmiller, personal communication).

Accumulation of S-100 does not occur in growing C₆ cells prior to contact with one another, but begins when the cells reach confluency and enter stationary phase. In contrast, CHB cells synthesize S-100 during exponential growth

(Lightbody et al., 1970). Inhibition of division of C₆ prior to cell contact is unable to initiate accumulation of S-100. Mixed culture experiments demonstrated that homologous cell contact is required for accumulation (Pfeiffer, Herschman, Lightbody, and Sato, 1970). C₆ cells adapted to suspension culture lose the ability to accumulate S-100. Loss of S-100 production is accompanied by loss of specific membrane antigens, suggesting a relationship between surface antigenicity and the expression of this differentiated function (Pfeiffer, Herschman, Lightbody, Sato, and Levine, 1971; Herschman, Breeding, and Nedrud, 1972). Hybrid clones of C₆ cells and mouse fibroblasts produce little or no S-100 protein (Benda and Davidson, 1971). A technique for specifically and quantitatively isolating radioactively labeled S-100 from cultures has been used to measure the synthesis and degradation of this protein in CHB cells (Herschman, 1971) and is currently being applied to C₆ cells.

C₆ cells have been used to evaluate glial contamination of various synaptosomal preparations. Fractionation of a C₆ mitochondrial preparation on discontinuous sucrose gradients yields large quantities of plasma membrane in the "synaptosomal" fraction. Fractionation on ficoll-sucrose gradients results in relatively little glial membrane in the synaptosomal region. These data suggest glial plasma membrane is more apt to be present in synaptosomal preparations isolated by the prior procedure (Cotman, Herschman, and Taylor, 1971).

Regulation of specific enzyme levels in cultured glial cells by agents which have similar effects either in vivo or on other neural preparations has been reported. Adrenalectomy and cortisol replacement therapy suggest that glycerol phosphate dehydrogenase (GPDH) is regulated in brain by cortisol levels (de Vellis and English, 1968). Cortisol induces GPDH accumulation up to 18 fold over a five day period in subclones of C₆ cells, but not in other cell lines, including CHB. Cortisol must be continuously present for maximal

induction. Cells either in exponential or stationary phase are inducible. Inhibitors of RNA and protein synthesis are able to block induction, suggesting that cortisol is acting at a transcriptional level. Several other hormones, including aldosterone, corticosterone, cortisone, and dexamethasone are active, while progesterone, estradiol, testosterone, insulin, thyroxine and cyclic AMP have little or no effect (de Vellis, English, and Galey, 1971). In C₆-mouse fibroblast hybrids GPDH is not induced by hydrocortisone (Davidson and Benda, 1970). Epinephrine and norepinephrine (1-10 µg/ml) increase lactic dehydrogenase in C₆ cells two to three fold (de Vellis *et al.*, 1971). This stimulation is sensitive to actinomycin D and cycloheximide. Cyclic AMP at high concentrations (0.5 mM) substitutes for norepinephrine in the LDH, but not GPDH induction (de Vellis and Brooker, 1972). Cyclic AMP, but not norepinephrine, is able to stimulate LDH activity in CHB cells.

Cyclic AMP levels increase dramatically in brain slices in response to biogenic amines, depolarizing agents, or electrical stimulation (Kakiuchi, and Rall, 1968; Shimizu, Creveling, and Daly, 1970). Several groups have reported a norepinephrine or isoproterenol induced increase in cyclic AMP in C₆ cells (Gilman and Nirenberg, 1971; Schultz, Hamprecht, and Daly, 1972; de Vellis and Brooker, 1972). Results with appropriate blocking agents suggest a β -receptor mechanism in these cells. Norepinephrine, epinephrine and isoproterenol increase adenyl cyclase activity in C₆ cell homogenates (Schimmer, 1971). Cyclic AMP does not increase in C₆ cells in response to other agents active in brain slices such as tyramine, histamine and dopamine, or to prostaglandins. De Vellis and Brooker suggest that cyclic AMP serves as the mediator in the norepinephrine induced rise in LDH in C₆ cells. Cyclic AMP levels also respond to potential neurotransmitters in a human glial line (1181N1) derived from a glioblastoma multiforme (Clark and Perkins, 1971). Like C₆,

1181N1 responds to norepinephrine and epinephrine. Log cells show a much greater response than stationary cells. In contrast to C₆ cells, 1181N1 is responsive to histamine. The latter response is not regulated by the age of the culture. Studies with propranolol suggest a β -adrenergic receptor for epinephrine and norepinephrine. In contrast, propranolol has no effect on the histamine induced increase by cyclic AMP. Although the functional significance of neurotransmitter-induced accumulation of LDH and/or cyclic AMP in glial cells is unclear, this stimulation is also observed in brain slices, and may perhaps reflect some aspects of neuron-glia interactions which can be analyzed in culture.

B. NEUROBLASTOMA CELLS

Tumors of neuronal origin are relatively rare in adults. Sympathetic neuronal tumors, or neuroblastomas, are common in childhood, however. Although primary culture of human neuroblastoma has been studied, only a few continuous strains from human tumors exist (Goldstein, Burdman, and Journey, 1964; Tumilowicz, Nichols, Cholon, and Greene, 1970; Lyon, 1970). Several laboratories, however, have described both suspension and monolayer culture of a murine neuroblastoma, C1300 (Klebe and Ruddle, 1969; Schubert et al., 1969; Augusti-Tocco and Sato, 1969). This spontaneous tumor, found in the abdominal cavity of a A/J mouse in 1940, grows as a relatively undifferentiated round-cell tumor in vivo. Tumors and suspension culture cells do not stain with the Bodian silver technique. Cells grown in monolayer extend long processes, or neurites (Fig. 6), and are positive to the Bodian stain. Initial biochemical studies demonstrated the presence of choline acetylase and tyrosine hydroxylase in clonal cell lines of C1300 cells (Augusti-Tocco and Sato, 1969; Schubert et al., 1969). Subsequently, lines which are cholinergic (i.e., high levels of choline acetyltransferase, low levels of tyrosine

Fig. 6

hydroxylase), adrenergic (low levels of choline acetyltransferase, high levels of tyrosine hydroxylase) and which do not synthesize either acetylcholine or catechols were isolated (Amano, Richelson, and Nirenberg, 1972). Electrophysiological studies were rapidly initiated to demonstrate unequivocally the functional differentiation of these cells. Intracellular recording demonstrated spontaneous and stimulated action potentials in monolayer cultures (Nelson, Ruffner, and Nirenberg, 1969; Nelson, Peacock, Amano, and Minna, 1971). Action potentials can be elicited by iontophoresis of acetylcholine (Harris and Dennis, 1970; Nelson, Peacock, and Amano, 1971).

Several groups have looked without success for synaptic activity in C1300 cells. A trophic interaction of neuroblastoma cells and clonal rat skeletal muscle cells exists, however. The muscle fibers formed in clonal culture exhibit supersensitivity to iontophored acetylcholine similar to that of denervated muscle. A region of localized increased chemosensitivity to iontophored acetylcholine is observed at the point where muscle and nerve cell make contact in mixed culture (Harris, Heinemann, Schubert, and Tarakis, 1971). These results are similar to initial stages of synaptogenesis in muscle.

6-Hydroxydopamine is selectively toxic to sympathetic neuroblasts in vivo, resulting in chemical sympathectomy. Neuroblastoma cells in culture retain this unique chemosensitivity (Angeletti and Levi-Montalcini, 1970). C1300 clones do not respond to NGF, and are not adversely affected by antisera to NGF, in contrast to sympathetic neuroblasts in vivo (Herschman, unpublished). Neurite outgrowth in response to NGF administration in two continuous strains of human neuroblastoma has recently been demonstrated, however (Goldstein, personal communication).

Induction of neuronal differentiation in C1300 clones has recently been the subject of a number of reports. The existence of a program of differen-

tiation in culture related to the degree of cell division is still an open question, however. The impetus for this interest was the observation that reduction of serum levels caused a decrease in cell division and a concomitant increase in the number of cells extending neurites (Seeds, Gilman, Amano, and Nirenberg, 1970). Neurite outgrowth has been assumed to be a neuron-specific event, and thus the expression of an organ-specific phenomenon induced in a less differentiated population. Dibutyryl cyclic AMP (Prasad and Hsie, 1971; Furmanski, Silverman, and Lubin, 1971) prostaglandins (Prasad, 1972), X-rays (Prasad, 1971), and inhibitors of phosphodiesterase (Prasad, 1972) have also been reported to promote neurite extension. Prostaglandins also stimulate the synthesis of cyclic AMP (Gilman and Nirenberg, 1971) in C1300 clones. Neurite outgrowth is also induced by 5-bromodeoxyuridine. This analogue can promote neurite extension in the absence of DNA synthesis, however (Schubert and Jacob, 1970).

Several biochemical parameters have been monitored in a search for characteristics indicative of a differentiative event other than neurite extension. The spectrum of proteins synthesized in suspension culture differs from that of monolayer cells with neurites (Schubert, Humphreys, DeVitry, and Jacob, 1971). A surface glycopeptide is present on C1300 neuroblastoma cells grown in 5-bromodeoxyuridine but not on cells grown on its absence (Brown, 1972). Neither phenomena, however, is necessarily neuron-specific. Neuroblastoma cell membranes but not membranes of C₆ or CHB contain the glycosphingolipids G_{M2}, G_{M1}, G_{D2} and G_{D1A} characteristic of neural tissue. Production of glycosphingolipids as a function of growth has not been reported, however (Dawson, Kemp, Stoolmiller, and Dorfman, 1972). The specific activity of acetyl cholinesterase increases 20-25 fold over a 48 hour period when neuroblastoma cells are shifted from normal to serum-free medium (Blume,

Gilbert, Wilson, Farber, Rosenberg, and Nirenberg, 1970; Kates, Winterton, and Schlessinger, 1971). An increase in acetylcholinesterase levels has also been observed after X-irradiation or addition of dibutyryl cyclic AMP (Prasad and Vernadakis, 1972). Increased specific activity of acetylcholinesterase is also observed in the stationary phase of growth, when neurite extension occurs (Blume et al., 1970). An inverse relation between cell division and differentiation for C1300 cells has been postulated on the basis of these results. Conditions can be found, however, in which division is prevented without neurite extension, or in which neurite extension and cell division occur concomitantly, suggesting cessation of cell division and neurite extension are not necessarily linked phenomena (Schubert et al., 1971). Cell division can also be halted in a manner which produces a rise in the specific activity of acetylcholinesterase, but no neurite outgrowth (Prasad and Vernadakis, 1972). Acetylcholinesterase production in serum-free medium seems to occur in two phases. An initial slow increase associated with neurite formation is followed by a second increase apparently associated with a reduction in viable cells (Schubert, Tarikas, Harris, and Heinemann, 1971). This latter increase occurs both in suspension cultures and in monolayer cells with neurites. The relationship between cell division, neurite outgrowth, and acetylcholinesterase levels in neuroblastoma cells is thus still somewhat obscure. The in vivo rate of conversion of tyrosine to dopamine, dopa and norepinephrine is similar in suspension and monolayer cultures (Schubert et al., 1969). "Shift down" from a serum-rich to serum-free medium does not increase the specific activity of tyrosine hydroxylase, choline acetylase or microtubule protein (Kates, Winterton, and Schlessinger, 1971). Cloned suspension cells of neuroblastoma, negative for the Bodian stain and possessing no neurites, are capable of producing an action potential (Heinemann and

Gilbert, Wilson, Farber, Rosenberg, and Nirenberg, 1970; Kates, Winterton, and Schlessinger, 1971). An increase in acetylcholinesterase levels has also been observed after X-irradiation or addition of dibutyryl cyclic AMP (Prasad and Vernadakis, 1972). Increased specific activity of acetylcholinesterase is also observed in the stationary phase of growth, when neurite extension occurs (Blume et al., 1970). An inverse relation between cell division and differentiation for C1300 cells has been postulated on the basis of these results. Conditions can be found, however, in which division is prevented without neurite extension, or in which neurite extension and cell division occur concomitantly, suggesting cessation of cell division and neurite extension are not necessarily linked phenomena (Schubert et al., 1971). Cell division can also be halted in a manner which produces a rise in the specific activity of acetylcholinesterase, but no neurite outgrowth (Prasad and Vernadakis, 1972). Acetylcholinesterase production in serum-free medium seems to occur in two phases. An initial slow increase associated with neurite formation is followed by a second increase apparently associated with a reduction in viable cells (Schubert, Tarikas, Harris, and Heinemann, 1971). This latter increase occurs both in suspension cultures and in monolayer cells with neurites. The relationship between cell division, neurite outgrowth, and acetylcholinesterase levels in neuroblastoma cells is thus still somewhat obscure. The in vivo rate of conversion of tyrosine to dopamine, dopa and norepinephrine is similar in suspension and monolayer cultures (Schubert et al., 1969). "Shift down" from a serum-rich to serum-free medium does not increase the specific activity of tyrosine hydroxylase, choline acetylase or microtubule protein (Kates, Winterton, and Schlessinger, 1971). Cloned suspension cells of neuroblastoma, negative for the Bodian stain and possessing no neurites, are capable of producing an action potential (Heinemann and

Schubert, personal communication). Other clones are reported to "provide evidence that regulation of the action potential occurs in neuroblastoma cells", however (Peacock, Minna, Nelson, and Nirenberg, in press).

We have looked for several brain specific antigens in C1300 clones without success. Recently, however, we have found that IMR-32, a cell strain derived from a human neuroblastoma (Tumilowicz et al., 1970) produces the brain-specific protein 14-3-2 (Moore/ 1968) and Perez, The level of this protein does

Fig. 7 not change as the cells progress from exponential to stationary phase (Fig. 7), in contrast to the results described for S-100 protein in C₆ cells. Similar results for IMR-32 and NJB, another human neuroblastoma line, have also been found by Kohler, Goldstein and Moore (personal communication).

Minna et al. (1971, 1972) have utilized clonal neuroblastoma-L cell hybrids in an attempt to characterize the control mechanisms which lead to expression of neuronal function. Hybrids of electrically excitable mouse neuroblastoma and electrically passive L cells with a characteristic electrical marker not present in neuroblastoma were selected by the HAT method. Electrophysiological characteristics of both parents could be demonstrated in many clonal hybrids (Minna et al., 1971). Generally elevated levels of acetylcholinesterase, increased levels of acetylcholinesterase in stationary phase relative to exponential phase, histochemical staining for acetylcholinesterase, neurite formation, and Bodian staining could also be found in many hybrids. A tentative sequence of steps involved in neuron maturation has been postulated, based on a cross-correlation of retained neuronal properties in the hybrids (Minna et al., 1972). No data have been reported on the presence of neurotransmitter synthesizing enzymes in either the drug-resistant neuroblastoma strains or in the hybrid clones.

VII. CONCLUSIONS

Cell culture studies will undoubtedly continue to play an expanded role in neurochemistry. The advantages offered by these various preparations for isolation of specific tissues from internal organismal interactions and the opportunity to control and manipulate the pharmacological and nutritional environment of tissue over extended periods of time are unique to this methodology. The nature of the problems approachable by cell and explant culture varies with the different in vitro preparations discussed, however.

The technology of explant cultures, either in Maximow slides or on flying coverslips, has not changed significantly in recent years. New information derived from studies of primary explant cultures will probably not result from dramatic alterations in culture technology, since this type of culture has by far the most extensive history of systematic study of optimization of culture conditions. Instead, advances in this field will emerge as a result of the application of microanalytical chemical techniques and recent technology developed for electron microscopy, such as freeze-fracture and freeze-etch studies of maturing cultures. The limited amount of tissue available in these cultures, as well as in dissociation and reaggregation cultures, will continue to put emphasis on the development of sophisticated analytical procedures. The systematic investigation of agents which block the maturation of various cell types in the nervous system (e.g. EAE serum, 6-hydroxydopamine) and a variety of pharmacological agents which affect neural function should provide extensive information on the necessity of various cell types and specific biochemical parameters for the achievement and maintenance of organotypic neural structure and function.

Studies of dissociated neural tissue and reaggregation cultures, in contrast to primary explant cultures, are still concerned to a large degree.

with optimization of culture conditions. With the exception of the work of DeLong and Sidman (1970), the studies carried out to date have primarily provided data on the maturation of in vitro preparations and have yet to produce a great deal of information not previously ascertained from in vivo investigations. These two culture techniques have now reached a point, however, where they should make substantial contributions to neurochemistry. Electrophysiological, electron microscopic, and microchemical analysis of individual maturing neurons in isolation should begin to provide an answer on the molecular as well as functional level to the questions of the ability of and program by which isolated neuroblasts develop into functional neurons. The major drawback to this as well as the other culture preparations, is our inability to date to observe specificity of cellular interaction. Systematic application of cell fractionation techniques prior to dissociation culture, and the dissociation culture of systems in which unique cells with specified connections exist (such as are known in a variety of invertebrates), may overcome this formidable drawback of cell and tissue culture and provide an isolated system in which study of the development of neural specificity will be possible. The use of cell fractionation techniques in conjunction with re-aggregation cultures will also provide a valuable experimental system to investigate questions of required cellular interactions in the histotypic and biochemical maturation of neural tissue.

Clonal cell cultures which retain neural properties have brought the powerful methodologies of molecular and cell biology to neurochemistry. The growth of large populations of cells with similar properties permits biochemical analyses not possible with cultures of limited numbers of cells of heterogeneous types provided by primary culture. The use of procedures developed for studies of cell biology, such as cell hybridization and variant

selection, currently permit an analysis of the regulation of genetic expression and the study of interacting genomes on some aspects of neural function. The problems arising from the study of neoplastic tissue, while certainly a consideration, will diminish as additional functional tumors are isolated, cultured, and compared. Once again, while providing a system in which biochemical parameters of neural maturation can be easily measured and manipulated, the current clonal cell culture systems obviously lack the characteristic cellular specificity at the heart of neural function. If functional neuronal tumors which are capable of forming synapses with target cells in culture can be induced, however, a powerful tool for the study of the biochemical basis of neural specificity will emerge.

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X. APPENDIX

APPENDIX A: Preparation of Rat Tail Collagen
(Bornstein, M.B. (1958) Lab. Invest. 7, 134)

1. Remove rat tails and store in freezer.
2. Soak tails in 95% ethanol for 15 minutes. All subsequent steps are carried out aseptically.
3. Fracture tails into small pieces (beginning at tip).
4. Pull each piece free, cut tendons and place in petri dish containing sterile distilled water.
5. Tease tendons into fine pieces with sterile forceps.
6. Transfer teased filaments from one tail to a sterile 250 ml centrifuge bottle containing 150 ml of 1:1000 acetic acid.
7. Seal bottle and store at 4° for 48 hours.
8. Centrifuge 2 hours at 2300 rpm.
9. Remove 40 ml of supernatant and store at 4°.
10. Add 30 ml 1:1000 acetic acid to residue.
11. Store residue at 4° for 24 hours.
12. Centrifuge again for 2 hours at 2300 rpm.
13. Remove 40-80 ml of collagen supernatant and store at 4°.
14. Shake residue to resuspend and store for additional 24 hrs. at 4°.
15. Centrifuge once again and remove acetic acid collagen solution (30-70 ml).

These solutions can be stored for up to ten months in the refrigerator.

16. Place 10 ml acetic acid collagen solution in autoclaved dialysis bag.
17. Dialyze against 750 ml sterile distilled water at 4°. Dialysis for 24 hrs.

produces a collagen solution which is easily spread. Longer periods of dialysis produce a solution of greater viscosity. Dialysis for more than 48 hrs. may produce a gel which cannot be spread. The water should be changed after 24 hrs. if a more viscous gel is desired.

18. Remove bag from flask and dry with a sterile towel.
19. Transfer collagen to a sterile glass container. This solution may be stored from 1 to 2 weeks at 4°.

APPENDIX B. BALANCED SALT SOLUTION FORMULATIONS

Component	Mg/L						
	<u>Earle's (1)</u>	<u>Gey's (2)</u>	<u>Hanks' (3)</u>	<u>Moscona's Ca⁺⁺ Mg⁺⁺ Free CMF (4)</u>	<u>Phosphate Buffered Saline (Dulbecco) (5)</u>	<u>Saline A (6)</u>	<u>Simms' (7)</u>
NaCl	6800	7000	8000	8000	8000	8000	8000
KCl	400	370	400	300	200	400	200
NaH ₂ PO ₄ ·H ₂ O	-	-	-	50	-	-	-
NaH ₂ PO ₄	125	-	-	-	-	-	213
Na ₂ HPO ₄ ·2H ₂ O	-	150	360	-	-	-	-
Na ₂ HPO ₄	-	-	-	-	1150	-	-
MgSO ₄	100	-	-	-	-	-	-
MgSO ₄ ·7H ₂ O	-	70	200	-	-	-	-
MgCl ₂ ·6H ₂ O	-	210	-	-	100	-	203
KH ₂ PO ₄	-	30	60	25	200	-	-
CaCl ₂	200	170	140	-	100	-	-
CaCl ₂ ·2H ₂ O	-	-	-	-	-	-	147
Dextrose	1000	-	-	-	-	-	1000
Glucose	-	1000	1000	2000	-	1000	-
NaHCO ₃	2200	2270	350	1000	-	350	1010

CONTINUATION OF APPENDIX B

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APPENDIX C. MEDIA FORMULATIONS

Component	Mg/L					
	<u>BME (1)</u>	<u>CMRL 1415 (2)</u>	<u>DME (3)</u>	<u>MEM (4)</u>	<u>F10 (5)</u>	<u>F12 (6)</u>
NaCl	6800.00	6800.00	6400.00	6800.00	7400.00	7599.00
KCl	400.00	400.00	400.00	400.00	285.00	223.00
KH ₂ PO ₄	-	-	-	-	83.00	-
NaH ₂ PO ₄	-	-	124.00	-	-	-
NaH ₂ PO ₄ ·H ₂ O	140.00	50.00	-	-	-	-
NaH ₂ PO ₄ ·2H ₂ O	-	-	-	150.00	-	-
Na ₂ HPO ₄	-	200.00	-	-	-	-
Na ₂ HPO ₄ ·7H ₂ O	-	-	-	-	290.00	268.10
CuSO ₄ ·5H ₂ O	-	-	-	-	0.00250	0.00249
FeSO ₄ ·7H ₂ O	-	-	-	-	0.834	0.834
Fe(NO ₃) ₃ ·9H ₂ O	-	-	0.10	-	-	-
MgCl ₂	80.00	-	-	-	-	-
MgCl ₂ ·6H ₂ O	-	-	-	200.00	-	122.00
MgSO ₄ ·7H ₂ O	-	240.00	200.00	-	152.80	-
CaCl ₂	200.00	140.00	200.00	200.00	-	-
CaCl ₂ ·2H ₂ O	-	-	-	-	44.10	44.11
ZnSO ₄ ·7H ₂ O	-	-	-	-	0.0288	0.864
Glucose	1000.00	500.00	1000.00	1000.00	1100.00	1801.60
Galactose	-	500.00	-	-	-	-
NaHCO ₃	2200.00	1000.00	3700.00	2000.00	1200.00	1176.00
L-arginine-HCl	210.00	-	84.00	-	211.00	210.70
L-arginine	-	500.00	-	105.00	-	-

	<u>BME(1)</u>	<u>CMRL 1415(2)</u>	<u>DME(3)</u>	<u>MEM(4)</u>	<u>F10(5)</u>	<u>F12(6)</u>
L-isoleucine	26.22	52.00	105.00	52.00	2.60	3.936
L-leucine	26.22	52.00	105.00	52.00	13.00	13.00
L-histidine	7.76	31.00	-	31.00	-	-
L-histidine-HCl·H ₂ O	-	-	42.00	-	-	20.96
L-histidine-HCl	-	-	-	-	21.00	-
L-lysine-HCl	-	-	146.00	-	29.30	36.54
L-lysine	29.22	47.00	-	58.00	-	-
L-methionine	7.46	15.00	30.00	15.00	4.48	4.48
L-phenylalanine	16.52	32.00	66.00	32.00	4.96	4.96
L-serine	-	12.00	42.00	-	10.50	10.51
L-threonine	28.82	48.00	95.00	48.00	3.57	11.91
L-tryptophan	4.08	10.00	16.00	10.00	0.600	2.04
L-tyrosine	18.20	36.00	72.00	36.00	1.81	5.44
L-valine	23.40	46.00	94.00	46.00	3.50	11.71
L-glutamine	292.00	292.00	580.00	292.00	146.20	146.20
L-cystine	12.15	24.00	48.00	24.00	-	-
L-cysteine-HCl·H ₂ O	-	-	-	-	-	35.12
L-cysteine	-	179.00	-	-	-	-
L-cysteine-HCl	-	-	-	-	31.50	-
L-asparagine·H ₂ O	-	-	-	-	15.00	15.01
L-proline	-	30.00	-	-	11.50	34.53
Hydroxy-L-proline	-	10.00	-	-	-	-
L-alanine	-	30.00	-	-	8.91	8.91
L-aspartic acid	-	10.00	-	-	13.30	13.30
L-glutamic acid	-	10.00	-	-	14.70	14.70
Glycine	-	17.00	30.00	-	7.51	7.51

	<u>BME(1)</u>	<u>CMRL 1415(2)</u>	<u>DME(3)</u>	<u>MEM(4)</u>	<u>F10(5)</u>	<u>F12(6)</u>
Hypoxanthine	-	-	-	-	4.08	4.08
Ascorbic acid	-	50.00	-	-	-	-
Putrescine	-	-	-	-	-	0.161
Sodium pyruvate	-	-	110.00	-	110.00	110.10
Thymidine	-	10.00	-	-	0.727	0.727
Choline·HCl	1.00	1.00	4.00	1.00	0.698	13.96
Biotin	1.00	1.00	-	-	0.024	0.00733
Ca-pantothenate	1.00	0.50	4.00	1.00	0.715	0.258
Niacinamide	-	-	-	-	0.615	0.0366
Nicotinamide	1.00	-	4.00	1.00	-	-
Pyridoxine·HCl	-	-	-	-	0.206	0.068
Pyridoxal HCl	1.00	-	4.00	1.00	-	-
Thiamine-HCl	1.00	-	4.00	1.00	1.012	0.337
Folic acid	1.00	1.00	4.00	1.00	1.32	1.32
Riboflavin	0.10	-	0.40	0.10	0.376	0.376
Vitamin B ₁₂	-	-	-	-	1.36	1.36
D-Inositol	-	2.00	7.20	2.00	0.541	18.02
Lipoic acid	-	-	-	-	0.200	0.206
Linoleic acid	-	-	-	-	-	0.0841
Coccarboxylase	-	1.00	-	-	-	-
Codecarboxylase	-	1.00	-	-	-	-
Diphosphopyridine nucleotide	-	7.00	-	-	-	-
Flavin adenine nucleotide	-	1.00	-	-	-	-
Glutathione	-	10.00	-	-	-	-

	<u>BME(1)</u>	<u>CMRL 1415(2)</u>	<u>DME(3)</u>	<u>MEM(4)</u>	<u>F10(5)</u>	<u>F12(6)</u>
Triphosphopyridine nucleotide	-	1.00	-	-	-	-
Uridine triphosphate	-	1.00	-	-	-	-
Deoxyadenosine	-	10.00	-	-	-	-
Deoxycytidine	-	10.00	-	-	-	-
Deoxyguanosine	-	10.00	-	-	-	-
5 methyldeoxycytidine	-	0.10	-	-	-	-

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3. Dulbecco's Modified Eagle's Medium, as prepared by Gibco: Dulbecco, R. and Freeman, G. (1959) Virology 8 396.
4. Minimal Essential Medium: Eagle, H. (1959) Science 130 432.
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6. Nutrient Mixture F12: Ham, R.G. (1972) in "Methods in Cell Physiology, Vol. 5" (D. Prescott, Ed) Academic Press, New York, London, p 37.

FIGURE LEGENDS

Fig. 1. Top and side views of a Maximow slide culture. The completed Maximow assembly includes (A) the depression slide, (B) a 40 mm square coverslip (C) a collagen-coated round coverslip 22 mm in diameter, (D) the tissue explant (E) culture media. Details of assembly and incubation are discussed in the text.

Fig. 2. Onset of myelination in newborn mouse cerebellar explants. Data are plotted as the percentage of new explants myelinating in each 24 hour in vitro period. Eight explants of equal size are prepared from a single newborn mouse cerebellum. Onset of myelination of single explants is illustrated in (A). When explants are paired randomly on coverslips, the onset of myelination is distributed in a biphasic manner (B). When explants are paired topographically in vitro (i.e. tissue fragments which were contiguous in vivo are paired in vitro) the onset of myelinogenesis is monophasic, and occurs over a relatively short time interval (C). Topographic pairing greatly increased both synchrony of myelination and number of myelin sheaths formed. Axons course extensively between topographically paired cerebellar explants. Data are redrawn from Allerand and Murray (1968).

Fig. 3. Effect of K^+ concentration on number of neurons in dissociated cell cultures of chick embryo dorsal root ganglia. DRG from 10-day old chick embryos, dissociated as described in the text, were plated on collagen-coated coverslips in media of various K^+ concentrations. Osmotic pressure was kept constant by adjusting the NaCl concentration. Neurons were counted after eleven days in culture. Each coverslip was sampled at 1 mm intervals (441 samples per coverslip). The results of three separate

experiments are presented. Data are redrawn from Scott and Fisher (1970).

Fig. 4. Reaggregation culture of normal and reeler isocortex from litter-mates. Fetal isocortexes from animals of 18.5 days gestation were dissociated as described in the text and allowed to aggregate in rotation culture for six days prior to sectioning and staining with Toluidine blue. Normal isocortex aggregates (A) have sorted into three relatively well-defined regions; a central area of poorly organized cells and fibers, a concentric middle layer of radially aligned cortical neurons with parallel processes, and an outer zone relatively poor of cells. In contrast the reeler aggregate (B) has a somewhat less clearly defined outer cell-poor zone and a second inner region of poorly oriented cells, with randomly directed processes, which extends to the center of the aggregate. Magnification is 120X. Data are taken from DeLong and Sidman (1970).

Fig. 5. Normal and reeler isocortical aggregates. Vertical columns of boxes represent successful cultures from a single litter. Each box is a single culture. Open boxes ($\square$) indicate poor aggregate organization. Filled boxes ($\blacksquare$) indicate excellent radial organization. Hatched boxes ($\square$ with diagonal lines) indicate good radial organization. Histological examination of intact cerebral cortex showed that all aggregates in this latter class were from wild-type animals. $\boxed{R}$ indicates culture derived from animals with reeler phenotype as indicated by histological examination of the intact cortex. All successful cultures of histologically identified reeler isocortex failed to show radial organization. $\boxed{Q}$ indicates an abnormal cortex not typical of reeler, which also failed to show radial organization in reaggregation culture. No normal animal produced aggregates similar to reeler. Aggregate organization in wild-type cultures was optimal for

samples prepared in the age range of 18-18.5 days of gestation. Reeler and wild-type cultures prepared from older fetuses or newborn animals were not distinguishable; aggregates were small. Aggregates of reeler and normal isocortex prepared from animals prior to 17.5 days of gestation were larger than those of 18.5 days, but were poorly organized and could not be distinguished from each other. Reeler and wild-type phenotypes could not be reliably distinguished histologically prior to 17.5 days of gestation.

Fig. 6. Neuroblastoma cells in culture. (A); Exponentially growing neuroblastoma cells, clone N18 in Dulbecco's Modified Eagle's medium (DME) with 10% fetal calf serum (B); stationary phase N18 cells grown on DME with 10% fetal calf serum. (C); N18 cells cultured in serum-free DME for 4 days.

Fig. 7. Production of the nervous system-specific protein 14-3-2 by human neuroblastoma line IMR-32. Cells were grown in Nutrient Mixture F10 plus 10% fetal calf serum, in 100 mm Falcon tissue culture plates. Medium was changed at 48 hour intervals. Cell number per plate, (o); Protein per plate, (●); 14-3-2 C' units per plate, determined by complement fixation (this unit, used because of the cross-reaction between beef and human 14-3-2, is defined as [reciprocal of the dilution of a soluble extract required to give 40% complement fixation in the antibody-excess region of the complement fixation curve with antisera to beef 14-3-2] X [volume of extract per plate]), (□); 14-3-2 C' units/μg protein, (⊗).

FIG. 2. (A) Section through the center of an aggregate of cells dissociated from the isocortex of a normal fetus at 18.5 days of gestation and aggregated in rotating culture for 6 days. Toluidine blue stain in this and all subsequent pictures. The young cortical neurons have formed a concentric middle layer, with a distinct outer margin and a less distinct but nevertheless definite inner margin. A central area, containing cell bodies and fibers without evident organization, is present. The young cortical neurons are seen to be aligned radially with their apical processes directed outward toward the periphery.

Fig. 1 Morphology of growing and non-growing neuroblastoma cells in culture. *a*, Log-phase growing culture; *b*, cells 3 days after removal of serum from the culture medium; *c*, 5 days after removal of serum.