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BIOELECTRIC ACTIVITY OF NEURONS IN TISSUE CULTURE: SYNAPTIC INTERACTIONS AND EFFECTS OF ENVIRONMENTAL CHANGES

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TABLE OF CONTENTS

ABSTRACT

Tissue cultures were prepared from cerebella and mid-brain of neonatal rats by embedding the explants in plasma clots on cover slips and incubating them in roller tubes at 36°C with a nutrient medium consisting of 75% balanced salt solution and 25% calf serum. Within about two to three weeks the cultures flattened so that the nerve cells eventually became arranged in a two-dimensional array surrounded by a felt-like matrix of glial cells and glial and neuronal processes. Under a phase contrast microscope, the living nerve cells could then be clearly seen and distinguished from other cells (glia, fibroblasts, etc.). Some of the structural features of neurons, such as Nissl substance and neurofibrils as revealed by histological methods, appeared quite normal. Structures resembling terminal boutons were commonly seen after Bodian's or Holmes' silver impregnation of unsectioned cultures. Myelin formed in the cultures usually after about 10 - 12 days *in vitro*.

By positioning microelectrodes under visual control near the soma of a neuron, spontaneous extracellular action potentials were recorded from the majority of cells tested. That the spikes originated in the soma of neurons nearest to the electrode and not from axons or dendrites in the vicinity was proven by showing that the spikes stopped when the neuron in question was killed. At room

temperature, the average frequency of spikes was generally between 50 and 300 spikes per minute, with bursts, doublets and triplet spikes frequently occurring.

The average spike frequency could be reduced to almost zero and the duration of the spike could be increased by a factor of almost two by lowering the temperature from 25°C to 16°C. At 36°C the spike frequency was up to twice as high and the duration of the spike was distinctly shorter than at room temperature (25°C). Above about 43 to 45°C the spike activity stopped irreversibly. The interspike interval histogram usually showed several peaks with rather narrow distributions, and in general did not follow a Gaussian or Poisson distribution, which seems indicative of neuronal circuits or some other non-random influence.

Spike trains simultaneously recorded with two microelectrodes from two neurons in the same microscopic field and separated by as far as 200 μ were usually loosely correlated, i.e., some spikes from one neuron were accompanied by a near-coincident spike from the second neuron, while there were also spikes from each neuron that did not correlate in time with spikes from the second neuron tested.

The average spike frequency could be increased and the discharge pattern drastically altered by the addition of 10 μg/ml

strychnine to the bathing medium during electrophysical recording. Reduction of the calcium concentration in the bathing medium (from 2.55 mM to 1.2 mM) resulted in a temporary high frequency burst of spikes followed by a depression of the spike frequency. Decreasing or increasing the magnesium concentration caused an increase or decrease, respectively, of the average frequency of unit spike activity.

Membrane potentials of up to -77 mv and subthreshold signals resembling EPSP's could be recorded with intracellular electrodes from neuronal somas.

From the non-random interspike intervals, from the high degree of correlation of two simultaneously recorded spike trains, and from the effects of strychnine and magnesium, it is concluded that the neurons in these thinly spread cultures form synaptically connected nets of interacting units. These thin cultures therefore seem to be a favorable preparation for the study of some problems of mammalian neurobiology, where simplification of the system, controllable environment, visibility, and accessibility of individual living neurons is desirable.

I. INTRODUCTION

Nervous tissue grown and maintained *in vitro* represents a model system for the study of some of the properties of neurons and their interactions under relatively simple and controllable conditions. Many investigations of cultured nervous tissue were and are still concerned with demonstrating the integrity of its crucial cellular and organotypic parameters, such as its cytological, morphological, ultrastructural, bioelectrical, and biochemical properties. As will be seen from a review of the relevant literature, most of the important structural and functional attributes of nerve cells are indeed maintained in culture; thus neurons differentiate and mature *in vitro*, their electrical properties correspond well to the *in vivo* situation, and they seem to retain at least some of their biochemical specificity.

A. STATEMENT OF THE PROBLEM

Obtaining successful cultures of nervous tissue seems to be as much an art as a science. The establishment, improvement, and refinement of culture techniques to obtain cultures of uniform differentiation and development was prerequisite to this study, consequently much time has been spent on this aspect of the problem. Next, a characterization of the spontaneous bioelectric activity was necessary be-

fore embarking upon the main goal of this investigation. This was first, to establish that the neurons in thinly-spread cultures have functional synapses forming nets of interacting units; and second, to explore some of the properties of these interactions with extracellular and intracellular microelectrodes under a variety of environmental conditions.

B. DEVELOPMENT OF NERVE TISSUE CULTURE TECHNIQUES

1. Advantages and Disadvantages of Tissue Culture

Long-term isolation of tissues has both experimental disadvantages and advantages. It implies a considerable disruption of the normal environment of the cells, as well as a distortion of the *in vivo* topographic and physiological relationships of the tissue. If the environment is changed too drastically, as for instance in established subcultured cell lines, the cells usually lose their characteristic morphology and function and dedifferentiate into a more embryological state (Harris, 1964). For some primary tissue cultures, where an attempt is made to allow the cells to be integrated into a tissue, as is the case in the cultivation of nervous tissue, dedifferentiation is usually not evident. Nevertheless, one has to be constantly on guard for such undesirable effects when dealing with cultured tissue. On the other hand, isolation and the ensuing simplification of the tissue in culture under suitable conditions allows

a) control of the physical and chemical environment (nutrients, ions, hormones, drugs, temperature, radiation, etc.);

b) isolation from the controlling and modifying influences (neural, humoral, hormonal) of other tissues in the body;

c) continuous observation of the cells in their living state under a suitable microscope (phase contrast, polarizing, interference, fluorescent, etc.) permitting study of morphology and cell dynamics;

d) accessibility of individual cells and even parts of cells to exploration with microelectrodes, microbeams, and other micro-instruments; and

e) the possibility of studying intracellular molecular synthesis and degradation with tracer and other techniques.

Compared to tissue slices and other similar short-term isolation techniques, long-term isolation in cultures often permits recovery from the dissection trauma and from the transient changes occurring in the tissue in its adaptation to the new environment. The recovery may not be complete, but hopefully at least some slowly changing condition is reached which allows meaningful experimentation.

All of these factors are of special significance in the study of nervous tissue, because the nervous system is an almost hopelessly complex organ consisting of various functionally and structurally different cell types. Simplification, coupled with the controllable environment, visualization, and accessibility made possible by the technique

of tissue culture, may therefore be one of the productive avenues to the study of some aspects of the nervous system.

2. Historical developments

A valuable guide to the older literature is the Bibliography of the Research in Tissue Culture compiled by Murray and Kopech (1953). Several recent books treat modern culture methods in detail and offer an introduction to various aspects of tissue culture research (Cameron, 1950; Parker, 1961; Penso & Balducci, 1963; Harris, 1964; Merchant, Kahn & Murphy, 1964; Paul, 1965; and Willmer, 1965). The reviews by Thomas (1956), Murray (1965), and Lumsden (1968) deal in particular with nervous tissue in culture.

Early attempts at culturing isolated tissue by von Recklinghausen (1866), Roux (1885), Ljunggren (1897), and many others, may only have resulted in a somewhat delayed death and decay of the tissues. But Jolly (1903), for instance, was able to maintain and observe amoeboid movement and cell divisions of leucocytes in vitro for about a month. In order to study the development of nerve fibers, Harrison (1907, 1910) developed a technique of culturing nerve tissue from embryonic frogs by embedding the explants in lymph clots on coverslips inverted and sealed over depression slides. In this way he observed the formation of fibers by protoplasmic outflowing from the central perikarya and thus substantially contributed to the confirmation of the neuron doctrine of nerve development.

Morphological studies continued from 1910 to the present, but the main effort went into experiments on the regenerative and proliferative capacities of explanted cells, using tissues of many origins. Until better methods had been developed, the study of nervous tissue was limited by the apparent inability of the cultures to survive for longer than a week or two, after which the newly grown fibers usually started to degenerate (Ingebrigtsen, 1913). Nevertheless, significant contributions to many problems of neurology were made during this time; e.g., Levi and Meyer (1945) on nerve regeneration; Weiss & Hiscoe (1948) on axoplasmic flow; on fiber orientation during development (summarized in Weiss, 1955); and on the existence of (non-artefactual) Nissl substance and neurofibrils in living neurons (reviewed by May and Courtey, 1966).

3. Modern Nerve Tissue Culture Methods

Any tissue culture method must attempt to provide the tissue with an environment that is close to the *in vivo* situation while allowing for the simplification and accessibility demanded by the particular experiment. Successful long-term cultivation of nervous tissue seems to be possible with a number of techniques using various combinations of vessels, substrates for cell attachment, and compositions of nutrient media. However, several critical requirements are common to all techniques:

a) An optimum size of the explant, usually about 1 mm^3, small enough to allow diffusion of nutrients and oxygen to all parts of the tissue, yet large enough to leave a maximum number of cells undamaged or with recoverable damage (Peterson, Crain & Murray, 1965; Lumsden, 1968).

b) A suitable substrate for cell attachment. Since cultures of nervous tissue do not adhere well to uncoated glass, several methods are commonly employed to hold the explants in place. Embedding the tissue in a plasma clot (chicken plasma clotted with chicken embryo extract) (Burrows, 1910) is one of the most convenient methods. Most neural tissues (except avian spinal ganglia) attach well to cover glass coated with a film of reconstituted collagen (Bornstein, 1958). Holding the explant under a strip of dialysis membrane has also been successfully employed (Pomerat, 1959); and culturing tissue on cellulose sponges has been reported (Cunningham & Estborn, 1958; Cunningham, 1962).

c) A nutrient medium consisting of a balanced salt solution (BSS) supplemented with serum and possibly other natural fluids (human placental serum; various fetal, newborn, or adult animal sera; various sera ultrafiltrates; and embryo extracts) (Murray, 1959; Peterson & Murray, 1960), with a high glucose concentration (Pomerat & Costero, 1956; Murray, Peterson & Bunge, 1962; Orr, 1965), and buffered

at a pH of 6.8 to 7.1 (Lumsden, 1968).

d) Sufficient oxygenation of all parts of the tissue.

e) Incubation at a temperature of 36-37°C (Peterson & Murray, 1960).

f) Lack of toxicity in all parts of the culture chamber, especially the cover glass (Peterson, Deitch & Murray, 1959).

g) Maintenance of strict sterility throughout all manipulations.

Based on the above requirements, several methods have emerged during the last two decades and are most commonly in use today:

a) The Maximow double coverslip assembly (Maximow, 1925), which was first used for nervous tissue by Murray & Stout (1942), consists of a small circular coverslip which bears the culture (either in a plasma clot or on a film of collagen) attached with a drop of balanced salt solution to a larger cover glass which is sealed with a petrolatum-paraffin mixture over a depression slide. The cultures are usually incubated in a "lying drop" position with a drop of rich medium (containing high concentrations of sera, embryo extracts, and ultrafiltrates) forming a thin film over the culture. This technique usually yields a kind of organ culture many cell layers thick which has retained a great deal of organization (Bunge, Bunge & Peterson, 1965, 1967); it

is well suited to allow continuous or repeated observation without disturbance. The cultures have to be opened frequently for feeding and washing and consequently require constant attention.

b) Roller tubes were used by Hogue (1947), and Costero and Pomerat (1951) added flying coverslips to carry the explants. The use of about 2 ml of nutrient medium allows much lower concentrations of serum and embryo extract in the medium and requires a much less frequent feeding schedule. The rolling action results in repeated draining, aeration, and refeeding of the cultures. Under these conditions, glial and mesenchymal migration seems to be encouraged; after approximately two weeks in culture the three-dimensional arrangement of neurons has become transformed into a two-dimensional array of nerve cells embedded in a matrix of dendrites, axons, glial cells, and glial processes thin enough to allow good visualization with the phase contrast microscope. Such cultures represent a much less complex system than the thick Maximow chamber cultures. Because of the favorable visibility and accessibility of the neurons, and because of the relative ease of preparation of the cultures, this technique has been selected for the present study and will be described and discussed in detail under "Materials and Methods".

c) The Rose perfusion chamber (Rose, 1954; Rose, Pomerat, Shindler & Trunnel, 1958) consists of two cover glasses on either side of an inert rubber gasket clamped between

two suitable metal plates. The cultures are usually covered with a strip of dialysis membrane (in order to encourage spreading) (Pomerat, 1959; Orr, 1965), but plasma clots or collagen coats can also be used; or a film of collagen can be combined with a dialysis membrane cover (Hendelman & Booher, 1966). Feeding is accomplished by inserting a sterile syringe needle through the rubber gasket, or permanent ports can be provided for perfusion for pharmacological and toxicological studies during observation (Pomerat, 1962).

Dissociation of cells represents an entirely different approach. Instead of trying to preserve organotypic organization as much as possible, cells -- usually of embryonic origin -- are dissociated by chemical and/or physical means immediately after explantation. They can then be cultured in suspension; plated on glass, collagen films, or in plasma clots; or recombined into small pellets. This system offers promising opportunities for studying purely cellular aspects; for assessing the importance of tissue integrity in cellular function, morphology, and differentiation; or for investigating the potential for reaggregation into histotypic structures (e.g., synapses). Only recently has this technique been successfully applied to some parts of the nervous system (St. Amand & Tipton, 1954; Nakai, 1956; Levi-Montalcini & Angeletti, 1963; Hillman & Sheikh, 1968). Some of the morphological and functional characteristics found in these cells

(Nissl substance, argyrophilic processes, large resting potentials, and excitable membranes with normal action potentials) demonstrate apparently normal neuronal differentiation (Scott, Engelbert & Fisher, 1969).

C. STRUCTURAL CHARACTERISTICS OF CULTURED NERVOUS TISSUE

The reviews of Murray (1965) and May and Courtey (1966) treat this subject extensively; the following discussion will consequently be restricted to highlighting some of the aspects relevant to this particular investigation. Cultures from different parts of the nervous system exhibit individual differences in their *in vitro* behavior. The patterns of development of cultured rat and kitten cerebellum have been described by Bornstein and Murray (1958) for Maximow slide cultures, and by Hild (1966) and Mamoon, Schlapfer and Tobias (1968) for roller tube cultures. These patterns consist essentially of an outgrowth of non-neuronal cells, a flattening of the cultures, and an appearance of myelin. Some neuronal migration has been reported in cultures of sympathetic ganglia (Murray & Stout, 1947) and fetal human cerebellum (Hogue, 1950), but it is now generally agreed that neurons of the cerebrospinal system are usually quite immobile. Under favorable conditions cultures have survived for up to six months without any noticeable deterioration (Lumsden, 1968), and rat sympathetic ganglia, for instance, have even been maintained for up to eleven months (Coidan, 1964).

Cultures of nervous tissue contain a variety of types of neurons, depending on the origin of the explant, in addition to various types of glial cells and mesenchymatous elements. In cultures of cerebellum of human fetuses, for instance, Hogue (1947) described granule cells, Golgi type II neurons, Purkinje cells, and giant dentate neurons. Similar observations were made by Pomerat and Costero (1956) in cultures of cerebellar folia of newborn kittens, and by Wolf (1964) in cultures of cerebellum of newborn mice, although it seems that cerebellar granule cells are difficult to distinguish from oligodendrocytes except for the retention of their neuronal electrical properties (Lumsden, 1968). Hild (1954, 1957c, 1966) has examined roller tube cultures from various other parts of the newborn mammalian brain, generally finding at least some of the neuronal types represented as in vivo. In cerebellum for instance, he found Purkinje cells (with slight morphological alterations, such as a reduction and simplification of the dendritic tree), and large multipolar cells from the deep nuclei; in brainstem cultures, he found large multipolar neurons from the lateral vestibular nucleus of Deiters and unipolar neurons from the mesencephalic Vth nucleus.

Among the structural characteristics specific to neurons are a) Nissl substance, b) neurofibrils, c) myelin, and d) synaptic structures. All of these are found in cultures in forms that are very similar to their in vivo counterparts, as the next section will review.

electron microscope, on the other hand, reveals clear and typical synaptic profiles in cultures of fetal rat spinal cord (Bunge, Bunge, Peterson & Murray, 1963; Bunge, Bunge & Peterson, 1965, 1967), rat cerebellum and brain stem (Callas & Hild, 1964; Lumsden, 1968), mouse cerebral cortex (Pappas, 1966), and chick embryo central nervous system (Meller & Haupt, 1967). The various types of axo-dendritic and axo-somatic synaptic structures with synaptic vesicles, mitochondria, and thickened presynaptic and post-synaptic membranes are commonly found, resembling the in situ structures very closely.

The question of whether synapses are simply carried over during the explanting operation or whether they are formed de novo in the cultures has attracted recent attention; de novo synthesis in cultures would suggest the possibility of studying problems of development and regeneration, as well as studying the mechanisms of learning.

Cultured fetal spinal cord can innervate isolated fragments of skeletal muscle (placed 0.5 to 1.0 mm apart), make functional connections (Peterson & Crain, 1968; Crain, 1968), and form structures having the characteristics of nerve-muscle junctions (James & Tresman, 1968). Dispersed embryonic spinal cord cells plated onto monolayers of muscle cells even develop structures resembling neuromuscular junctions (Shimada, Fischman & Moscona, 1969). Neuronal synaptic profiles formed de novo in culture were demonstrated by Stefanelli, Zacchei, Caravita, Cataldi & Ieradi (1967) using 4-day

chick embryo retinal cells dissociated by trypsin and reaggregated into pellets before culturing for twenty-five days. Similar experiments on synapse formation in cultured pellets of previously disaggregated neurons from fetal rat brain have not yet been successful (Jacobs, Andrews & Mak, 1969). In thick cultures of embryonic rat spinal cord explanted prior to synapse formation (14 days *in utero*) synaptic structures closely resembling the *in situ* structures have been shown to develop (Crain, Bunge, Bunge & Peterson, 1964; Bunge & Bunge, 1965; Bunge, Bunge & Peterson, 1967); and similar observations have been made in cultures of mouse cerebral cortex (Pappas, 1966). There is consequently no doubt that synaptic structures can and do form in culture, at least under certain conditions. Electrophysiological evidence for the onset of synaptic function will be reviewed below (page 18).

4. Non-neuronal Cells

As *in situ*, the neurons in culture are wholly or partially embedded, depending on the thickness of the culture, in a matrix of glial cells and their processes. The identification and classification of the various types of neuroglia (oligodendroglia, fibrous and protoplasmic astrocytes) has been the subject of several recent treatises (Nakai, 1962; Nakai & Okamoto, 1963).

Ciliated cells from the ependymal linings of the ventricu-

lar system have been repeatedly observed in cultures (Weiss, 1934; Nakai & Okamoto, 1963; Lumsden, 1968). The ciliary beatings of these cells has been described and documented with motion pictures by Hild (1957b).

Mesenchymatous and fibroblastic cells from meninges and walls of blood vessels, as well as microglia (macrophages), are always present in cultures of nervous tissue.

D. BIOCHEMICAL PARAMETERS OF NERVE TISSUE CULTURES

The few aspects of this important subject that have been studied generally indicate a good correspondence between the _in vitro_ and _in situ_ biochemical activities, e.g., glucose metabolism (Bornstein & Hochstein, 1962; Cechner, Geller & Fleming, 1969), oxygen consumption (Luk'yanova, Shungskaya, Yenenko & Donskova, 1968), pyrimidine metabolism (Appel & Silberberg, 1968), and nucleic acid and protein synthesis (Utakoji & Hsu, 1965).

Oxidative enzyme activities retain their characteristic temporal and spatial patterns (Yonezawa, Bornstein, Peterson & Murray, 1962), acetylcholinesterase activity is localized in neurons (Hansson, 1966), the uptake of catecholamines is restricted to sympathetic nerve cells (Burdman, 1968; England & Goldstein, 1969), and choline is incorporated into peripheral nerve myelin (Hendelman & Bunge, 1969).

E. ELECTROPHYSIOLOGICAL STUDIES ON CULTURED NEURONS

Crain, Grundfest, Mettler & Flint (1953) were the first to study the bioelectric activity of cultured neurons by recording extracellular action potentials from chick embryo dorsal root ganglion cell bodies after electric stimulation of their neurites. This early work was extended with intracellular microelectrodes (Crain, 1956) to explore membrane potentials, graded or local response, all-or-none action potentials; impulse propagation along neurites, post-spike soma refractoriness, and temporal summation of subliminal stimuli of cultured chick embryo dorsal root ganglion cells; all these parameters were found to correspond closely to similar <u>in situ</u> material. Hild and Tasaki (1962) recorded intracellularly from somas and dendrites of cultured neurons from cerebella of neonatal rats and kittens and found membrane potentials of about 50 mv, excitable dendritic branches and soma membranes, as well as some spontaneous firing. In similar cultures, resting potentials of up to 75 mv have subsequently been obtained, and spontaneous action potentials are seen quite regularly (Raj & Lumsden, 1968). Klee & Hild (1967) investigated various membrane properties of cultured neurons and neuroglia (membrane potentials, action potential amplitude, membrane time constants, membrane resistance and capacitance). In cultured dissociated chick embryonic spinal ganglion cells, Scott, Engelbert & Fisher (1969) were able to record membrane potentials of 40-55 mv and evoked action potentials for up to five weeks in culture.

A great deal of work has been done with extracellular electrodes, investigating the bioelectric activity of thick organ cultures obtained with the Maximow technique. Complex bioelectric potentials with long-lasting (100 msec. up to many seconds), diphasic, oscillatory (7-15 per second) afterdischarges can be evoked by brief, single, electric stimuli in older cultures of rat, chick, and human fetal spinal cord (Crain & Peterson, 1963, 1964; Peterson, Crain & Murray, 1965), and neonatal cerebral cortex of mouse (Crain, 1964b, 1964c; Crain & Bornstein, 1964). In cultures of spinal cord explanted together with an attached spinal ganglion, local stimulation to the ganglion evokes a complex response in the spinal cord tissue (Crain & Peterson, 1964; Crain, 1966). These complex evoked potentials develop during maturation of the cultures from simple spike potentials to more and more complex patterns (Crain, 1966). In young cultures of fetal material explanted before electron microscopic evidence for synaptic structures can be found, only simple spikes can be evoked during the first few days in culture; but as the cultures mature, complex activity with afterdischarges begins to appear. The onset of this complex activity coincides with the appearance of synaptic profiles in electron micrographs (Crain & Peterson, 1965, 1967; Bunge, Bunge & Peterson, 1967).

Functional interneuronal connections develop even between explants of various mammalian central nervous tissue separated by gaps of about 1 mm. Neuritic bridges form between such explants, and

stimulation of one explant often elicits complex responses in the other one. Some of the characteristics of these newly-formed connections have been examined, for instance in paired spinal cord cultures (Crain & Peterson, 1965), and in cultures of spinal cord - brainstem, spinal cord - cerebellum, and cerebellum - medulla (Crain, 1967; Crain, Peterson & Bornstein, 1968).

Spontaneous bioelectric activity in cultures of nervous tissue was first reported by Cunningham, Dougherty & Rylander (1960), who explanted central nervous tissue onto platinum electrodes for continuous recording during incubation. Complex repetitive patterns varying in time were recorded from explants of chick embryo telencephalon and adult human cerebellum (Cunningham, 1961a, 1961b, 1962), suggesting the interaction of many cells. In later microelectrode studies spontaneous unit spikes were recorded both extracellularly and intracellularly (Cunningham, O'Lague, Rojas-Corona & Freeman, 1966).

In some of the long-term organotypic Maximow cultures of spinal cord and cerebrum, spontaneous oscillatory potentials occur sporadically (Crain, 1966).

In contrast to the above EEG-type activity recorded with relatively large microelectrodes in thick cultures, spontaneous action potentials can be recorded with extracellular microelectrodes placed under visual control near individual nerve cell bodies in thinly-spread roller tube cultures of rat and mouse cerebellum (Hild & Tasaki, 1962; Cechner & Fleming, 1967; Cechner, 1967; Lumsden, 1968). These spon-

taneous spikes may occur at irregular intervals with frequencies ranging from 15 per second to one per several seconds (Hild & Tasaki, 1962); they have been reported to be "usually characterized by a Gaussian interspike interval histogram," and it was found that "units physically close together have discharge patterns which, to date, appear to be uncorrelated" (Cechner & Fleming, 1967). Later, however, Cechner (1967) sometimes found bursts of activity, and Lumsden (1968) reported bursting spontaneous activity (groups of 5-6 spikes at about 2 second intervals) in Purkinje cells of rat cerebellum.

F. EVIDENCE FOR THE PRESENCE OF FUNCTIONAL SYNAPSES IN CULTURES

In thick long-term organ cultures obtained by the Maximow technique, there is, by now, overwhelming evidence for the presence, and even de novo formation, of functional synapses in cultures. To summarize the preceding review briefly, it has been most elegantly shown that

a) complex bioelectric activity develops during maturation of the tissue in vitro with the transformation from simple evoked responses to complex evoked potentials coinciding with the morphological development of synaptic structures, and

b) functional interneuronal connections form between separated explants of various parts of the nervous system.

In thinly flattened cultures obtained with the roller tube technique, on the other hand, the tissue has undergone considerably more reduction and simplification and the survival of synapses has been questioned (Hild, 1966). In electrophysiological experiments with intracellular microelectrodes, Hild & Tasaki (1962) found no evidence for functional synapses, and their results indicate quite clearly that they were dealing with functionally isolated cells. Since they were interested in the bioelectrical properties of single cells and their dendrites, they probably chose neurons in extremely thin areas of their cultures, where the cellular processes are clearly visible under the phase contrast microscope. However, electron microscopic evidence of synaptic structures was found in similar cultures (Callas & Hild, 1964; Lumsden, 1968), but their functionality remained in question (Hild, 1966).

Cechner (1967) presented some data possibly suggestive of synaptic interactions in his cultures. Klee & Hild (1967) peripherally mentioned the occurrence of "miniature-EPSP's" but apparently did not attempt to investigate them further. And finally, Lumsden (1968) inferred primitive "nerve net" properties from the long latencies between stimulus and evoked response in similar thinly-spread roller tube cultures.

G. CONCLUSIONS

The preceding review of the literature amply demonstrates that nervous tissue cultured under suitable conditons retains most of its organotypic structure and function.

As has been pointed out, the Maximow double coverslip technique favors the maintenance of a maximum of structural and functional integrity, including de novo myelin and synapse formation, in thick long-term organ cultures. While such cultures are valuable test objects for a variety of physiological, biochemical, and pharmacological experiments relating to nervous system development and differentiation, their thickness (100-300 μ), which seems imperative for optimal development, renders them opaque to phase contrast (Wolf, 1964; Crain & Peterson, 1964; Bunge, Bunge & Peterson, 1965); and the use of bright field illumination (Peterson, Crain & Murray, 1965) does not seem to allow sharp visualization of the contours of perikaria and cellular processes (dendrites, etc.) within the explant. The surface of the explant becomes covered with a neuropil consisting of neuritic and neuro-glial processes. Consequently, it is very difficult to place microelectrodes accurately near individual neurons, and most electrophysiological experiments have been carried out with large extracellular electrodes recording from a large number of cells simultaneously.

At the other extreme, the cultivation of dissociated cells

from the nervous system sheds light on the cellular aspects of nervous tissue function. But even under these conditions, some organotypic interactions (e.g., synapses and neuromuscular junctions) can occur, demonstrating the endogenous cellular potential in this regard. The proper function of these structures has, however, not yet been demonstrated.

Finally, the roller tube technique, which favors a rapid flattening of the culture into a two-dimensional layer of nerve cells embedded in a matrix of glial cells and their processes, allows good visibility of individual neurons and their processes and accurate placement of extracellular or intracellular microelectrodes. In spite of the transformation of the tissue from a cubical to a membrane-like flat arrangement, with considerable distortion of the original topography, most of the significant organotypic structures (myelin and synapses) can still be found, and the functional aspects (membrane potentials, action potentials, etc.) seem to be well-preserved. However, the normal architecture of the explanted tissue is largely destroyed and the integrative mechanisms of the tissue segment are therefore altered considerably.

In order to use roller tube cultures as simplified models of the nervous system, the properties of these cultures have to be examined in great detail so that a baseline for experimentation can be established. The present study is an attempt to contribute to this baseline information by making an inquiry into the characteristics

of spontaneous bioelectric activity and the properties of synaptic interactions in these cultures.

II. MATERIALS AND METHODS

A. TISSUE CULTURE TECHNIQUES

Cultures of nervous tissue are always primary explants and they are therefore considerably more demanding than the established cell lines usually found in a tissue culture laboratory. Media, glassware, and instruments demand special attention with regard to sterility, purity, and absence of toxicity. The culturing techniques represent modifications of a method of preparing plasma clot cultures on "flying coverslips" in roller tubes, originally developed by Costero and Pomerat (1951) and adapted to the present material by Hild and Tasaki (1962).

The nerve tissue culture laboratory here was established by Mr. Abdel-Megid Mamoon. He laid the foundation and developed the initial skills which were necessary to make this project a success. The culture methods finally adapted are the result of subsequent improvements suggested primarily by Mr. Mamoon and tested and developed jointly by Mr. Mamoon and the author. Most morphological observations on cultured neurons reported in this dissertation were arrived at in conjunction with Mr. Mamoon.

1. Biological Material

Cultures were prepared from the cerebellum and midbrain (corpora quadrigemina) of three to five day old rats (Sprague-Dawley, Simonsen albinos). Strict sterile conditions were observed throughout the explanting procedure. The rats were decapitated, the roof of the cranium was removed, and the entire midbrain and cerebellum was cut out with iridectomy scissors and placed in a drop of balanced salt solution (BSS). Without delay, both cerebellum and midbrain were cut with triangular knives made from stainless steel razor blades into pieces of approximately 1 - 2 mm^3; the cerebellum was cut sagitally into 6 - 8 pieces, the midbrain into 10 - 15 pieces. The cut explants were then transferred to a new drop of BSS, where they were allowed to stay for about twenty minutes. Two to three pieces each were then placed in a drop of heparinized chicken plasma (Hyland Laboratories # 65-080 or Baltimore Biological Laboratories # 70-025G) on a 12x50 mm #1 Gold Seal coverslip and clotted with a drop of chicken embryo extract (Baltimore Biological Laboratories # 70-027G). After the clot had solidified, the coverslips were inserted into Falcon Plastic culture tubes (Falcon Plastic # 3026) to each of which 2 ml of culturing medium was added.

2. Media

Gey's balanced salt solution (Microbiological Associates # 10-505), enriched with glucose to a final concentration of 5.5 mg/ml, was used during explanting and during most electrophysiological experiments. Several nutrient media compositions were used, but the best cultures so far have been obtained with a medium consisting of 75% Gey's balanced salt solution and 25% heat inactivated (56°C for 30 min) calf serum (Hyland Laboratories # 65-200), with glucose concentration increased to approximately 5.5 mg/ml. No antibiotics were used in the media. In most cultures, the medium was exchanged weekly, but some cultures were fed after seven days and biweekly thereafter.

3. Incubation

The cultures were incubated in roller drums at 36.5°C ± 0.5°C in a Rollertherm Incubator (New Brunswick Scientific Co.). The drums containing the culture tubes were tilted about 4-5°, with respect to the horizontal axis, in order to confine the medium to the bottom of the tube. They were rotated at 1/5 rev/min, providing the cultures with a well-mixed and aerated nutrient medium. Since Falcon Plastic culture tubes are permeable to CO_2, the atmosphere in the incubator was kept at approximately 4-5% CO_2 in humid air to

insure control of the bicarbonate buffered medium at a pH of between 6.9 and 7.1.

Most cultures were used for experiments after 15 - 30 days *in vitro*. By that time they had thinned enough for clear phase contrast visualization of nerve cell bodies, myelinated axons, and some dendrites.

4. Comments about the Nerve Tissue Culture Technique

To obtain satisfactory cultures, meticulous attention has to be given to the details of the culturing methods. Prior to use in experiments, the cultures were examined with a Zeiss phase contrast microscope, usually after 15 - 30 days *in vitro*; they were judged "satisfactory" if they conformed to the following criteria:

a) sufficient thinning (good outgrowth) to allow observation of cellular detail;

b) lack of necrotic areas, little decay and cellular debris;

c) relatively small population of macrophages;

d) presence of neurons with centrally located round nuclei (figs. 5 to 15, and 18);

e) abundant myelin (figs. 16 and 17) in an apparently healthy state.

While it is not possible specifically to pinpoint all the

factors that contributed to the final success after initial failures to obtain satisfactory cultures, some of the most important elements were:

a) Glassware.

All materials used that come in contact with the cultures or the media, especially the coverslips, should be free of toxic ingredients (Peterson, Deitch & Murray, 1959). A constant source of trouble are traces of detergent left on the glass from the washing procedure. Our cultures improved markedly when we shifted from glass test tubes to sterile disposable Falcon Plastic culture tubes. The reason for this improvement was not investigated in detail, but it could have been due to traces of detergents or other toxic materials on the surfaces of our glassware, which disappeared with the shift to the disposable tubes. An additional advantage of the plastic culture tubes is that the coverslips bearing the cultures do not slip in plastic tubes and consequently receive a much more uniform exposure to media and air, whereas they slide in the glass roller tubes during the rolling action. All glassware (petri dishes and sterile coverslips for holding the explants during the explanting operation, pipettes for feeding, etc.), with the exception of the coverslips,

was subsequently replaced with Falcon Plastic material. The coverslips were boiled in several changes of 3X distilled water, immersed in redistilled 95% alcohol for several days, air dried one by one over a hot plate, and heat sterilized.

b) Instruments used for explanting.

Explanting instruments (scissors, iridectomy scissors, forceps) should be scrupulously clean. They were usually cleaned with an ultrasonic cleaner and boiled in distilled water.

c) Explants

Animals aged 3 - 5 days were used for starting material, and no attempt was made to investigate the effect of the age of the animal on the cultures. The need for an optimum size of the explant has been repeatedly pointed out (e.g., Peterson, Crain & Murray, 1965; Lumsden, 1968). A major factor in obtaining good cultures seems to be a clean and sharp cutting of the explants without tearing or shearing. For this reason, new knives made out of stainless steel razor blades were always used in each explanting session, and care was taken not to damage the delicate tips of the knives.

d) Plasma clot.

Chicken plasma and chicken embryo extract (CEE) from

various suppliers, including material we prepared ourselves, were tested. Large differences between the various brands were found, but lyophilized chicken plasma from Hyland Laboratories (cat. # 65-080), clotted with fortified lyophilized CEE from Baltimore Biological Laboratories (cat. # 70-025G) has given the best results so far.

e) Nutrient media.

During the early phases of this investigation the nutrient medium used was similar to the one favored by Hild and Tasaki (1962), namely 45% Gey's BSS, 50% heat inactivated (56°C for 30 min) calf serum from Hyland Laboratories (cat. # 65-200), and 5% CEE, with the glucose content increased to 600 mg/100 ml. Great variations exist between different batches of calf serum, even from the same supplier; a new lot is consequently always tested before ordering or reserving a larger quantity of serum. It is not known why these differences exist, but toxic effects of high serum concentrations in nutrient media have been reported (Olmsted, 1967).

Omission of chicken embryo extract from this original formulation did not seem to have a deleterious effect; in fact, a slight improvement in the overall appearance

of the cultures was noted. A further amelioration was achieved by lowering the serum concentration to 25% in BSS, whereas a still further reduction to 15% resulted in a worsening of the overall appearance of the cultures.

Experimentation with different media [such as substituting basal medium Eagle (Microbiological Associates (MBA) # 12-104) for Gey's BSS, adding 1.5 mM glutamine (MBA # 17-605F), 0.5 mg/ml sodium pyruvate (MBA # 13-115), or 0.75% of 100X essential amino acid mixture (MBA # 13-602)] has resulted so far in no apparent improvement of our cultures. Further experimentation along these lines is planned.

B. HISTOLOGICAL METHODS

Representative cultures were stained as whole mounts with Nissl stains (cresyl violet, toluidine blue, Einarson's gallocyanin-chromalum, thionine and methylene blue-azure blue), with silver stains (Bodian's protargol and Holmes' silver impregnation), and with Sudan black B and Luxol Fast blue for myelin sheaths. Standard histological methods for Nissl staining were used (Conn, 1960; Culling, 1963). The Bodian protargol method (Bodian, 1936) has been repeatedly used on whole mount nerve tissue cultures (Murray &

Stout, 1947; Lumsden, 1951; Hild, 1957c, 1966) to demonstrate myelinated and unmyelinated axons. For Holmes' silver impregnation (Holmes, 1947), the procedure of Wolf (1964) has been followed, and myelin was stained with Sudan black B according to the method given by Peterson & Murray (1955). After examination of the living culture with phase contrast optics, a desired area was often photographed and a circle inscribed on the coverslip with a diamond marker mounted on the microscope. In this fashion, identification of the same neuron or group of neurons after fixation and staining was greatly facilitated.

C. ELECTROPHYSIOLOGICAL METHODS

1. Mechanical and Optical Arrangements

A modified Bausch and Lomb phase contrast microscope with a fixed stage and a 40X Zeiss objective with a 1/4 wavelength phase plate was used to view the cultures during electrophysiological experiments. The microscope and three micromanipulators (one Brinkmann and two Narishige model MD-4) were attached to a heavy base plate (loaded with approximately 100 lbs. of lead bricks) which rested on shock-absorbent material (ordinary packing material) on a two-inch laminated plywood table top. Asbestos vibration pads separated the table top from the sturdy frame of the table, which was in turn isolated from the floor by shock mounts (Barry Hi-Damp). In order

to eliminate electrical interference, the whole setup was surrounded by a well-grounded electrostatic shield (constructed of copper mesh) which served as a common ground for all electronic equipment.

The coverslip bearing the culture was mounted, culture down, on a bridge similar to the one used by Hild, Chang & Tasaki (1958) and Hild & Tasaki (1962), which allowed the positioning of microelectrodes under visual control through the open sides (fig. 1). The bathing solution (approximately 0.65 ml -- usually Gey's balanced salt solution enriched with glucose, or modifications thereof) was held in the chamber by surface tension and was exchanged frequently in order to avoid pH changes and accumulation of waste products, and to provide freshly aerated medium. Ports for perfusion and exchange of the bathing solution were provided through the bridge mounts. A manual syringe pump consisting of two pairs of back-to-back 10 ml plastic syringes allowed the exchange of the bathing solution during electrophysiological experiments. Although most experiments were carried out at room temperature, the preparation could be warmed by a thermocouple-controlled shielded heater coil mounted around the microscope condenser, or cooled by flowing cooling liquid through a loop of copper tubing under the chamber; and the perfusate could be prewarmed or precooled before entering the recording chamber.

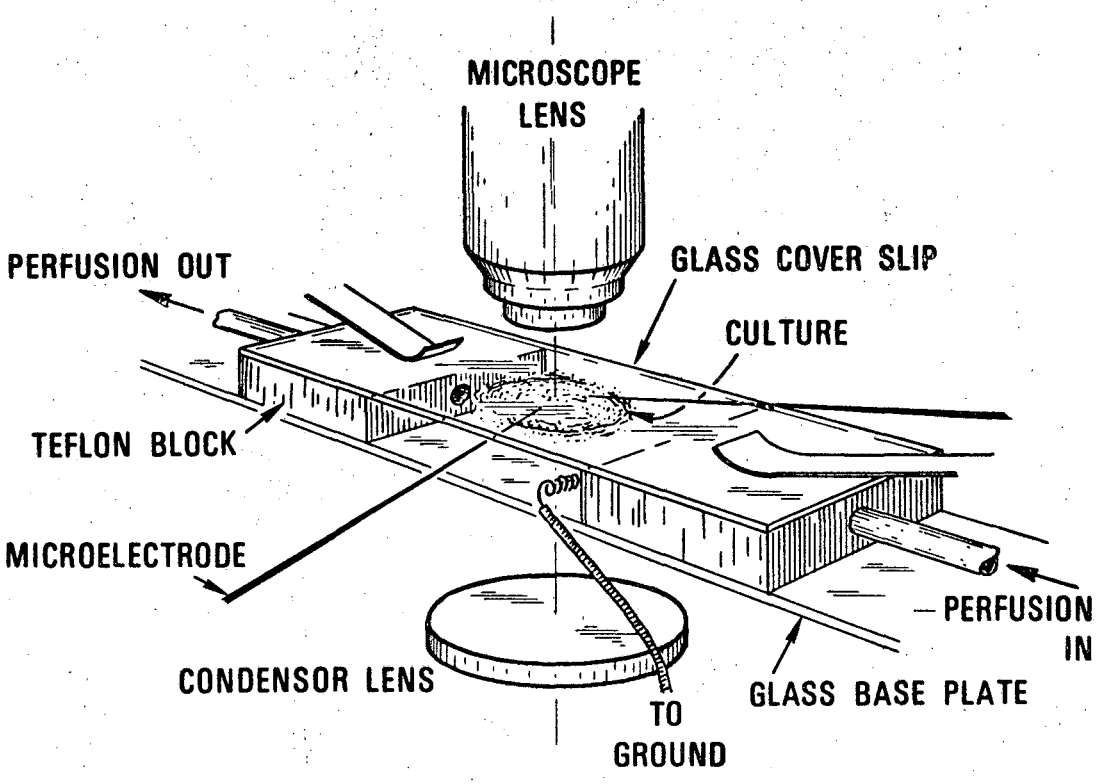

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Fig. 1: Microscope stage for electrophysiological experiments. The chamber consists of a glass base plate onto which two teflon blocks have been glued with bee's wax. The coverslip bearing the culture forms the roof of the chamber. The chamber is filled with balanced salt solution (or modifications thereof) held by surface tension.

2. Recording of Extracellular Potentials (Fig. 2)

Glass micropipettes with tips of 4 - 6 μ were manufactured on a horizontal electrode puller (John Keefe Assoc., Cambridge, Mass.) from Kimax capillary tubes (O.D. 0.7 to 1.0 mm) and filled with 0.9% NaCl simply by immersing the tips in saline and then filling the shafts with a fine syringe needle. Such electrodes have a resistance of approximately 10 megohms. Two recording microelectrodes could be placed into the culture simultaneously. A platinum wire inserted into the shaft of each microelectrode was connected to a low noise a.c.-coupled FET (field effect transistor) preamplifier (Applied Cybernetics model 4UAH) mounted very close to the microelectrodes. This preamplifier has a frequency response of .3 Hz to 100 kHz, and input impedence of 500 megohms, and a peak-to-peak noise (measured) of 30 μv at full bandwidth and with grounded input. A coil of platinum wire in the bathing solution served as the indifferent electrode. The outputs of the preamplifiers were hooked to a four-trace storage oscilloscope (Tektronix model 564) and each, in parallel, to two Tektronix 122 amplifiers; the output of the first one was fed directly into one channel of an 8-channel tape recorder (Precision Instruments model PI-6208), while the output of the second one went to a discriminator and a pulse generator. The pulses were a) examined on the oscilloscope to make sure that the discriminator level was set correctly (i.e., that each pulse corresponded to an extra-

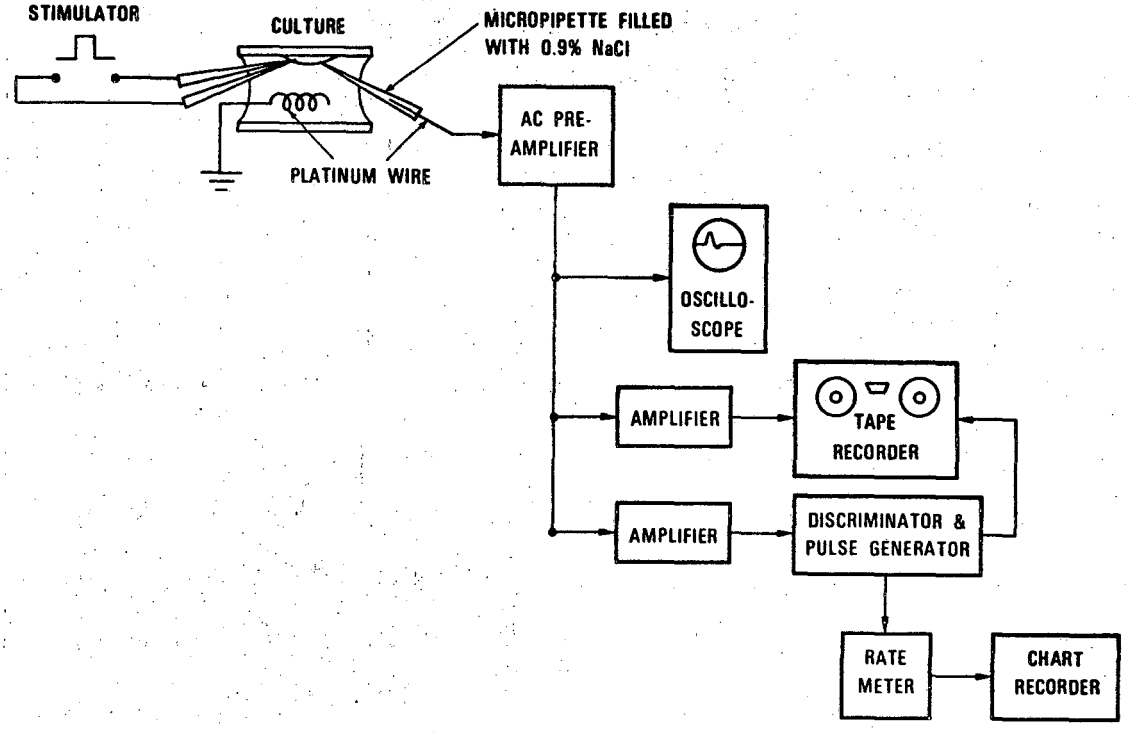

Fig. 2: Experimental arrangement for recording extracellular potentials from cultured neurons.

cellular spike), b) recorded on the tape recorder for later computer analyses of the interval distribution and correlation, and c) fed into a rate meter (Nuclear Chicago model 1620) with a chart recorder to plot automatically the average spike frequency.

3. Intracellular Recordings (Fig. 3)

Hyperfine micropipettes (0.1 to 0.5 μ tip diameter) were drawn from well-cleaned Kimax capillary tubes and filled with 3M KCl by immersing the tips into freshly filtered (0.22 μ Millipore) KCl solution and filling the shafts with distilled water. They were then left in a vertical position (tips down dipped into 3M KCl) overnight under a heat lamp which caused the bubble in the tip to move up into the shank or shoulder. The next day, the distilled water could be exchanged with 3M KCl with a fine syringe needle, and if the bubble still persisted it could usually be removed with a fire-etched tungsten wire (O'Lague, 1969). Microelectrodes manufactured in this fashion usually have a resistance of 40 - 80 megohms. The electrodes were then used within 2 to 3 days.

The micropipettes were coupled with polyethylene tubing, filled with a gel of 2% agar in saline, to a reservoir of saline containing a large chlorided silver wire; this was in turn connected to a d.c.-coupled high input impedance, negative capacitance preamplifier (Winston Electronic Co., model S-857; Transidyne General Corp., model

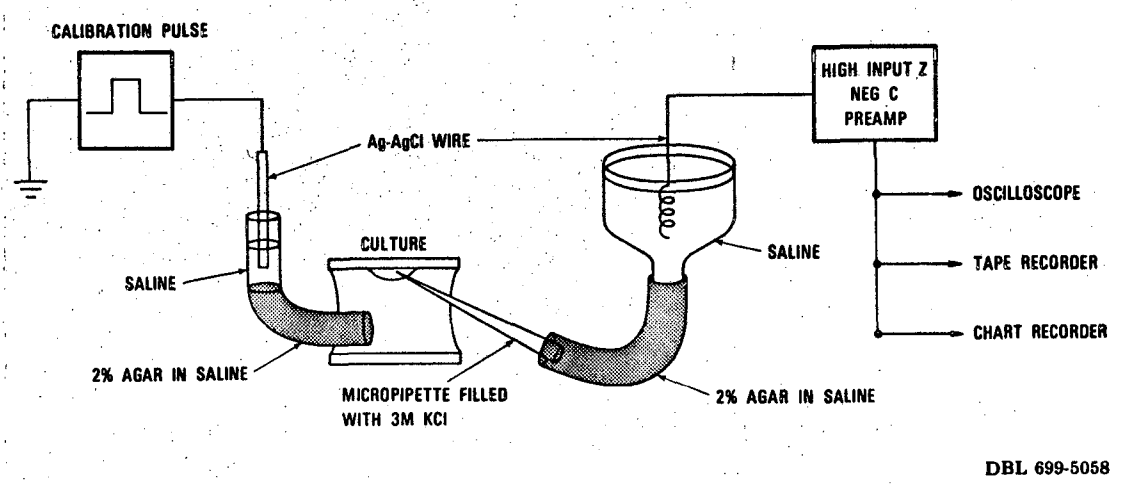

Fig. 3 Experimental arrangement for intracellular recordings from cultured neurons.

MPA-2; or a homemade FET input amplifier). The preamplifier output was fed in parallel to a storage oscilloscope (Tektronix model 564), a chart recorder (Mosely model 680), and an FM channel of the tape recorder. The indifferent electrode consisted of an Ag-AgCl wire stuck into a saline pool above a saline-agar gel in a glass tube making contact with the bathing solution in the experimental chamber. Before and after each experiment, a calibration pulse from a Grass stimulator (model S4G) was used to measure the total amplification of the recording system.

4. Analysis of Electrophysiological Data

Some of the spike trains recorded with extracellular electrodes were analyzed with a general purpose computer (PDP 8/1). The interspike intervals could be measured and displayed as a time interval histogram. For records that involved two channels (two separate microelectrodes recording from two neurons), a first-order "cross-interval histogram" could also be displayed (Perkel, Gerstein & Moore, 1967). The diagram in Fig. 4 defines the intervals that were measured and displayed.

The complete program with flow diagrams is included as an Appendix .

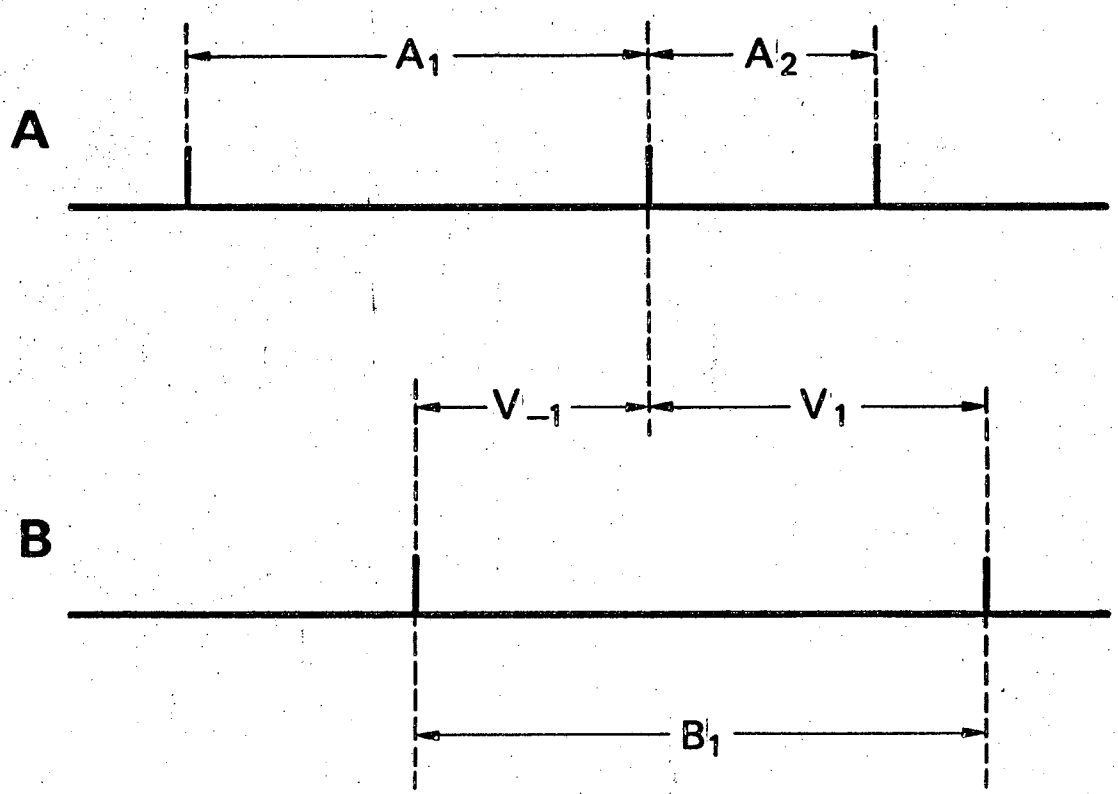

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Fig. 4: Definition of intervals used for computer analyses of spike trains. A_1 and A_2 are interspike intervals of spike train A: B_1 is an interspike interval of spike train B; and V_{-1} and V_1 are the first-order backward and forward recurrence times, respectively, of any one spike in train A with respect to the nearest spikes in train B (Perkel, Gerstein & Moore, 1967).

III. RESULTS AND DISCUSSION

A. MORPHOLOGICAL CHARACTERISTICS OF CULTURED NERVOUS TISSUE

Within 24 hours after explantation, mesenchymal cells derived from the meninges and blood vessels, glial cells, and possibly neurites started emerging from the explant. The tissue flattened as macrophages engulfed debris and as non-neuronal cells migrated peripherally with nerve cells generally staying behind. Flattening was sufficient for transillumination for phase contrast microscopy starting about the twelfth day *in vitro*, and by the third week, most parts of the culture were usually thin enough for considerable cellular detail to become visible. In most areas, the nerve cell bodies became arranged in a two-dimensional layer embedded between glial cells and an extensive network of glial and neuronal processes. Hild (1957a, 1966), Cechner (1967), and Lumsden (1968) have described the morphological development of cerebellar cultures that are very similar to the ones used in this study.

1. Identification of Neurons in Culture

Some of the neurons in living cultures observed under phase contrast could be identified by their large size compared to the other cells present, by their phase-dark cytoplasm (appearing dark

under the phase contrast microscope), and by their phase-light large rounded or oval nuclei with a distinct dark nucleolus. Neurons were usually found in groups or nests (figs. 5 and 6), and often in rows (figs. 7 and 8), within the confines of the original explant. Dendrites were often visible in areas of lower cellular density and could sometimes be followed for some distance from the perikarion (fig. 9). The tentative identification of cells as neurons in phase contrast can be confirmed by staining the culture as whole mounts with Nissl stains (cresyl violet, toluidine blue, thionine, Einarson's gallocyanin-chromalum, azure blue) (figs. 6 and 7) or by silver impregnation (Bodian or Holmes) (figs. 10 and 18). Some cells resembled neurons in phase contrast but failed to stain with either silver or basic stains. With some experience, and repeated comparison of stained whole mounts with photographs of the same areas taken with phase contrast, a high degree of confidence in the proper identification of many neurons can be acquired.

Although no attempt was made to rigorously classify neurons according to their histological and morphological types as seen in situ, neurons resembling, and probably derived from, Purkinje cells could be seen in almost every cerebellar culture. Their characteristics include a) location in rows, or groups, usually toward the periphery of the explant (figs. 7 and 8), similar to their in situ distribution; b) often a pear-shaped cell body with a large oval nucleus (fig. 11); and c) a dendritic tree, usually to one side of the cell

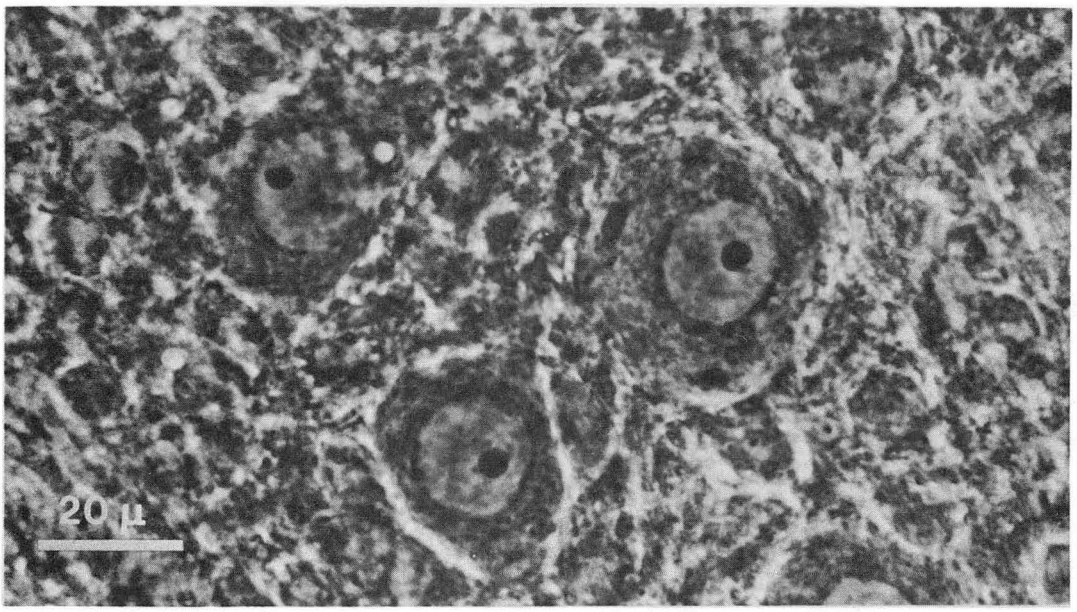

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Fig. 5: Group of living neurons (Purkinje cells?) from cultured cerebellum of newborn rat. 21 days in vitro. Phase contrast. Note the distinct dark nucleoli and the lighter rounded nuclei.

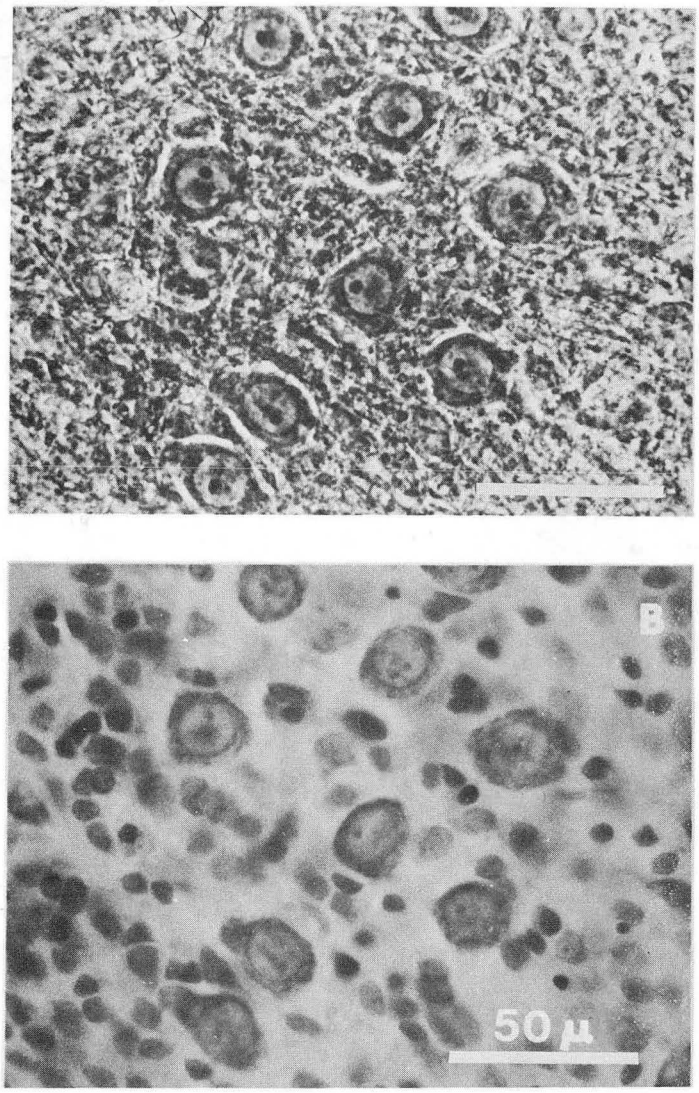

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Fig. 6: Group of nerve cells embedded between glial cells. 19-day old cerebellar culture. A: phase contrast; B: the same area after staining as whole mount with cresyl violet (Stains Nissl substance in neuronal cytoplasm, as well as glial nuclei).

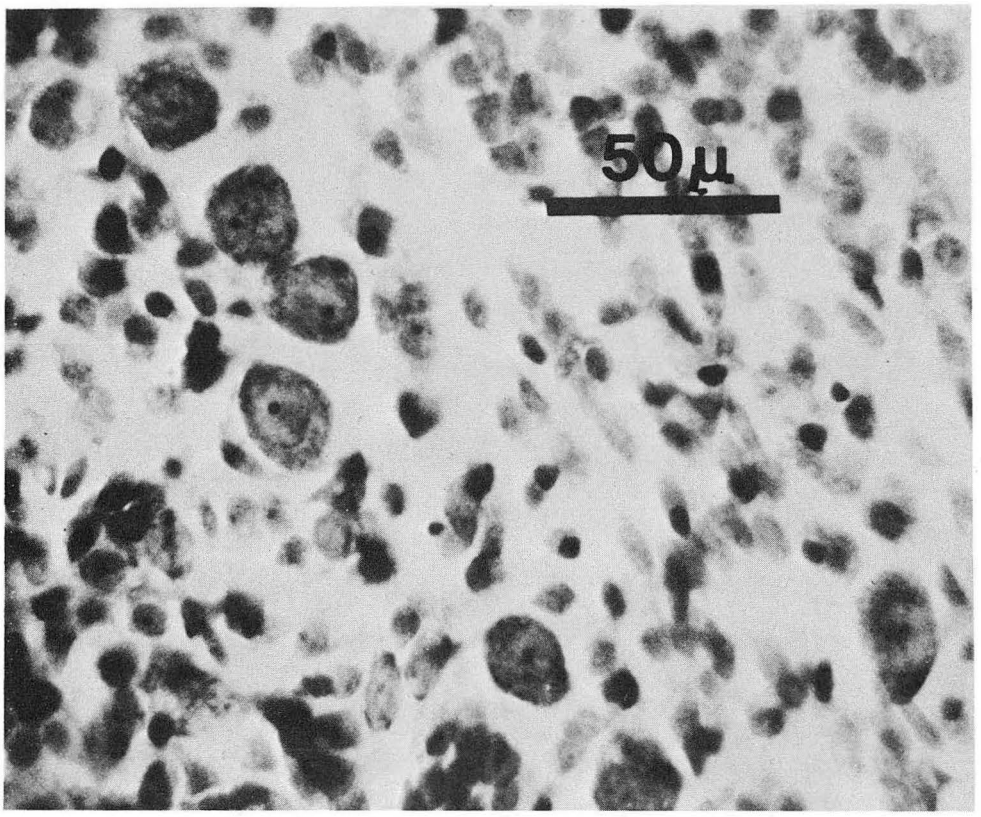

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Fig. 7: Row of neurons (Purkinje cells?) surrounded by glial cells in 17-day old cerebellar culture. Stained as whole mount with toluidine blue (Basophilic stain, stains primarily nucleic acids, such as contained in Nissl substance, nucleoli and nuclei).

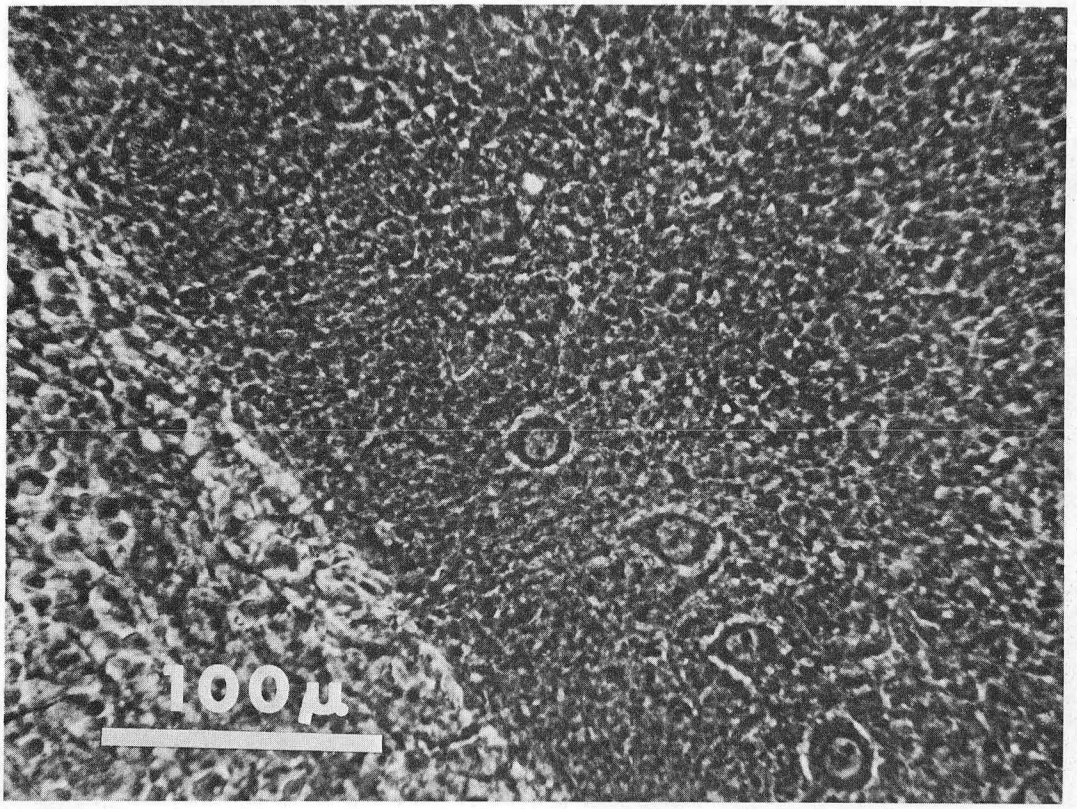

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Fig. 8: Row of living neurons (Purkinje cells?) from cerebellar culture. 26 days *in vitro*. Phase contrast.

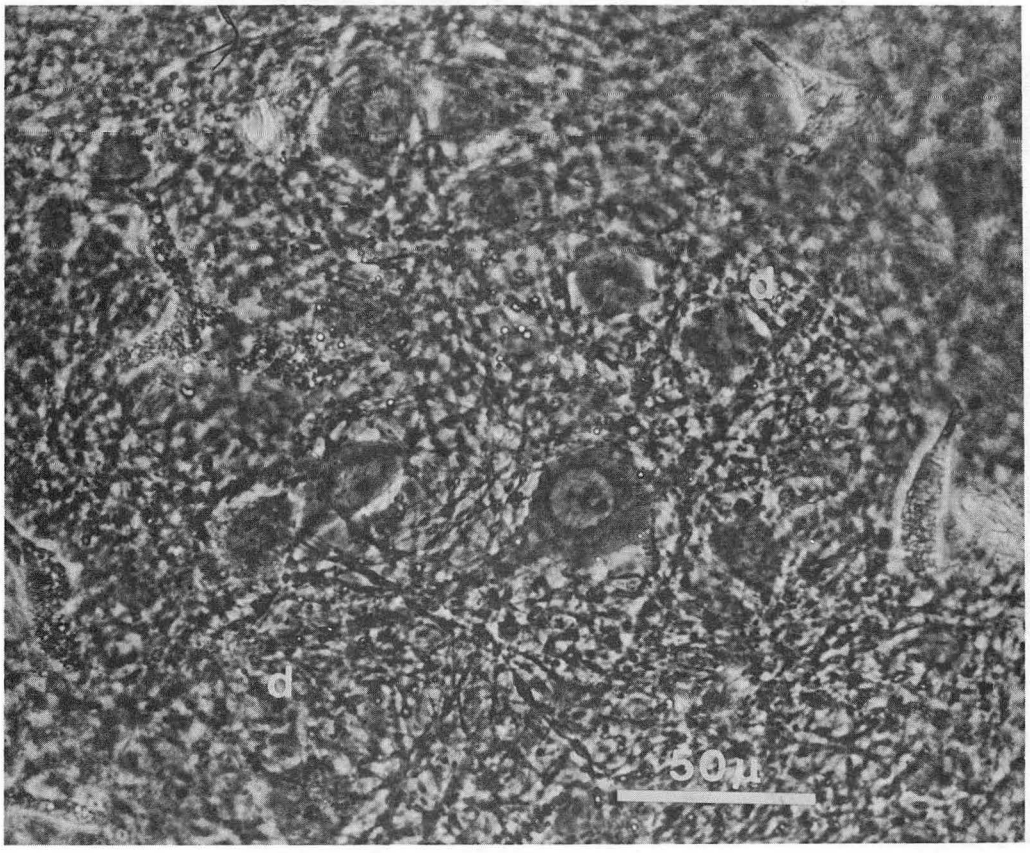

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Fig. 9: Living neuron with three long dendrites. 21-day old mid-brain culture. Phase contrast.

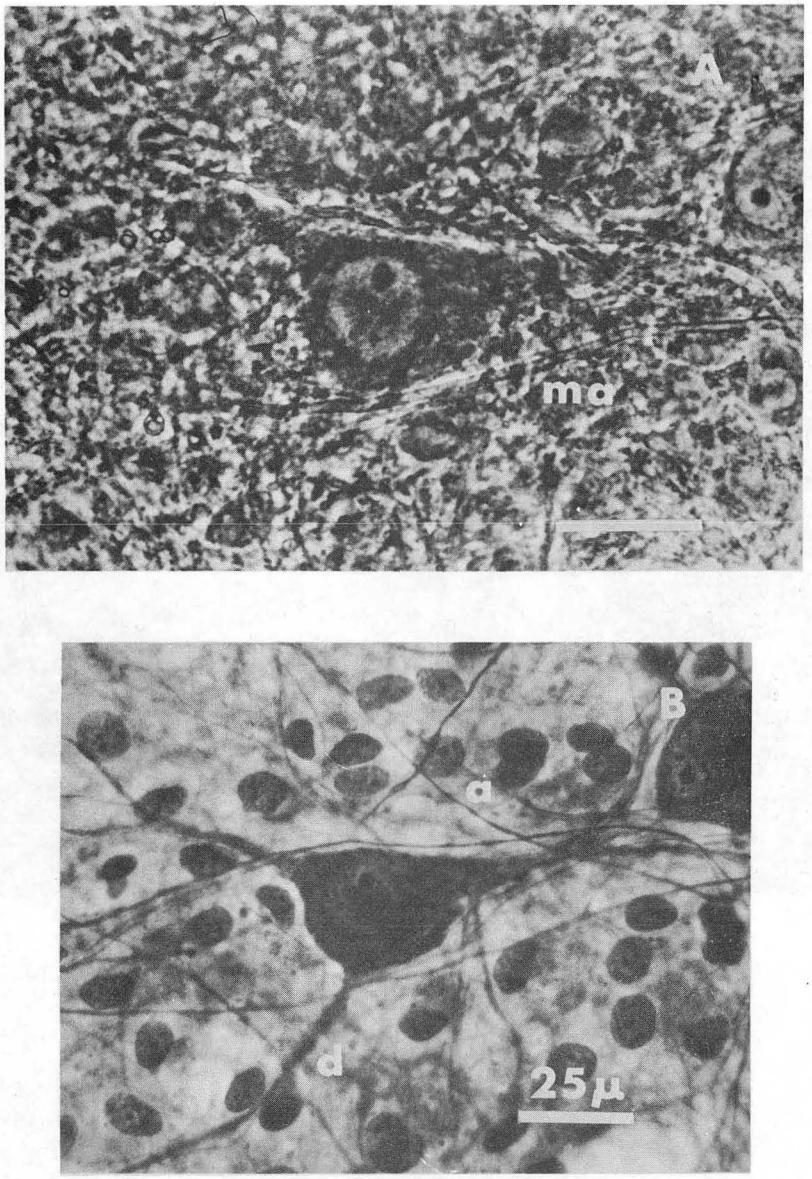

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Fig. 10: Nerve cell from a deep nucleus of a cultured cerebellum. 20 days in vitro. ma: myelinated axon; a: axon; d: dendrite. A: living, phase contrast; B: Bodian.

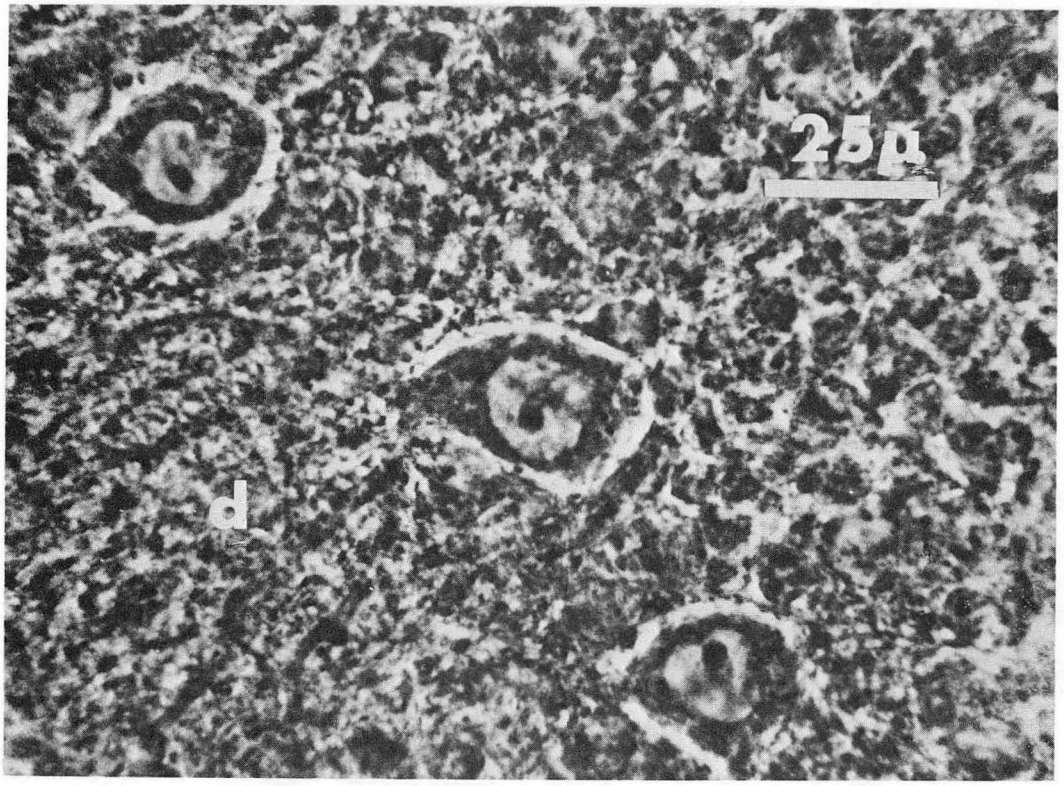

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Fig. 11: Living neurons (presumably Purkinje cells) from 26-day old culture of cerebellum. Note characteristic dendritic tree under (d), pear-shaped cell bodies and large rounded nuclei. Phase contrast. Same culture as fig. 8.

body, but in a generally much reduced form compared to the in vivo morphology (figs. 11 and 12). The culture environment may have induced structural alterations, but some of the features of the Purkinje cells could still be distinctly recognized (Addison, 1911; Dadoune, 1966). In central areas of the cerebellar cultures, large multipolar cells, often surrounded by myelinated axons, and presumably derived from the deep nuclei of the cerebellum, were regularly found (fig. 10). The other neuronal cell types of the cerebellum (basket cells, stellate cells, granule cells) could not be identified with certainty.

In cultures of midbrain no attempt was made to classify the neurons, but representative cells are shown in figs. 9, 13, 14 and 15.

2. Axons

Unmyelinated axons usually could not be discerned in a living culture during phase contrast observation. Myelinated axons, on the other hand, were quite distinct; the myelin sheath appeared as dark double lines under phase contrast and birefringent in polarized light (figs. 16 and 17). Staining with Sudan black B or Luxol Fast blue confirmed the presence of myelin (fig. 16c). Myelin usually appeared after about twelve days in culture.

Both Bodian's and Holmes' silver impregnations are well-

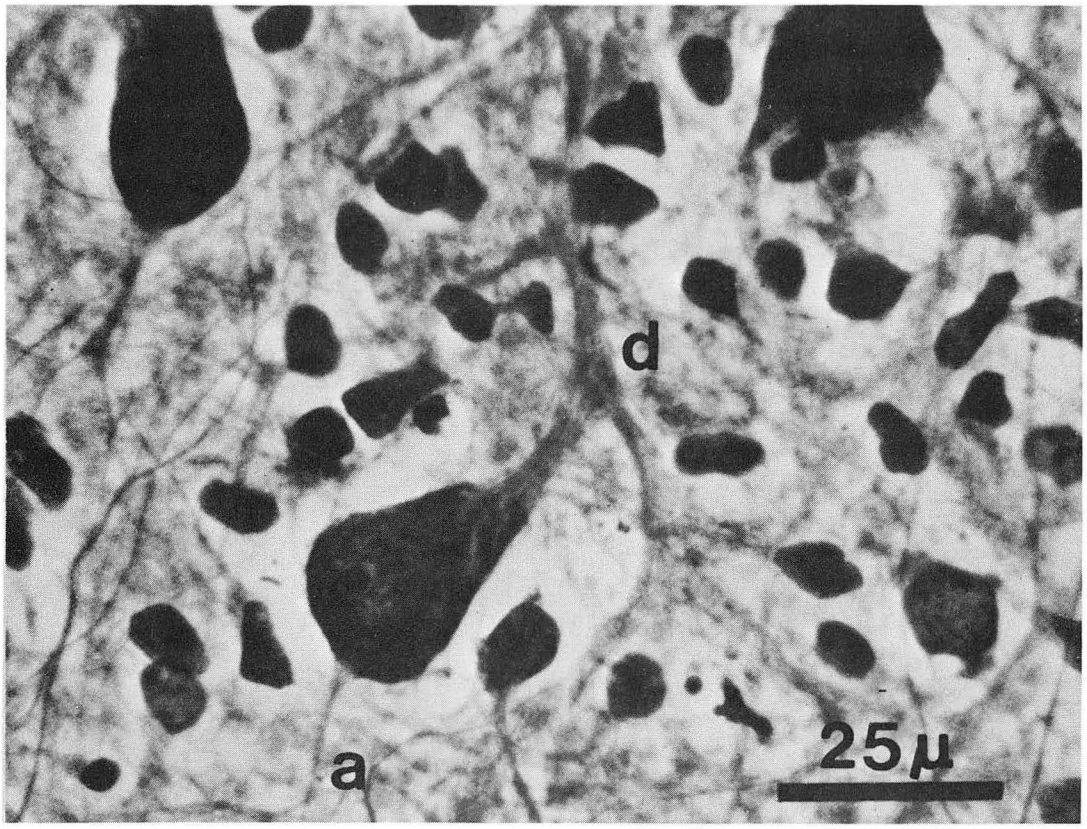

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Fig. 12: Bodian stained culture of cerebellum. 11 days in vitro. Note dendritic tree (d) and axon (a) emerging from soma.

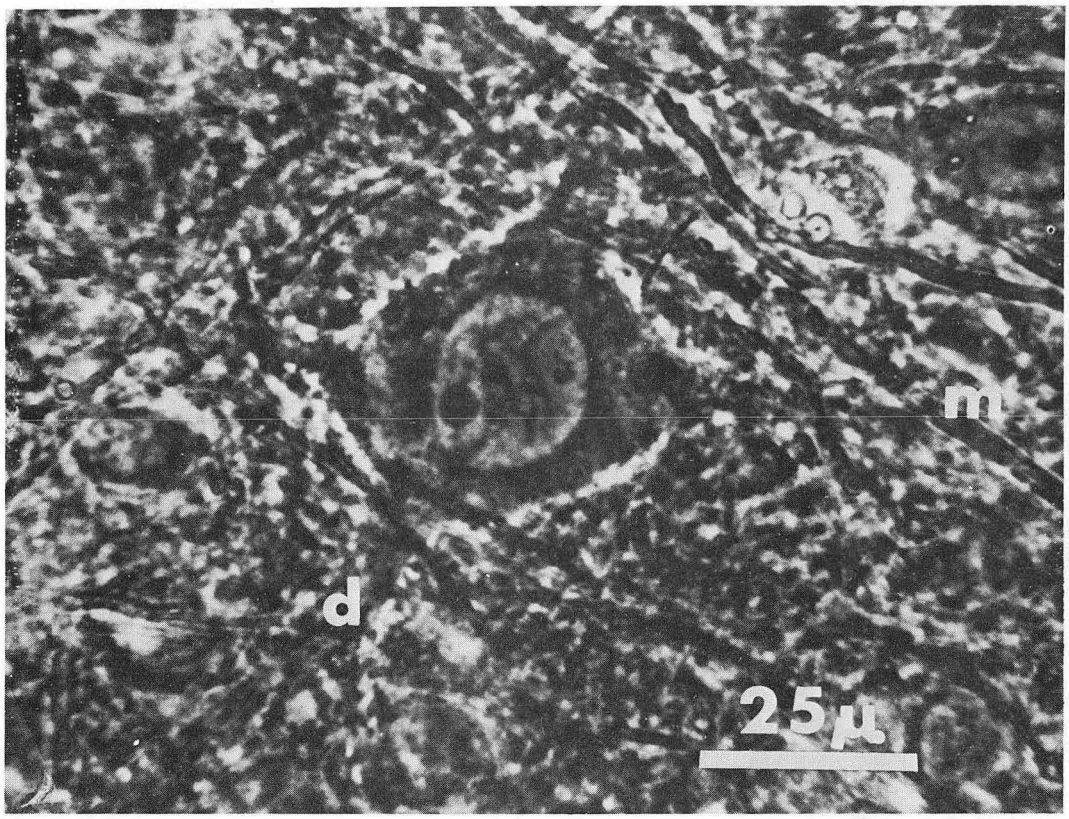

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Fig. 13: Living multipolar neuron from midbrain culture of 36 days in vitro. Note four dendrites (d) and several myelinated axons (m). Phase contrast.

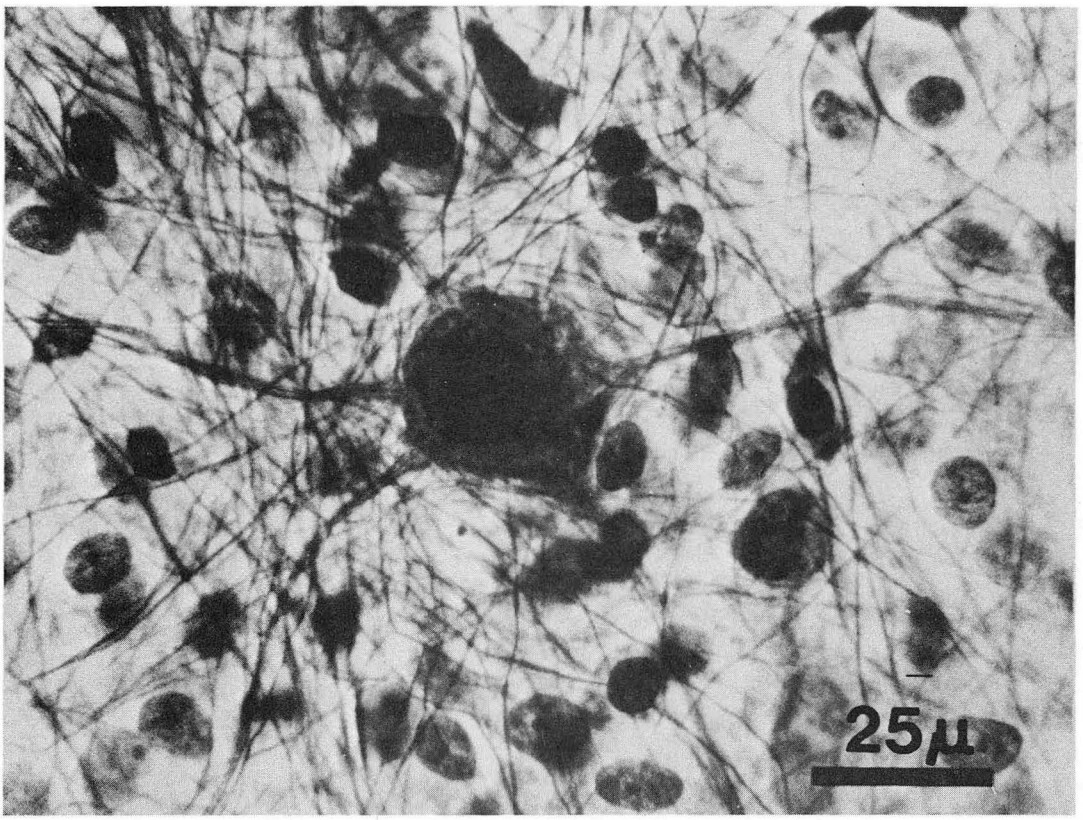

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Fig. 14: Bodian stained neuron from a 17-day old midbrain culture. Note several dendrites emerging from the soma, some of them branching. Dark lines criss-crossing are silver impregnated axons.

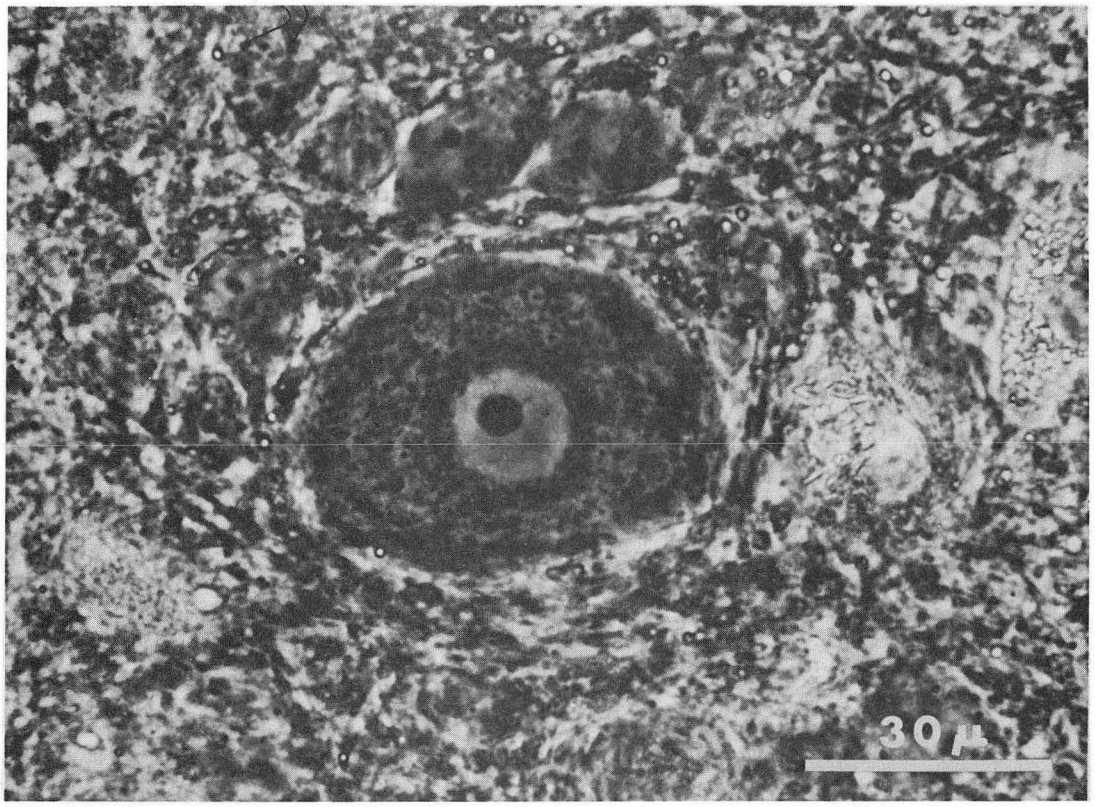

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Fig. 15: Living neuron from 21-day old midbrain culture. Phase contrast.

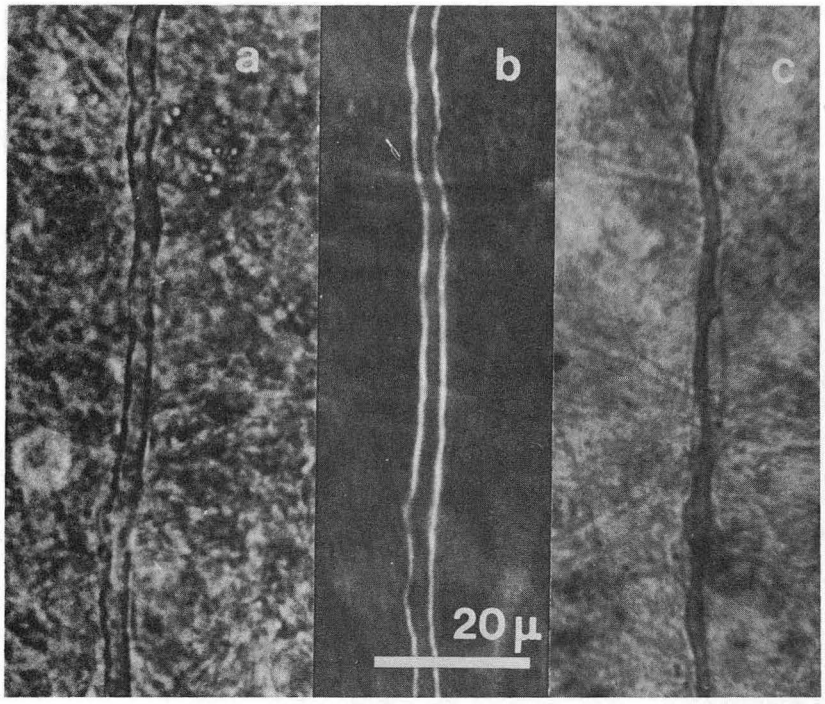

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Fig. 16: Myelinated axon in a 55-day old culture of midbrain. a) phase contrast, b) same axon in polarized light, and c) same axon after staining with Sudan black B.

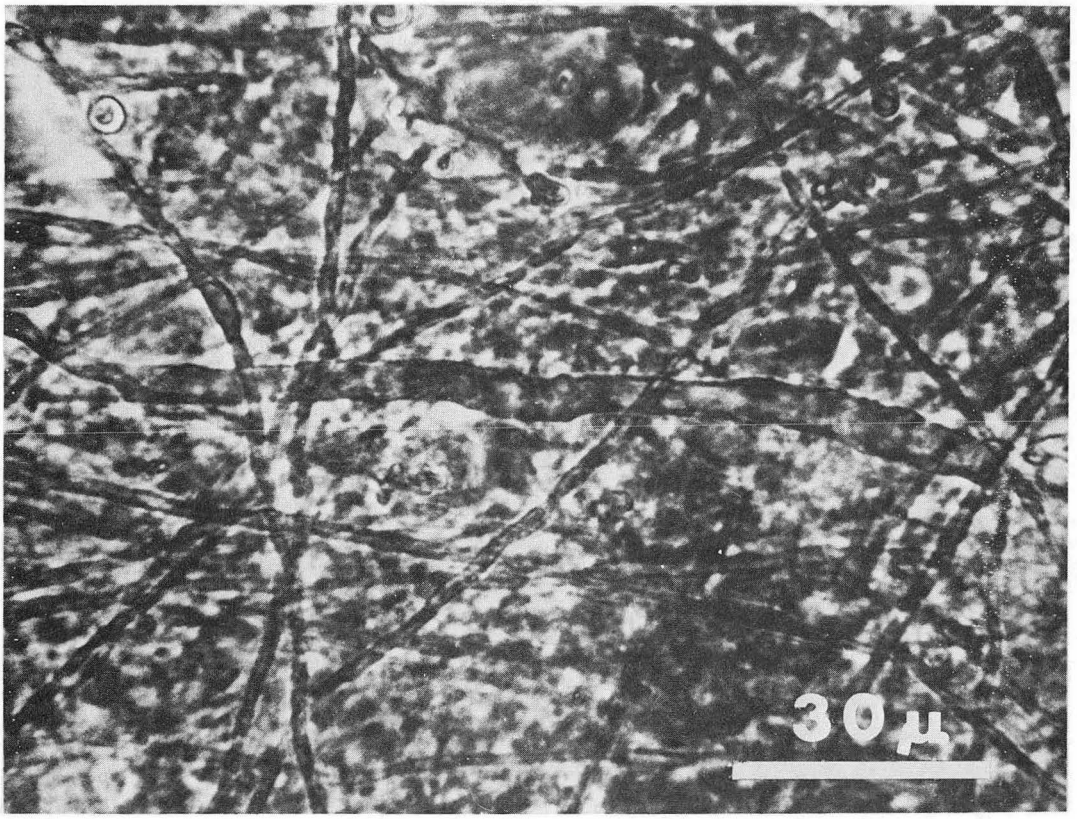

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Fig. 17: Myelinated axons of a midbrain culture, 33 days in vitro. Phase contrast.

suited for demonstrating nerve cells and their processes, especially axons, since neurofibrils (extending throughout the entire length of neurons) are usually deeply stained. The nuclei and nucleoli of all cells are also stained, beautifully revealing the entire architecture of the culture (figs. 10, 12, 14 and 18). Many axons that could not be seen in phase contrast appear very clearly after silver impregnation. In some areas of the cultures, the axons formed a felt-like network similar to the neuropil (figs. 17 and 18). Axons were commonly observed to bifurcate repeatedly, and the meandering and seemingly erratic path of an axon can often be followed for a great distance. Axons almost never ventured into cell free areas of the clot, nor into outgrowth areas covered primarily with mesenchymal elements. This is in marked contrast to spinal cord cultures, in which axons push peripherally into the meningeal outgrowth (Peterson, Crain & Murray, 1965). Quite often, axons wound around neuronal cell bodies, possibly making contact with the somas (fig. 18).

3. Terminal Boutons

Structures resembling terminal boutons could often be seen after Bodian or Holmes silver impregnation, appearing sometimes as dark balls of about 1 μ diameter, sometimes as ring-shaped terminals, and sometimes as club-like endings (figs. 18, 19 and 20). Occasionally such a bouton-like ending was seen in a Bodian stained

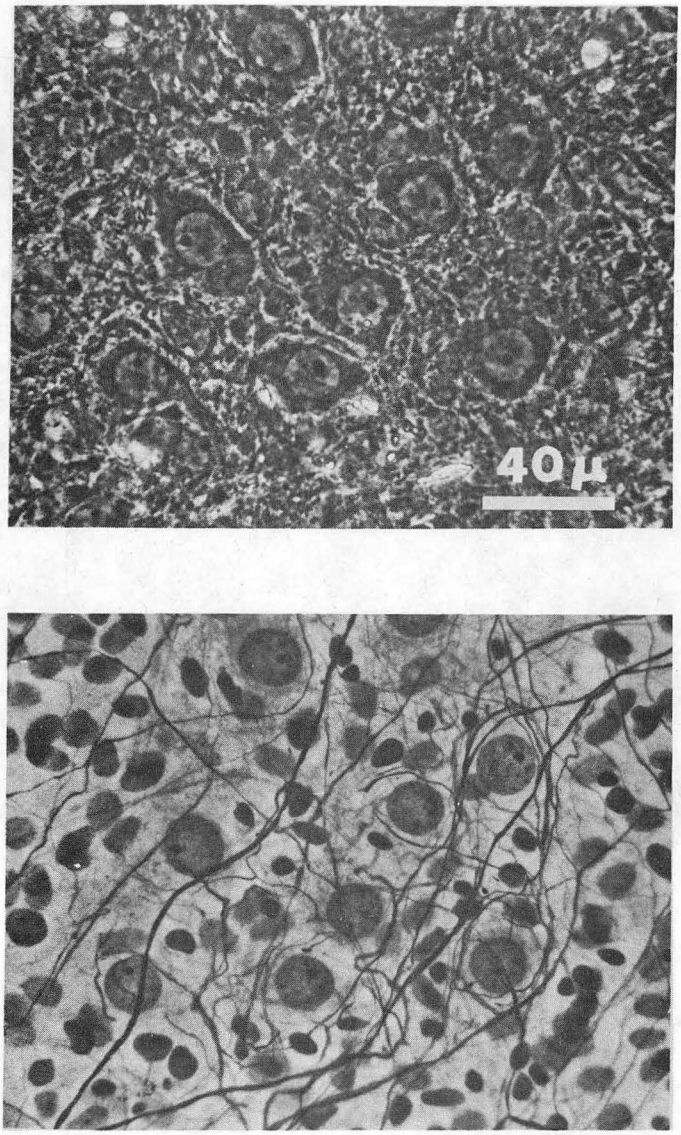

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Fig. 18: Group of cerebellar neurons (Purkinje cells?), 20-day old culture. Top: Living, phase contrast. Bottom: Same area after fixation and staining with Holmes' silver impregnation. Note the rather dense axonal network (dark lines) with many terminals surrounding the neurons. The cytoplasm is stained only very lightly but nuclei of nerve cells (large, rounded) and glia (small, oval) are densely stained.

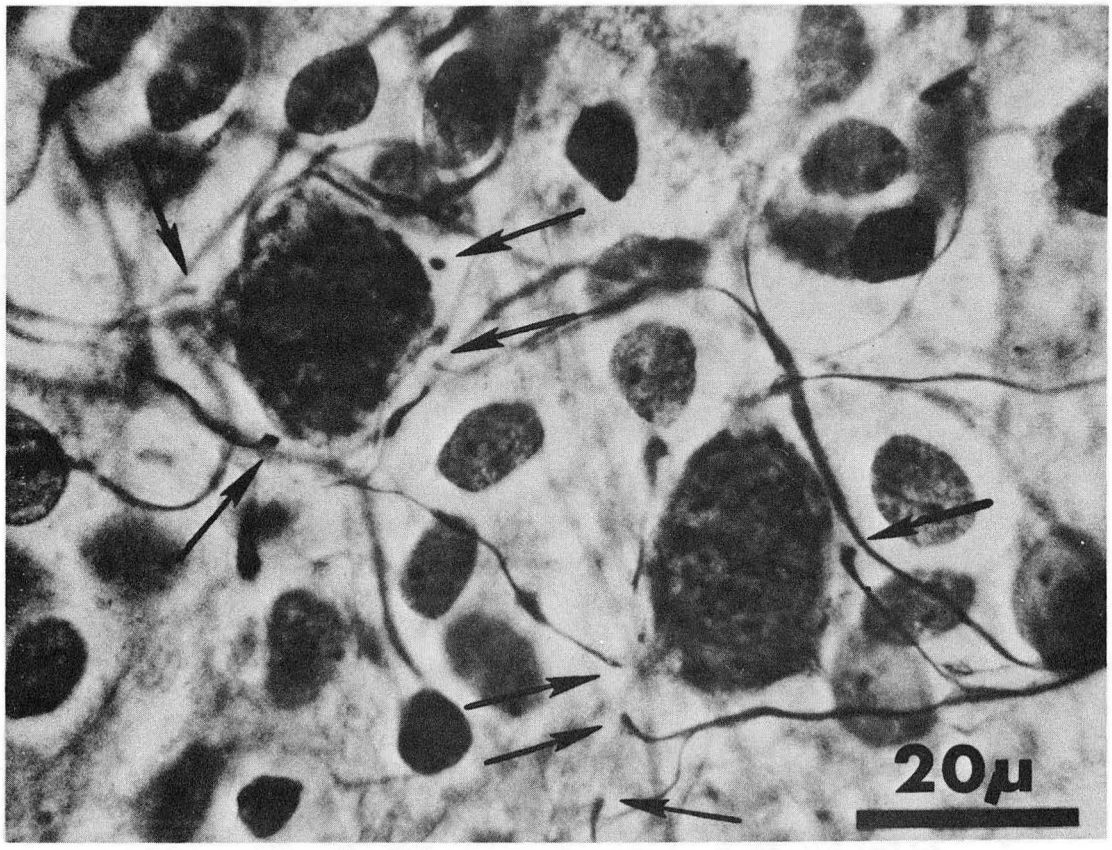

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Fig. 19: Cerebellar neurons with several axons and presumed terminal boutons (arrows). 34 days *in vitro*. Bodian. Oil immersion.

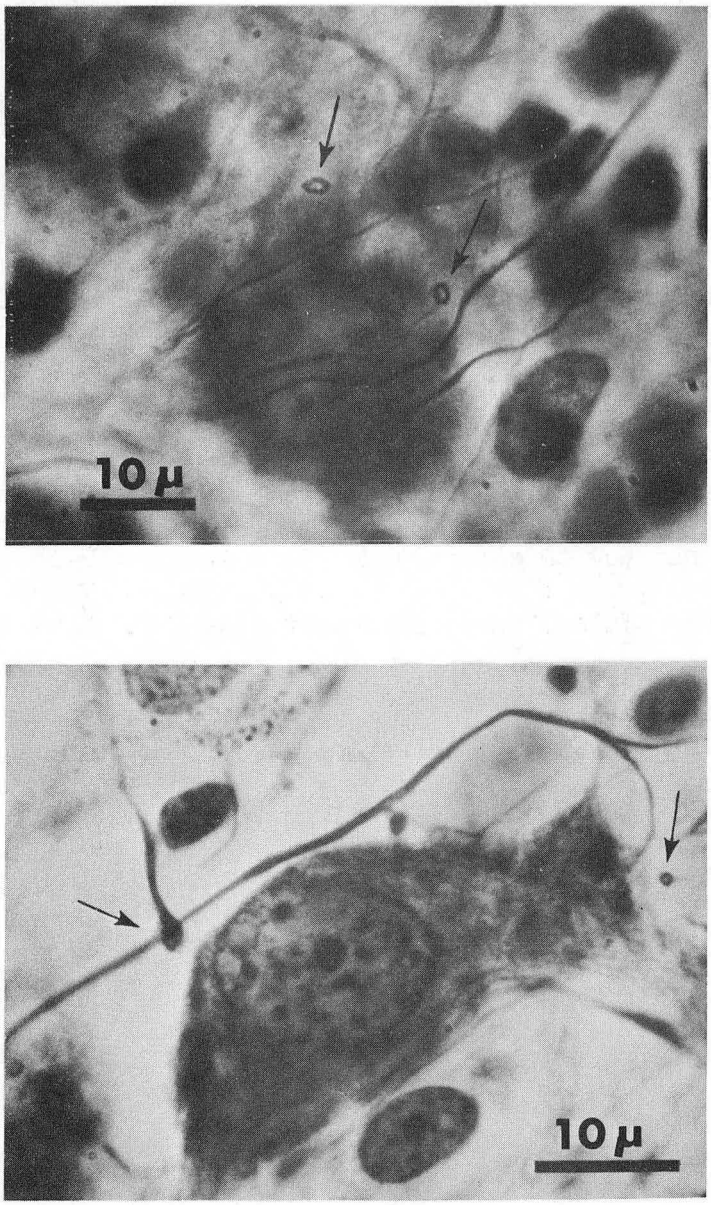

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Fig. 20: Cerebellar neurons with ring-shaped and club-like terminal boutons (arrows). Bodian. Oil immersion. Top: 21 days *in vitro*. Bottom: 16 days *in vitro*.

culture not associated with any visible neurons, but it is of course not known whether contact was made with some unstained dendritic branch. Such branches may reach some hundreds of micra from the cell body and, especially in denser areas, may not be discernible even after Bodian staining. Cell bodies that were densely surrounded by axons (fig. 18) could have possibly provided contacts in the form of boutons-en-passage. Whether these various morphological structures, as revealed by silver impregnation, really represent functional synapses could not yet be determined. Such structures have now been seen in silver stained preparations of tissue cultures by Lumsden (1951), Wolf (1964), Kim (1965), Hild (1966), and Cechner (1967), but except for the case of thick organ cultures (Peterson, Crain & Murray, 1965; Bunge, Bunge & Peterson, 1965), correlated ultrastructural and functional studies are still lacking and a cautious interpretation is warranted (Hild, 1966). However, in view of the electrophysiological data presented below, they could possibly be identical with functional synapses.

4. Discussion

In general the morphological observations reported here fully agree with the ones published by Hild (1966). The flat, membrane-like nature of the cultures used in this study is apparent from an inspection of the stained whole mount (non-sectioned) preparations (figs. 6, 7, 10, 12, 14, 18, 19 and 20). At an age of between about 13 and 25

days in vitro, glial nuclei are often seen to overlap, but neuronal cell bodies are mostly laid down in a more or less dense two-dimensional array. Exceptionally thick areas can, of course, be found, more often in younger cultures. Neurites (axons and dendrites) and glial processes, on the other hand, overlap considerably, and the cultures can therefore not be considered as monolayers. The cultures thin out progressively as they get older, and an increasing number of them start to degenerate. Some cultures, however, have been maintained in an apparently healthy state for up to two months.

The development of roller tube cultures is thus radically different from the thick organ cultures obtained with the Maximow double coverslip technique. The accessibility both for phase contrast visualization and for microelectrode placement is a major advantage, even if it has to be bought with additional distortion of the organotypic structure. This advantage is especially valuable if one is interested in the function at the cellular level (e.g., Hild & Tasaki, 1962), or in the interactions of a relatively small number of cells.

No experiments have yet been performed to determine the reason for the distinctly more pronounced thinning of roller tube cultures compared to Maximow cultures. In fact such experiments may be impossible to carry out since the various aspects of the fluid motion (aeration, waste product removal, etc.) may be hard to dissociate from other differences, such as the requirements for a different nutrient composition, amount of nutrient medium, schedule of feeding,

oxygen tension, concentration of CO_2, and many more. Even the gravity compensation of the rolling arrangement may play a role! In any case, one may speculate that the constant movement of the nutrient medium in the roller tubes plays an important part. In addition to thinning due to the peripheral migration of mesenchymal and glial cells, and to the macrophagic removal of debris from cells injured during the explanting surgery or from cells degenerating during the adaptation to the culture environment, thinning may be enhanced due to the rolling action of the tubes by washing away many cells that would have remained part of the tissue in an undisturbed system.

B. SPONTANEOUS BIOELECTRIC ACTIVITY

1. Extracellular Recordings from Individual Neurons

By positioning the tip of a microelectrode under visual control within a few micra of the soma of a neuron (fig. 21), spontaneous extracellular action potentials were recorded from a majority of the nerve cells tested. Spontaneous activity was found in cultures of 13 to 34 days *in vitro*; younger cultures were usually still too thick to allow accurate visual placement of electrodes; and older ones usually became spread too thinly and often started degenerating, or the supply of cultures simply was exhausted. 34 days *in vitro* is consequently not to be considered an upper limit for the detection of spontaneous bioelectric activity. With respect to a remote electrode,

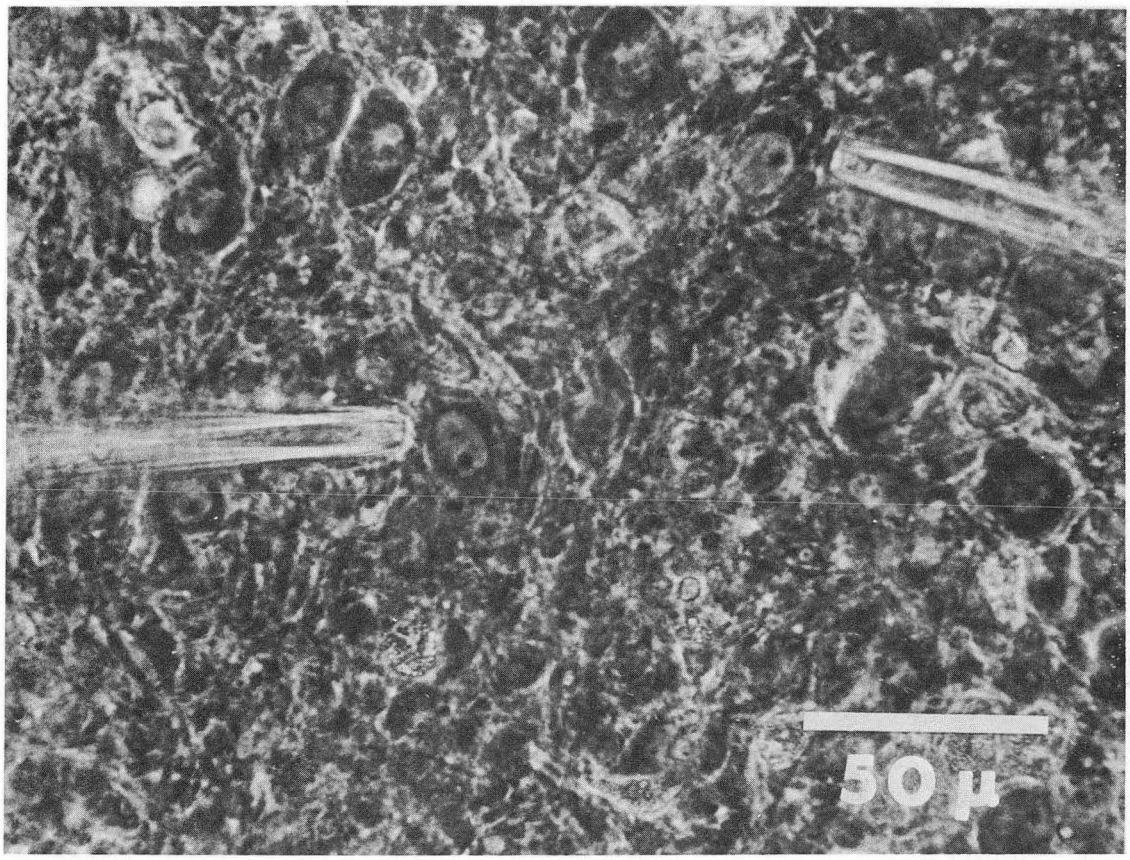

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Fig. 21: Typical arrangement of the extracellular recording electrodes in a cerebellar culture (17 days *in vitro*).

the spikes were sometimes negative (fig. 22a), most often biphasic negative-positive (fig. 22b), or triphasic with a small positive deflection preceding the major negative-positive potential (fig. 22c), frequently with an inflection in the negative swing. At times, large positive (fig. 22d) or positive-negative potentials (fig. 22e) were seen.

The shape and duration of the biphasic negative-positive extracellular spike was very similar to the in vivo recordings from cerebellar Purkinje cells (Eccles, Llinas & Sasaki, 1966); it corresponded well to the theoretically calculated potential distribution due to an action potential of a spherical cell body with dendrites in a conducting medium (Rall, 1962). Triphasic extracellular action potentials are commonly seen in vivo (e.g., Fatt, 1957); the initial positive deflection is due to a remote depolarization, and the large negative potential is due to the depolarization of the soma membrane near the electrode, with the inflection reflecting the lower threshhold for excitation in the initial segment (axon hillock), and the delayed depolarization of the soma (Fuortes, Frank & Becker, 1957). The rarer positive-negative spikes (fig. 22e), were characterized by a much larger amplitude (up to 2 mv peak-to-peak to date, compared to at most 0.6 to 0.8 mv for spikes with a negative major leading phase) and by an absence of other spikes (from other nearby units). Such extracellular "giant spikes" have been repeatedly reported, though at times with much greater amplitudes (Granit & Phillips, 1956). These

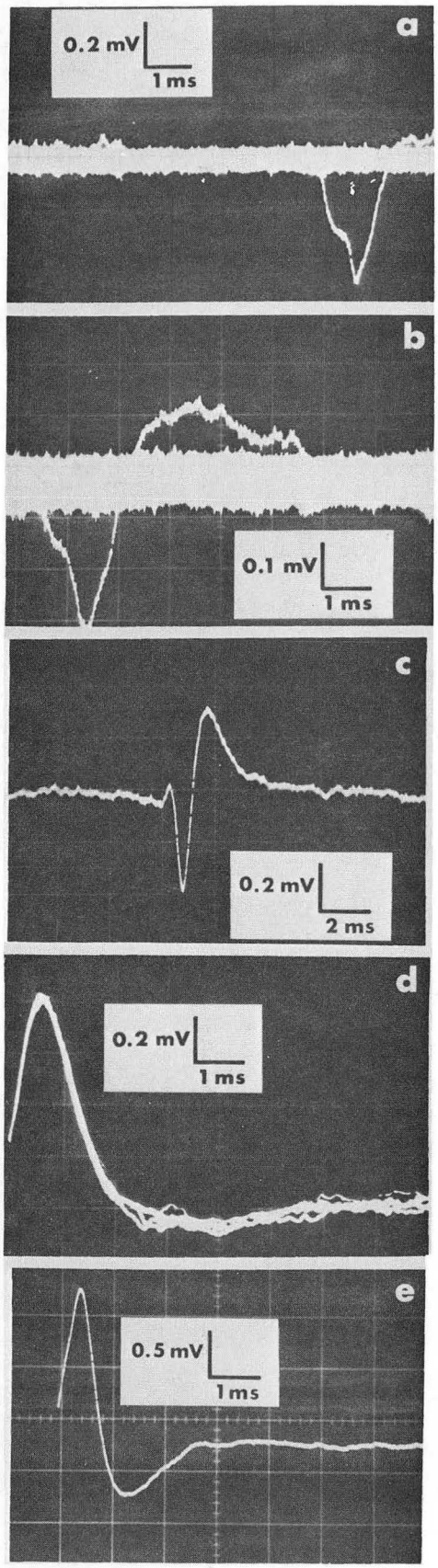

Fig. 22:

Examples of extracellular action potentials.

a) Midbrain, 27 days in vitro (DIV),

b) cerebellum, 20 DIV,

c) cerebellum, 25 DIV,

d) midbrain, 27 DIV,

e) midbrain, 22 DIV.

In this and all following oscilloscope records, positivity is indicated by an upward deflection. a, b & d are multiple trace photographs.

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positive-negative spikes resembled the extracellular potentials seen by Woodward, Hoffer & Lapham (1969) in the Purkinje cell layer of rat cerebellum, and both Crain (1956) and Hild & Tasaki (1962) have reported both negative-positive and positive-negative extracellular unit spikes recorded from cultured neurons. The differences in the observed potentials are probably caused by differences in the relative position of the recording microelectrode with respect to the electrogenic structure. These large positive or positive-negative spikes were not encountered often enough in this investigation to permit conclusive experimentation as to the relationship of the electrode position to the polarity of the extracellular potential. More experiments are planned since the visibility of the nerve cells and the recording electrode tip under culture conditions seem ideal for the investigation of this problem. Upon approaching in situ motoneurons with extracellular microelectrodes, the spikes, in response to antidromic stimulation, change from a triphasic positive-negative-positive shape (similar to fig. 22c) to a positive-negative giant extracellular spike (Nelson & Frank, 1964). Similarly, the negative extracellular spikes from pyramidal cells in the cat cortex change to larger positive-negative potentials, depending on the position of the electrode (Rosenthal, Woodbury & Patton, 1966).

Because of the dense network of axons that is seen to wind around some cell bodies after silver impregnation (fig. 18), a large extracellular microelectrode (4 - 6 μ tip diameter) may pick up small

potentials from many cells; one can usually not be sure that the potentials recorded are really from the cell that is seen in phase contrast. While most records had spikes of uniform height, and thus were considered to originate from a single neuron, a few showed spikes of various amplitudes (fig. 23), and at times quite complex potentials were seen, probably resulting in part from activity in a "miniature neuropil" (fig. 18) near the cell body whose bioelectric activity was sought (fig. 24).

2. The Physiological Origin of Extracellular Spikes

Even though the shape, duration, and amplitude of the above-described spikes corresponded closely to the well-documented spikes reported in the literature, several experiments were carried out to determine whether they were indeed identical to neuronal action potentials.

a) Effect of the distance of the electrode from the cell membrane. When the extracellular microelectrode was withdrawn from the immediate vicinity of the neuron soma, the amplitude of the spike decreased rapidly with increasing distance. Fig. 25 shows an example of such an experiment. Beyond a certain distance (more than 10 to 15 μ with electrode just sitting in balanced salt solution), no signals have ever been detected, nor have any

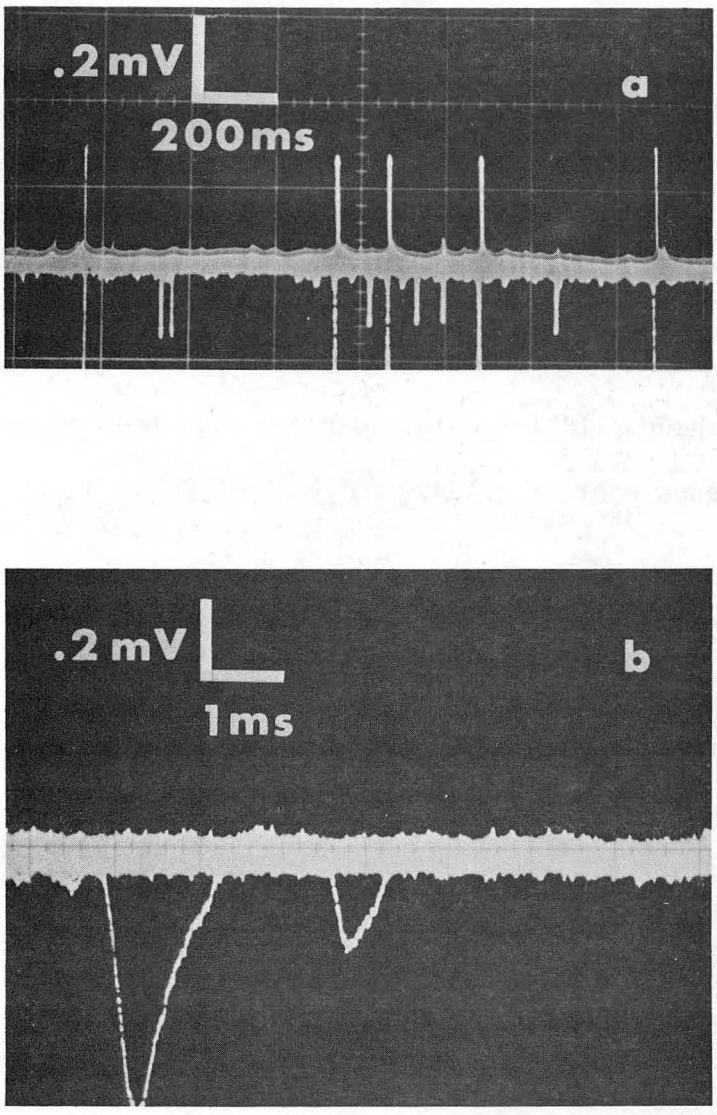

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Fig. 23: Extracellular spikes from two different cells recorded with a single electrode. a) Cerebellum (Purkinje cells?), 23 days *in vitro* (DIV), b) midbrain, 27 DIV.

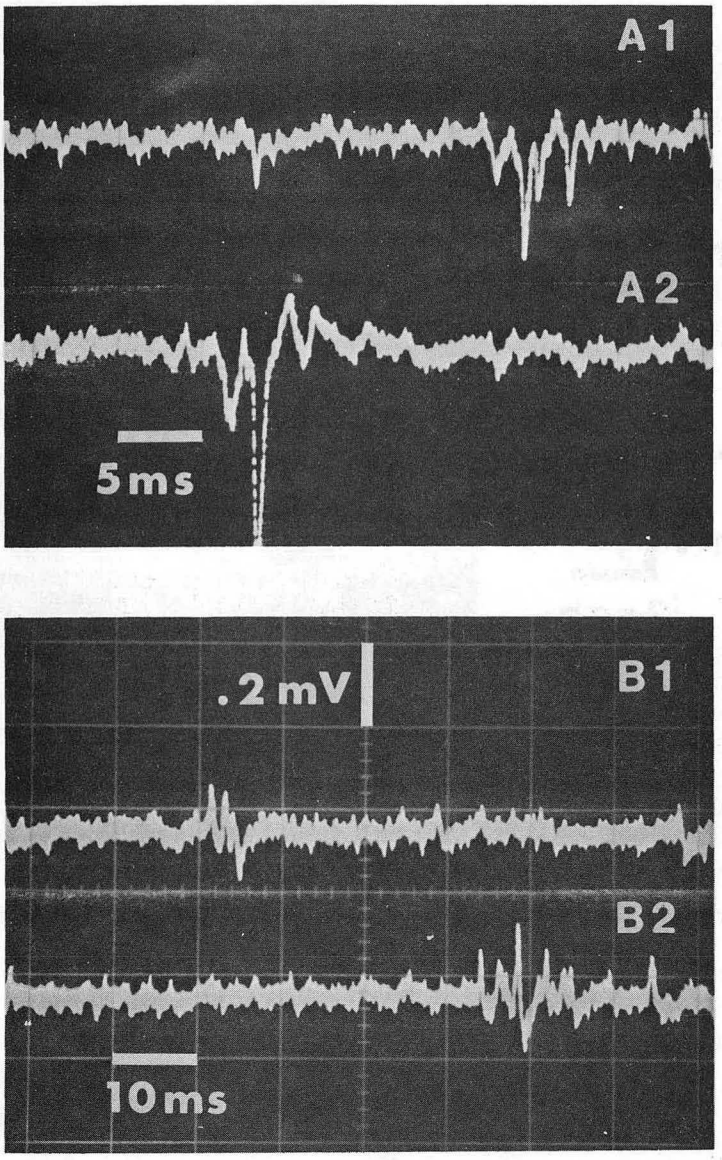

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Fig. 24: Complex potentials recorded with a single extracellular electrode. Midbrain, a) 27 DIV, b) another culture, midbrain, 26 DIV.

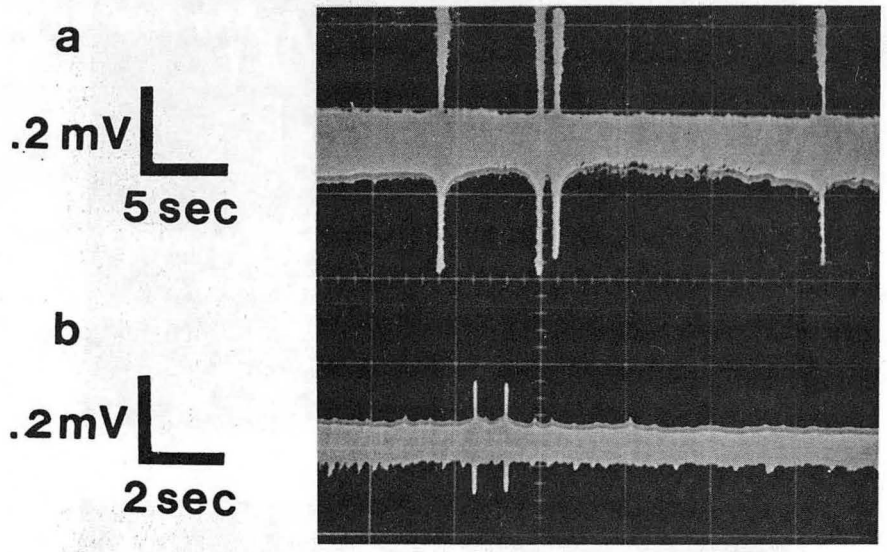

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Fig. 25: Extracellular recording from cerebellum culture, 16 DIV. a) electrode very close to cell membrane, b) electrode removed 5 - 8 μ.

signals ever been found by placing the electrode onto presumed glial or mesenchymal cells.

b) Irreversible injury to neurons during recording. In order to assess the origin of the extracellular spikes, the cell body near the recording electrode was damaged by puncturing it with a second (ultrafine) electrode from the opposite side. In every case the large extracellular action potentials (as shown in fig. 22) disappeared following the entry of the damage-inflicting electrode tip, at times immediately, at other times after a few high frequency "injury" discharges. The injured cell body could then be seen to disintegrate (it turned rapidly granular and phase-light) and seemed subsequently to disappear. Occasionally, smaller irregular and complex potentials, at times barely above the noise level, persisted; this is similar to activity that can sometimes (not very often) be detected by placing the recording electrode into the tissue away from visible nerve cells. On the other hand, large spikes have so far never been found, except when the electrode is very close to a neuronal cell body. Thus it can be assumed with reasonable certainty that the large spikes seen in most records are actually from the cell body near the electrode tip and not from any other small undetectable neurite.

c) Effect of temperature on average spike frequency and shape of extracellular action potential.

Warming the bathing medium from room temperature (25°C) to 36°C increased the average spike frequency by a factor of up to 2. The increase varied from preparation to preparation. If the temperature was raised even more, the spike frequency increased progressively and the neuron often fired prolonged high frequency bursts, until at approximately 43 - 45°C all bioelectric activity stopped suddenly and irreversibly. Extracellular action potentials had a distinctly shorter duration at 36°C than at room temperature; the Q_{10} for the spike duration between 25°C and 36°C was about 2 (fig. 26). Lowering the temperature to 15°C decreased the spike frequency to almost zero and lengthened the duration of the extracellular action potential (fig. 27) with a Q_{10} (24 to 16°C) of about 2.7. Only preliminary experiments have been performed to examine the effect of temperature variation on the electrical activity of cultured neurons. More experiments are planned, especially with respect to the reversibility of the observed phenomena.

The effect of temperature on the action potential duration is well-known for other preparations (Schoepfle & Erlanger, 1941; Hodgkin & Katz, 1949; Schoffeniels, 1958), and has also been noted in intracellular record-

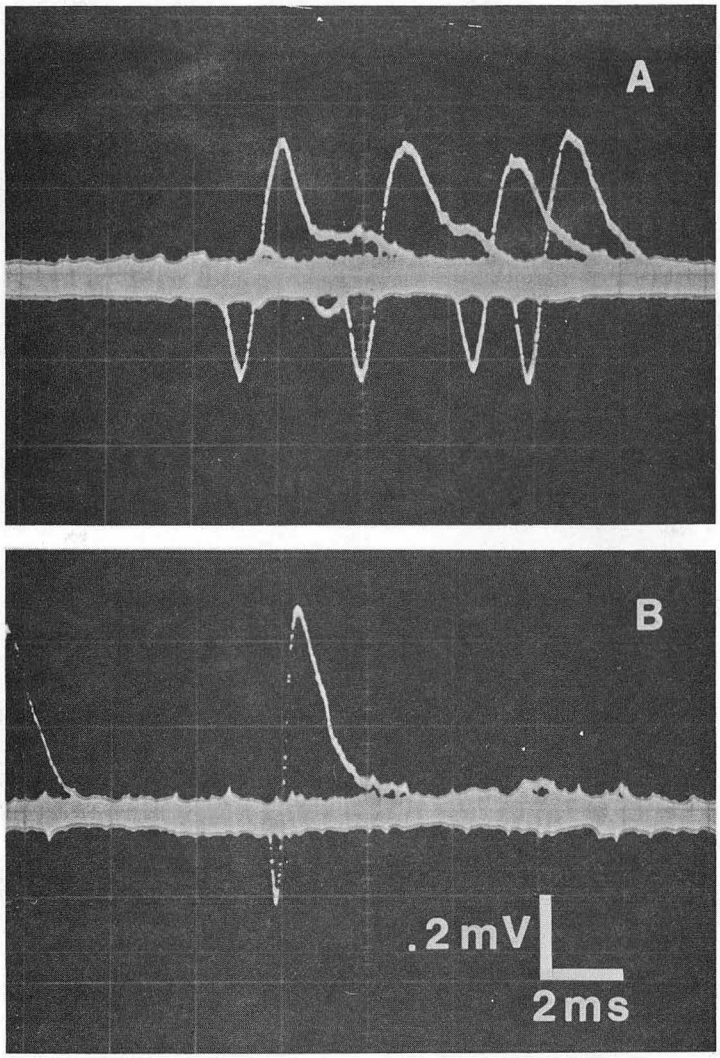

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Fig. 26: Extracellular spikes from a 15-day old cerebellar culture. a) at 25°C, b) at 36°C recorded from the same neuron (same electrode position). Notice the shorter duration of the spike at 36°C.

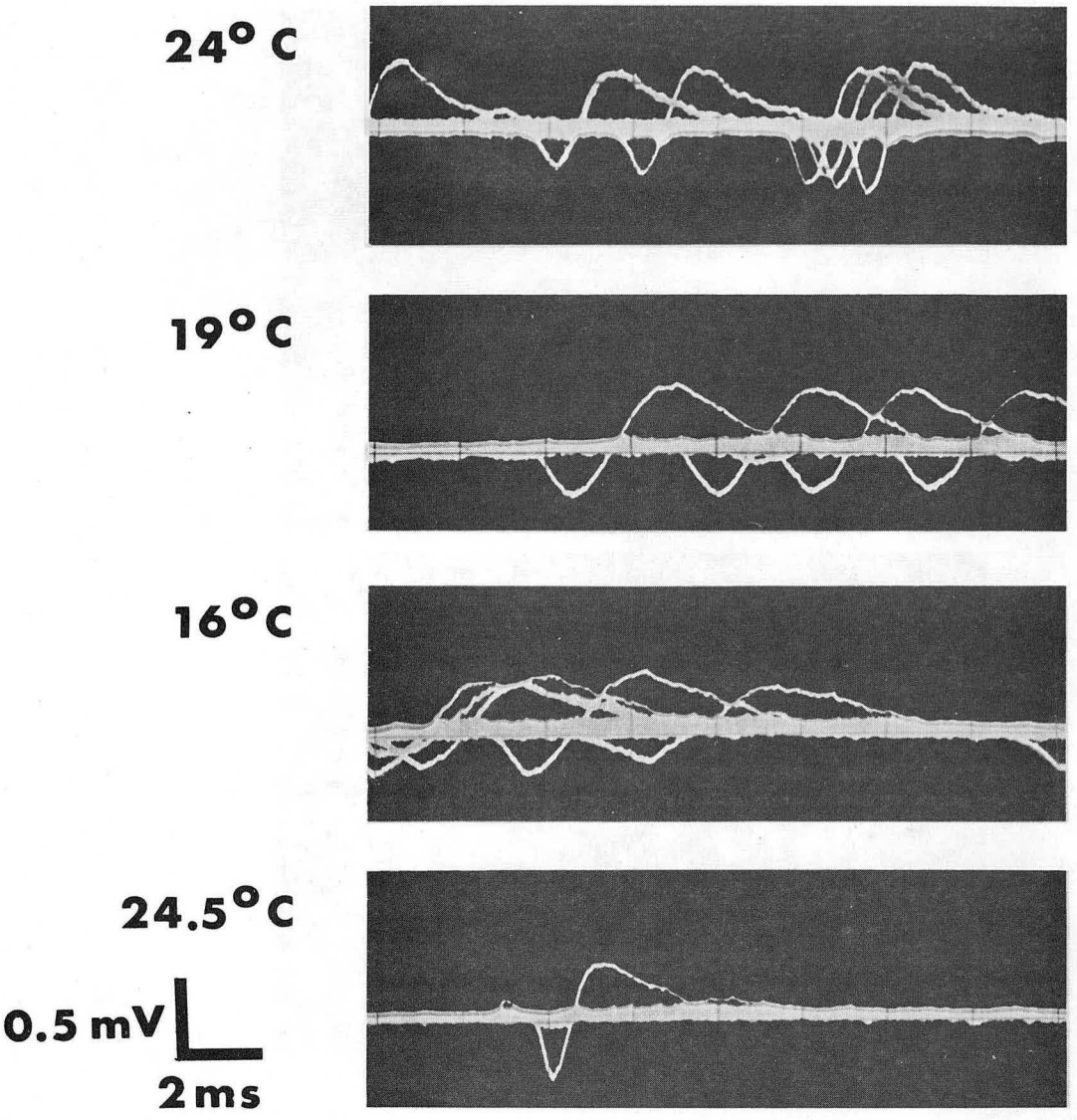

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Fig. 27: Extracellular action potentials recorded from the same nerve cell while lowering the temperature from 24°C to 16°C and then raising it again to 24.5°C. Cerebellum. 14 days in vitro. Notice the change in duration of the extracellular action potential.

ings of neurons in tissue culture (Hild & Tasaki, 1962).

d) Effects of strychnine, calcium, and magnesium concentration in the bathing solution.

The marked effect of strychnine on the spike pattern, and the sensitivity of the average spike frequency to changes in the concentration of calcium and magnesium in the external medium, well attest to the physiological nature of the observed spikes. This subject will be treated in section III, D.

3. Patterns of Spontaneous Bioelectric Activity

After placing the culture under the microscope and positioning the electrodes successfully, activity could be recorded undiminished and presumably from the same unit, for up to five hours or longer at room temperature in balanced salt solution. Good activity could usually be found in nests of neurons (presumably Purkinje cells) of cerebellar cultures, as shown in figs. 5 and 6, and from neurons in denser areas of midbrain and cerebellar cultures profusely surrounded by myelinated axons (figs. 13 and 14). In neurons located in very thin areas of the explant, where the density of cells and cellular processes is low, spontaneous spikes have, so far, been detected only infrequently by the extracellular recording method. It appears that spontaneous activity may require a critical cellular

density or, alternatively, it is possible that a high cell density is essential for the maintenance of functional synapses that mediate the bioelectric activity. The relationship between cellular density (neuron-glia interaction?) and spontaneous electrical activity warrants further study.

The average spike frequency at room temperature ranged from a few spikes per minute to about 10 to 15 spikes per second, but frequencies of 50 to 300 per minute were most often found, with interval distributions that were usually irregular and non-random. Spikes often occurred in trains, with silent periods of sometimes several seconds between bursts (fig. 28), and spike frequencies of up to 100/sec. within the bursts. At times, a very regular spike pattern was found (fig. 29), and often doublet and triplet spikes were recorded in both cerebellar and midbrain cultures, with the second and third spikes usually somewhat smaller in amplitude than the first one (fig. 30). Fig. 31 shows an excerpt of the record and the interspike interval histogram of a culture with a doublet firing pattern. Notice that, while there are some intervals smaller than 5 msec., there is a large peak in the 5 - 10 msec. and 10 - 15 msec. bins. Almost no counts were accumulated for intervals between 15 msec. and 140 msec., with subsequent peaks at around 200 msec. and 425 msec. The interval histogram of another culture showed a sharp peak at a small interval, and a broad secondary peak at a much larger interval (fig. 32). Two other examples of non-random interval distributions are

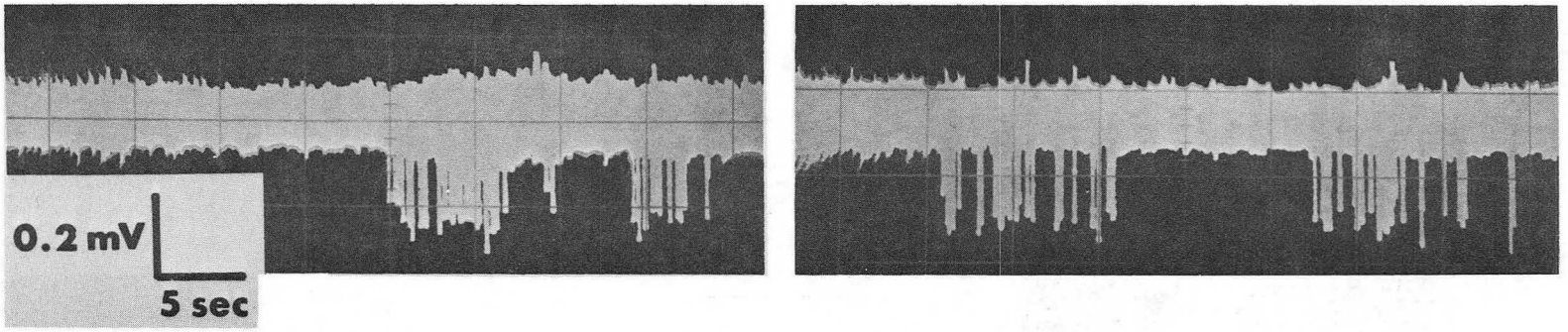

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Fig. 28: Bursting spike activity from a 17-day old midbrain culture.

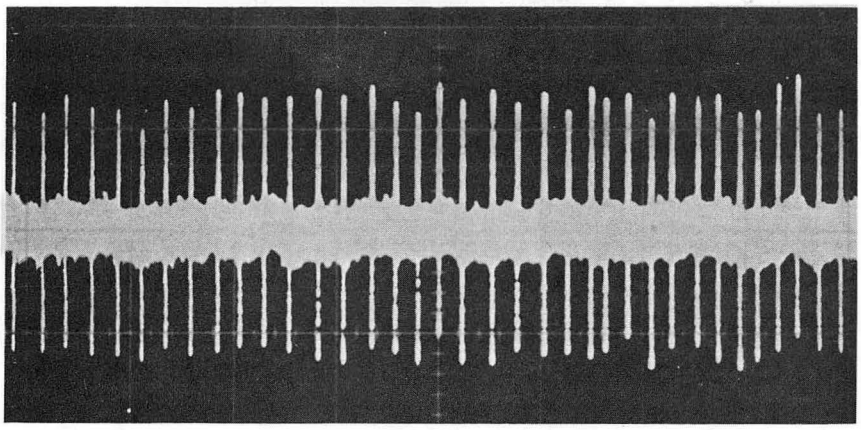

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Fig. 29: Fairly regular extracellular action potentials from Purkinje cells (?) of 16-day old cerebellar culture.

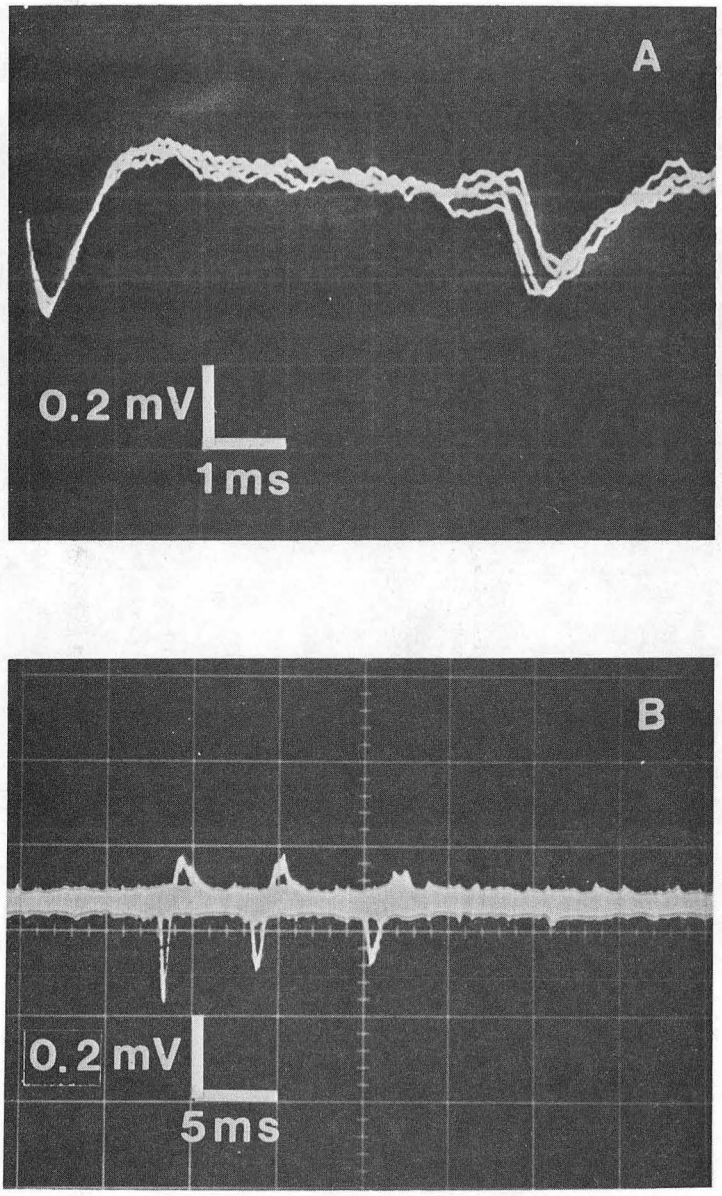

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Fig. 30: Examples of doublet and triplet spikes of decreasing amplitude. A: cerebellum, 14 days in vitro. B: 18-day old cerebellar culture.

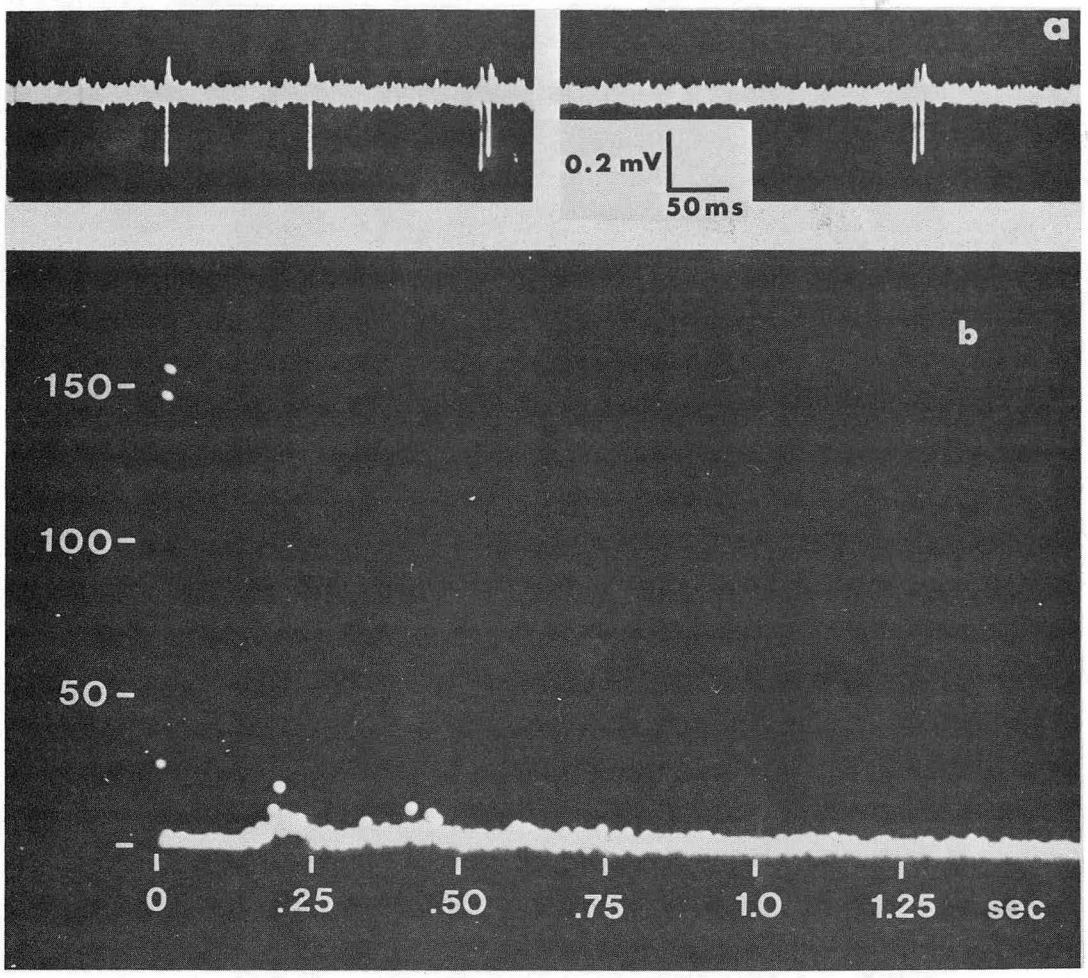

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Fig. 31: a) Excerpts from original record showing single and double spikes. b) Interspike interval histogram. Number of occurrences versus time. Bin size: 5 msec. Sampling interval: 5 min. Number of counts plotted: 957. Cerebellum, 30 days in vitro.

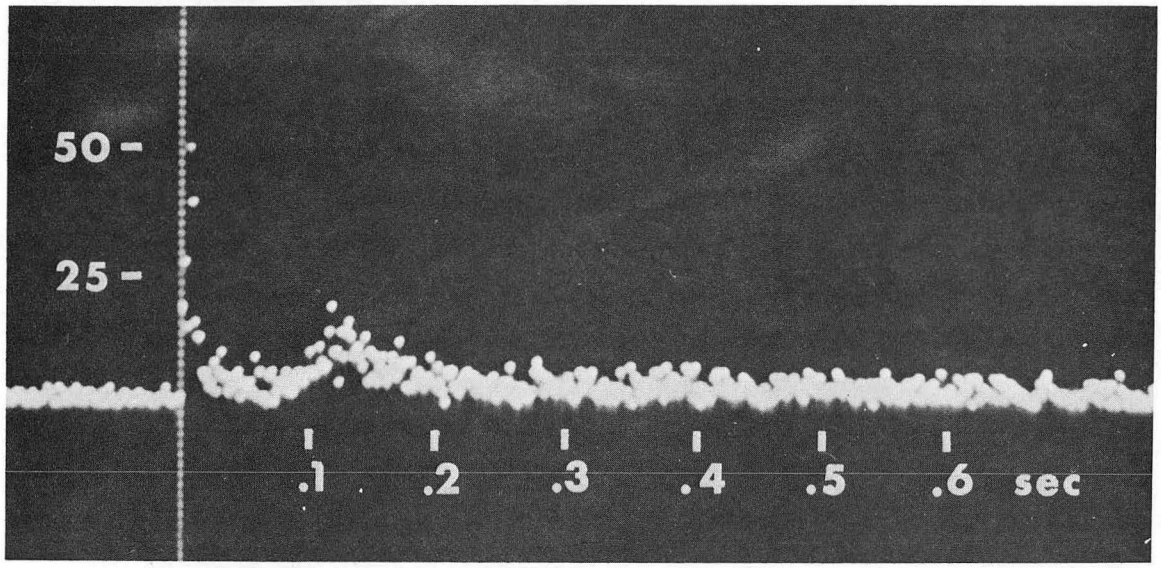

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Fig. 32: Interspike interval histogram of a 22-day old midbrain culture. Ordinate: number of occurrences. Bin size: 2 msec. N = 2053. Sampling interval: 16 min. 25 sec.

given in the histograms of fig. 33 and 34. In the former, the firing pattern has preferred spike intervals at about 10 msec. and at about 70 msec., with a tail of long intervals; in the latter, short intervals are conspicuously absent and the preferred intervals seem to be near 160, 300, and 450 msec., again with a large number of much longer intervals.

A variety of different discharge patterns are thus found in cultures of cerebellum and midbrain. These may reflect not only the heterogeneity of cell types in these cultures, but also the somewhat unorganized and distorted growth and maturation of the cultures. *In situ* architecture, topographic relations, and anatomical connections are changed during the explanting operation and the subsequent readjustment to the new environment, and possibly new interneuronal relations are developed.

However, certain patterns mimicking the *in vivo* activity are repeatedly found. The bursting activity, for instance, has been found in intact and decerebrate cat cerebellum (Brookhart, Moruzi & Snider, 1950; Granit & Phillips, 1956), in intact rat cerebellar cortex (Woodward, Hoffer & Lapham, 1969), and in isolated cat cerebellar folia (Snider, Teramoto & Ban, 1967). Depending on the state of development, the cerebellar Purkinje cells may be spontaneously active at a rather regular slow frequency, may fire doublets, or may be bursting (Woodward, Hoffer & Lapham, 1969). Comparison with the thick long-term organ cultures of Crain may not be easy, but the sporadic, spontaneous, long-lasting complex potentials detected with

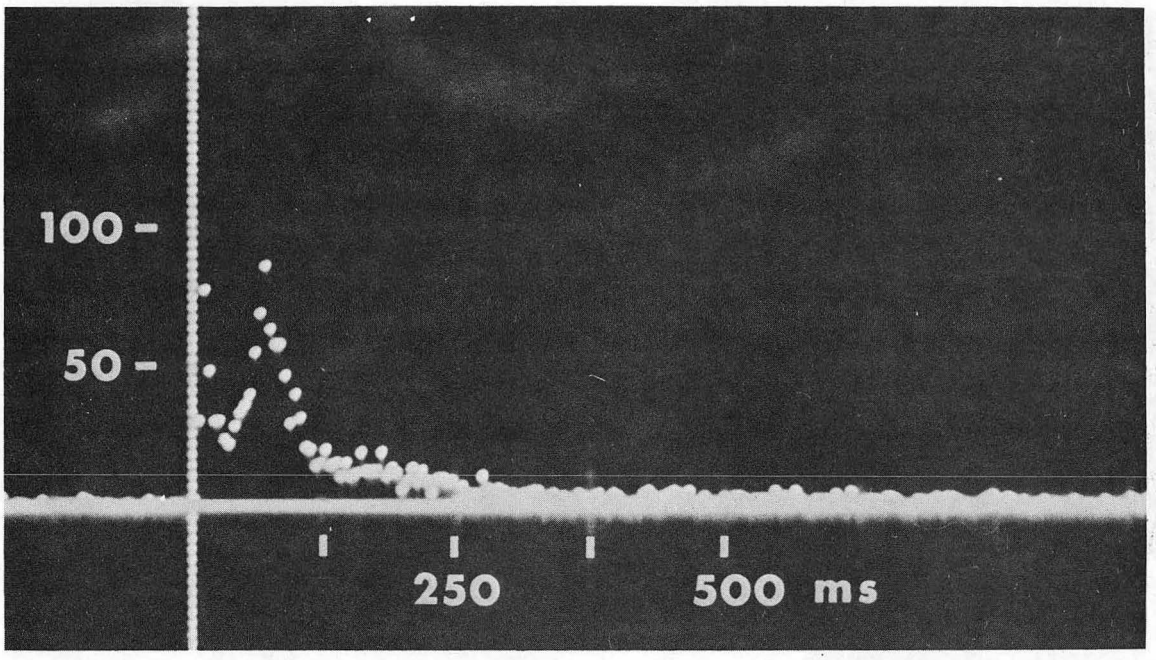

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Fig. 33: Interspike interval histogram of a midbrain culture, 20 days in vitro. Ordinate: number of occurrences. Bin size: 5 msec. N = 1765 counts. Sampling interval: 5 min. Note peaks at about 10 and 70 msec.

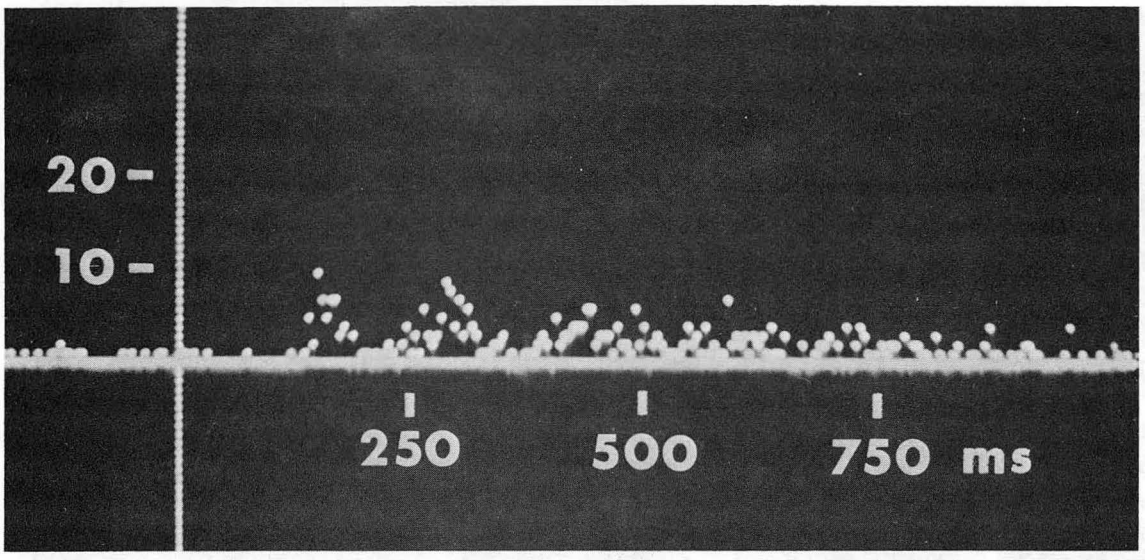

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Fig. 34: Interspike interval histogram of 20-day old cerebellar culture. Bin size: 5 msec. N = 509. Sampling interval: 5 min. 30 sec.

Note absence of short intervals and three peaks.

larger electrodes (Crain, 1966) may be a manifestation similar to the bursting activity reported here. The rhythmic activity of the organ cultures of Cunningham (1962) may have similar origins.

So far no differences with respect to average frequency or interval distribution could be detected between cerebellum and midbrain, nor could any differences be found as a function of age of the culture. Such differences may well exist, but they may be difficult to find due to the heterogeneity of the cultures. As work progresses on this system, it may become possible to study the development of bioelectric activity from specific cultured cell types.

4. Simultaneous Extracellular Recording from Two Neurons

When unit activity was simultaneously recorded with two extracellular microelectrodes from two randomly selected neurons, the somas of which were 20 μ to 200 μ apart (fig. 21), their spike activities were very often correlated. Such correlation was found in almost all cases where both cells showed any activity at all.

The probability of accidentally recording simultaneously with two electrodes from two different points on the same cell (the cell body and some distant neurite that happens to run near the second cell body) is extremely low. Since the action potential could be abolished by damaging the cell body (described above), there was no doubt that the electrodes recorded from two different cells.

Fig. 35 shows the correlated bursting activity of two nearby neurons. The bursts started and ended almost simultaneously, with common silent periods between bursts. Within the bursts the spike correlation was not one-to-one; at times spikes could be seen in one neuron and not in the other. The correlation in another culture, one without marked bursting activity, is demonstrated in figs. 36 and 37. A common characteristic should be noted here; the spikes from neuron A fell near a spike from neuron B more often than expected under chance conditions. The "cross-interval histogram" generated by the PDP-8 computer (see appendix) dramatically expresses the non-random distribution of the spikes from the two neurons (fig. 37). It should also be noted that, while the pattern is not symmetrical around points of coincidence, neuron B often fired with very small intervals before and after neuron A. The closely correlated activity of another culture is shown in fig. 38. In this case, spikes in cell B almost exclusively followed spikes in cell A by a latency of around 5 to 10 msec. The cross-interval histogram of another culture is given in fig. 39, showing yet another distribution around the point of coincidence. For comparison, fig. 40 shows a cross-interval histogram similarly generated from two random pulse generators (two radioactive sources), with average pulse and frequencies comparable to the average spike frequencies found in cultured neurons. During the course of this investigation, simultaneous extracellular recordings with two electrodes were made from 19 pairs of neurons

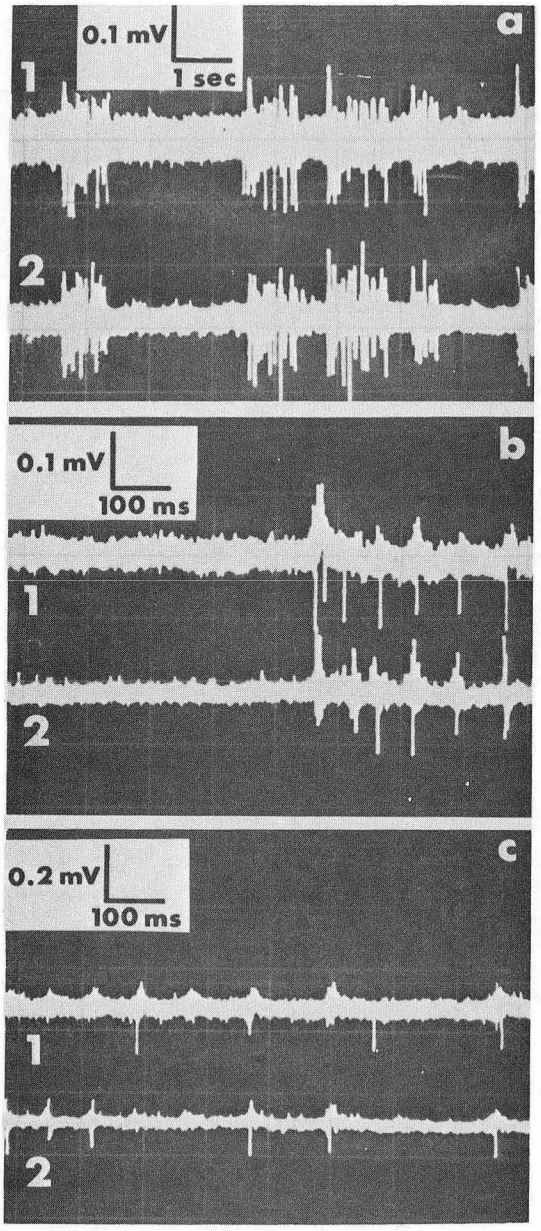

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Fig. 35: Correlated burstin activity simultaneously recorded with two extracellular microelectrodes from two neurons (traces 1 and 2, respectively) approximately 50 μ apart. Cerebellum, 25 days in vitro. A: bursts, B: beginning of a burst, C: within a burst.

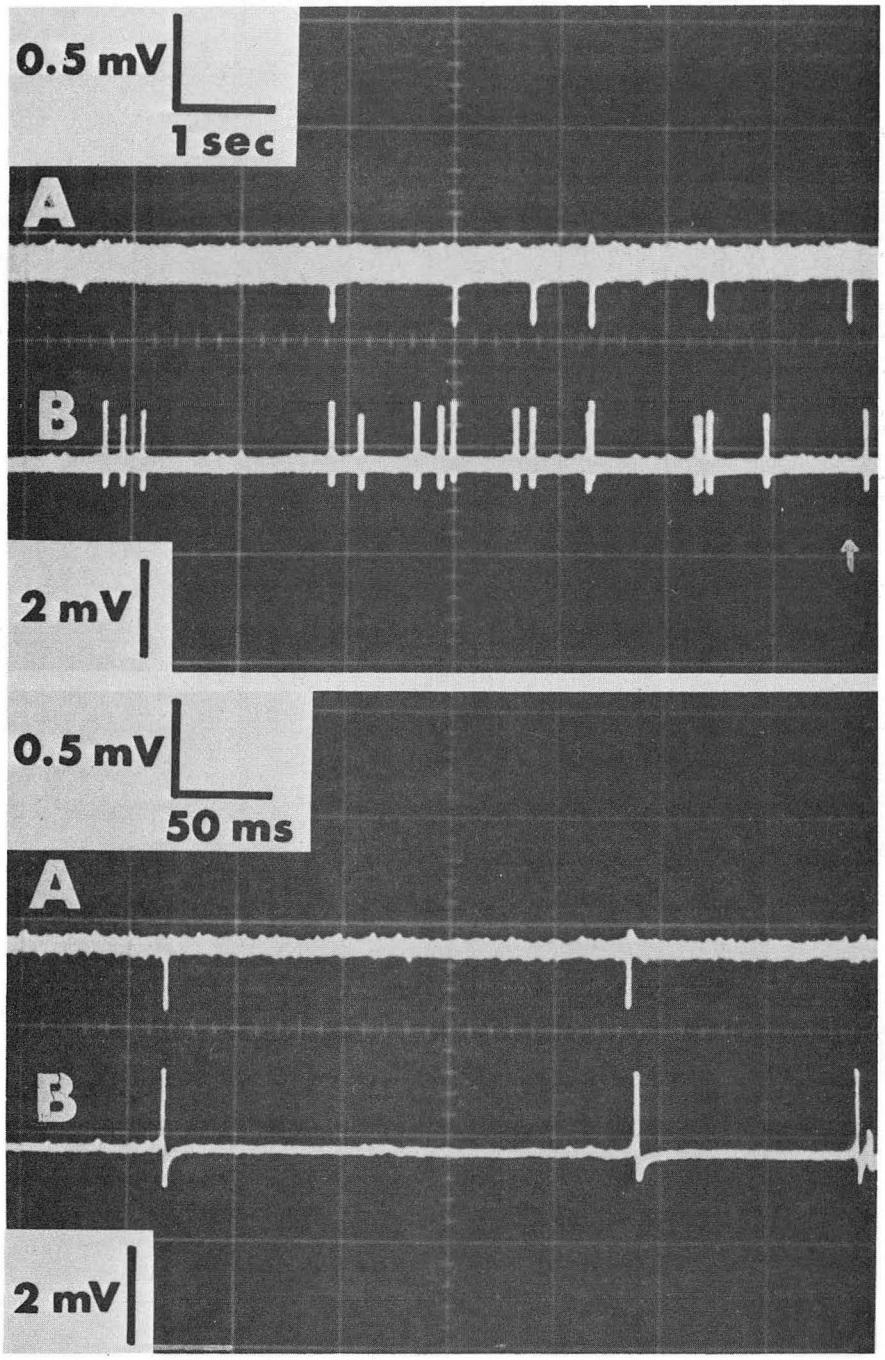

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Fig. 36: Correlated spike activity from two neurons (simultaneously recorded with two extracellular microelectrodes). Midbrain, 22 days *in vitro*.

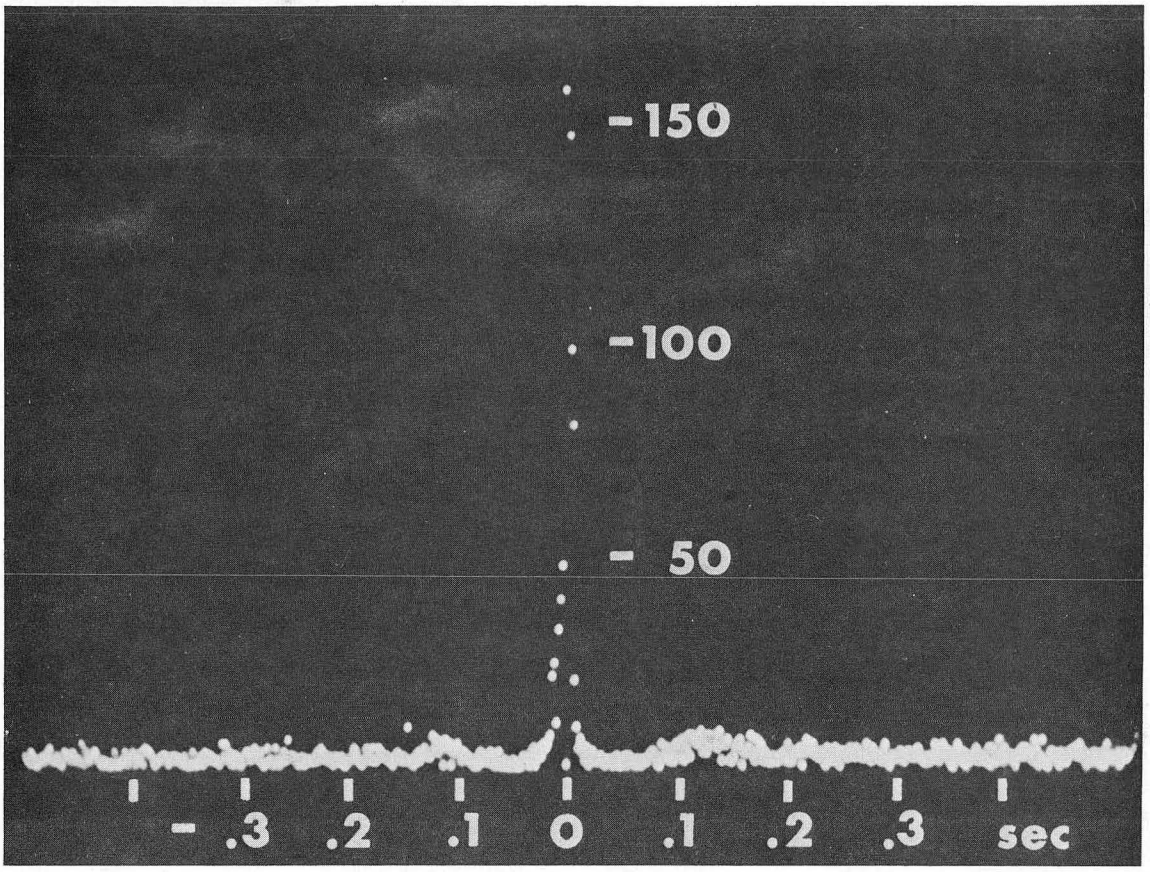

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Fig. 37: Cross-interval histogram of the record of the same culture as fig. 36. Ordinate: number of occurrences. Bin size: 2 msec. Sampling interval: 16 min. 25 sec. Number of counts in train A: 1095, in train B: 2053. The intervals of a spike in train B following any spike in train A are plotted to the right of 0 (forward recurrence times); the intervals of a spike in train B preceding any spike in train A are plotted to the left of 0 (backward recurrence times). Note the uneven distribution around coincidence (= zero).

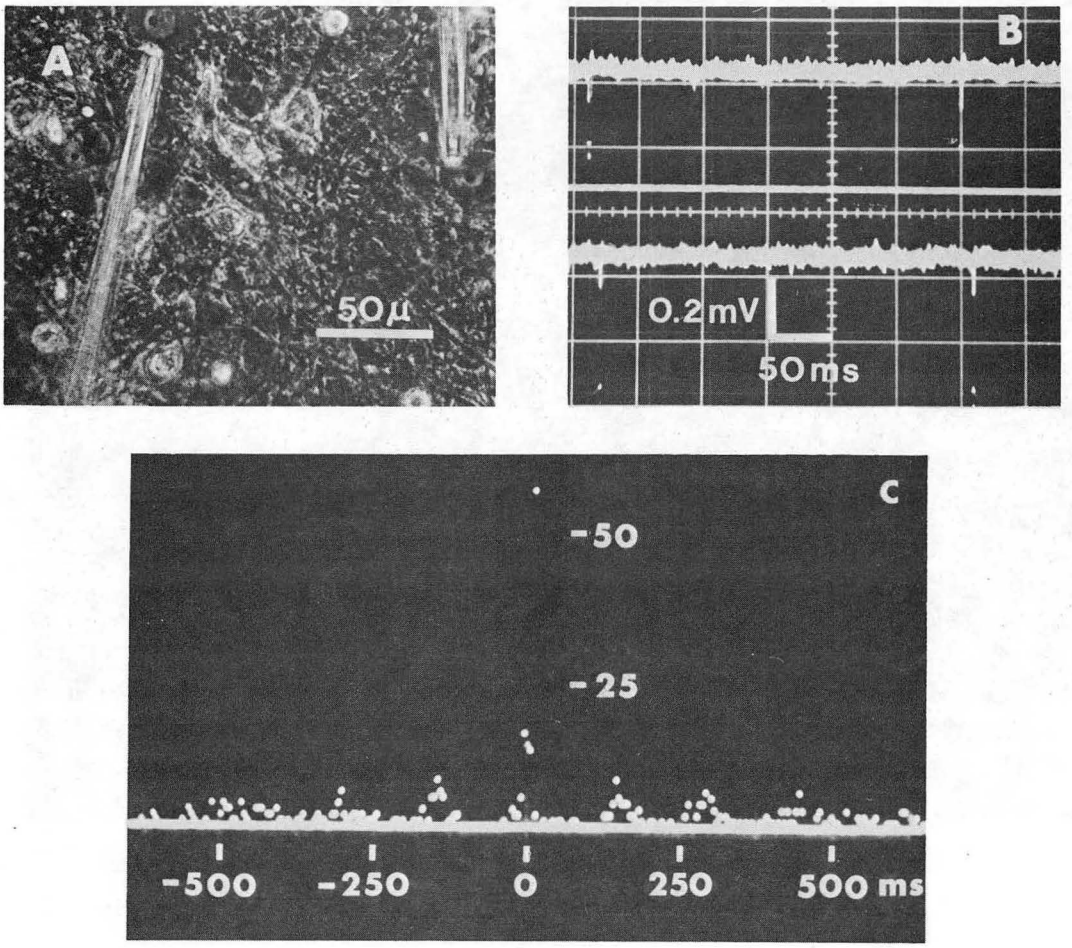

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Fig. 38: 20-day old culture of cerebellum. A: position of electrodes; B: sample record of the spikes simultaneously recorded from the two neurons shown in A. Notice the more or less constant time relationship of the spikes in the upper trace to the spikes in the lower trace. The discriminator outputs (10 volt pulses used for the computer analysis) appear as dots below each spike; C: cross-interval histogram. Bin size: 5 msec.; sampling interval: 5 min. 30 sec.; counts in train A: 509, in train B: 255. Note the maximum point at 5 - 10 msec. to the right of zero, indicating the high probability of a spike in train B following a spike in train A with a latency of 5 - 10 msec.

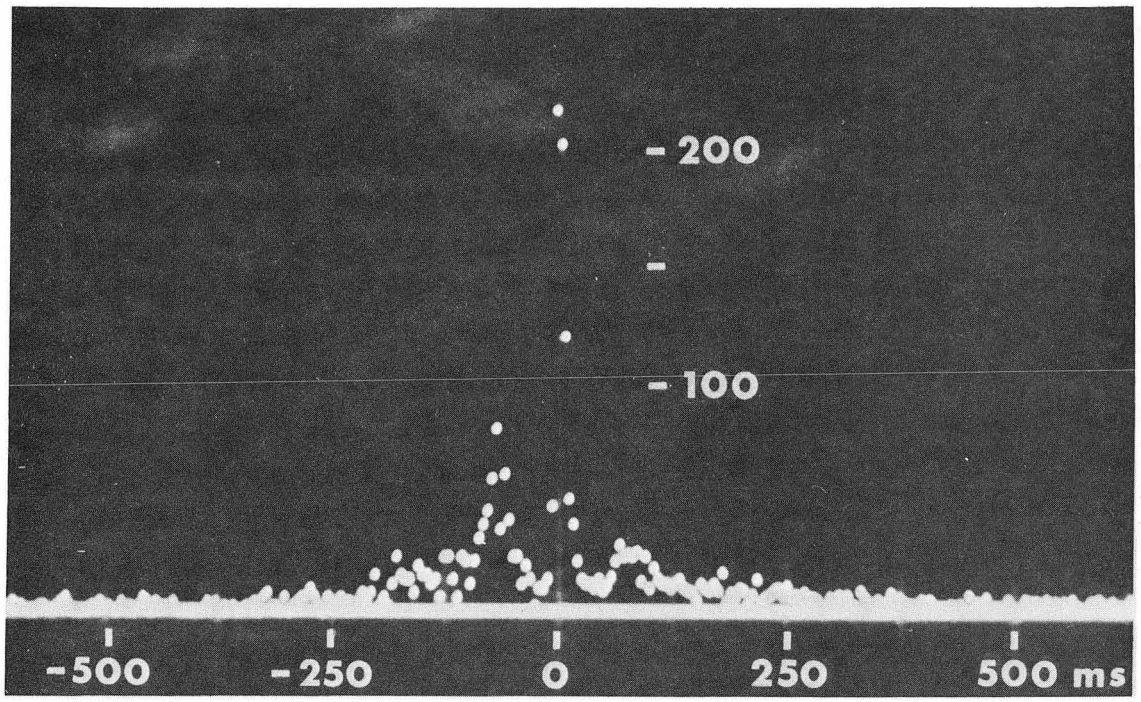

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Fig. 39: Cross-interval histogram of the simultaneous extracellular recording from two neurons in a 20-day old midbrain culture. Bin size: 5 msec.; sampling interval: 5 min.; counts in train A: 727, in train B: 1765.

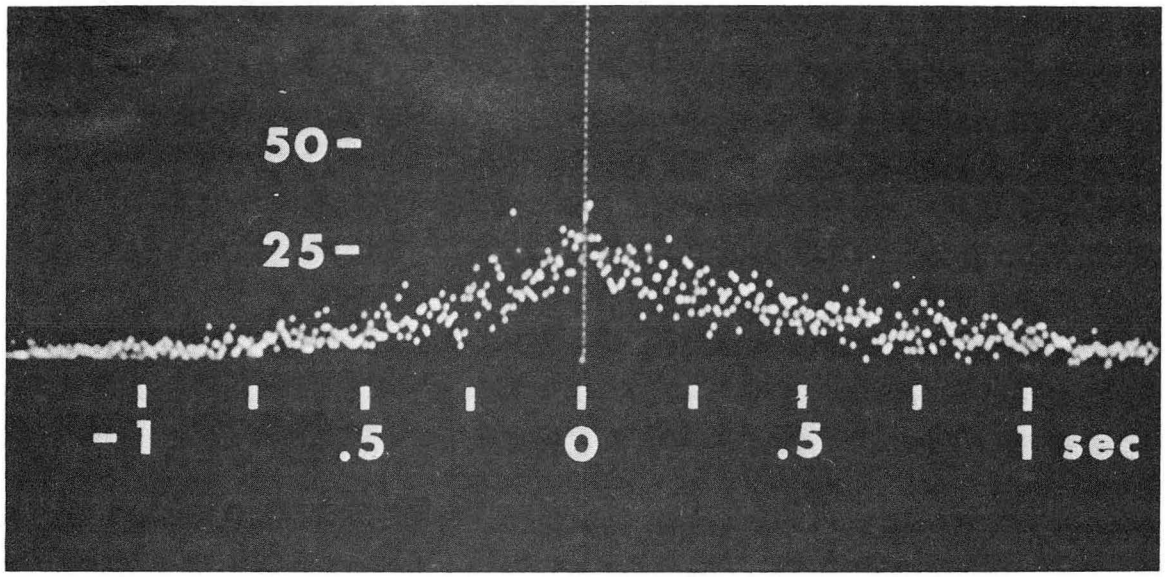

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Fig. 40: Cross-interval histogram of two simultaneous, uncorrelated, random pulse trains obtained by displaying Geiger counter outputs recorded from two radioactive sources (count rates: 106 and 182 counts per minute, respectively). Bin size: 5 msec.; sampling interval: 18 min.; counts in train A: 1914, in train B: 3279.

(from 19 cultures) and in 17 cases the correlation was clear-cut; in the other 2 cases no obvious correlation existed, but detailed computer analysis may still reveal an interaction in these latter cases.

Various degrees of correlation between the unit spikes of neurons in the same microscopic field can thus be seen. A kind of loose correlation may exist, with one neuron firing very often near (shortly before or after) a spike in the other neuron. The two units may be active at different frequencies, and a more or less large number of spikes in one neuron may not have counterparts in the other neuron (fig. 36). At other times, a clear and more or less fixed time relation between the two neurons can be seen (fig. 38), again with the possibility of spikes missing in the record of either nerve cell.

5. Discussion

At least four different interpretations can be given for the non-random interspike interval distributions (bursting patterns, preferred intervals, doublets, triplets) found in most cultures tested so far. The particular cell may have an endogenous mechanism that, in some way, can give rise to the observed firing patterns; local fluctuations in the surrounding medium (ionic composition) may produce bursts, doublets or triplets; the cell may be driven by one or several inputs from other endogenously non-randomly firing neurons; or

it may be part of a self-reexciting network containing excitatory and inhibitory pathways, with at least some cells acting as pacemakers, connected in such a way as to generate the observed patterns. Aspects of all four mechanisms may play a role, and none can be excluded on the basis of analysis of single spike trains. For example, the bursts shown in figs. 28 and 35 may be triggered by one or several neurons that fire spontaneously at a low frequency (one per several seconds, as is often seen in cultures) and which then synaptically relay their activity through possibly very complex nets to a cascading series of neurons to produce bursts in a fashion similar to the long-lasting complex potentials which can be elicited with a single short electrical stimulus in thick organ cultures of central nervous tissue (Crain, 1966). Alternatively, the bursts may be due to long-lasting or repetitive inhibitory inputs impinging on one or many spontaneously active neurons so as to produce relatively long silent periods. Similarly the common occurrence of preferred spike intervals (figs. 33 and 34) may result from excitatory networks, where a neuron can get reexcited periodically with intervals depending on the size of the net, the degree of connectivity and the type of connections (excitatory or inhibitory). Such a model has been investigated with a computer by Farley (1965) who found that oscillatory patterns are generated after stimulation of a single element in a net of nerve analogues with synapse-like connection. On the other hand, these preferred intervals may also be produced by one or many inhibitory

inputs which suppress otherwise spontaneously active cells for some time.

The close correlation (more or less simultaneous firing) of the individual spikes from two neurons in the same microscopic field of a culture suggests the presence of nets of interacting units, with at least some (and possibly many) nerve cells firing spontaneously (pacemaker activity) and transmitting their activity through complex nets to other neurons. The units sampled with microelectrodes represent two nearby points in the postulated net, possibly receiving inputs from common and from independent sources, in addition to their possible endogenous spontaneous activity. A great many combinations are therefore feasible, varying from culture to culture; these result in a rather loose correlation with individual units often exhibiting a degree of independence, such as the lack of fixed time relation or the presence of spikes in one unit without a corresponding spike in the other.

Various kinds of interneuronal interactions are possible. One interpretation for the observed correlation might be a non-synaptically mediated threshhold alteration due to the electric field from the firing of a nearby neuron (Terzuelo & Bullock, 1956). Thus, the fact that one neuron fires an action potential may increase the probability of a spontaneous spike in another neuron even in the absence of synaptic inputs. It seems, though, that such action would only work at very close distances and would need a relatively small

extracellular space to limit current shunting effects. Such an arrangement is generally not present in the cultures used in these experiments. Not only have the neurons (and generally all cells) become spread apart, but the extracellular space has been expanded tremendously. Isolated pockets of close neuronal contact do, of course, exist, and this type of interaction cannot be excluded at present. However, such a situation should be relatively rare and two neurons selected at random in a microscopic field should ordinarily not be so situated. Good correlation is found, however, in the vast majority of cases whenever the activity of two neurons is recorded simultaneously.

A second possibility would be the modification of a neuron's firing pattern by ionic changes in the extracellular space due to the activity of another neuron in a way similar to the depolarization observed in glia (Orkand, Nicholls & Kuffler, 1966). Again such effects are probably only working at very close distances and seem unlikely, though not impossible, under culture conditions. In the spinal cord, for instance, exploration with a microelectrode reveals that the soma of a gastroenemius motoneuron may be as close as 50 μ to 100 μ to a biceps-semitendinosus motoneuron, and yet these two neurons exhibit quite distinctive responses to afferent volleys. The dendrites of such cells may be as long as 1 mm, so that there is a large overlap of the dendritic fields (Romanes, 1953).

A further alternative could be the existence of electrotonic

junctions between neurons (Furshpan & Potter, 1959). In lobster cardiac ganglia, for instance, correlated bursting activity with a similar rather loose correlation (a spike in one cell is not always accompanied by a spike in the second neuron) has been observed (Watanabe, 1958); in that case it is due to electrical connections between some of the ganglion cells. Similar interneuronal electrotonic junctions have not yet been observed in the mammalian nervous system, at least not *in situ*. Nerve tissue cultures similar to the ones used in this study have recently been tested for electrotonic junctions by Walker and Hild (1969), who found widespread electrotonic coupling between glial cells and a certain class of neurons. The electrotonically coupled neurons were morphologically indistinguishable from other (non-coupled) neurons, but their functional properties were quite unusual; they did not seem to be excitable, thus they were not spontaneously active and no action potentials could be evoked with applied electrical stimulus. The electrical response of these cells was similar to that of cultured glial cells (Hild & Tasaki, 1962; Hild, Takenaka & Walker, 1965). Cultured neurons with physiological properties similar to *in vivo* neurons (excitable membranes, etc., as described in the preceding pages) did *not* seem to be coupled electrotonically to each other or to any other cells. While much work is needed to confirm and further investigate this challenging finding of Walker and Hild, it seems, at least at present, that electrotonic coupling is not instrumental in the correlation of discharges from

"normal" excitable cultured neurons.

This leaves the most likely interpretation, namely that neurons showing good bioelectric activity (located in fairly dense areas of the explant) are part of a synaptically connected network. This interpretation is strengthened by the common occurrence in culture of terminal boutons (infra vide) and synaptic structure (Lumsden, 1968).

The exact connections probably vary greatly from culture to culture but one may assume that both excitatory and inhibitory synapses are involved. In addition to multiple synaptic inputs to the cultured neurons, at least some, if not most, cells in these cultures may be spontaneously active. Thus the spontaneous action potentials may be synchronized loosely due to synaptic inputs, or, alternatively, a sufficiently strong synaptic input may cause depolarization below threshold for the firing of an action potential. Such complex interactions can easily give rise to the non-random firing patterns possibly of an oscillatory nature.

Simple, synaptically connected nets of neurons of other biological preparations often exhibit similar characteristics. Some experimental preparations of locust flight motor neurons, for instance, show loosely coordinated discharge patterns due to common synaptic inputs, synaptic interactions between interneurons and motor neurons, and possibly longer term inhibitory interactions (Waldron & Wilson, 1969).

C. MODIFICATIONS OF THE SPONTANEOUS BIOELECTRIC ACTIVITY

Nerve tissue cultures are a convenient material for the study of environmental effects on the cells and their interactions. In order to a) confirm the physiological origin of the potentials recorded; b) investigate some of their properties; c) explore some of the characteristics of the interneuronal interactions; and d) assess the feasibility of future pharmacological investigations, a series of experiments were performed to test the effects of environmental changes.

1. Effects of Strychnine

The addition of 10 µg/ml strychnine to the bathing medium increased the frequency and altered the pattern of the spike activity of single neurons in cultures. Unit spike activity of a low frequency changed to a bursting pattern within less than a minute after the beginning of the drug injection into the recording chamber. The pattern was rather stereotyped (fig. 41B), consisting of a single spike followed by a slow positive wave and a series of 6 - 12 spikes. Such spontaneous bursts occurred at intervals of 4 - 8 seconds (fig. 41C).

The effect of strychnine on the bioelectric activity of thicker cultures has been described by Crain (1964c) and is essentially

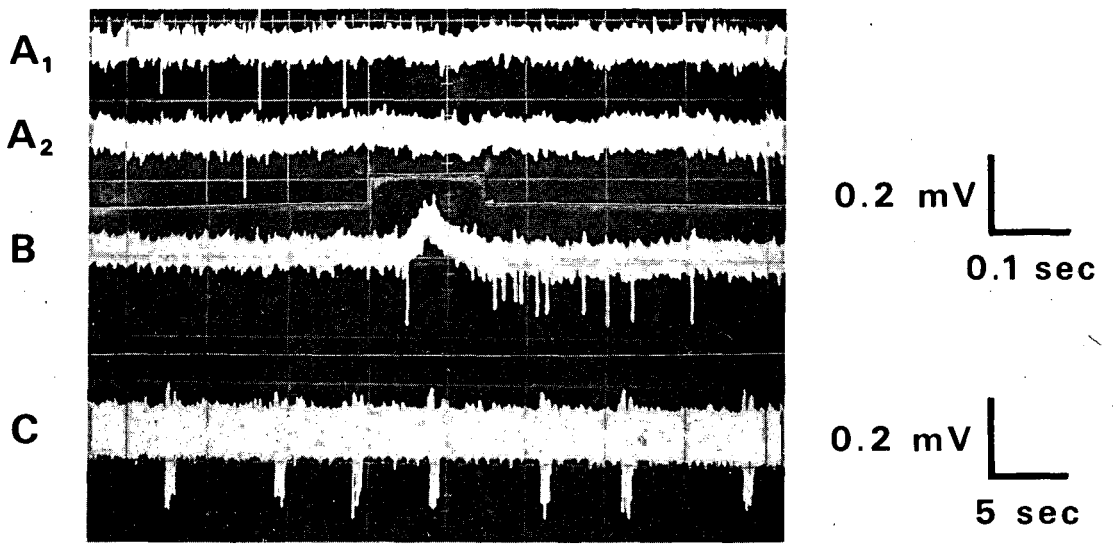

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Fig. 41: Effect of injection of 10 µg/ml strychnine in balanced salt solution. A: control with single spikes of low frequency. B and C: after injection of strychnine.

similar. Convulsive outbursts can be recorded in situ from single units in the cerebellar cortex after strychnine administration (Bremer & Gernandt, 1954; Brookhart, Moruzzi & Snider, 1950).

The action of strychnine points to the presence of inhibitory synapses, since it is thought that strychnine selectively inhibits these synapses (Bradley, Easton & Eccles, 1953).

2. Effects of Varying the Concentration of Calcium

Lowering the calcium concentration from 2.55 mM (normal amount of Ca^{++} in Gey's balanced salt solution) to 1.2 mM increased the unit spike frequency by a factor of 3 to 5. A reduction to 0.5 mM resulted in a large transitory increase of the firing rate, lasting about a minute, followed by a depression to a level lower than the control value (fig. 42). A return to balanced salt solution with 2.55 mM Ca^{++} restored the spike frequency to more or less control level. Exchanging the BSS with an identical solution except for the absence of Ca^{++} caused a short burst of activity of even higher frequency with a subsequent cessation of the spontaneous activity. The activity could be restored by returning to the control Ca^{++} level within a few minutes. Longer periods in Ca-free bathing medium were accompanied by irreversible changes in the cellular appearance and, of course, permanent loss of all bioelectric activity.

No change in spike activity was noted after an increase of the calcium concentration from 2.55 mM to 3.55 mM.

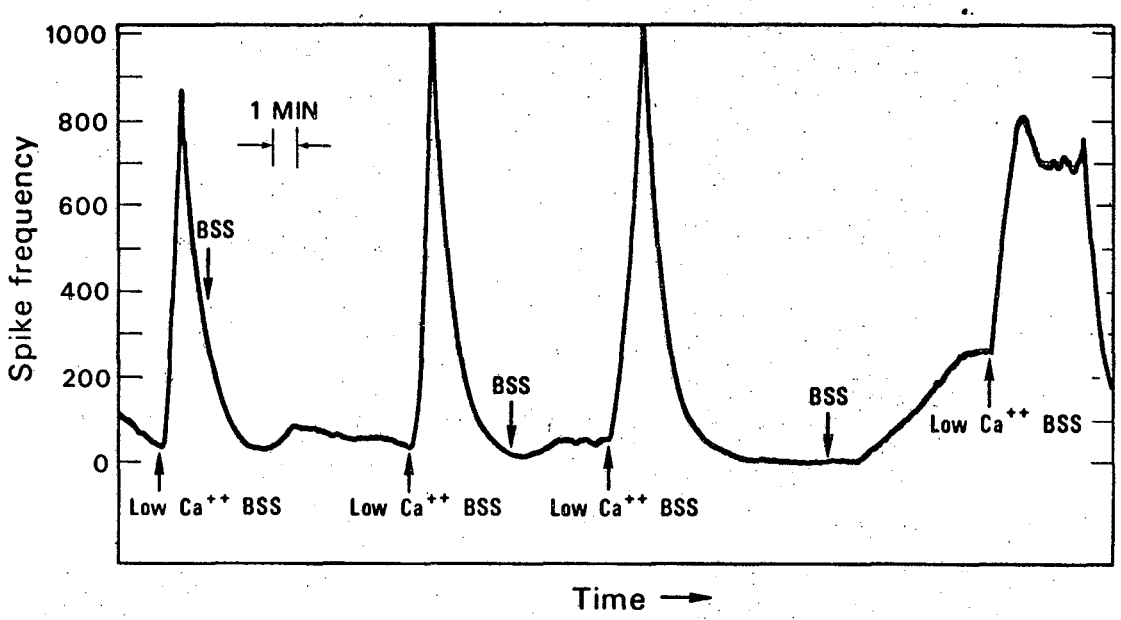

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Fig. 42: Effect of lowering the calcium concentration of the balanced salt solution (BSS) from 2.55 mM to 1.2 mM (midbrain culture, 20 days in vitro).

The effect of the Ca^{++} level on bioelectric activity is thought to be due to an instability in the membrane structure at low Ca levels, with a resulting increase in the excitability. This has been extensively discussed in recent reviews (e.g., Koketsu, 1969), and the action on cultured neurons is entirely as expected.

3. Effects of Magnesium Concentration

Varying the concentration of magnesium in the bathing medium in the presence of Ca^{++} had a much less pronounced effect than varying the calcium concentration alone. Reduction of the magnesium concentration from its normal level (1.07 mM in Gey's BSS) to zero resulted in a reversible increase of the unit spike frequency, while an increase from 1.07 to 2.14 mM caused a reversible decrease of the frequency by a factor of about 2. An addition of 6 mM of Mg^{++} led to a sharp drop of the average spike frequency (fig. 43).

While magnesium affects the excitability of the neuronal membrane, its main action is presumably on the synaptic transmission (Somjen & Kato, 1968; Desmedt, 1963). The action of magnesium ions is thus entirely compatible with the presence of neuronal nets in our cultures.

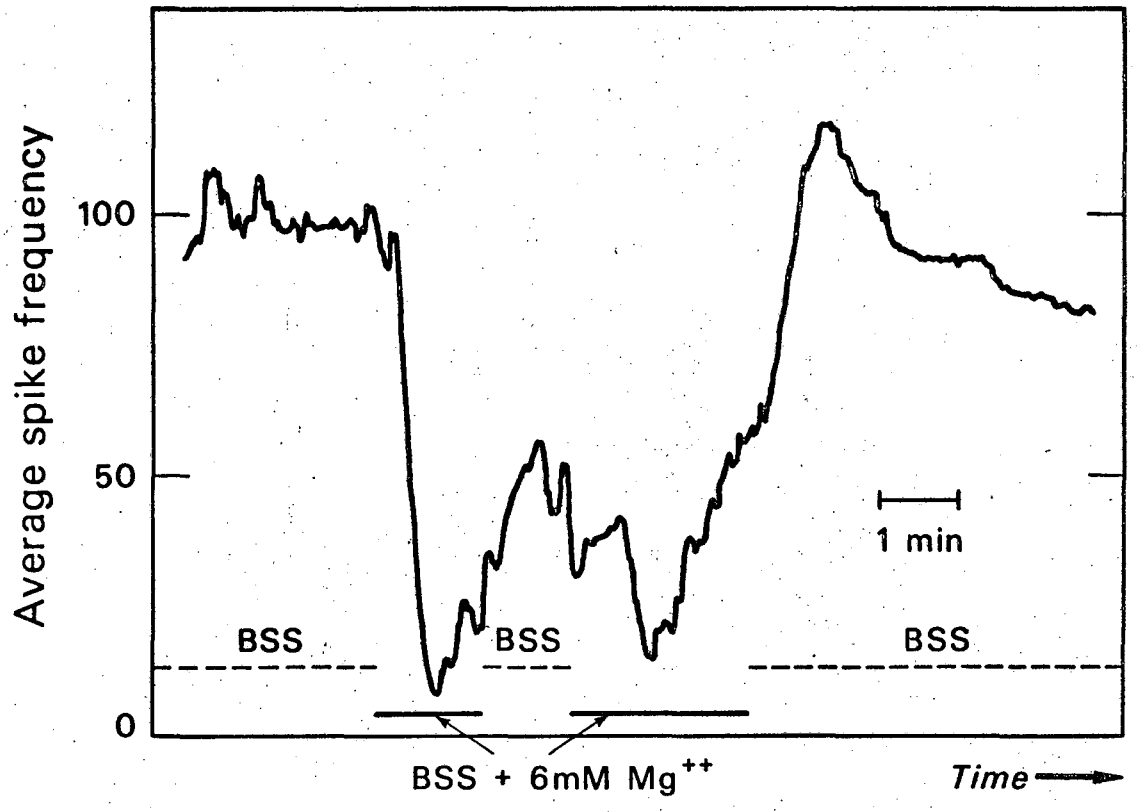

Fig. 43: Addition of 6 mM Mg^{++} to the bathing medium (BSS) (cerebellum, 14 days *in vitro*).

4. Discussion

These experiments demonstrate the feasibility of using cultures for further studies of the actions of various environmental factors (transmitter substances, drugs, ionic composition) on individual nerve cells in the absence of such complicating factors as the blood brain barrier and other control mechanisms. Experiments such as lowering the concentration of an ion are difficult to carry out *in situ*. Estimates of the effective concentrations of ions and drugs in contact with individual cells are hard to make *in vivo* due to a variety of compartments (of unknown size and composition), diffusion barriers, and selective and active transport mechanisms. Iontophoretic application of various chemical substances has been successfully used to study their effect on individual neurons in the intact animal, but it remains very difficult to accurately know the final concentration of the test substance applied (Salmoiraghi & Stefanis, 1967). Nerve tissue cultures may be ideal to supplement pharmacological studies on intact animals. In addition, a more detailed study of the "reversibility" following alteration in the environment (temperature, chemicals, etc.) may give some clues to the adaptive capabilities of neurons.

In the interpretation of the experiments with calcium and magnesium, the data analysis was limited to the average spike frequency. In future experiments, a more detailed analysis of the dis-

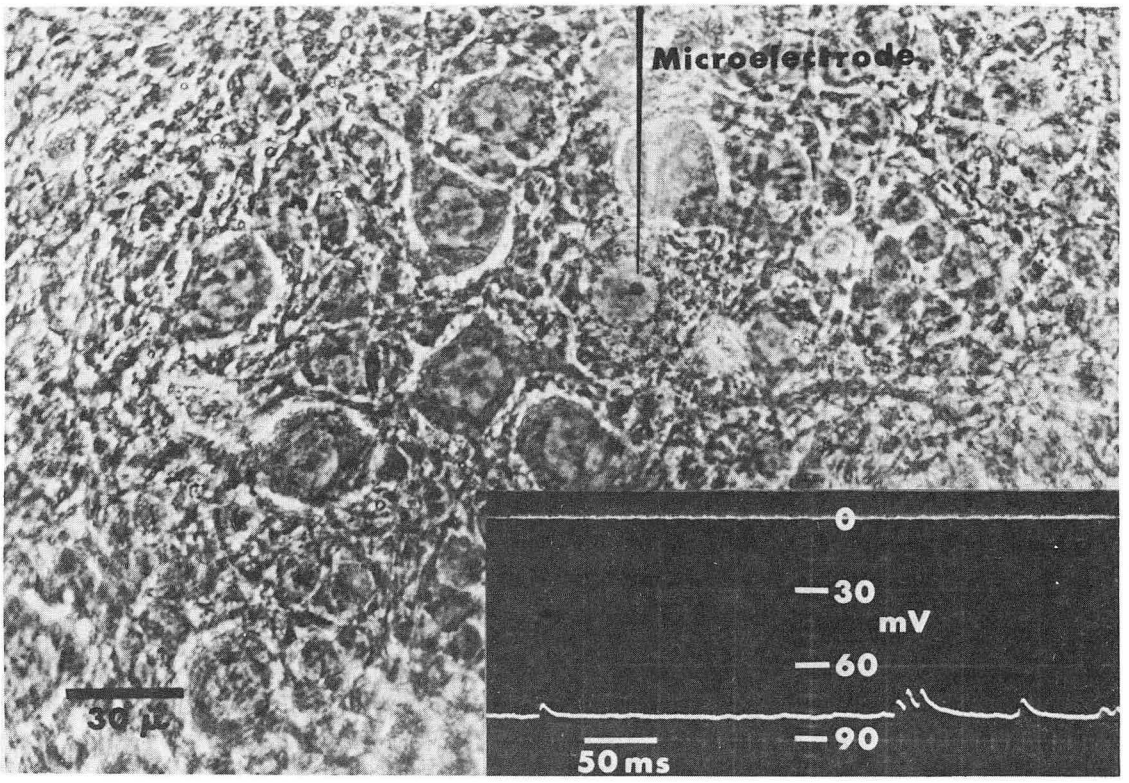

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Fig. 44: Group of living neurons (phase contrast) from cerebellum, 19 days in vitro, showing position of intracellular microelectrode. Inset: intracellular record from one neuron (arrow) showing membrane potential of -77 mV and subthreshold signals (presumably EPSP's).

ical modifications, etc.) have yet been done. Similar signals have, however, been mentioned by Klee and Hild (1967), but without any further comments.

IV. CONCLUSION AND OUTLOOK

The value of simple nervous systems for the study of some of the fundamental problems of neurobiology (integrative mechanisms, plasticity, learning, memory, etc.) has been pointed out repeatedly (Bullock, 1966; Burns, 1968; Giacobini, 1969). Phylogenetically simple organisms (e.g., annelids, molluscs, arthropods, etc.) have yielded a wealth of knowledge, especially since many of these lower animals have conveniently segmented nervous systems, often with large nerve cells. However, it is not yet clear whether these relatively simple preparations will contribute to the elucidation of such problems as neuronal plasticity since their behaviour is usually rather stereotyped and unadaptive. One of the ultimate aims of man's quest for knowledge is to explore his own mind and consequently the study of the nervous system of higher animals (and in particular, mammals) has an inherent appeal. The cultivation of nervous tissue of mammalian origin is an attempt to combine the advantages of simple nervous systems with the complexities of higher animals and to achieve simplifications of and accessibility to an otherwise hopelessly complex organ. Serious disadvantages and problems, however, still plague the preparation: the cultures are still very complicated and their non-uniformity (each culture is different) and somewhat disorganized growth represent major drawbacks. In addition, the neurons are usually relatively small compared to many

invertebrate preparations and very labile to electrode impalement.

This investigation has shown that even after transformation of the explant from a three-dimensional block of tissue into a membrane-like structure, some synaptic interactions between the neurons in culture persist. Whether these synapses have simply survived the explanting operation or whether they have grown de novo during the development of the culture remains to be shown. Future investigations should also examine the functional properties of such cultured synapses especially in regard to their similarity with the corresponding in situ structures. This can be done by pursuing the intracellular studies or by further, more detailed analyses of extracellular recordings, both in conjunction with environmental changes.

The cultures lend themselves quite naturally to the investigation of the development of intercellular interactions, such as formation of synapses, under controlled environmental conditions. This might bear upon the problems of neuronal specificity and plasticity, and more generally upon the problems of learning and memory. For example, long-term stimulation (electrically and/or chemically) during the development of the cultures might lead to changes in their response to electrical stimulation, changes in their gross morphology or ultrastructure, or changes in their synthetic activities (RNA, protein, etc.). Even short-term, acute electrical stimulation might induce detectable changes in their bioelectric response to stimulation, or in their biochemical synthetic activities.

V. SUMMARY

1. Explants from the cerebellum and midbrain of newborn rats were cultured in plasma clots on flying coverslips in roller tubes.

2. Neurons differentiated and matured in cultures and they seemed to maintain a normal morphological appearance. After 2 to 3 weeks in culture, the nerve cell bodies became largely arranged in a flat array embedded between glial cells and neuronal and glial processes. Many of the axons became myelinated and silver impregnations often revealed terminal boutons.

3. Spontaneous bioelectric spike activity was recorded in the majority of the cultures from single neurons by placing extracellular microelectrodes near the neuronal soma. It was shown by killing the cell nearest to the electrode tip that the spikes originated in living neurons whose somas were immediately adjacent to the tip of the electrode.

4. The patterns of unit spike activity included bursts with irregular silent periods of sometimes several seconds, doublet and triplet spike patterns with intervals as short as 4 - 5 msec., and at times regular firings. At room temperature, the average frequency ranged from a few spikes per minute to up to 100 spikes per second in some

of the bursts. 50 - 300 spikes per minute was the most common. In general, the interspike interval histogram showed variations in the distribution of intervals that was much more complex than a simple Gaussian or Poisson distribution.

5. Reducing the temperature from 25°C to 16°C decreased the average spike frequency to almost zero and lengthened the duration of the spikes, while raising the temperature to 36°C increased the average spike frequency by a factor of up to 2 and shortened the duration of the spikes. Spike activity stopped at temperatures higher than 43 - 45°C.

6. Spikes simultaneously recorded with two microelectrodes from two neurons in the same microscopic field and separated by as far as 200 μ showed varying degrees of correlation with each other, i.e., spikes from one neuron were accompanied within a small time interval by spikes from the other neuron more often than expected on the basis of chance.

7. 10 μg/ml strychnine increased the average spike frequency and changed the discharge pattern of single neurons in culture in a way similar to the changes seen <u>in situ</u>.

8. Lowering the calcium concentration in the bathing medium resulted in a transient high frequency burst followed by a decrease of the average spike frequency. Irreversible damage was caused by

removal of all calcium from the bathing medium for longer than a few minutes.

9. Decreasing or increasing the magnesium concentration resulted in an increasing or decreasing average frequency of unit spike activity, respectively.

10. Membrane potentials of up to -77 mv and subthreshold signals resembling EPSP's were recorded with ultrafine intracellular microelectrodes from the somas of cultured neurons.

11. From the non-random interspike intervals, from the close correlation of simultaneous spike trains from two neurons, as well as from the action of strychnine and magnesium, it is concluded that the neurons in these thin cultures of rat cerebellum and midbrain are synaptically connected to form complex nets of interacting units. They represent a much simplified, isolated neural net accessible to visual observation and electrode exploration under controllable environmental conditions. This work demonstrates that this preparation may be suitable for the correlative study of function and structure in the mammalian central nervous system which might possibly lead to a better understanding of neuronal development, plasticity, and the mechanisms of learning and memory.

VI. ACKNOWLEDGMENTS

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This manuscript is dedicated to my wife Sonja, whose endurance and encouragement was invaluable.

VI. APPENDIX

Computer Program for Analyses of Spike Trains

Two channels of an 8-channel tape recorder were used to record the 10-volt square pulses that were produced by two discriminator - pulse generators for each neuronal spike (see fig. 2). The two simultaneous pulse trains were later analyzed with a general purpose computer (Digital Equipment Corp. PDP-8/I) to construct time interval histograms and cross-interval histograms as defined in fig. 4. The computer simultaneously generated four histograms and displayed them in the four quadrants of the oscilloscope screen: a) the intervals of train A (from neuron A), A_1, A_2, etc. (designated histogram AA); b) the intervals of train B (from neuron B), B_1, etc. (designated histogram BB); c) the first-order backward recurrence times between a spike in train A and the next previous spike in train B (V_{-1}) (designated histogram BA); and d) the first-order forward recurrence times between a spike in train A and the next following spike in train B (V_1) (designated histogram AB) (see fig. 4). Each histogram could consist of a maximum of 512 by 512 points (1000_8 by 1000_8).

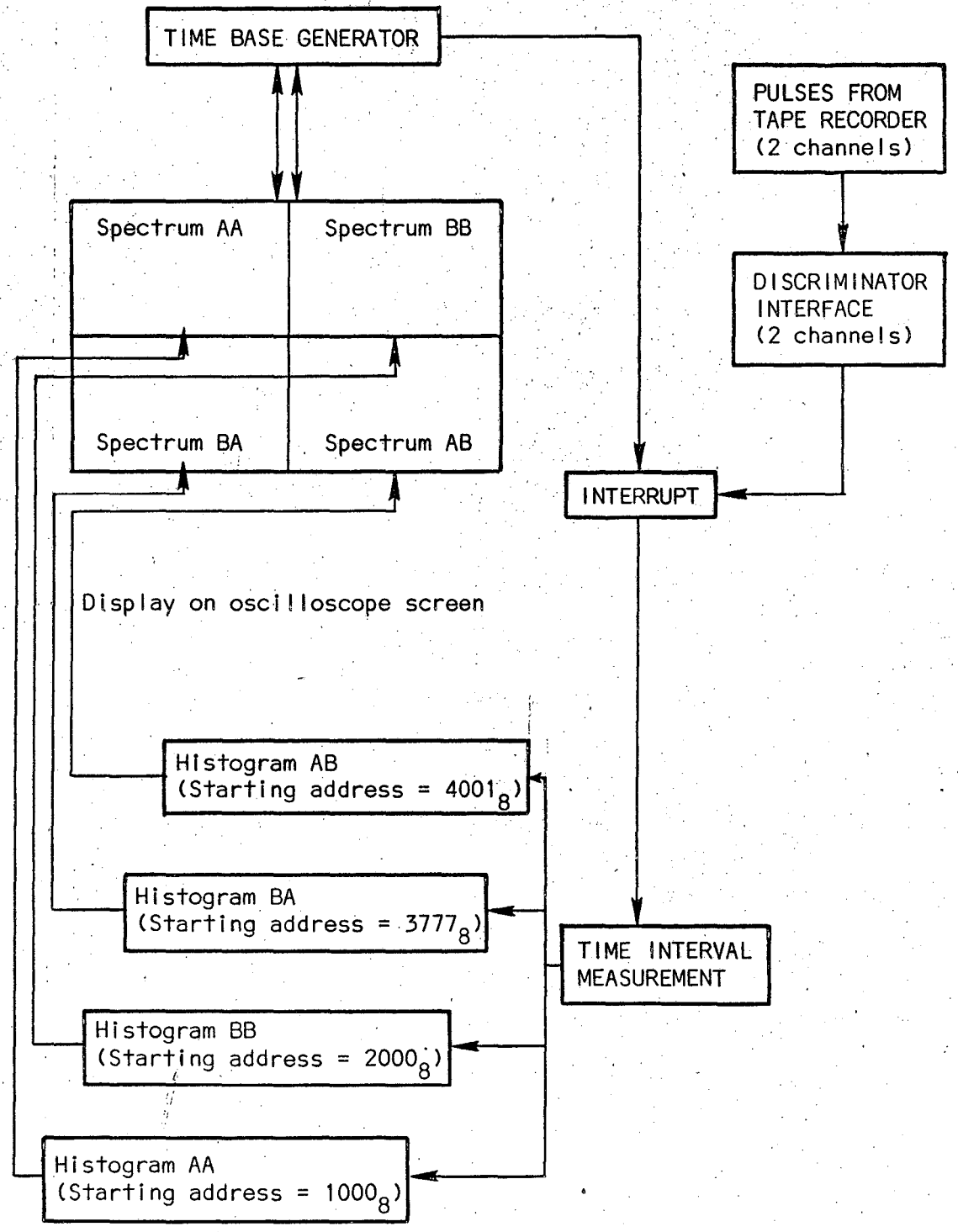
The cycle time (40 μsec.) for displaying one point of the display routine was used as a unit of time for the measurement of time intervals. The basic display consisted of two horizontal lines made up of 1024 points each (baselines for spectra AA, BB, and BA, AB, respectively).

The bin size could be selected by choosing an appropriate divisor to convert the number of display point cycles to the desired bin duration.

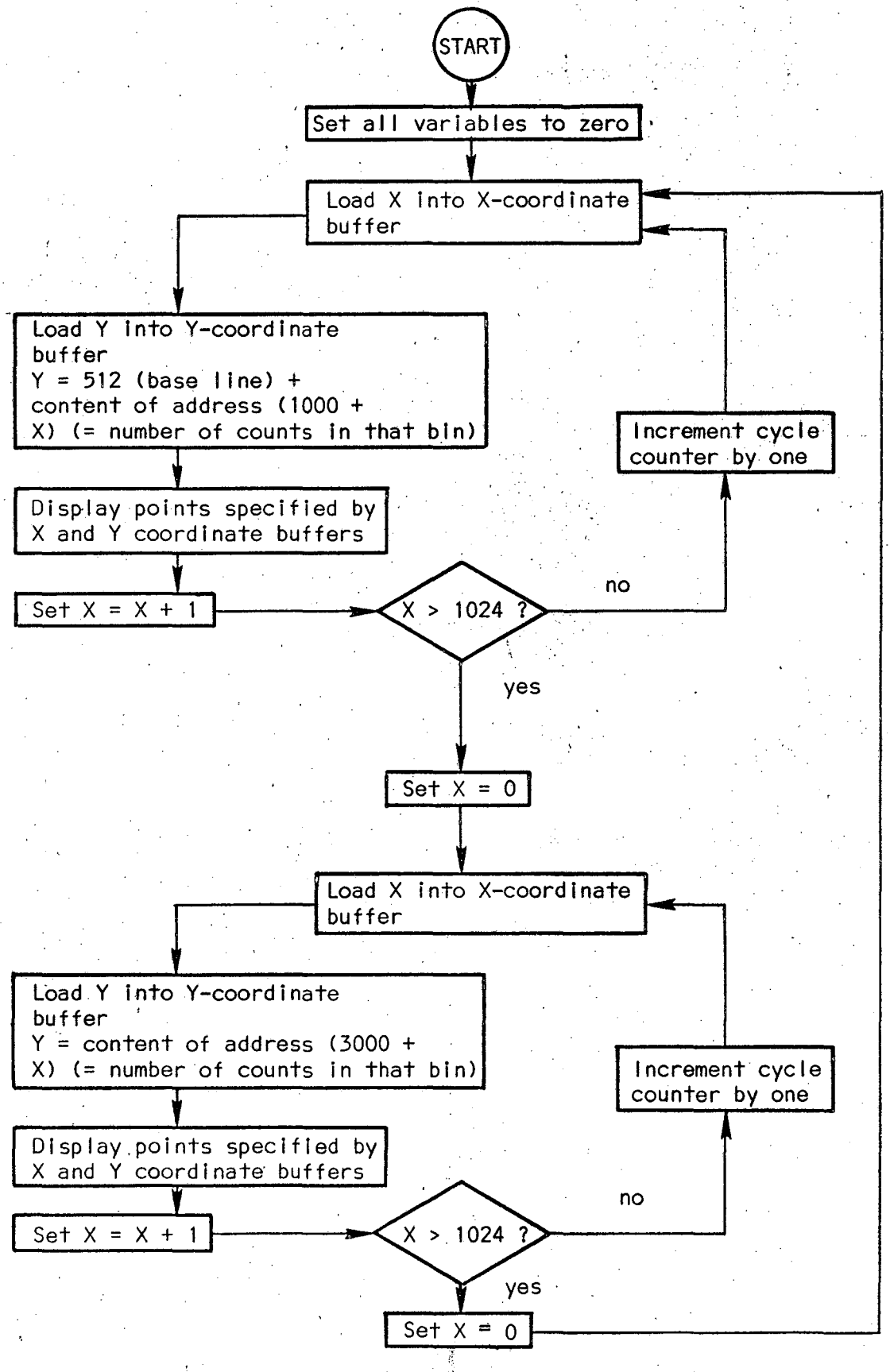
Each occurrence of a spike (= a pulse from the discriminator interface) caused the display routine to be interrupted and the computer program to be transferred from the display routine to the main program with a subsequent resumption of the display routine. The overall logic diagram and the flow diagrams for both the display routine and the main interrupt program are given below.

Figs. 31-34 are examples of interspike interval histograms (AA or BB), figs. 37-40 are cross-interval histograms (BA and AB).

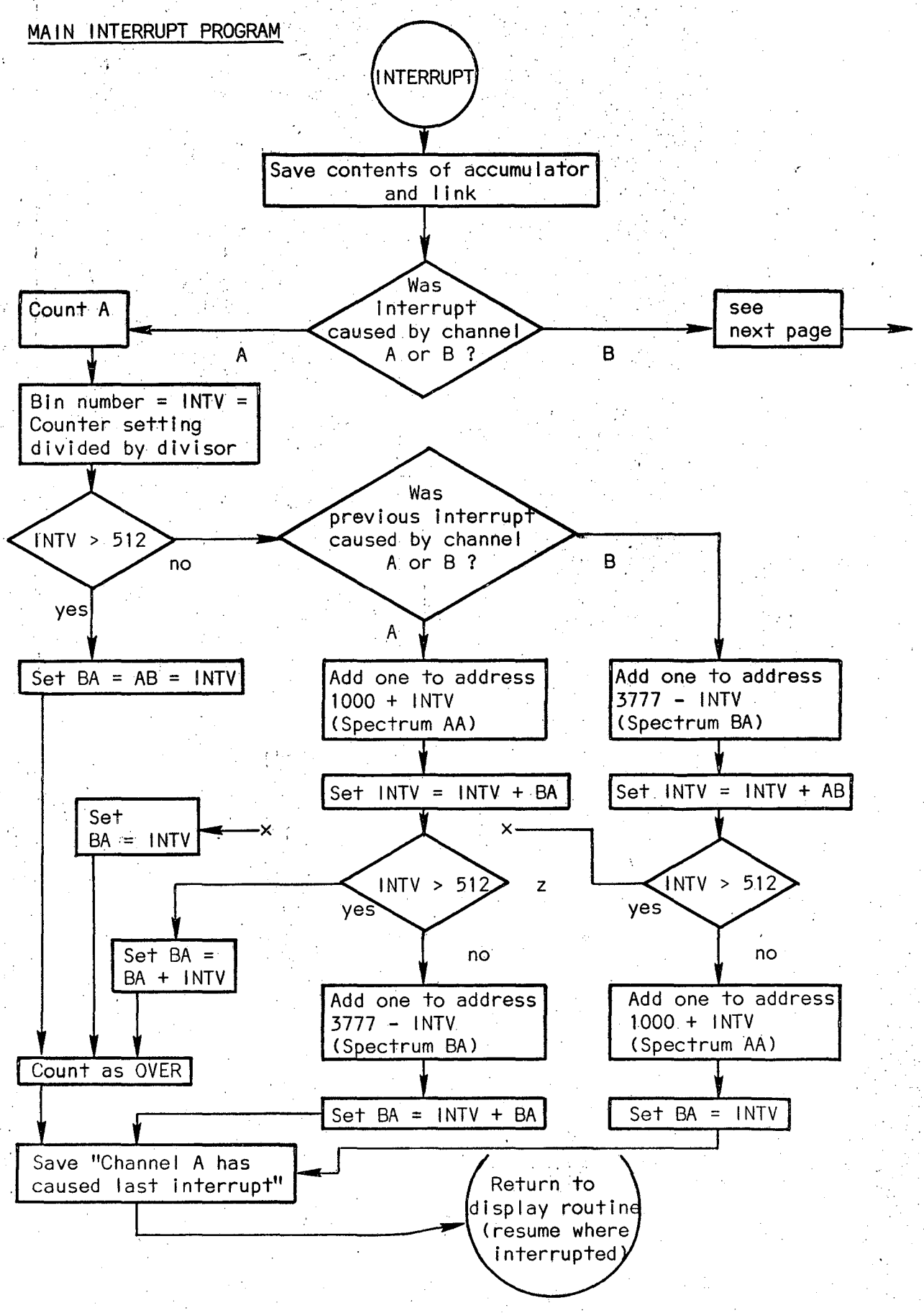
OVERALL LOGIC DIAGRAM



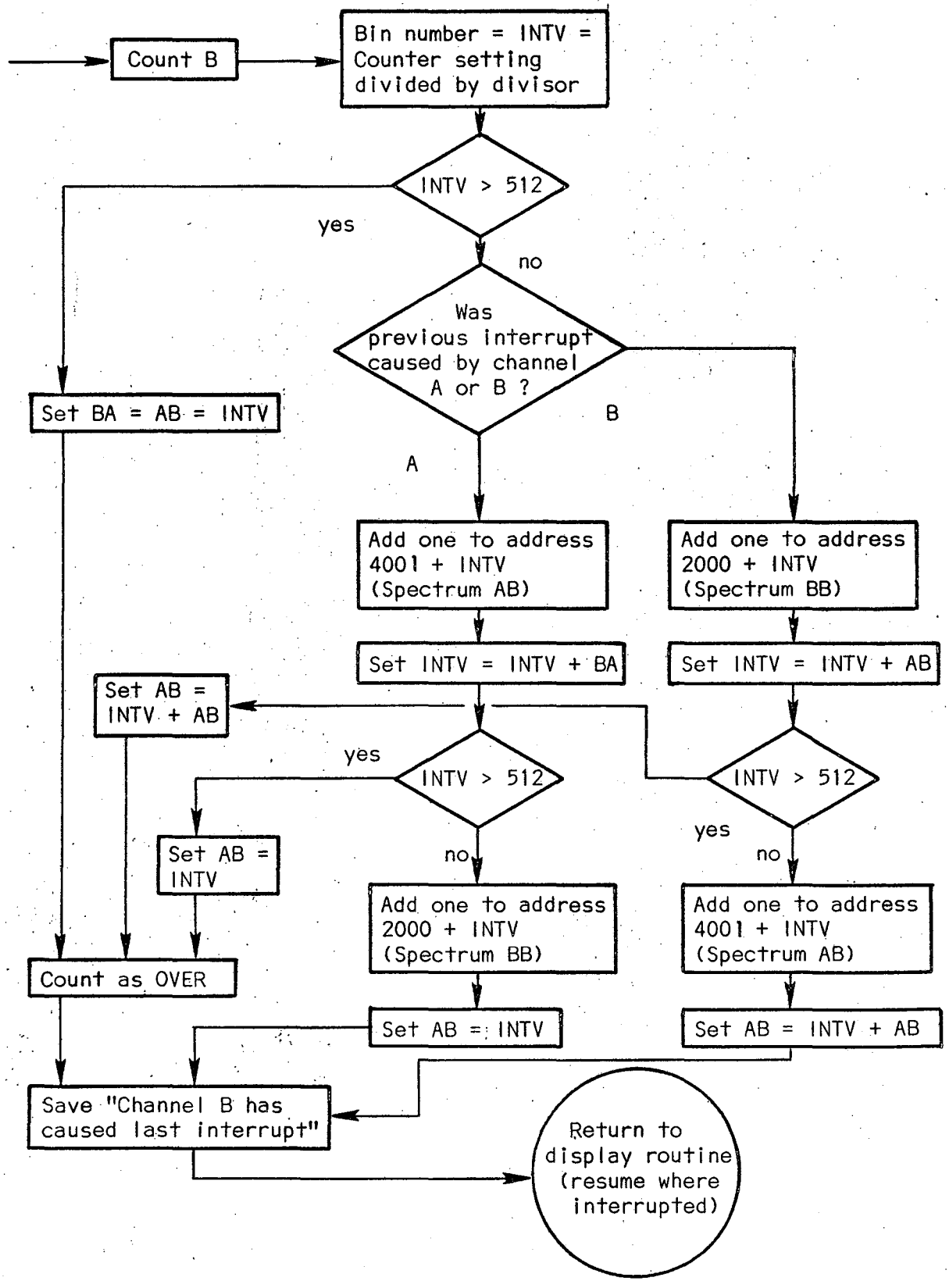
DISPLAY and TIMING ROUTINE



MAIN INTERRUPT PROGRAM



MAIN INTERRUPT PROGRAM (cont'd.)



Computer Program

```
                    DXL = 6053
                    DYS = 6067
                    MQL = 7421
                    DVI = 7407
                    MQA = 7501
                    ADOWN = 6102
                    BDOWN = 6534
                    DASF = 6101
                    DBSF = 6531

                    *0000
0000  0000                0000
0001  5402                JMP I 2
0002  0600                NTRUPT

                    *0010
0010  0000  INDEX,   0000

                    *0100
0100  0000  AC,0
0101  0000  LINK,0
0102  0000  INTV,0
0103  0004  TIM10,4
0104  0000  TIM20,0
0105  0000  LAST,0
0106  0000  INTVAA,0
0107  0000  INTVBB,0
0110  0000  INTVAB,0
0111  0000  INTVBA,0
0112  0000  BA,0
0113  0000  AB,0
0114  3777  SPKBA0,3777
0115  4001  SPKAB0,4001
0116  2000  SPKB0,2000
0117  1000  SPKA0,1000
0120  1000  RANGA,1000
0121  0000  STOR,0
0122  0000  OVER2,0
0123  0000  OVER1,0
0124  0000  ACNT2,0
0125  0000  ACNT1,0
0126  0000  BCNT2,0
0127  0000  BCNT1,0
0130  0001  X0,0001
0131  0001  INCX,0001
0132  1000  YA0,1000
0133  0777  INDEX0,777
```

```
0134  0000  TIM1,0
0135  0000  TIM2,0
0136  0400  MESAGI,MESAGE
0137  0460  UDPRTI,UDPRNT
0140  7000  SPKAI,SPKA
0141  7055  SPKBI,SPKB
0142  7036  SPKABI,SPKAB
0143  7016  SPKBAI,SPKBA
0144  6000  RESET,-2000
0145  0000  CNTR,0000
0146  0000  X,0000
0147  0000  OUTSWT,0
0150  7400  FOURH,-400
0151  0004  FOUR,0004
```

```
                *200
0200  7300            CLA CLL
0201  6042            TCF
0202  4365            JMS CLEAR
0203  3125            DCA ACNT1
0204  3124            DCA ACNT2
0205  3127            DCA BCNT1
0206  3126            DCA BCNT2
0207  3134            DCA TIM1
0210  3135            DCA TIM2
0211  3105            DCA LAST
0212  3106            DCA INTVAA
0213  3107            DCA INTVBB
0214  3110            DCA INTVAB
0215  3111            DCA INTVBA
0216  3113            DCA AB
0217  3112            DCA BA
0220  3123            DCA OVER1
0221  3122            DCA OVER2
0222  3147            DCA OUTSWT
0223  6102            ADOWN
0224  6534            BDOWN
0225  6001            ION

0226  1133  DISPA0,   TAD INDEX0
0227  3010            DCA INDEX
0230  1144            TAD RESET
0231  3145            DCA CNTR
0232  1130            TAD X0
0233  3146            DCA X
0234  1146  DISPA,    TAD X
0235  6053            DXL
0236  1131            TAD INCX
0237  3146            DCA X
0240  1410            TAD I INDEX
0241  1132            TAD YA0
0242  6767            DYS
0243  7200            CLA
0244  2145            ISZ CNTR
0245  7410            SKP
0246  5254            JMP DISPB0
0247  2134            ISZ TIM1
0250  7410            SKP
0251  2135            ISZ TIM2
0252  7000            NOP
0253  5234            JMP DISPA
0254  1144  DISPB0,   TAD RESET
0255  3145            DCA CNTR
0256  1134            TAD X0
0257  3146            DCA X
0260  1146  DISPB,    TAD X
0261  6053            DXL
0262  1131            TAD INCX
```

```
0263  3146          DCA X
0264  1410          TAD I INDEX
0265  6067          DYS
0266  7200          CLA
0267  7000          NOP
0270  7000          NOP
0271  7000          NOP
0272  2145          ISZ CNTR
0273  7410          SKP
0274  5302          JMP END
0275  2134          ISZ TIM1
0276  7410          SKP
0277  2135          ISZ TIM2
0300  7000          NOP
0301  5260          JMP DISPB
0302  7404  END,     OSR
0303  7700          SMA CLA
0304  5226          JMP DISPA0
0305  6002          IOF
0306  1120          TAD RANGA
0307  6053          DXL
0310  7200          CLA
0311  1150          TAD FOURH
0312  3145          DCA CNTR
0313  6067          DYS
0314  1151          TAD FOUR
0315  2145          ISZ CNTR
0316  5313          JMP .-3
0317  7200          CLA
0320  1147          TAD OUTSWT
0321  7710          SPA CLA
0322  5226          JMP DISPA0
0323  4536          JMS     I MESAGI
0324  4543          4543                    /CR  LF
0325  0317          0317                    /C O
0326  2516          2516                    /U N
0327  2440          2440                    /T .
0330  0140          0140                    /A SPACE
0331  7540          7540                    /= . (SPACE)
0332  ~~7777~~ ØØØØ 0000                    END
0333  4537          JMS I UDPRTI
0334  0124          ACNT2
0335  4536          JMS I MESAGI
0336  4543          4543                    /CR LF
0337  0317          0317                    /C O
0340  2516          2516                    /U N
0341  2440          2440                    /T SPACE
0342  0240          0240                    /S SPACE
0343  7540          7540                    /= SPACE
0344  0000          0000                    /END
0345  4537          JMS I UDPRTI
0346  0126          BCNT2
0347  4536          JMS I MESAGI
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0350  4543            4543                    /CR LF
0351  1726            1726    /O V
0352  0522            0522                    /E R
0353  4075            4075                    /SPACE =
0354  4000            4000                    /SPACE END
0355  4537            JMS I UDPRTI
0356  0122            OVER2
0357  4536            JMS I MESAGI
0360  4543            4543                    /CR LF
0361  0000            0000                    /END
0362  7040            CMA
0363  3147            DCA OUTSWT
0364  5226            JMP DISPA0

0365  0000  CLEAR,    0000
0366  1133            TAD INDEX0
0367  3010            DCA INDEX
0370  1144            TAD RESET
0371  1144            TAD RESET
0372  3145            DCA CNTR
0373  3410            DCA I INDEX
0374  2145            ISZ CNTR
0375  5373            JMP .-2
0376  5765            JMP I CLEAR
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*400

/DIGITAL 8-18-U
/MESSAGE
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                *600
0600   3100  NTRUPT, DCA AC
0601   7004         RAL
0602   3101         DCA LINK
0603   6101         DASF
0604   7410         SKP
0605   5212         JMP A
0606   6531         DBSF
0607   7410         SKP
0610   5252         JMP B
0611   7402         HLT

0612   6102  A,     ADOWN
0613   2125         ISZ ACNT1
0614   7410         SKP
0615   2124         ISZ ACNT2
0616   4322         JMS DIV
0617   7710         SPA CLA
0620   5743         JMP I OVERAI
0621   1105         TAD LAST
0622   7710         SPA CLA
0623   5236         JMP AA
0624   1102         TAD INTV
0625   3111         DCA INTVBA
0626   4543         JMS I SPKBAI
0627   1102         TAD INTV
0630   1113         TAD AB
0631   3106         DCA INTVAA
0632   4540         JMS I SPKAI
0633   1102         TAD INTV
0634   3112         DCA BA
0635   5247         JMP OUTA
0636   1102  AA,    TAD INTV
0637   3106         DCA INTVAA
0640   4540         JMS I SPKAI
0641   1102         TAD INTV
0642   1112         TAD BA
0643   3111         DCA INTVBA
0644   4543         JMS I SPKBAI
0645   1111         TAD INTVBA
0646   3112         DCA BA
0647   7040  OUTA,  CMA
0650   3105         DCA LAST
0651   5311         JMP OUT
```

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0652  6534  B,      BDOWN
0653  7200          CLA
0654  2127          ISZ BCNT1
0655  7410          SKP
0656  2126          ISZ BCNT2
0657  4322          JMS DIV
0660  7710          SPA CLA
0661  5742          JMP I OVERBI
0662  1105          TAD LAST
0663  7710          SPA CLA
0664  5277          JMP ABS
0665  1102          TAD INTV
0666  3107          DCA INTVBB
0667  4541          JMS I SPKBI
0670  1102          TAD INTV
0671  1113          TAD AB
0672  3110          DCA INTVAB
0673  4542          JMS I SPKABI
0674  1110          TAD INTVAB
0675  3113          DCA AB
0676  5310          JMP OUTB
0677  1102  ABS,     TAD INTV
0700  3110          DCA INTVAB
0701  4542          JMS I SPKABI
0702  1102          TAD INTV
0703  1112          TAD BA
0704  3107          DCA INTVBB
0705  4541          JMS I SPKBI
0706  1102          TAD INTV
0707  3113          DCA AB
0710  3105  OUTB,    DCA LAST

0711  1103  OUT,     TAD TIM10
0712  3134          DCA TIM1
0713  1104          TAD TIM20
0714  3135          DCA TIM2
0715  1101          TAD LINK
0716  7010          RAR
0717  1100          TAD AC
0720  6001          ION
0721  5400          JMP I 0

0722  0000  DIV,     0
0723  1134          TAD TIM1
0724  7421          MQL
0725  1135          TAD TIM2
0726  7407          DVI
0727  0250  DIVISR,  250
0730  7430          SZL
0731  7402          HLT
0732  7701          CLA MQA
0733  3102          DCA INTV
0734  1102          TAD INTV
0735  7000          NOP
0736  7000          NOP
0737  7041          CIA
0740  1121          TAD RANGA
0741  5722          JMP I DIV
0742  7174          OVERBI,OVERB
0743  7174          OVERAI,OVERA
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                *7000
7000  0000  SPKA,  0
7001  1106        TAD INTVAA
7002  7041        CIA
7003  1120        TAD RANGA
7004  7710        SPA CLA
7005  5330        JMP OVRAA
7006  1106        TAD INTVAA
7007  1117        TAD SPKA0
7010  3121        DCA STOR
7011  2521        ISZ I STOR
7012  2521        ISZ I STOR
7013  7000        NOP
7014  7000        NOP
7015  5600        JMP I SPKA

7016  0000  SPKBA, 0
7017  1111        TAD INTVBA
7020  7041        CIA
7021  1120        TAD RANGA
7022  7710        SPA CLA
7023  5314        JMP OVRBA
7024  1111        TAD INTVBA
7025  7041        CIA
7026  1114        TAD SPKBA0
7027  3121        DCA STOR
7030  2521        ISZ I STOR
7031  2521        ISZ I STOR
7032  7000        NOP
7033  7000        NOP
7034  7200        CLA
7035  5616        JMP I SPKBA

7036  0000  SPKAB, 0
7037  1110        TAD INTVAB
7040  7041        CIA
7041  1120        TAD RANGA
7042  7710        SPA CLA
7043  5322        JMP OVRAB
7044  1110        TAD INTVAB
7045  1115        TAD SPKAB0
7046  3121        DCA STOR
7047  2521        ISZ I STOR
7050  2521        ISZ I STOR
7051  7000        NOP
7052  7000        NOP
7053  7200        CLA
7054  5636        JMP I SPKAB

7055  0000  SPKB,  0
7056  1107        TAD INTVBB
7057  7041        CIA
7060  1120        TAD RANGA
7061  7710        SPA CLA
7062  5336        JMP OVRBB
7063  1107        TAD INTVBB
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7064  1116          TAD SPKB0
7065  3121          DCA STOR
7066  2521          ISZ I STOR
7067  2521          ISZ I STOR
7070  7000          NOP
7071  7000          NOP
7072  7200          CLA
7073  5655          JMP I SPKB

7074  2123  OVERA,  ISZ OVER1
7075  7410          SKP
7076  2122          ISZ OVER2
7077  1102          TAD INTV
7100  3113          DCA AB
7101  1102          TAD INTV
7102  3112          DCA BA
7103  5744          JMP I OUTAI

7104  2123  OVERB,  ISZ OVER1
7105  7410          SKP
7106  2122          ISZ OVER2
7107  1102          TAD INTV
7110  3113          DCA AB
7111  1102          TAD INTV
7112  3112          DCA BA
7113  5745          JMP I OUTBI

7114  2123  OVRBA,  ISZ OVER1
7115  7410          SKP
7116  2122          ISZ OVER2
7117  1111          TAD INTVBA
7120  3112          DCA BA
7121  5744          JMP I OUTAI

7122  2123  OVRAB,  ISZ OVER1
7123  7410          SKP
7124  2122          ISZ OVER2
7125  1110          TAD INTVAB
7126  3113          DCA AB
7127  5745          JMP I OUTBI

7130  2123  OVRAA,  ISZ OVER1
7131  7410          SKP
7132  2122          ISZ OVER2
7133  1102          TAD INTV
7134  3112          DCA BA
7135  5744          JMP I OUTAI

7136  2123  OVRBB,  ISZ OVER1
7137  7410          SKP
7140  2122          ISZ OVER2
7141  1102          TAD INTV
7142  3113          DCA AB
7143  5745          JMP I OUTBI

7144  0647          OUTAI, OUTA
7145  0710          OUTBI, OUTB
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