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EFFECTS OF IONIZING RADIATIONS ON MYELIN FORMATION
IN RAT BRAIN CULTURES

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(Ph.D. Thesis)

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EFFECTS OF IONIZING RADIATIONS ON MYELIN FORMATION
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December 31, 1969

ABSTRACT

Organotypic cultures from newborn rat brains of different postnatal ages were maintained in an apparently healthy condition for at least two months. Explants (about 1 mm^3 in volume and 1 mm thick) from cerebella and midbrains were used. The explants were embedded in a plasma clot and incubated in roller tubes. An incubation temperature of 36°C and glucose concentration of 6.5 mg/ml. of the feeding medium resulted in the highest percentage of all incubated explants becoming myelinated. Blood vessels and any preexisting myelin degenerated upon explantation. At 15 days postincubation over 90% of the incubated explants had developed abundant and apparently normal myelination. Examination of the cultures at 15 days in vitro by phase contrast and polarization microscopy, histological techniques, scanning electron microscopy, whole mount autoradiography using uridine-2- ^{14}C and by electrophysiological methods showed that many nerve cells were differentiated, many axons myelinated and in general much of the structural and functional characteristics of in vivo nerve tissue were retained in vitro. These observations denote the considerable recovery that had taken place from dissection damage (involving anoxia, ischemia and physical disruption) that is reflected in low Q_{O_2} of the fresh explants ($1.25 \mu\text{l/mg wet weight/hr.}$) and only about 10% survival of the large nerve cells originally present. The cultures were used as a model system for in vivo nerve tissue in investigating the damage

to myelin formation by ionizing radiations.

The effects of ionizing radiations on myelin formation in the cultures were studied using 145 Kvp x-rays, 36 Mev helium ions, 54 Mev helium ions and 30 Mev deuterium ions. Freshly dissected explants were exposed to radiations and the number of myelinated explants at 15 days in vitro compared to the total number irradiated was used as an index for myelination damage. The modification of such damage by radiation dose and age of animal used was investigated. The results showed, that although the radiations used differed in their LET and dose rates, they were not significantly different in their effectiveness. The mean radiation dose that resulted in only 50% of the explants (compared to the controls) becoming myelinated was 1727 ± 115 rads (mean $\pm$ LSD). A dose above 400 rads was needed in order to produce observable effect on myelination and the damage increased with the dose. At 4000 rads all exposed explants did not develop myelin. No recovery of myelination was observed in amyelinated cultures during an observation period of one month. Explants from 8 day old animals were found to be significantly more radio-resistant to myelination damage when compared to those from 1 day old animals.

Many nerve cells ($> 20 \mu$) in most cultures even after exposure to 4000 rads were apparently unaffected, irrespective of age of animal used, when examined at 15 days postirradiation. The axons were similarly unaffected. In contrast, small cells (5-15 μ) which include small nerve cells and glia were markedly reduced in density by 4000 rads.

It is concluded that damage to myelination in vitro is due to direct glial cell damage. The chance for affecting more of these cells increases with the dose. Since these cells occur in greater density in explants from 8 day old animals than in those from 1 day old animals (myelination gliosis), a higher dose will be needed to damage enough glial cells in the former ex-

plants in order to produce myelination damage. Although radiation damage to blood vessels in vivo may initiate or contribute to myelination damage, the present findings show that glial injury, in absence of blood vessels leads to myelination damage, at doses comparable to those used in vivo.

I. INTRODUCTION

A. Statement of the Problem

Ionizing radiation decreases and delays myelin formation in irradiated neonatal mammalian nerve tissue in vivo sometimes to a very profound degree. The effect depends on the radiation dose and age of the animal. Because of the radiosensitivity of blood capillaries, the possibility exists that the damage to the myelination process is associated with capillary damage impeding the supply of oxygen and nutrients to the irradiated tissue. The primary goal of this study is to investigate the effect of radiations on myelination and glia-neuron relationship in a capillary free system, i.e. in culture. However before this study could be attempted, cultures from newborn rat brains of different neonatal ages, with at least the crucial structural and functional integrity of nerve tissue in vivo, had to be established. Thus a study of the factors involved in the development, differentiation and maintenance of apparently healthy myelinated cultures is the secondary goal in this inquiry. Factors necessary for maintenance of neurons in culture will be described and the characteristics of nerve tissue when in culture are demonstrated. Should irradiation of freshly dissected explants from nerve tissue, prove damaging to myelin formation in the culture, then the cells responsible for the myelination process itself must be directly damaged. Modification of such damage by radiation dose and age of starting material will be explored. Information obtained along these lines could provide a new avenue for quantitative studies of the cellular process of myelination itself. In addition, knowledge about the factors involved in the maintenance of healthy myelinated cultures can help provide the investigator of myelin disturbances with a model system of nerve tissue, that is simpler, yet well organized, and amenable

to controlled variation of its environment. Disturbances in myelin maintenance, sometimes follow radiotherapy and occur in several demyelinating diseases, for instance multiple sclerosis. The cause of multiple sclerosis remains obscure (Adams, 1965). There is no reliable preventive or effective therapy for the basic disease process. In the fresh lesion the myelin sheath is damaged while the axons are more or less preserved. The oligoglia, which are responsible for myelination in the CNS (Bunge, 1968) appear to be directly involved in the disease. Investigations of the responses of myelination in tissue culture to different environmental insults, e.g. ionizing radiations, may prove to be rewarding in furthering our understanding about myelin disturbances.

B. Nerve Tissue Culture Techniques

1. Tissue Culture as a Research Method

The tremendous complexity of living tissues in terms of the multitude of structures they possess and their mutual interactions is well known. Thus it becomes difficult to understand the mechanisms involved in a certain tissue structural and functional reaction to an experimental treatment. Investigators have sought to simplify the situation by doing their experiments on model systems for living tissues with varying degrees of approximations of the real in vivo situation.

Tissue culture techniques (Penso & Balducci, 1963, Parker, 1961) attempt to study the structure and function of living tissues at a level intermediate between the cell culture model systems (Paul, 1965) and organ culture model systems (Willmer, 1965). The cell culture technique involves chemically or mechanically dissociating the tissue into its component cells and propagating selected cell types in vitro. Tissue organization is completely lost. Organ culture technique, on the other hand involves culturing whole organs or organ rudiments that are within 1.5 mm. in thickness. The size limitation is due to the short range of oxygen and nutrient diffusion through tissues. Tissue organization is optimal in organ cultures. The cell culture technique has limited success in case of nerve tissue for two reasons. Firstly it involves severe trauma to the tissue because of the disruption of the intimate associations between the different cell types. It is believed that maintenance of the association between neurons and its satellite cells is fundamental to their extended survival and normal function. Secondly, postnatal division of nerve cells is restricted and neuroglial cells have low turnover rates, hence their propagation in a cell culture pattern is difficult under normal conditions.

Our nerve tissue cultures are organotypic cultures. Instead of the whole organ, a small piece of the tissue, about a mm. in thickness (explant) is excised and cultured. The trauma of excision results in some disorganization of the tissue structure. Normal blood circulation is lost and replaced by nutrient fluids. This results in a complex series of phenomena including degradation and loss of cells as well as stimuli for cell proliferation and emigration. Cellular organization in tissue cultures becomes less than optimal. The recent advances in tissue cultures techniques have helped to minimize the trauma of dissection. But since the in vitro conditions do not provide the ideal environment and feed back controls present in vivo, this results in some modulation of the cultured cells (Harris, 1964) with varying degrees depending upon the cell type, age in vitro and culture conditions.

On the other hand these limitations are outweighed by the preservation of many fundamental structural and functional properties of the cultured tissue cells as well as their maintenance in a degree of organization allowing cell interactions. Among the advantages of tissue cultures are the following:

- (a) Accessibility of the culture for inspection during its entire life by light microscopy. Cell development, maturation and tissue organization can be closely observed at all the stages involved. Convenient preparations for either electron microscopy or scanning electron microscopy can be made in the form of sections or whole mounts respectively.
- (b) Ability to subject the cultures to different experimental treatments without involving other tissues, as well as a degree of freedom

from the modifying influences of humoral, hormonal and neural factors that arise in vivo from the rest of the intact organism.

- (c) Probes of different kinds (microelectrodes, laser beams, radiation microbeams) can be directed to certain cell types, or to sub-cellular organelles after their microscopic localization. This cannot be done in vivo except at skin and outer surfaces.
- (d) Ability to observe tissue interactions by culturing explants from different tissues close together, for e.g. nerve and muscle tissue.
- (e) Migratory movements of cells from the cultured tissue can be studied in detail. This is important for nervous tissue where glial movements were long assumed to exist, but have not been documented in detail.
- (f) The ability to vary at will the physical parameters of external environment and to observe the consequences of such changes. Physical environmental factors that are particularly amenable to study are temperature, radiation and electrical and magnetic fields. Chemical factors include composition of nutrients, ionic concentrations, excitatory and inhibitory substances, and a variety of pharmacological agents.
- (g) The technique as we have developed it is suitable for tracing de novo synthesis intracellularly by adding labelled precursors to the nutrient medium. Autoradiography is a suitable technique for detection of the localization of such substances, e.g. protein or nucleic acid precursors.

It seems then that tissue cultures can be used advantageously to investigate many fundamental biological problems, and provided that the cultured

tissue is found to preserve most of its important structural and functional differentiation, the results can help considerably in shaping our understanding about tissues in vivo.

2. Early Achievements

The early reports concerning nerve tissue culturing are included in a survey by (Thomas, 1956) and in a more complete bibliography by (Murray and Kopech, 1953). Nerve tissue culture history essentially starts with the culturing by Harrison of a fragment of frog's neural tube in a drop of clotted lymph on a coverslip that was inverted and sealed over a depression slide (Harrison, 1907). Not only did Harrison make the first nerve tissue culture, but he also used it to provide strong evidence favoring the neuron doctrine which was debatable at that time. This he achieved by showing that nerve fibers did come out from the neuron perikaryon and were not a product of the extracellular matrix. This study in morphogenesis was followed by more or less similar studies by others using nerve tissues from various sources and in general directed towards studying the regeneration of nerve fibres in culture and the survivability of nerve cells. For example (Burrows, 1911) cultured chick embryo neural tissue, (Ingebrigtsen, 1913) studied degeneration and regeneration of axis cylinders in vitro, (Lewis et al., 1912) cultured sympathetic nerves from the intestine of chick embryo, (Levi, et al., 1934) observed the morphological changes in cultured sympathetic ganglia, (Meyer and Jablonski, 1937-38) were able to improve the survivability of cultured nerve cells, although the nerve cells appeared abnormal.

Factors determining the course of nerve fiber outgrowth were studied by (Weiss, 1934). Neurofibrils were observed by (Weiss et al., 1936) in cultured ganglion cells of the chick. Mechanisms of nerve growth were studied by (Weiss and Hiscoe, 1948) in cultured nerve tissue. Nissl bodies true existence in living nerve cells was confirmed more recently by (Deitch

et al. 1956 and 1957).

From this brief review of the early achievements of nerve tissue culture method, it can be seen that early investigators made substantial contributions to neurobiological knowledge and indeed laid the foundations for the eventual improvements in nerve tissue culture techniques and its wider applications.

3. Recent Advances

The earlier tissue culture techniques were improved upon in three general areas by many investigators. Better dissection techniques were established, a culture environment closer to that in vivo was provided and culture vessels that allow greater accessibility of the cultures for observation were designed. Details of these improvements can be found in books by (Cameron, 1950; Parker, 1961; Penso and Balducci, 1963; Harris, 1964; Merchant, Kahn and Murphy, 1964; Paul, 1965 and Willmer, 1965). A book devoted exclusively to nerve tissue cultures appeared recently (May, 1966). Reviews by (Thomas, 1956; Murray, 1965 and Lumsden, 1968) treat nerve tissue culture development and its applications.

These improvements resulted in the adoption of several techniques which produced apparently healthy, differentiated and long surviving cultures. Certain basic requirements, the importance of which was not sufficiently realized by earlier workers, are satisfied by all the techniques:

- (a) Minimal trauma to the tissue during its dissection for explantation.

Undoubtedly large numbers of cells die because of physical injury received by them however gentle the dissection is. However use of sharp instruments that allow clean and quick cutting of the tissue minimizes its tearing and crushing that can be detrimental to tissue organization and viability (Parker, 1961).

- (b) Explants should be about 1 mm^3 . Dissection involves severing the blood capillaries resulting in cessation of the supply of nutrients and oxygen to the tissue. This immediately results in tissue ischemia and anoxia. Since in in vitro, oxygen and substrates are available only by diffusion which has a limited range, hence the size limitation. Although many cells are damaged beyond recovery during the dissection, yet with an explanted tissue volume of 1 mm^3 , enough relatively undamaged cells that can recover remain, (Lumsden, 1968).
- (c) Tissue cultures have to have a support to grow upon. They were not successfully grown in suspension. A simple uncoated glass coverslip by itself cannot keep the explant attached to it. A lymph clot substrate was used by early workers (Harrison, 1907). A plasma clot substrate (chicken plasma clotted with chicken embryo extract) was used by (Burrows, 1910). Avian plasma produces clear clots that are more stable than clots made from mammalian plasma. However avian plasma clots may require patching to replace the liquified clot digested by proteolytic enzymes present, (Peterson and Murray, 1955). Reconstituted rat tail collagen (Bornstein, 1958) is used extensively as a substrate for nerve tissue cultures. It is more stable than the plasma clot. An uncoated glass coverslip supporting the explants supplemented with a strip of dialysis membrane laid on top of the explants was used successfully to culture nerve tissue (Pomerat, 1959). A cellulose sponge matrix was used as the substrate for viable nerve tissue culture, (Cunningham, 1962).

- (d) Maintenance and differentiation of tissue cultures depend on the nutritive value and physiological parameters of the medium used. Firstly, the dissected explants should be maintained before actual explantation, not in just physiological saline, but in a balanced salt solution, for example Gey's balanced salt solution (BSS). Secondly, the feeding medium used during incubation should have the substrates needed for the various synthetic activities and energy requirements of the culture. Completely synthetic media are insufficient for nerve tissue cultures (Murray, 1965). Media of varying composition made up from different percentages of BSS supplemented with biological fluids (sera from different mammalian sources, sera ultrafilterates and embryo extracts) were developed, (Pomerat and Costero, 1956; Peterson and Murray, 1960; Orr, 1965). A glucose enrichment was found needed for differentiation of cultured nerve tissue, (Pomerat and Costero, 1956; Murray, Peterson and Bunge, 1962; Orr, 1965). Frequent changes of the medium during the life of the culture are needed to replace used substrates and to get rid of elaborated waste products. The medium pH should be maintained at a pH of 6.8-7.1 (Lumsden, 1968).
- (e) Mechanical disturbances affecting the culture chamber should be minimized. This allows the establishment of a relatively stable microenvironment around the culture conditioned by its metabolic activities. In this regard unnecessary changing of the medium should be avoided.
- (f) The culture should not be too deeply submerged in the feeding medium. This allows the culture to be within the range of oxygen diffusion from the ambient atmosphere.

- (g) Temperature of incubation should be maintained within 36-37°C (Murray, 1959; Peterson and Murray, 1960; Murray, 1965).
- (h) Scrupulously clean instruments and glassware should be used (Lumsden, 1968; Peterson, Deitch and Murray, 1959).
- (i) Sterile conditions should be available during all phases of tissue preparation and incubation. Antibiotics may be used in the feeding medium (Murray, 1965).

The above requirements are satisfied by different culture techniques, all of them give rise to apparently healthy nerve tissue cultures:

(a) True organotypic nerve tissue cultures were possible after the adaptation of the Maximow technique (Maximow, 1925) to nerve tissue cultures by (Murray and Stout, 1942 and 1947). The Maximow assembly consists of a depression slide, a small circular coverslip and a larger one. The explants are deposited on plasma or collagen coated small coverslip. They are then fed with a drop of medium rich in biological fluids. The small coverslip is then made to adhere to the large cover slip with a drop of BSS, then the depression slide is inverted over the coverslip. Petrolatum jelly-paraffin mixture is used to seal the large coverslip to the depression slide. The cultures are incubated in the lying drop position. Because of the small volume of medium used, this technique requires frequent feeding and washing of the cultures. The cultures produced have a high degree of differentiation and can be kept for several months (Peterson and Murray, 1955, 1960).

(b) The roller tube technique (test tube supplied with a flying coverslip to carry the explant embedded in a clot) (Pomerat, 1951) was found to allow good differentiation and maintenance of nerve tissue in culture (Hild et al, 1962). Instead of a drop of feeding medium, as in Maximow technique,

two ml. of medium are used. This permits a relatively longer interval between successive feedings, thus minimizing the chances for contamination and saves the cultures from the trauma of frequent feedings. The tubes are inserted in a rotating drum inside an incubator, allowing the cultures to be exposed intermittently to the ambient atmosphere and to the feeding medium. In this type of culture cell migration has been observed to occur resulting in thin cultures about the end of the second week in vitro. By that time nerve cells, glia and other cellular elements can be easily observed by phase contrast microscopy. Although the degree of preservation of tissue organization is less with this technique than with Maximow slide yet a great deal of organization evidenced by myelin and synapse formation is present. The present study has adopted the roller tube method as the culture technique, details of which will be discussed under materials and methods.

(c) A device that has the potentiality for culturing nerve tissue and carrying out perfusion studies was introduced by (Rose, 1954). A white rubber or silicone rubber gasket with a central hole, is enclosed between two No. 1 thickness cover glasses. One of the cover glasses carries the explants covered by a strip of dialysis membrane to hold them in place. The assembly is enclosed between two metal plates that have central holes. Tightening screws between the two metal plates hold the assembly firmly together, thus creating a leak proof culture chamber. The cultures are fed 1 or 2 ml. of medium, by inserting sterile syringe needle through the gasket. Removal of used medium is done in the same way. The nerve tissue cultures produced are well differentiated (Rose, Pomerat, Shindler and Trunnel, 1958). Accessibility of the cultures for observation by phase contrast microscopy is excellent. This technique was employed by (Quastel et al., 1963; Pomerat et al., 1964) for radiation studies of cultured

nerve tissue. Collagen coating of the cover glass carrying the explants seems to improve the differentiation and maintenance of the cultures (Hendelman and Booher, 1966).

C. Structural Characteristics of Cultured Nerve Tissue

Dissection of nerve tissue in preparation for explantation subjects the tissue to a trauma from which many cells apparently recover with little or no observable damage. In addition to the physical injury to cells and cell processes, interruption of the blood supply immediately results in tissue ischemia and anoxia. Indications of anoxic damage can be seen in the majority of cells during the first three days in vitro, (Lumsden, 1968). Nuclear pyknosis and cytoplasmic vacuolation and granulation are present in many cells. Any preexisting myelin degenerates (Hild, 1959). Near the end of the second week in vitro, most of the cells that have survived show evidence of recovery and differentiation. At that time the culture has a well developed outgrowth zone containing the migratory cell types, i.e. neuroglia and mesenchymal cells mainly, as well as cell processes. The inner zone of the culture contains nerve cells, glia, mesenchymal cells as well as myelinated and non myelinated axons and glial processes. The literature provides detailed reviews of the cellular and subcellular structures of cultured nerve tissue from different neural sources (Murray, 1965; May and Courtney, 1966; Lumsden, 1968). Since the present study concerns itself with myelination, literature evidence for myelin formation in vitro, as well as the presence of related nerve tissue elements and their apparent integrity will be reviewed.

(1) Nerve Cells

Nerve cells can be identified in cultures on the basis of their morphological and/or functional characteristics. A variety of nerve cell types

may be present in the cultures depending upon the source of the tissue. The number of cells of each type present presumably reflects the original numbers present in vivo, provided that the different cell types have equal viability in culture. Mature nerve cells in vitro generally are sedentary in spite of the conditions favoring migration, (Lumsden, 1968). Nerve cells from sympathetic ganglia do migrate however (Murray and stout, 1947), but these cells have little functional capacity (Murray, 1965). Migration of nerve cells was reported by (Costero and Pomerat, 1951) in cultures from mammalian brain. The migration was sluggish compared to that of glia, and the nerve cells were poorly differentiated. Staining by silver stains helps in the identification of nerve cells. Some nerve cells show affinity for supravital staining by methylene blue (Costero and Pomerat, 1951) but the affinity cannot be relied upon as specific for nerve cells. Hogue (1947, 1950, 1953) cultured brain tissue from adult and fetal human material. No mitosis of nerve cells were observed. This is a general fact about adult nerve cells, the only exception being nerve cells from sympathetic ganglia (Murray, 1965). Very large, granular and extensively branched cells were identified as nerve cells. These cells have a vesicular nucleus and single large nucleolus. Costero and Pomerat, 1951 applied histological procedures to help identify possible nerve cells in the outgrowth zone. Pomerat and Costero, 1956 reported on cultures from cerebellar folia of newborn kittens. A glucose enrichment of their medium was favorable to the general health of the cultures. Large Purkinje cells and Golgi II nerve cells remained inside the culture and were apparently healthy. Granule cells were found along with glial cells in a zone intermediate between the explant proper and the outgrowth zone. Stellate cells were present in the peripheral zone of outgrowth. Hild (1954, 1957, 1966) observed in his cultures from

different areas of neonatal mammalian brain nerve cells of different types (Purkinje cells, large multipolar nerve cells and unipolar neurons) generally maintaining most of their respective in vivo morphology. Cerebella from newborn mice were cultured by (Wolf, 1964) and apparently healthy nerve cells of different types were observed.

Beside the preservation of nerve cell somas in vitro as reported in the above studies, other structural characteristic of nerve cells were observed to be present.

Axon regeneration and maintenance occurred in cultures from dorsal root ganglia of embryonic chick (Lumsden, 1951, 1968). Fasciculation of the nerve fibres into bundles was frequent in his cultures. Boyde et al., 1968 observed also fasciculation of nerve fibres in his scanning electron microscopic study of cultured chick dorsal root ganglia.

Nissl substance (ribonucleoprotein) in a well developed condition was found by (Pomerat and Costero, 1956) in Purkinje cells in their cultures. Deitch and Murray, 1956 and Deitch and Moses 1957 also observed apparently normal pattern of Nissl substance in healthy living nerve cells. Most nerve cells examined in a culture few days post dissection and incubation appear to have little Nissl substance or have an abnormal pattern of it (chromatolysis), (Murray, 1965). Chromatolysis is believed to be due to the severance of axons and processes during the dissection process. This abnormal state of Nissl substance is common in immature and developing nerve cells as well as in some pathological conditions.

Neurofibrils are refractile filaments present in the cytoplasm and axon of nerve cells. They were observed by (Weiss and Wang, 1936) and by (Levi and Meyer, 1937) in living chick spinal ganglia. The neurofibrils are believed to endow the nerve cell with its affinity for silver stains.

Occurrence of what appears to be synaptic terminals in living nerve tissue cultures was reported by Hild (1966) and Coidan (1964). Silver staining by Holmes and Bodian techniques reveals ring shaped structures that look like synaptic endings seen in vivo (Wolf, 1964; Kim, 1965, Hild 1966; Cechner, 1967). The possibility that these endings may be ultra-structurally damaged and not formed in vitro but simply carried over in the explanted material (Hild, 1966) was eliminated by further studies, (Lumsden, 1968).

Although the above light microscopic evidence, concerning nerve cell somas, axons and neurofibrils, Nissl substance and synaptic terminals are suggestive of their maintenance in vitro in an apparently healthy condition, yet ultrastructural confirmation was necessary. Conclusive evidence for the close similarities between these structures and their in vivo counterparts was provided by electron microscopy, (Bunge, Bunge, Peterson and Murray, 1963; Bunge, Bunge and Peterson, 1965, 1967 a,b) and (Lumsden, 1968).

(2) Neuroglia

Central nerve tissue has in addition to neurons other "supporting" cells collectively called glia. These include oligodendrocytes, fibrous and protoplasmic astrocytes, ependymal cells and macrophages. Microglia are different from the rest of glia and nerve cell in being mesenchymal in origin, the others being ectodermal. Oligodendrocytes are believed to be of three varieties, interfascicular, perivascular and perineuronal. The interfascicular variety is the one responsible for myelinating axons in central nervous tissue (Bunge, 1968). Astrocytes in vivo intervene between the blood capillaries and neurons, and some authors believe that the neuronal supply of water and substrate molecules has to go through

the astrocyte first (Lumsden, 1955, 1958) and that this forms the so-called blood brain barrier. This is not completely proven. However, in vitro, blood vessels degenerate and the barrier breaks down. Early in the culture life the three main types of glia can be seen in the outgrowth zone and their forms are then closest to that in vivo (Pomerat 1952, 1958; Lumsden, 1958; Murray, 1958; Wolfgram and Rose, 1957; Nakai and Okamoto, 1963). However as the cultures advance in age, modulation of oligodendrocytes forms takes place into a more protoplasmic type intermediate between typical oligodendrocytes and typical astrocytes (Lumsden, 1951; Murray, 1965). Multiplication rates of oligoglia and astrocytes, are low even in cultures from neonatal animals while ependymal cells apparently increase in number and frank mitosis of microglia can be seen (Lumsden, 1968). Pomerat, 1958 showed a movie record of oligodendrocytes and astrocytes in division, in vitro.

After about a day of incubation, oligoglia and astrocytes emigrate from the explant closely following the microglia. Oligodendroglia tend to predominate in the outgrowth. (Murray, 1965).

Ependymal cells from the lining of the ventricles are easily noticed in cultures because of their beating cilia (Hild, 1957a; Lumsden, 1968).

3. Myelin Formation

Myelin, a sleeve of lipoprotein membranes wrapped around and ensheathing the nerve cell axon, is formed anew (any preformed myelin degenerates) in cultures from peripheral as well as central nerve tissue. Myelin formation in peripheral nerve tissue cultures was reported by (Peterson and Murray, 1955; Murray, 1959; Bunge et al., 1967a) and the first report on myelination in central nervous tissue cultures was by (Hild, 1957b). Successful myelination of cultures from central nerve tissue was also reported by (Born-

stein and Murray 1958; Hild, 1959; Perier, 1959; Hild, 1960; Bornstein, 1963 and 1964; Mamoon et al., 1969).

Myelin appears in cultures from neonatal nerve tissues about two weeks post explantation (Murray, 1965). The myelin sheaths formed are initially very thin but thickening occurs with increasing age in culture. The myelin makes its first appearance as a short "cuff" around the axon (Hild, 1959). It increases later in length on both sides but it remains segmental in nature and not all the axons present get myelinated. Nodes of Ranvier (interruptions in the myelin sheath) that occur in vivo are present in vitro in both central and peripheral myelin, (Perier, 61; Murray, 1965). Myelin is easily seen in the cultures by both phase contrast microscopy and in polarized light microscopy (Hild, 1959; Murray, 1965; Bunge et al. 1967a). It appears as two more or less parallel glistening lines that may show some irregularities. These optical properties of in vitro myelin are exhibited in vivo as well as its specific staining with Sudan Black B.

Peripheral myelin in vitro is formed by spiral wrapping of Schwann cell cytoplasm around the nerve cell axon (Peterson and Murray, 1955; Peterson et al. 1960). Central myelin is formed in vivo by spiral wrapping of the oligodendroglial cytoplasm around the axon (Bunge, 1968). In vitro, myelination was suggested by (Hild, 1957c) to be directly produced by the axoplasm because it appeared that there is no regular immediate relationship between the myelinating axons and neuroglia. Bornstein and Murray, 1958 showed evidence however for the involvement of neuroglia in myelination. Hild in 1960 abandoning his earlier thesis adopted the same view since there was no instance in which naked axons were seen to myelinate. The current general consensus is that neuroglia in a certain minimal density should be present in the culture for myelination to occur (Perier, 1961;

Murray, 1965). A healthy axon seems to be needed for successful myelination since it was noticed to contain an increased number of mitochondria in areas of active myelination, suggesting an elevated metabolic rate (Hild, 1960). In addition no myelination was ever observed about other cellular processes, for example astroglial processes, (Lumsden, 1968). Along these lines it should be mentioned that a recent report by (Ernyei et al., 1966) about myelin formation by Schwann cells in absence of any axons was not confirmed (Lumsden, 1968). Thus it appears that differentiated nerve cells, axons and a certain density of neuroglia are needed for myelination to occur. In fact, McIlwain, 1966 reported that in the rat brain active myelination starting about ten days postnatally is preceded by maturation of the nerve cell (accumulation of Nissl substance), growth of axons and proliferation of glial cells. In addition glial cells also undergo changes in several of their enzymatic activity during myelination in vivo (Friede, 1966). Similarly (Yonezawa et al., 1962) observed enzymatic changes in cultures of developing cerebellum concordant with active myelination.

The nature of the glial cell involved in myelination in vitro is difficult to ascertain because of the modulation of the typical forms of these cells shortly after culturing (Murray, 1965; Lumsden, 1968). The neuroglial cells involved appear to be intermediate in form between oligodendrocytes and astrocytes.

Myelination in vitro appears to follow the same steps irrespective of the age of the starting material and type of neuron involved, (Lumsden, 1968). Cechner, 1967 observed myelination in his cultures from different brain areas from rats aged 4-8 days, about the same time in vitro.

Certain physical and nutritional conditions are apparently required for successful myelination in vitro. An incubation temp of 31°C is not

conductive to either maturation of neuron soma or to myelination (Murray 1959, 1965). The inhibitory effect of low temperature can be overcome by supplementing the medium with thyroxine (Hamburgh and Bunge, 1964). Enrichment of the feeding medium with glucose (3 mg/ml) had a favorable effect on myelination (Pomerat and Costero, 1956). Currently the glucose enrichment mostly used is 6 mg/ml (Murray, 1965). A high concentration of serum and other biological fluids in the feeding medium is needed for myelination to occur (Murray, 1962).

The structural similarity between in vitro myelin and in vivo myelin was revealed by electron microscopy (Perier and De Harven, 1961; Ross et al. 1962; Bunge et al., 1965).

D. Functional Characteristics of Cultured Nerve Tissue

1) Biochemical Parameters

In general cultured nerve tissue reflects biochemical properties of in vivo nerve tissue. Thus the general health of a culture and the extent of myelination are improved by glucose enrichment of the feeding medium (Pomerat and Costero, 1956; Orr, 1965; Murray et al., 1962). Glucose metabolism is of central importance to nerve tissue in vivo. Compared to tissue slices, cultures have a higher metabolic rate (Hoskin et al., 1968), making them of potential value for studying the associations between intermediary metabolism, development and function. Evidence suggesting the functioning of Embden-Meyerhof route in cultures was provided by (Hoskin et al., 1968; Cechner et al., 1969). Histochemical studies on cultures from central and peripheral nerve tissue show increase in activity of oxidative enzymes in glia concomitant with myelination (Yonezawa et al., 1962 and 1966), similar to the changes observed in vivo (Friede, 1966). Evidence for the presence of different metabolic activities and their localization was provided by different studies. For example pyrimidine

metabolism was studied by (Appel et al., 1968), oxygen uptake by (Lukyanova et al., 1968), incorporation of choline into peripheral nerve myelin (Hendelman et al., 1969), localization of acetylcholinesterase activity in retinal nerve cells (Hansson, 1966) and catecholamines in sympathetic nerve cells (Burdman, 1968).

In addition to the above evidence suggesting biochemical "differentiation" of cultured nerve tissue, metabolites of certain diseases affecting nerve tissue in vivo produce more or less equivalent damage in vitro (Silberberg, 1969).

(2) Electrophysiological Parameters

From the preceding sections it is clear that the morphological parameters involved in the display of electrophysiologic behavior of nerve tissue in vivo are maintained in vitro. Thus nerve tissue culture techniques allow nerve cells to mature and differentiate (Bunge et al. 1967a; Hild, 1966), myelination to occur (Peterson et al., 1955; Hild, 1957a) and synaptic structures are present (Bunge et al., 1963; Callas et al. 1964).

Resting and action potentials from cultured chick embryo spinal ganglion corresponded well to the in vivo situation (Crain, 1956). Hild and Tasaki, 1962 recorded intracellularly from neonatal mammalian cerebella and found membrane potentials of about 50 mv. Spontaneous action potentials as well as resting potentials up to 75 mv were observed by (Raj et al., 1968) on neonatal mammalian cerebellar cultures.

Complex bioelectric activity is absent from young cultures of fetal material which can be shown by electron microscopy to lack synaptic structures (Bunge et al., 1967b, Crain, 1967). Only simple spikes can be evoked in the first few days of culturing. Later, complex activity with after-

discharges start to appear concomitant with the presence of distinct synaptic structures.

Extracellular microelectrodes placed near nerve cells, under visual control, permit more detailed studies of the spontaneous activity than is possible with relatively large microelectrodes and thick cultures, a combination that results in an EEG-type activity. Spontaneous action potentials from thinly spread roller tube cultures were observed by (Hild et al, 1962; Cechner, 1967; Lumsden, 1968; Schlapfer et al, 1969).

E. Comments on Nerve Tissue Cultures

The preceding review of the literature shows that much of the structural and functional integrity of nerve tissue is indeed maintained in vitro. The explanted tissue is able to recover from the dissection trauma, adapt to the abnormal in vitro environment and retain a high degree of its original characteristics.

Structurally, myelin sheath and synaptic structures form in vitro. Myelinated axons have a tendency to fasciculate into bundles. Nerve cells develop differentiated patterns of Nissl substance and neurofibrils. The close similarity between all these structural elements and their in vivo counterparts is revealed even at the ultrastructural level. Functionally, cultured nerve tissue has biochemical and electrophysiological properties that correspond well to those in vivo. Apparently then, the tissue culture model system can be relied upon to investigate problems in biology that are too difficult to do in vivo because of the much greater complexity of in vivo tissue and its environment. On the other hand since tissue culture is a simplified model of in vivo tissue it should be expected that the tremendously complex panorama of integrated functions associated with the three dimensional structure of in vivo tissue and its environment

will be restricted in vitro. However this simplification still permits many fundamental neurobiological problems to be investigated advantageously in vitro.

The degeneration of any preexisting myelin early after explantation is probably due to the physical trauma of dissection in addition to the sudden cessation of the blood supply. After the cells have adjusted to the new environment the culture becomes suitable for studies of the essential conditions for de novo myelin formation. Radiation effects on nerve tissue in general and myelin formation in particular is the goal of the present study.

F. Radiation Effects on Nerve Tissue

Ionizing radiations whether electromagnetic (x and gamma radiations) or particulate (e.g. ^4He , ^{12}C and ^2H ions) can inflict biological damage that is out of proportion with the actual amount of energy absorbed. Energy is absorbed from the radiations during their interactions with atoms and molecules in their path resulting in excitations, ionizations and free radicals. Virtually all of the biological damage is produced by interactions between electrons produced secondarily after the primary ionization, and cell molecules. The degree of damage inflicted may be influenced by physical and biological factors. Physical factors include absorbed dose, dose rate and radiation quality. Biological factors include cell type, its physiological state and its environmental conditions including the degree of hydration and oxygenation, at the cellular level; type of tissue (in terms of its cellular composition and frequency of each cell-type) and volume irradiated, and species, individual and age differences at the organism level.

Certain aspects of radiobiological damage are unique while others can be duplicated by radiomimetic agents (Hicks, 1953).

At the cellular level the following types of cytological damage may be seen acutely or after a delay, temporarily or permanently and with a frequency that is governed by physical and biological factors: (a) Mitotic inhibition (b) Mitotic retardation (c) Giant cell formation (d) Chromosome abnormalities (e) Mitotic or delayed death (f) Non-mitotic or immediate death.

Tobias et al., 1960 summarised the problem of radiosensitivity by noting that radiation effects manifest themselves most clearly in two kinds of tissues: those that have a high rate of mitotic cell division, like epithelial cells, bone marrow and some tissues of the reproductive system and those that regenerate very slowly like nerve tissue. Nerve tissue cells (nerve cells, glial cells and endothelial cells of blood vessels) respond to radiations in two ways: they are either killed by the radiations or may suffer a sublethal damage from which they may or may not recover, and which will alter their functional performance. A lethal or sublethal damage to the endothelial cells will indirectly affect nerve and glial cells even when these cells are not directly damaged.

The literature on the physical properties of ionizing radiations, their biological effects and the response of nerve tissues is voluminous. The review attempted here will be necessarily limited to the relevant findings. Books by (Andrews 1961; Hollaender, 1954-56; Errera, 1960-61; Bacq and Alexander, 1961 and Casarett, 1968) discuss the physical and biological properties of ionizing radiations. Radiation effects on nerve tissue is the subject of books by (IAEA, 1962; Haley and Snider, 1962, 1964; Lebedinsky, 1963; Van Cleave, 1963; Kimeldorf and Hunt, 1965). Articles by (Haymaker, 1962, 1969; Zeman, 1961, 1968; Tobias, 1954, 1961, 1964) discuss radiopathology of nerve tissue and innovations in irradiation methods. Applications of tissue cultures in radiobiological investigations

are reviewed by (Trowell, 1966).

The following discussion of the literature on the effects of ionizing radiations on nerve tissue is divided into two parts:

- (1) Dose-dependent radiation effects
- (2) Age-dependent radiation effects

1. Dose Dependent Radiation Effects

Leboucq, 1934 exposed areas of the spinal cord of newborn rats to x-ray doses of 1000 French R units, repeated 1 to 4 times and observed damage to nerve cells and inhibition of myelination in the irradiated area. Clemente et al., 1960 irradiated heads of newborn rats with doses varying from 125 to 1000r and found abnormal changes in nerve cells, glia and blood vessels as early as 48 hours post-irradiation. The degree of damage depended on the dose. Petechial hemorrhages were observed in animals irradiated at 1-5 days of age after a dose of only 500r. In this regard, (Rose, 1958) found evidence for blood vessel damage with doses as low as 100r. He investigated cerebral capillary permeability of irradiated (100-1500 r) and control rabbits using radioactive sodium. His data show an increased penetration of Na^{24} into the brain and cerebro-spinal fluid of irradiated animals, even though histologic changes were minimal at the higher dose used.

Gilmore, 1963a irradiated a limited area of the spinal cord of 3-day old rats with 4430r of soft x-rays (50kv) and examined the irradiated areas through the two post-irradiation weeks. She observed a marked reduction in the number of neuroglia in the irradiated area as early as 2 days post-irradiation, but could not find signs of hemorrhages at that time. However, signs of hemorrhages were present at about 9 days post-irradiation. At about two weeks post-irradiation, the irradiated areas have

greatly reduced amounts of neuroglia or were absent. The same areas were either hypomyelinated or amyelinated while the unirradiated areas immediately above or below the irradiated ones had normal amounts of neuroglia and myelination. The majority of nerve cells and axons in the irradiated areas appeared normal. The reduction in the amount of neuroglia was confirmed by (Gilmore, 1964a). Brownson et al, 1963a,b, noticed the high radiosensitivity of oligodendroglia. Pyknotic forms of these cells were found scattered throughout the irradiated area, few hours after irradiation with a dose of 150r given to limited portion of rat head. Meanwhile nerve cells in the irradiated areas appeared normal or little affected, except granule cells, even after a dose of 10,000r. Furthermore Brownson found that the acute necrosis of oligodendroglia was directly correlated with the dose given.

Rodgers, 1965 irradiated limited portion of the spinal cord of 3-day old rats with different doses of soft x-rays. He found that a dose of 4000r produced at two weeks post-irradiation reduction or absence of myelination in the irradiated area accompanied with a reduction or absence of neuroglia. Hemorrhages were present in the irradiated area about nine days post-irradiation. A dose of at least 1000-2000r was found to be necessary to produce this effect. Again like Gilmore, he found this effect limited to the irradiated area only and nerve cells there appeared mostly normal. Animals irradiated with only 2000r showed recovery of the irradiated area, in both myelination and neuroglial content, at about 30 days post irradiation. This was confirmed by (Gilmore, 1966).

Gilmore, 1964b irradiated limited areas of the spinal cord of neonatal rats with 185 Mev protons and examined the early and late effects (till 23 days) of a single dose of 6000 rads. Her histopathologic findings generally resemble those found by her and Rodgers after high doses of

x-irradiation including presence of hemorrhages. Up till near the end of the second post-irradiation week nerve cells and axons appeared mostly normal. At about 23 days post-irradiation obvious damage to nerve cells and axons was present in the irradiated area as well as a generalized necrosis. Her interpretation was that these late degenerative changes were brought about by progression of vascular damage, initially observed at about 9 days post-irradiation, which leads to the late severe damage.

Gilmore, 1965 irradiated limited areas of spinal cords of neonatal rats with doses ranging from 0.75 k rad to 12 k rad of 185 Mev protons. She found that only doses above 1.5 k rad produced observable damage including amyelination or hypomyelination, absence or decrease of glia and presence of hemorrhages. The degree of damage observed was generally dose dependent. Gilmore et al 1967 studied the histopathologic changes in blood vessels in the limited area of spinal cord of 3 day old rats irradiated with 4000r x-rays. She suggested that irradiation at that age could impair vascular development resulting in decreased number of vessels and vasodilation later. This leaves open the question whether the noted early effect on neuroglia is due to direct damage or due to vessel damage, or both.

Schjeide et al. 1966 irradiated heads of young rats with 750r x-irradiation and observed effects on myelination and glial content generally similar to those of Gilmore and Rodgers mentioned earlier. Cerebellum proved to be more radiosensitive than other areas examined. Retardation of oligodendrocyte division or outright destruction was believed to be responsible for the effects observed. Schjeide et al., 1966, 1968 made also interesting biochemical studies of his irradiated rats. Lipid synthesis was found inhibited by irradiation and the normal dehydration of nerve

tissue that accompanies development during the postnatal period was also inhibited or retarded. Further biochemical analysis showed that there was not only a quantitative decrease in lipid synthesized by irradiated animals, but also the proportions of unsaturated fatty acids and long chain fatty acids were less in irradiated animals. It was suggested that derangement or deficiencies of specific dehydrogenases may be responsible. De Vellis et al, 1967 investigated the effects of post natal irradiation (750 r to head of 2 day old rats) on development of enzymes, for example, glycerophosphate dehydrogenase, that undergo large increase in their activity concomitant with active myelination. He found that the activity of this enzyme, as well as others, was inhibited. They postulated that this inhibition may be among the factors involved in radiation inhibition of myelination.

Quastel et al, 1963 irradiated already differentiated cultured rat dorsal root ganglia with different doses up to 40,000r. The degree of damage observed and the time of its appearance varied with the dose given. Pomerat et al., 1964 irradiated rat dorsal root ganglia in vivo with high doses and noticed the relatively higher radioresistance of in vitro irradiated ganglia. Masurovsky et al., 1967 a,b, irradiated differentiated cultures of chick dorsal root ganglia with 40,000r of x-rays and noticed damage, that progressed with time, in all cellular elements including nerve cells, and concluded that nerve cells are directly damaged. Masurovsky et al., 1967c irradiated differentiated mouse cerebellar cultures with 5 and 10 k rad of fission neutrons and noticed damage that increased with dose in all cellular elements present. Small cells (presumably granule cells and oligodendroglia) decreased in number in the irradiated cultures. Goldring, 1956 investigated cultures of chick dorsal root ganglia irradiated in vivo

and in vitro with high x-ray doses and noticed radioresistance of the cultures irradiated in vitro.

2. Age Dependent Radiation Effects

Prenatal nerve tissue is known to be very radiosensitive (Hicks et al., 1961, Rugh, 1962). Alterations in development occurred subsequent to irradiation as well as postnatally, including growth retardations and malformations. Postnatal biochemical changes in CNS lipids were observed following prenatal irradiation with low x-ray doses (Vernadakis et al., 1968).

Neonatal nerve tissue is more radiosensitive than adult tissue. Hicks et al. 1963 found that low x-ray doses, applied at the first postnatal day can cause abnormal ramification of Purkinje cells dendritic tree. Altman et al., 1968, 1969 observed destruction of external granule cells of the cerebellum and alterations in the morphological development of Purkinje cells after irradiating newborn rats with low doses of x-rays. Biochemical changes were induced in newborn born rats by 500r x-rays, and accompanied electrophysiological changes produced by the same dose (Timiras et al., 1964).

Yamazaki et al., 1960 investigated the effects of different x-ray doses to the heads of rats varying in age between 8 hours and 15 days. He found that while nearly 70% of the rats irradiated under 5 days of age had neurologic abnormalities, only 15% of those irradiated after the fifth postnatal day had such abnormalities. Also the adult weight of those animals irradiated during the first five days was lower than that of animals irradiated after five days. Clemente et al., 1960, using the same doses and animal ages as used by Yamazaki et al., 1960 found a very high correlation between the observance of neurologic findings and brain pathology. Alterations in neurons, glia and blood vessels were seen. The brains of animals irradiated during the first 3-5 days of life proved to be more

radiosensitive than those of animals irradiated during the latter part of the postnatal week. The lesions were most frequent in subcortical white matter and cerebellum.

Effects of ionizing radiations on nerve cells, myelination, quantity of glia and blood vessels in neonatal rats of different postnatal ages were studied by Gilmore, 1963b (using 4430r on a limited portion of spinal cord); Gilmore, 1964b, 1965 (using 185 Mev protons on a limited portion of spinal cord); Schjeide et al., 1966 (using 750r x-irradiation of the head of newborn rats) and they all reported rapid increase in radioresistance after the few early postnatal days.

During postnatal development the radiosensitivity of the retina decreases rapidly. In the mouse, the sharpest decline is observed between days 4 and 8 (Lucas, 1961); and at neonatal day 13 a single dose of 3800 rads reduces the number of visual cells by only 50% while the bipolar nerve cells and interstitial glia are almost unaffected. Rat brain acquires adult radioresistance by 16-17 days postnatally, (Haymaker, 1962).

Adult nerve cells, in general, do not seem to suffer changes after moderate doses of ionizing radiations, when examined by the light microscope, early post-irradiation (Bailey, 1962). Cerebellar granule cells show early pyknosis, that is apparently reversible, after 7500r (Alvord, 1957). Even at higher doses (30,000 rad Bragg peak dose) Estable-Puig et al., 1964 observed at 96 hours post-irradiation signs of myelin degeneration while nerve cells and their axons were relatively intact. Delayed radionecrosis, with high selectivity for white matter, may develop later after a period of months or years following a dose about 3000 r or less (Arnold et al., 1954). Histochemically demonstrable glycogen was observed in glia after irradiation with few thousand rads (Klatzo et al., 1961).

II MATERIALS AND METHODS

Newborn rats of different postnatal ages (1-8) are used to provide brain explants for the study of myelination and its radiosensitivity. The rat brain at birth is relatively immature. Cell nuclei are larger and more hydrated than adult nuclei (Folch-Pi, 1955). Nerve cells and glia have not yet assumed neither their adult shapes nor the adult neuron/glia ratio. Most of the microneurons (granule cells and stellate cells) are produced postnatally (Altman, 1967). During the first ten postnatal days active cell growth and cell division are taking place (McIlwain, 1966). Nissl substance makes its appearance which is a sign of nerve cell maturation. Axons and dendrites begin to mature and cell connections become more complex. About the tenth postnatal day myelin appears and myelination is very active during the next ten days. These extensive synthetic activities, and the need for maintenance of ion distributions across greater and greater areas of cell membranes, for bioelectric activities, require greater expenditure of metabolic energy. Thus energy production by respiration contributes, with passage of time after birth, greater and greater share in energy metabolism beside glycolysis. In short the first 2-3 postnatal weeks are a period of great biochemical, functional and morphological differentiation.

A. Tissue Culture Methods

1. Biological Material

The roller tube culture technique originally developed by (Costero and Pomerat, 1951) and described by (Hild and Tasaki, 1962) was used in the present study. New born rats aged 1-8 days (Sprague Dawley, Simonsen albino) that appeared normal were selected. The animal was decapitated and the cerebellum or midbrain quickly and gently dissected out under sterile conditions. The tissues were transferred to few drops of Gey

balanced salt solution (BSS) in a sterile plastic petri dish (Falcon Plastics). The cerebellum was used more frequently than the midbrain in this study. The tissues were then washed free of adhering blood and body fluids by immersion in several drops of (BSS). The meninges and any visible blood vessels were removed as much as possible. The tissues were then cut into small uniform pieces (explants), each about 2 mm in diameter and about 1 mm thick. Duplicate washing of the cut explants in fresh changes of BSS was done next to remove debris and released fluids. Explants destined to become controls and those that will be subjected to the different treatments, were always made from symmetrical areas of the tissue and were made as close as possible in uniformity and thickness. Explants that will receive radiation are so treated at this stage.

One or two explants were then placed on a No. 1 12 x 50 mm glass coverslip (Gold Seal) previously coated with two drops of heparinized chicken plasma (Hyland Laboratories 65-080 or Baltimore Biological Laboratories 70-025G). One or two drops of chicken embryo extract (Baltimore Biological Laboratories 70-027G) were then added to the coverslip and mixed quickly with the plasma. Clotting of the plasma took place in few minutes. After the clots had hardened the coverslips carrying the explants were placed in sterile plastic tissue culture test tubes (Falcon Plastics 3026) and fed with 2 ml of medium.

2. Medium

A glucose enriched BSS (net glucose concentration = 5 mg/ml.) was used as the bathing solution during dissection, irradiation and when viewing the cultures by phase contrast microscopy. The standard feeding medium was composed of 25% heat inactivated (30 min. at 56°C) calf serum (Hyland Laboratories 65-200) and 75% BSS. The medium was fortified with glucose

to a net concentration of approximately 6.5 mg/ml. This feeding medium gave consistently good results. For the study of effect of glucose enrichment on myelination, similar medium but with different net concentrations of glucose was employed. Net glucose concentrations used were approximately 2 mg/ml., 3 mg/ml., 4 mg/ml, 6.5 mg/ml. (the standard medium) and 11.5 mg/ml. The medium was changed weekly. No antibiotics were used.

3. Incubation

The culture tubes were inserted into the holes of a rotating drum assembly tilted 5 deg. with respect to the horizontal axis and rotating at about 10-12 revolutions per hour. The rotation of the drum at this speed allows the cultures to be exposed to the ambient atmosphere for most of the time. The tilting of the drum allows the culture to be covered by a thin layer of medium during the part of the time when it is submerged by the medium. The rotating drum was kept inside a 36°C incubator (New Brunswick Sci. Co.) having an atmosphere of 5% CO₂ in humidified air. Because the plastic culture tubes were somewhat permeable to CO₂ and water vapour, such an atmosphere was needed to help maintain the bicarbonate buffered medium at a pH close to 6.9-7.1 and to prevent loss of water from the medium. The effect on myelination of incubation temperatures of 30°C and 32°C were investigated.

4. Examination of Cultures

The cultures were viewed by phase contrast optics utilizing a bridge mount at different times of incubation for inspection of growth and first signs of myelination. The cultures can be viewed under sterile conditions and returned to the incubator for further incubation. At 15 days in vitro or earlier, control and treated cultures were inspected for presence or absence of myelination by both phase contrast and polarization microscopy

and mean percent myelination recorded. Several thousand cultures were involved in the present study.

5. Factors Affecting Culture Success

When a culture contains many healthy nerve cells, abundant and normal myelination at 15 days in vitro, few macrophages, absence or few necrotic areas and few debris, it is regarded as a generally healthy or successful culture. Such a culture if fed regularly can usually be maintained differentiated and in apparent good health for several months.

A long experience with nerve tissue culturing proved it to be one of the most demanding biological techniques. Many factors, some of them became known, affect the ultimate failure or success of a culture. Among these factors are the following:

- a. Strict sterile conditions should be maintained during all phases of dissection, culturing, feeding and examination of living cultures that will be further reincubated.
- b. Quick, gentle and clean dissection of the tissues. Tearing and crushing of the tissues during dissection and cutting out of explants will invariably lead to damaged cultures.
- c. Scrupulously clean and exceedingly sharp instruments must be used, and wiped clean of blood and debris immediately after dissection. Heamosol solution is effective in removing traces of blood adhering to the instruments. After soaking in hemosol for few hours, the instruments are boiled once in 3x distilled water, once in a solution of 7x (Microbiological Laboratories), then three times in fresh changes of 3x distilled water, then wiped dry and stored away from dust.

- d. Glassware should be scrupulously cleaned free of traces of used detergent or heavy metals. In the early phases of culture preparation for this study glassware was used extensively. Traces of detergent used, heavy metals etc. were apparently not removed completely since most of the cultures showed signs of toxicity and frank degeneration. When sterile plastic (Falcon Plastics) culture petri dishes, test tubes, pipettes were used the cultures became healthy, differentiated and maintainable for several months in vitro. The only glassware item that was still used, was the glass coverslip. The coverslips were cleaned by boiling in fresh changes of 3x distilled water three times. They were then immersed in redistilled 95% alcohol over night. This was repeated once more. A final rinsing with redistilled 95% alcohol was followed by drying the coverslips individually over a hot plate. Heat sterilization of the coverslips was done next.
- e. Different media were tried. They varied in their content of biological fluids (sera, sera ultrafilterates, whole egg ultrafilterates, chicken embryo extracts etc.) No completely synthetic medium produced a successful culture. One medium composition (50% heat inactivated calf serum, 45% BSS, 5% chicken embryo extract, with a net glucose conc. of 6 mg/ml.) was used successfully in the early phases of culturing. However it was replaced by the medium adopted for this study (see page 31) because although it has less biological fluids in it (thus less chance for variability and possible toxicity) yet it gave equal or better results consistently.
- f. The explants should be cut with extreme care to avoid as much as possible tissue disorganization and damage. The thickness of

explants should not be larger than the range of diffusion of oxygen, nutrients into the explant. The same applies for waste leakage from the explant. When one dimension of the explant is maintained within one mm in thickness, the above processes can be satisfied. A thick explant will develop necrotic areas in the center resulting from lack of oxygen and nutrients and accumulation of waste products.

- g. Plasma and chicken embryo extract from different commercial sources as well as preparations we made ourselves were compared as to their efficiency in producing a suitable clot. Bad clotting or quick lysis of the clot after incubation will result in detachment of the culture from the substrate. This was obtained in a number of cases. In other cases although a firm clot was obtained, the cultures were not well differentiated. Lyophilized chicken plasma (Hyland Laboratories 65-080) and fortified chick embryo extract (Baltimore Biological Laboratories 70-025G) produced when mixed in about equal proportions good clots that always maintained successful cultures.

B. Histological Methods

The tentative identification of elements of cultured nerve tissue by phase-contrast microscopy, polarization microscopy and supravital staining techniques is ascertained by the use of classical staining methods (Culling, 1963). Control and irradiated cultures were stained by methods for Nissl substances, axons, synapses and myelin sheaths. Comparison was then made between the appearance of these nerve tissue elements in the control and experimental groups. Neurofibrils were stained by Bodian protargol method (Bodian, 1936), thus revealing axons,

neuron somas, dendrites and synaptic boutons. Myelin sheaths were stained by sudan Black B (Peterson and Murray, 1955). Comparison was often made between photographs taken of certain elements in the culture before fixation and staining and afterwards and in many cases the tentative identification was upheld. The staining was improved when the cultures were routinely washed free of adhering medium before fixation.

Supravital staining with methylen blue (1:50,000 in BSS) was used in some cases (Costero and Pomerat, 1951). Incubation of a culture in such a solution for 20 min. at 36°C results in staining of many nerve cells and their processes.

C. Scanning Electron Microscopy

The retention of the in vivo three dimensional shape and organization of nerve tissue elements in culture was investigated by the use of scanning electron microscopy (SEM). These investigations were carried out with the help of Dr. Helge Dalen to whom the author is indebted.

Five to six weeks old cultures from newborn rat cerebellum were used. By that time most of the plasma clot has apparently lysed, especially at areas near the edge of the culture, and the surface of many cells and processes were free from the overlying clot layer.

The cultures were viewed with phase contrast microscopy and areas of interest were marked and photographed at different magnifications. The coverslip bearing the culture is washed twice by immersion in 0.1M phosphate buffer at pH 7.2. The culture is first fixed for 5-10 min. in OsO₄ vapour followed by 24 hours fixation in 2% glutaraldehyde made up in 0.1M phosphate buffer at pH 7.2. This is followed by post-fixation for one hour in 1% OsO₄ made up in the same buffer. The culture is then dehydrated in increasing concentrations of alcohol followed by air drying.

A conducting surface was obtained by depositing a thin layer of Pd/Pt on the preparations as they were rotated in a vacuum evaporator. Thereafter the coverslip bearing the culture was mounted on the stage of the scanning electron microscope and surface structure observations were carried out by using the secondary electrons of the SEM. (Model JSM manufactured by Japan Electron Optics Laboratory Co. Ltd.) The instrument had a 45° inclined sample stage and was operated at 25 Kv with specimen current equal to 2×10^{-10} amp. Photographs of the images were made on Polaroid film.

D. Autoradiography

Whole-mount autoradiography by the dipping technique (Rogers, 1967) was done on a few of the cultures to investigate uptake of uridine-2 ^{14}C and capacity of glia and nerve cells for synthesis of RNA in vitro.

Healthy 16 day old cerebellar and midbrain cultures from 2 day old rats were used. Uridine-2 ^{14}C (Sp. Oct. 55 mCi/mmol) was added to the culture medium at a final concentration of 1.25 $\mu\text{Ci/ml}$ and the culture incubated for 30 min. at 36°C . The culture was then washed three times with BSS to remove any adhering, not incorporated, radioactive precursor. The culture was then fixed in 10% formol saline. Autoradiography was carried out according to the following steps:

1. A dark room equipped with Kodak series 2 filtered light was used during all light sensitive steps. Two to three spoonfuls of Kodak NTB2 Nuclear Track Emulsion were transferred into a dipping jar. The jar is then placed in a 40°C water bath for 30 min. to one hr.
2. A slide carrying the coverslip culture is dipped in the emulsion and then allowed to drain for five min. The slide is then stored in a light-proof box, that has a few drierite capsules, in a refrigerator. An exposure period of about two weeks produces good results.

3. Development was done in the dark room:

Dektol developer	2 min.
Rinse	1-2 min.
Fixer	5 min.
Rinse	30 min. to one hour

4. Air drying in a dust free atmosphere.

5. Staining by Einarson's technique for nucleic acids (Culling, 1963).

6. Rinse several times

7. Air dry

8. Xylene for 5 min. followed by mounting in permount.

E. Electrophysiological Methods

Limited investigation of the existence of spontaneous bioelectric activity in few control and irradiated cultures from newborn rat cerebellum was done. Cultures about 15 days in vitro with apparently healthy nerve cells were used.

Electrophysiological investigations of cultured nerve tissue were established here by Mr. Werner T. Schlapfer. The electrophysiological investigations were done in collaboration with Mr. Schlapfer to whom the author is indebted (Schlapfer et al., 1969).

A modified Bausch and Lomb microscope with Zeiss phase-contrast optics was used to view the cultures during the experiments. The coverslip bearing the culture was mounted on a bridge which allowed the positioning of microelectrodes under visual control through the open sides. Ports for perfusion with the bathing solution (Gey BSS enriched with glucose) were provided through the bridge mounts. A syringe pump facilitated the perfusion of the bathing solution. The experiments were done at room temperature. The recording (extracellular) of bioelectric activity was

done with glass capillary microelectrodes having tips about 3-6 μ and filled with 0.9% NaCl. The electrodes were connected through low-noise ac preamplifiers (Applied Cybernetics Model 4 UAH) to a storage oscilloscope and a tape recorder.

F. Irradiation Methods

Investigations of the effect of ionizing radiations on myelination in vitro were done utilizing x-rays and heavy charged particle radiations. Dose dependent radiation effects were investigated after irradiation with x-rays and heavy charged particles. Only 2-day old animals were used. Age dependent radiation effects were investigated after irradiation with x-rays only. Animals aged, 1,5 and 8-days were used. Controls and explants to be irradiated were made from the same animal using symmetrical areas of cerebellum or midbrain. While most of the experiments were done after in vitro irradiation, few were done after in vivo irradiation.

1. X-Ray Dosimetry:

Fricke ferrous sulfate dosimetry was used for estimating the dose rate. Pure, analytical grade, reagents were used and triple distilled water was used to dissolve the salts. An average value for G (Fe^{3+}) of 15.0 was used. A molar extinction coefficient for ferric ions equal to 2174 optical density units was employed. The value used for density of dosimeter solution was 1.024g/cm³. A Beckman Model DU spectrophotometer was used for measuring optical density of dosimeter solutions after irradiation with different doses. One-cm quartz cells were used and optical density was read at a wave length equal to 304 m μ .

Two ml of dosimeter solution were placed in a 6-cm plastic petri dish (Falcon Plastics), the same type of dish used for irradiating the explants. The volume used of the dosimeter was enough to cover the dish bottom with

a layer of solution about the same thickness as that of BSS used when irradiating explants. The dosimeter was irradiated through the dish covers. Irradiation of dosimeter was repeated several times at different dose levels and an average optical density for each dose level was calculated. The x-ray machine used (Norelco MG 150) was operated at 145 Kvp, 12 mamp with 1mm Al added filtration. The distance from tube target to dosimeter solution was approximately 13.5 cm.

The calculated mean absorbed dose by the dosimeter per min was found to be $800 \pm 2\%$ rads/min. The HVL was approximately 2.1 mm Al.

2. In vitro X-Irradiation:

Freshly cut and BSS washed explants were transferred to few drops of BSS in a 6-cm sterile plastic petri dish (Falcon Plastics). Most of the explants were from cerebellum of newborn rats aged 1,2,5 and 8-days. Few explants from newborn 2-day old midbrain were used. The explants were then irradiated through the dish cover with different doses. Different groups of explants were exposed to a net dose equal to 200,400,800,1200,1600, 2000,2400,2800,3200,3600 and 4000 rads. Controls were always sham irradiated. After irradiation, the treated and control explants were washed twice in fresh changes of BSS and then explanted separately. In few cases one control and one irradiated cerebellum explant were cultured together. All irradiations were done at room temperature.

3. In vivo X-Irradiation:

2-days old animals were used. The head was irradiated with 4000 rads surface dose. The rest of the body was shielded with 2mm thick lead shield. The animals were made immobile by taping them to the support with scotch tape around the trunk. Although the animal could move his head a little yet it was well within the radiation field. The irradiation conditions

were the same as in the in vitro situation with the exception that the distance from x-ray target to animal head was approximately 13 cm. After irradiation the animals were quickly dissected and explants from the cerebellum were made and explanted as usual.

4. In vitro Heavy Charged Particle Irradiations:

Different particles that have different ranges in tissue were used. Helium and carbon ions (approximately 9 Mev/nucleon) were obtained from the heavy ion linear accelerator (Hilac) at LRL. Helium ions (54 Mev) and deuterium ions (30 Mev) were obtained from the LRL 88-inch cyclotron. The dose rate used with the former ions was 1500 rad/min and for the latter ions 4000 rad/min. Description of the 88-inch accelerator, dosimetry methods, beam characteristics are discussed in detail by (Fulton et al., 1963). Description of the heavy ion linear accelerator, dosimetry methods, beam characteristics are discussed in detail by (Todd et al., 1968).

Since the accelerator beams are horizontal, the explants had to be irradiated in vertical position. To keep the explants from slipping during irradiation, the freshly cut and BSS washed explants from rat cerebella (aged 2 days) were deposited on the surface of already clotted and hardened plasma clot in the center of sterile plastic petri dish (Falcon Plastics). The explants did not become covered with the clot and no two explants were allowed to overlap each other. For Hilac irradiations, the mammalian cell irradiator (Todd et al. 1968) was used to hold the petri dishes. For cyclotron irradiations a very similar device capable of holding twelve 60-mm dishes instead of ten 35-mm. dishes was used. A humidified atmosphere was provided inside the dishes in both cases. Doses varying from 200 to 4000 rads were used with the different ion irradiations. Controls were sham irradiated. After irradiation the explants were peeled off the

clot very gently and washed twice in BSS and then explanted as usual. All irradiations were done at room temperature.

G. Microrespirometry

The metabolic rate of freshly cut explants, in terms of its O_2 uptake, was estimated by microrespirometry. An all-glass differential microrespirometer (Microchemical Specialities Co. #2570) was used. The instrument consists of a capillary attached to two vessels; a respiration vessel in which the tissue plus medium etc. is kept and a compensation vessel. An index droplet (purified kerosene) is introduced into the capillary and serves to indicate the change in the volume of gas inside the respirometer during a certain interval of time. The apparatus and the technique were described in detail by (Grunbaum et al., 1955).

Freshly cut explants (total wet weight about 8 mg.) from cerebellum of 2-day old rats were used. Gey BSS was used as the medium, and oxygen uptake was measured at $37^\circ C$ at hourly intervals. A 0.5 mm capillary was used.

The change in gas volume is given by Kxd where d is the distance covered by the index droplet and K is derived from

$$K = \frac{273}{T} \left(\frac{P - P_w}{P_o} \right) A \left(\frac{V_g}{V_{g'}} + 1 \right)$$

where T = temperature (absolute)

P = atmospheric pressure (mm Hg)

P_w = vapor pressure of water at T (mm Hg)

P_o = standard atmospheric pressure (mm Hg)

A = cross section area of capillary bore

V_g = volume of respiration

$V_{g'}$ = volume of compensation vessel

When the two vessels are equal in size and the gas volume change is small compared to the vessel volume, the gas volume change is approximately equal to:

$$\Delta V = 2 A d$$

The oxygen consumption is expressed as μl (microliter) per mg wet weight per hour.

III RESULTS AND DISCUSSION

A. Structural and Functional Characteristics of Cultured Nerve Tissue

Results 1. Time Related Growth Patterns

The growth patterns of our cultures closely resembled that obtained by (Hild, 1966; Cechner, 1967; Lumsden, 1968) using similar techniques. In general the degenerative changes that start after dissection, due to ischemia and anoxia, gradually subside and by 15 days in vitro most cultures have many apparently healthy nerve cells and abundant myelination.

One day in vitro: At about 24 hours post-explantation, glia (oligodendrocytes and astrocytes) and mesodermal elements (epithelial cells, fibroblasts and macrophages) as well as neurites start emerging from mid-brain and cerebellar explants. Outgrowth from explants obtained from 5-8 day old animals is relatively slower than that from 1-2 day old animals.

Seven days in vitro: During the next few days more cells emigrate and make complex connections between each other (fig. 1). Neither blood vessels nor myelin can be seen anywhere. Any pre-existing myelin must have degenerated and new myelin has not formed yet as evidenced by negative results with Sudan Black B staining (fig. 2). Small cells (5-15 μ in diameter) with dense cytoplasm, presumably oligodendrocytes, predominate in the outgrowth. No identifiable nerve cells can be seen in the outgrowth. This is because neurons migrate least if at all and so remain inside the explant.

Ten days in vitro: Nerve cells (fig. 3) become visible inside the explant zone after about 10 days or less in vitro, when the explant has become thin enough for transillumination. The nerve cells appear embedded in an extensive network of glial and neuronal processes. Along with nerve cells, glial cells and mesodermal cells are also present. In case of

cerebellar cultures many more of the "small" cells are present than in mid-brain cultures. Some of the small cells, then, in cerebellar cultures are presumably granule cells, the rest being oligodendrocytes, which are present in both midbrain and cerebellar cultures. The nerve cells are easily spotted because of their characteristic shapes, their usually large size (about 20-25 μ at this stage) compared with the majority of other cells in the culture, and their very distinct usually single nucleolus. The nerve cells are sedentary and are present in groups. At this stage the nerve cell nucleus may be found to occupy in some cells a peripheral location (fig. 3) and Nissl substance may still be not well formed. However many nerve cells have a central nucleus and are apparently healthy. Also about ten days in vitro many axons acquire a myelin sheath which is easily visible due to its high refractivity in ordinary light and its birefringence in polarized light (fig. 4a, 4b). Myelin is usually seen in areas with a high density of glia. Only a small segment of an axon is apparently myelinated at first, then gradually longer lengths of myelin sheaths appear. The myelin sheath is only about 1-1.5 μ in thickness when it is first observed.

Fifteen days in vitro: At about two weeks in vitro myelin is present in abundant amounts (fig. 5a). Almost all explants that will myelinate at all will have myelin by 15 days in vitro. Less than 1% of the cultures that had no myelin by 15 days, became myelinated later. The number of explants that myelinate is upwards of 90% of the total number explanted. (fig. 5b to 8) irrespective of the age of animal used. Nodes of Ranvier can be seen clearly after staining with myelin stains (fig. 9). Myelin sheath increases in thickness with time and is about 2.5 μ at two weeks in vitro. The sheath usually has a uniform thickness but may show some irregularities and almost always survives intact for the entire life of

the culture as long as adequate nutrition is provided and waste products removed. Myelin is shown in a 55-day old culture (fig. 10) examined by phase and polarized light as well as by myelin staining. Early in the third week in vitro many apparently healthy nerve cells (fig. 11) with normal amount and distribution of Nissl substance (fig. 12a,b) can be seen in most of the cultures. Staining by Bodian technique for neurofibrils delineates the cell soma, its processes and ring type synaptic boutons (fig. 13a,b). The very large number of axons present in any one culture, as revealed by Bodian staining when compared with the number of myelinated axons in the same culture shows that many unmyelinated axons are present. The axon sometimes bifurcates but never ventures into cell free areas. Unmyelinated axons are difficult to identify in the living culture. Methylene blue solution, used as a supravital stain is taken up by some but not all nerve cells and helps to reveal hidden cell processes in the thick areas of living cultures (fig. 14).

Frequently some areas in cultures from both midbrain and cerebellum exhibited ciliary activity (fig. 15). The cilia are believed to be of ependymal origin and were observed by (Hild, 1957a). Ependymal cilia are thought to be involved in the movement of cerebrospinal fluid in the brain ventricles. The ciliary beating is greatly slowed when the culture temperature is lowered to 4°C. The activity resumed, however, when the culture temperature was raised to 37°C.

One month in vitro: At about one month in vitro the cultures seem to be stabilized and no major further development occurs. Dead cells and debris are phagocytized by macrophages at all stages of the culture development. The cultures in general can be maintained in an apparently healthy condition for a period of about two months, a period long enough for in-

vestigating the effects of many factors on the structure and function of nerve tissue. However chromatolysis of the neuron soma followed by degeneration, as well as fragmentation of the myelin sheaths, generally occurs more frequently in older cultures. Thus, while chromatolytic nerve cells in 15 day old cultures are about 5% of the large nerve cells, the incidence increases to about 20% in 2 months old cultures (fig. 16).

No neurons were ever seen in division. Occasionally binucleated nerve cells (less than 1 per 1000 nerve cells) were seen (fig. 17a). Nerve cell sizes vary between 25-50 μ in diameter. About 60% of them have a diameter of 25 μ , 30% are 30-35 μ in diameter and 10% have a diameter about 40-50 μ . Small cells presumed to be oligodendrocytes, which are known to undergo mitosis in vivo, are infrequently seen in what appears to be cell division (fig. 17b). Purkinje cells of the cerebellum often are present in a row-like fashion like in vivo (fig. 18). Other large nerve cell types of cerebellum and midbrain that could not be classified as to type were also present (fig. 19).

Cultures from 5-8 day old animals generally show better differentiated cells by 10 days in vitro (fig. 20) and the nerve cells usually appeared generally larger in size than in similar cultures from 1-2 day old animals. Myelin may appear a little earlier, about 8-9 days in vitro in cultures from 5-8 day old animals.

Our cultures developed from explants that had an average oxygen uptake at 37°C approximately equal to 1.25 μ l/mg wet weight /hr. This is lower than in vivo values which are about 2 μ l/mg wet weight/hr. Cell damage during the dissection process could contribute to the observed low QO_2 provided that oxygen diffusion throughout the explant is not impaired. No measurement was made of QO_2 of the cultures. However another parameter, namely the approximate number of large (> 20 μ) healthy nerve cells, was

Fig. 1 Glial cells (g) in the outgrowth zone at 7 days in vitro (div).

Edge of cerebellar explant (e) is seen, phase, 2 day old rats used.

Fig. 2 Sudan Black B staining of explant at 7 div. Notice absence of any myelin. 2 day old rats used. Bar represents 20 μ .

Fig. 1

Fig. 2

XBB 6912-8113

XBB 6912-8107

Fig. 3. Cerebellar culture at 10 div. Notice two nerve cells (n) and peripheral nuclei. Myelin (m) is present. Phase. 2 day old rats used.

Fig. 4a Myelin in a cerebellar culture at 10 div. Phase. 2 day old rats used. Bar represents 25 μ .

Fig. 4b Same culture as in fig. 4a but viewed with polarized light. Myelin (m). Bar represents 25 μ .

Fig. 4a

XBB 6912-8112

Fig. 4b

XBB 6912-8124

Fig. 5a. Abundant and apparently normal myelination at 15 div. Myelin (m) and small cells (s). Phase. 2 day old rats used. Bar represents 20 μ .

Fig. 5b

DBL 6911 5151

Development of myelination in explants from 1 day old rat cerebellum.

The explants were inspected daily, under sterile condition for presence or absence of myelin, then were reincubated. The mean percentage of myelinated explants averaged from 3 groups of explants (each composed of 25 explants) is plotted against incubation time. The mean percent myelination had a standard deviation about 10% of the mean at the different incubation days. At 15 div about 90% of the explants had myelin. The cultures were inspected by phase and polarized light optics.

Fig. 6

DBL 6911 5152

Development of myelination in explants from 2 day old rat cerebellum. The explants were inspected daily, under sterile condition for presence or absence of myelin, then were reincubated. The mean percentage of myelinated explants averaged from 3 groups of explants (each composed of 25 explants) is plotted against incubation time. The mean percent myelination had a standard deviation about 10% of the mean at the different incubation days. At 15 div about 90% of the explants had myelin. The cultures were inspected by phase and polarized light optics.

Fig. 7

Incubation period, days

DBL 6911 5153

Development of myelination in explants from 5 day old rat cerebellum.

The explants were inspected daily, under sterile condition for presence or absence of myelin, then were reincubated. The mean percentage of myelinated explants averaged from 3 groups of explants (each composed of 25 explants) is plotted against incubation time. The mean percent myelination had a standard deviation about 10% of the mean at the different incubation days. At 15 div about 90% of the explants had myelin. The cultures were inspected by phase and polarized light optics.

Fig. 8

DBL 6911 5154

Development of myelination in explants from 8 day old rat cerebellum.

The explants were inspected daily, under sterile condition for presence or absence of myelin, then were reincubated. The mean percentage of myelinated explants averaged from 3 groups of explants (each composed of 25 explants) is plotted against incubation time. The mean percent myelination had a standard deviation about 10% of the mean at the different incubation days. At 15 div about 90% of the explants had myelin. The cultures were inspected by phase and polarized light optics.

Fig. 9 Sudan Black B staining of a cerebellar culture at 15 div. Notice node of Ranvier (n) 2 day old rats were used. Bar represents 20 μ .

Fig. 10 Apparently normal myelin at 55 div in a midbrain culture. 2 day old rats were used. (a) Myelinated axon. Phase. (b) The same myelinated axon viewed by polarized light. (c) The same myelinated axon after fixation and staining with Sudan Black B.

Fig. 9

Fig. 10

XBB 6912-8111

XBB 6912-8125

Fig. 11. Many apparently normal nerve cells (n) at 15 div. Small cells (s) can be seen. Phase. 2 day old rat cerebellum was used. Bar represents 50 μ .

Fig. 12a Nerve cell in a cerebellar culture at 15 div. Notice dendrite (d) and well distributed Nissl substance (n). Phase. 2 day old rats used. Bar represents 10 μ .

Fig. 12b Nerve cell in a cerebellar culture at 15 div. Cresyl violet staining. Well developed Nissl substance (n) can be seen. Notice distinct nucleolus (s) inside a prominent nucleus (n). 2 day old rats were used. Bar represents 10 μ .

Fig. 12a

Fig. 12b

XBB 6912-8116

Fig. 13a Nerve cells in a cerebellar culture at 15 div. Bodian staining. Notice axon (a), dendrite (d) and neurofibrils (f). 2 day old rats were used. Bar represents 10 μ .

Fig. 13b Nerve cell in a cerebellar culture at 15 div. Bodian staining Notice ring like synaptic bouton (s). 2 day old rats were used. Bar represents 10 μ .

Fig. 13a

Fig. 13b

XBB 6912-8117

XBB 6912-8122

Fig 14. Cerebellar culture at 15 div. Supravital Methylene blue staining. Upper picture: Nerve cell soma and dendrites (d) stained by the dye. Since no phase optics were used, unstained cells are not clear. Lower left picture: two nerve cells stained by the dye. Phase. Lower right picture: the same two nerve cells viewed without phase optics. 2 day old rats were used.

Fig. 15 Cerebellar culture at 15 div. The rapidly beating ependymal cilia (c) at the edge of the explant (e) appear blurred in the still picture. Phase. 2 day old rats were used. Bar represents 50 μ .

Fig. 16 Nerve cell in a cerebellar culture at 15 div. Notice uneven distribution of Nissl substance (n) in the chromatolytic nerve cell. Cresyl violet staining. 2 day old rats were used. Bar represents 10 μ .

Fig. 15

Fig. 16

XBB 6912-8115

Fig. 17a Binucleated nerve cell in a cerebellar culture at 15 div.
Cresyl violet staining. The nuclei (n) are surrounded by
well distributed Nissl substance. Bar represents 10 μ .

Fig. 17b Midbrain culture at 15 div. A small cell (g) presumably an
oligodendrocyte appears to be dividing. Phase. 2 day old
rats were used. Bar represents 20 μ .

Fig. 17a

Fig. 17b

XBB 6912-8114

XBB 6912-8106

Fig. 18. Cerebellar culture at 15 div. Purkinje cells (p) arranged in a row. Phase. 2 day old rats were used. Bar represents 30 μ .

Fig. 19 Midbrain culture at 15 div. Notice nerve cell (n), dendrite (d) and small cell (s). Phase. 2 day old rats used. Bar represents 20 μ .

Fig. 20 Cerebellar culture at 10 div. Notice a relatively large nerve cell (n) and small cells (s). Phase. 8 day old rats were used. Bar represents 30 μ .

Fig. 19

Fig. 20

XBB 6912-8129

estimated and compared with that for in vivo tissue. One mm^3 of newborn rat brain tissue has approximately 1000 large nerve cells (Purkinje cells, Golgi cells etc). These nerve cells can be easily seen and identified in vitro. At 15 days in vitro, the mean culture area (average radius measured with an ocular micrometer) was $50 \pm 5 \text{ mm}^2$ (mean of 50 cultures). This is about 16 times original explant area. The culture was essentially one cell layer thick in terms of the large nerve cells. The number of these cells vary in individual cultures. The mean number found for cerebellar cultures (25 cultures counted) was 96 ± 9 cells. It appears then that about 10% of the original large nerve cells are able to survive the dissection trauma and adjust to the in vitro conditions. Undoubtedly many glial cells also die, yet because glia divide and are stimulated to proliferate (gliosis) in response to injury, no meaningful comparison can be made with the in vivo situation. In addition some small glial cell types (oligodendrocytes) are difficult to differentiate from small nerve cells. However since it was noticed that in the irradiation experiments (page 120) the density of the small cells (5-15 μ) was markedly decreased by the higher doses used, an estimate of the density of these cells in control cultures was attempted. At 15 days in vitro the small cells in the peripheral area of cerebellar cultures from 2-day old rats occur mostly in a sparsely populated single cell layer while they are present in densely populated 1-2 cell layers in the central thicker part of the culture. The mean density of the small cells was found to be (based on counting ten areas each $400 \mu^2$ randomly selected throughout the culture and averaging the results from 25 cultures) 2 ± 0.5 cells/ $400 \mu^2$.

2. Scanning Electron Microscopy

Examination of the cultures by scanning electron microscopy reveals that much of the three dimensional shape of nerve cells, glia and their

Fig. 21 Upper picture: Scanning electron microscopic image of a cerebellar culture at 40 div. Notice that the two nerve cells (n) are not flattened against their two dimensional support and prominent nucleolus (n1). 2 day old rat was used. Bar represents 20 μ . Lower picture: The same two nerve cells (n) in the upper picture as seen by phase optics. The phase picture is an inverted mirror image of the scanning picture. Bar represents 40 μ .

Fig. 21

XBB 6912-8130

Fig. 22 Upper picture: Scanning electron microscopic image of a cerebellar culture at 40 div. Notice nerve cell (n) and small cells (s). The small cells have a distinct globose form. Notice cylindrical shape of cell processes. 2 day old rat used. Bar represents 30 μ . Lower picture: Same culture as in the upper picture, a larger area is shown, as seen by phase optics. Notice nerve cell (n) and small cells (s). Bar represents 50 μ .

XBB 6912-8132

Fig. 22

Fig. 23a Upper picture: Cerebellar culture at 40 div. Notice myelinated axons (m) crossing holes in the culture. Phase. 2 day old rat used. Bar represents 20 μ . Lower picture: Scanning electron microscopic image of a similar area in the same culture. Notice myelinated fibre (b) showing ridges suggesting it is a bundle of axons. Bar represents 30 μ .

Fig. 23a

XBB 6912-8131

Fig. 23b Right picture: Scanning electron microscopic image of two glial cells in the outgrowth zone of a cerebellar culture at 40 div. Notice the glial cells (probably astrocytes) show membranous swellings of their surfaces (a). Notice also the ramification of the cylindrical glial processes into a complex network (b). 2 day old rat was used. Bar represents 20 μ . Left picture: The same glial cells as seen by phase contrast optics. The phase picture is a mirror image of the scanning picture. Bar represents 40 μ .

Fig. 23b

XBB 698-5061

processes is retained in vitro in spite of the essentially two dimensional array of the cells and the disruption in their geometrical relationships. Comparison between phase contrast pictures of living cultures and the fixed and scanned cultures was used in order to exclude artifacts and identify the different structures in the scanning images. Nerve cells (fig 21) appeared elevated above the surface i.e. they have not flattened completely on the substrate. Small cells that are probably a mixture of granule cells and oligodendrocytes had a very distinct globose form and cylindrical processes (fig. 22) that varied in number between 2-7 processes. Myelinated axons, crossing "holes" in the explant showed ridges presumably due to fasciculation of several axons into a bundle as occurs in vivo (fig. 23a). Glia, presumably astrocytes showed membranous swellings (fig. 23b).

3. Functional Activity

a. Biochemical: Synthesis of RNA from labeled uridine-2 ^{14}C was active in nerve cells, glia and mesenchymal cells in the third week in vitro as shown by autoradiography and nucleic acid staining. No studies of RNA synthesis during the first two weeks in vitro were done. The precursor (Sp. Act. 55 mCi/mmal) was added to the culture medium at a final concentration of 1.25 $\mu\text{Ci/ml}$ and the culture was incubated for 30 min. at 36°C . Digestion with ribonuclease was not done. Labelling of nerve cells was visible only in areas near the edge of the explant. Most of the grains appeared over the nuclei of the labeled cells (fig. 24-25). Presumably, in the relatively thick central part of the culture, the weak beta particles of the ^{14}C -labelled RNA precursor did not reach the overlying emulsion layer. Grain counting was not attempted because of the nature of the preparation used for autoradiography, namely whole mounts instead of sections. Since the thickness of plasma clot over the labeled cells is not uniform, the number of beta particles absorbed by it varies thus in-

Fig. 24 Autoradiograph of a cerebellar culture at 16 div. Einarson staining for nucleic acids. Uridine - 2^{14}C was used as RNA precursor. See text for details of labelling. Notice silver grains are mostly over nuclei of nerve cells (n) and glia (g). 2 day old rat was used. Bar represents 20 μ .

Fig. 25 Autoradiograph of a midbrain culture at 16 div. Same conditions as in fig. 24. Again nerve cells (n) and glia (g) show labelling. Bar represents 20 μ .

Fig. 24

Fig. 25

XBB 6912-8128

fluencing the number of grains observed.

b. Bioelectric

Spontaneous action potentials were observed by extracellular electrodes at room temperature and in BSS in most nerve cells tested in 15 day old cultures (fig. 26a,b). The average spike frequency ranged from a few spikes per minute to about 10 spikes per second, but frequencies of 50 to 300 per. min. were most often found, with interval distribution that were quite often irregular and non random. Spikes often occurred in trains with silent periods of sometimes several seconds between bursts. The spontaneous activity usually lasted for up to 5 hours. No spontaneous extracellular spikes were detected in nerve cells in very thin areas of the culture. These observations were made with the assistance of Mr. W.T. Schlapfer.

4. Effect of Temperature on Myelination

Incubation of the explants at 30°C instead of the standard 36°C resulted in absence of myelination at 15 days in vitro in all cultures. At an incubation temperature of 32°C only about 20% of the cultures had myelin at 15 days in vitro, while about 90% of the controls were myelinated (fig. 27). Coupled with retardation of myelin emergence was a thinner than usual myelin sheaths at 15 days in vitro. Many of the visible nerve cells were less differentiated than usual at this stage (fig. 28), but the axons appeared intact. Eventually, at about 3-4 weeks most of the cultures showed myelination, the myelin sheath average thickness, however remaining less than the controls, and by that time, most of the nerve cells appeared normal. These cultures were observed for only one month.

5. Effect of Glucose Concentration on Myelination

Media with net glucose concentration approximating 2 mg/ml, 3 mg/ml., 4 gm/ml., 6.5 mg/ml. (the standard medium) and 11.5 mg/ml. were used. In

Fig. 26a Spontaneous bioelectric activity recorded by extracellular electrodes positioned close to the nerve cell soma. Recording was done at room temperature. Horizontal side of each square represents 500 msec., vertical side of each square represent 200 μ v. Cerebellar culture at 15 div. 2 day old rat was used.

Fig. 26b Spontaneous bioelectric activity recorded by extracellular electrodes positioned close to the nerve cell soma. Recording was done at room temperature. Multiple trace. Horizontal side of each square represents 2 msec., vertical side of each square represents 200 μ v.

Fig. 26a

Fig. 26b

XBB 6912-8119

DBL 6911 5149

Fig. 27

Effect of incubation temperature on the percentage of incubated explants that became myelinated by 15 div. Means of three experiments, each involving 75 explants (25 explants at each of the three incubation temperatures tested). Notice absence of myelination at 30°C. The standard deviation was approximately 10% of the mean values.

XBB 6912-8104

Fig. 28. A nerve cell with abnormal distribution of Nissl substance (n) typical of many nerve cells in a 15 day old cerebellar culture incubated at 32°C. Crysyl violet stain. Bar represents 10 μ .

Fig. 29

DBL 6911 5150

Effect of glucose concentration in the medium on the percentage of myelinated explants at 15 div. More of the incubated explants become myelinated as the glucose concentration is increased. Mean of three experiments each involving 100 cerebellar explants (twenty at every glucose level). Standard deviations were about 10% of the means. Notice maximum percent myelination attained at about 6.5 gm glucose/ml. medium. 2 day old rats were used.

Fig. 30 A cerebellar explant at time of explantation. Phase. 2 day
old rat used. Bar represents 2 mm.

Fig. 31 The same cerebellar explant in fig. 30 after 15 days of incu-
bation. Difference in areas is highly significant. Bar repre-
sents 2 mm.

Fig. 31

Fig. 32

XBB 6912-8134

general percent incidence of myelination at 15 days in vitro rapidly increased with glucose concentration in the medium up till about 6.5 mg/ml., then remained stable after that (fig. 29). At a glucose concentration of 6.5 mg/ml, myelination, at 15 days in vitro, occurred in 90-95% and sometimes 100% of the cultures. Further increase in glucose concentration (11.5 mg/ml.) did not seem to further improve the per cent myelination. These cultures were kept under observation for only one month.

Discussion

Nerve tissue in vitro apparently can recover to a large degree from the trauma of dissection that includes severing of axons, anoxia and ischemia, and adapt successfully to the abnormal in vitro conditions. Part of the reasons for this success lies in the adoption of less traumatic dissection methods, quick incubation, culture vessels that can help maintain near physiological conditions, and enriched media. In addition, the great inherent capacity of nerve cells for regeneration of its Nissl substance and axons, provided they are not severed too close to the soma, explains the ability of nerve cells to differentiate and mature in vitro (fig. 11, 12a,b). The organotypic culture conditions adopted subjects the organization of nerve tissue cells to little disruption as evidenced by the occurrence of myelination (fig. 5a) and synaptic terminals (fig. 13a,b). Although about 90% of the large nerve cells on the average apparently suffer too much trauma during the dissection to permit them to survive, yet a hundred or so nerve cells may be visible in an ordinary culture after 15 days post incubation. The apparent low incidence (about 5%) of chromatolytic nerve cells in most cultures by the end of two weeks in vitro means that in vitro conditions permitted the recovery of most slightly injured nerve cells and or the differentiation and maturation of many

neuroblasts that are undoubtedly present in large numbers particularly in the tissues from 1-2 day old animals. The ability of Purkinje cells to remain in many cultures in their characteristic single row arrangement (fig. 18) testifies to the little disruption of organization in vitro. The emergence of myelination at about ten days in vitro, and its apparent health, long survival and presence of nodes of Ranvier are evidence for maintenance of not only the in vivo neuronal-glia cooperation, but also its characteristic temporal coordination. Scanning electron microscopy showed still another level of structural integrity in vitro. The maintenance of the three dimensional in vivo shape of nerve cells, glia and their processes is evidence against the supposition that the essentially two dimensional in vitro substrate will flatten the cells and their processes. Also fasciculation of axons into bundles in vitro which was shown also by (Boyde et al., 1968), means that this is a basic property of axons that is maintained even outside the body.

In addition to the apparent structural integrity of cultured nerve tissue, important functional characteristics which underlie and accompany the presence of healthy nerve tissue, are demonstrated in vitro. Thus, at the biochemical level, RNA synthesis from its precursors was active in nerve cells, glia as well as other cells. This is similar to the situation in vivo when active RNA and protein synthesis are characteristics of nearly all cells in the neuraxis with the exception of astrocytes which are relatively sluggish in this regard (Koenig, 1964). These synthetic activities are probably concerned with the continuous formation of axoplasmic constituents and with the replacement of oxidative and other enzymes consumed in normal usage in the case of neurons. In the case of glia, RNA is involved in the formation of myelin, the synthesis of respiratory and

other enzymes and the biosynthetic activities accompanying reactive gliosis. Whole mount autoradiography can only indicate the functioning of RNA synthetic machinery. To make a quantitative study of the rate of synthesis at different stages of development in vitro, and under various experimental conditions, sections of the culture should be made and studied, after autoradiography and staining, by grain count and spectrophotometry. At the bioelectrical level a characteristic property of the neuronal membrane, namely spontaneous bioelectric activity is exhibited in vitro. Demonstration of such a highly specialized nerve cell function in vitro denotes the high degree of structural integrity that is possible inspite of the abnormal conditions. In addition, bioelectrical observations by (Schlapfer, et al., 1969) made on our cultures suggest the presence of simple nerve cell nets in areas of the culture that have high nerve cell density.

The effect of low incubation temperatures (30°C and 32°C) on the percent incidence of myelination generally agrees with observations by (Peterson et al., 1960; Hamburgh, 1966). However our results for myelination incidence at 32°C shows about 20% myelination at 15 days in vitro. The difference could be due to different culture techniques and media used. A low temperature could decelerate the rate of various synthetic activities. Recovery of injured nerve tissue elements will be impaired. Maturation and differentiation of uninjured nerve cells and glia will be retarded with concomittant delay in emergence of myelination. Since the percent incidence of myelination eventually becomes equal to that of the controls kept at 36°C, but at a later time (which was also observed by Hamburgh, 1966) the effect of incubation at 32°C apparently does not result in permanent damage, at least during the observation period.

The positive effect of increasing glucose concentration on myelination incidence found in the present study supports, in general, findings by

(Murray 1959, 1965). However, the author did not observe an inhibitory effect on percent myelination by increasing glucose concentration in the medium above 6 mg/ml medium, as was reported by Murray, 1965). A glucose concentration as high as 11.5 mg/ml medium, though it did not ensure 100% myelination, did not lower the percent incidence of myelination than that observed at 6.5 mg/ml medium.

The large increase in the surface area of a culture (fig. 30, 31) by 15 days in vitro is due to several factors:

- (1) Flattening of the explant against its support.
- (2) Centrifugal migration of cells.
- (3) Cell division
- (4) Increase in cell size
- (5) Centripetal migration
- (6) Death of cells
- (7) Decrease in cell size

The contribution of each of these factors to the increase in area in an individual culture is variable and the relative contribution of each is difficult to assess. However, since a significant reduction in culture area had been consistently produced by high doses of radiation, in the present study, estimation of the area of control culture was made in order to compare it with that of irradiated cultures at 15 days in vitro.

B. Dose Dependent Radiation Effects

Results 1. X-irradiation

Freshly dissected explants from 2-day old rat brain were irradiated with different doses. Fifty explants (in five groups each composed of ten explants) were used for each dose level and for controls. The percent incidence of myelinated cultures compared to the total number irradiated

depended on the dose used. Radiation doses up to 400 rads produced no observable effect on myelination percent incidence in the irradiated explants compared to the controls (fig. 32). Examination of such irradiated cultures by phase contrast microscopy, polarization microscopy as well as by staining for Nissl substance, axons and myelin (fig. 33-37a) at 15 days in vitro did not reveal apparent differences between them and the controls. This does not exclude the possibility that some damage may have occurred but remained latent.

At doses between 400 and 4000 rads irradiated explants had lower percent incidence of myelination than the controls (fig. 32, 37b at 15 days in vitro). Myelin when present in some of the irradiated cultures, was apparently normal but less abundant than in a control culture. Thus while a control culture may have several hundred myelinated axons, a culture that received 3000 rads may have only about ten myelinated axons and it remained less myelinated than a control culture even after a month of incubation. A dose of 4000 rads produced essentially complete inhibition of myelination, at 15 days in vitro, in all irradiated explants. No recovery of myelination occurred in such amyelinated cultures even after one month of incubation. The dose that caused inhibition of myelination in 50% of exposed cerebellar cultures was 1800 ± 100 rads (mean ± 1 S.D.). For midbrain cultures the dose for 50% myelination incidence was 1900 ± 130 rads which is not significantly different from that for cerebellar cultures (fig. 38). Myelination in vitro of in vivo irradiated cerebellar tissue appeared to be more sensitive than when irradiation was done in vitro. The dose for 50% myelination was 1640 ± 110 rads which is significantly different (at the 5% level) from that for in vitro irradiated cerebellar tissue (fig. 39).

Cerebellar cultures from explants that received 4000 rads and showed 100% inhibition of myelination at 15 days in vitro were further examined

by optical, histological and electrophysiological methods. The following observations were made:

1. The mean area of the culture (mean of 50 cultures) was $19 \pm 4 \text{ mm}^2$. The reduction in area, when compared to that of control cultures ($50 \pm 5 \text{ mm}^2$) was highly significant (fig. 40,41).
2. The density of small cells was markedly reduced (fig. 42a,b). In contrast to a density of $2 \pm 0.5 \text{ cells}/400 \mu^2$ for control cultures, the amyelinated cultures had a density of $0.2 \pm 0.1 \text{ cells}/400 \mu^2$ (based on counting ten areas each $400 \mu^2$ randomly selected throughout the culture and averaging the results from 25 cultures).
3. Most cultures had normal abundance of large nerve cells (about a hundred cells). The majority of cells had normal distribution of Nissl substance (fig. 43a). The incidence of chromatolytic nerve cells (fig. 43b) did not differ significantly from that of the controls (about 5%). In irradiated cultures, however, "white" patches sometimes were seen in the cytoplasm of chromatolytic nerve cells (fig. 44).
4. The row like arrangement of Purkinje cells was less marked and some Purkinje cells had bizarre shaped dendrites (fig. 45).
5. Cell debris and macrophages were more conspicuous in irradiated than in control cultures, and were especially prominent at 7 days post irradiation (fig. 46a,b).
6. Neurofibrils, axon cylinders were, like in control cultures, unfragmented (fig. 47). Synaptic boutons were present and silver staining showed them to be apparently similar to those in control cultures (fig. 48). Control and irradiated explants (4000 rads) cultured together showed no cross effects.
7. Spontaneous bioelectric activity was retained by most of the nerve cells tested extracellularly (fig 49, 50). As in the case of the controls,

Fig. 32

Effects of X-irradiation on the percentage of myelinated explants at 15 div. Cerebellar explants from 2 day old rats were used. Above 400 rads, some of the exposed explants did not develop myelin and their percentage increased with the dose. Controls were sham irradiated. Exposure of the explants to 4000 rads resulted in failure of myelination in all exposed explants when examined at 15 div. The means of five experiments each involving ten explants at every dose level used and as controls, are plotted against dose received. The means had standard deviations about 10% of the mean values. Dose that resulted in only 50% of the irradiated explants becoming myelinated (control explants considered as 100% myelinated) was 1800 ± 100 rads (mean $\pm$ 1S.D.).

DBL 6911 5142

XBB 6912-8105

Fig. 33. Cerebellar culture from an explant that received 400 rads x-rays.
15 div. Notice apparently normal myelination (m) and nerve cells (n).
Phase. Bar represents 20 μ .

Fig. 34 Cerebellar culture from an explant that received 400 rads x-rays.
15 div. Normal myelin (m). Polarized light. Bar represents 20 μ .

Fig. 35 Cerebellar culture from an explant that received 400 rads x-rays.
15 div. Normal myelin (m). Sudan Black B staining. Bar represents 20 μ .

Fig. 34

Fig. 35

XBB 6912-8127

Fig. 36 Cerebellar culture from an explant that received 400 rads x-rays.
15 div. Apparently normal neurofibrils (f) and axon cylinders (a).
Bodian staining. Bar represents 20 μ .

Fig. 37a Cerebellar culture from an explant that received 400 rads x-rays.
15 div. Notice apparently normal Nissl substance (n). Dendrites
appear to lack Nissl substance. Cresyl Violet staining. Bar
represents 10 μ .

Fig. 36

Fig. 37a

XBB 6912-8121

XBB 6912-8123

Fig. 37b. Cerebellar culture from an explant that received 2000 rads x-rays. 15 div. Notice absence of myelin in the explant (3). Sudan Black staining. Bar represents 20 μ .

Fig. 38 Effects of X-irradiation on percentage of myelinated explants at 15 div. Midbrain explants from 2 day old rats were used. Percentage of explants that did not develop myelin increased with dose received. The means of five experiments, each involving ten explants at every dose level used and as controls, are plotted against dose received. The means had standard deviations about 10% of the mean values. Dose that resulted in only 50% of the explants becoming myelinated was 1900 ± 130 rads (controls considered 100% myelinated).

DBL 6911 5147

Fig. 38

DBL 6911 5148

Fig. 39

Effect of X-irradiation on the percentage of myelinated explants at 15 div. In vivo irradiation was done. Cerebellar explants from 2 day old rats were used. The means of five experiment, each involving ten explants at every dose level used and as controls, are plotted against dose received. The means had standard deviations about 10% of the mean values. Dose that resulted in only 50% of the irradiated explants becoming myelinated (control explants considered as 100% myelinated) was 1640 ± 110 rads.

Fig. 40 Control cerebellar culture (e) at 15 div. Bar represents 2 mm.

Fig. 41 Cerebellar culture at 15 div, from an explant that received 4000 rads. This explant was cultured close to the one that gave rise to the culture in Fig. 40. Notice marked reduction in the area of irradiated culture. Bar represents 2 mm.

Fig. 40

Fig. 41

XBB 6912-8109

Fig. 42a Control cerebellar culture at 15 div. Notice nerve cell (n) and small cells (s). Phase. Bar represents 25 μ .

Fig. 42b Irradiated cerebellar culture (4000 rads to the explant) at 15 div. Notice absence of small cells, but apparently normal nerve cells (n) are present. Phase. Bar represents 25 μ .

Fig. 42a

Fig. 42b

XBB 6912-8120

Fig. 43a Nerve cell, representative of many in a culture that received 4000 rads. Notice apparently normal Nissl substance (n). Cresyl violet staining. Bar represents 10 μ .

Fig. 43b Chromatolytic nerve cell in a cerebellar culture that received 4000 rads. Notice "pale" staining of Nissl substance (n) and its abnormal distribution. The incidence of these cells, at 15 div, in the irradiated cultures did not differ significantly from their incidence in the controls. Cresyl violet staining. Bar represents 10 μ .

Fig. 43a

Fig. 43b

XBB 6912-8110

Fig. 44. Chromatolytic nerve cell in a cerebellar culture that received 4000 rads. Notice "white" patch in the cytoplasm. Cresyl violet staining. Bar represents 10 μ .

Fig. 45. Nerve cell (n) in a cerebellar culture that received 4000 rads. 15 div. Notice the disorientation of dendrites (d). Phase. Bar represents 20 μ .

Fig. 44

Fig. 45

XBB 6912-8185

Fig. 46a Control cerebellar culture at 7 div. Notice glia (g) and few debris (d). Phase. Bar represents 50 μ .

Fig. 46b Irradiated cerebellar culture (4000 rads) at 7 div. Notice relatively more debris (d) than in fig. 46a and many macrophages (m). Phase. Bar represents 50 μ .

Fig. 46a

Fig. 46b

XBB 6912-8108

Fig. 47 Irradiated cerebellar culture (4000 rads) at 15 div. Notice apparently normal (unfragmented) neurofibrils (f) axons (a) and dendrites (d). Bodian staining. Bar represents 20 μ .

Fig. 48 Irradiated cerebellar culture (4000 rads) at 15 div. Notice synaptic bouton (s) adjacent to a nerve cell soma. Bodian staining. Bar represents 10 μ .

Fig. 47

Fig. 48

XBB 6912-8133

Fig. 49. Spontaneous bioelectric activity as recorded extracellularly in the vicinity of a nerve cell in a cerebellar culture that received 4000 rads. 15 div. Notice that the spontaneous activity is still present inspite of the irradiation and appears to be not different from that of the controls (fig. 26a). Horizontal side of each square represents 500 msec. and vertical side of each square represents 200 μ v.

Fig. 50 Spontaneous bioelectric activity in an irradiated culture (4000 rads) recorded with extracellular electrodes in the vicinity of a nerve cell. Multiple trace. Notice pulse shapes apparently are similar to those from unirradiated cultured nerve cells (fig. 26b). Horizontal side of each square represents 2 msec. and vertical side represents 200 μ v.

XBB 6912-8118

Fig. 49 (top picture)

Fig. 50 (bottom picture)

the extracellular recording was done in the vicinity of the nerve cell soma. This may explain the apparent little difference observed in either the frequency of pulses or their shape. However, since few irradiated cultures were so tested, no conclusions can be made at this stage concerning quantitative differences if any between bioelectric activity of control and radiation amyelinated cultures.

2. Particle Irradiation

Helium ions 36 Mev and 54 Mev (from Hilac and 88-inch cyclotron respectively) as well as 30 Mev deuterium ions at a surface dose of 4000 rads inhibited myelin formation in all irradiated explants at 15 days postirradiation. Although the dose rates used varied and entrance LET of the different ions were different from that of used X-rays (approximate LET's: x-rays = $3\text{ Kev}/\mu$, Hilac Helium ions about $20\text{-}25\text{ Kev}/\mu$ and cyclotron deuterium ions about $5\text{-}6\text{ Kev}/\mu$), no difference in morphological damage was obvious at the light microscope level between x-rays and particle irradiation of equal dose. Thus the effect of 4000 rads particle radiations on culture area, density of small cells, and appearance of nerve cells, Nissl substance, neurofibrils, axon cylinders, dendrites and synaptic boutons were generally similar to those produced by 4000 rads x-rays. Again, as with x-irradiation a dose above 400 rads was needed in order to produce an observable effect on myelination at 15 days in vitro (fig. 51-53). Between 400 and 4000 rads the number of cultures that had myelin compared to the total number irradiated decreased with dose increase. The doses that caused inhibition of myelination in 50% of irradiated explants were $1620 \pm 132\text{ rads}$, $1710 \pm 118\text{ rads}$; $1780 \pm 110\text{ rads}$ for 36 Mev helium ions, 54 Mev helium ions and 30 Mev deuterium respectively. The differences between these doses are not statistically significant. On comparing these doses for 50%

Fig. 51 Effects of different doses of 36 Mev helium ions on percentage of myelinated explants at 15 div. Cerebellar explants from 2 day old rats were used. Controls were sham irradiated. Above 400 rads the percentage of myelinated explants at 15 div decreased with the dose received. Fifty explants (in five groups each containing ten explants) were used as controls and at each dose level used. The mean percent myelination of the five groups for each case is plotted against the dose used. The standard deviation was about 10% of the mean value in each case. The dose that resulted in only 50% of the exposed explants becoming myelinated (controls considered as 100% myelinated) was 1620 ± 132 rads.

DBL 6911 5143

Fig. 51

Fig. 52 Effects of different doses of 54 Mev helium ions on percentage of myelinated explants at 15 div. Cerebellar explants from 2 day old rats were used. Controls were sham irradiated. Above 400 rads the percentage of myelinated explants at 15 div decreased with the dose received. Fifty explants (in five groups each containing ten explants) were used as controls and at each dose level used. The mean percent myelination of the five groups for each case is plotted against the dose used. The standard deviation was about 10% of the mean value in each case. The dose that resulted in only 50% of the exposed explants becoming myelinated (controls considered as 100% myelinated) was 1710 ± 118 rads.

DBL 6911 5144

Fig. 52

Fig. 53 Effects of different doses of 30 Mev deuterium ions on percentage of myelinated explants at 15 div. Cerebellar explants from 2 day old rats were used. Controls were sham irradiated. Above 400 rads the percentage of myelinated explants at 15 div decreased with the dose received. Fifty explants (in five groups each containing ten explants) were used as controls and at each dose level used. The mean percent myelination of the five groups for each case is plotted against the dose used. The standard deviation was about 10% of the mean value in each case. The dose that resulted in only 50% of the exposed explants becoming myelinated (controls considered as 100% myelinated) was 1780 ± 110 rads.

DBL 6911 5145

Fig. 53

effect on myelination with that obtained with x-irradiation, no significant difference was found except in the case of 36 Mev Helium ions. Although the difference is significant at the 5% level, yet because the particle radiation doses used are surface doses, the actual dose delivered inside the explant is higher and there is probably no difference. No observable effect on myelination was produced by 4000 rads of Hilac carbon ions. It should be mentioned that while Hilac helium ions used had a range in tissues of about 1 mm. and 88-inch cyclotron helium ion used had a range in tissues of over 1mm, the range for used carbon ions is only about 0.5 mm. Thus the absence of myelination with carbon ion irradiation is due to incomplete irradiation of the explants.

Discussion

Dose dependent nerve tissue damage was investigated at both the neurological and histopathological levels by several investigators, (Yamazaki et al., 1960; Clemente et al., 1960; Gilmore, 1965; Rodgers, 1965). At the histopathological level, reduction or absence of neuroglia and reduction or absence of myelination and blood vessel damage were all observed. Rose, 1958 found evidence for cerebral blood vessel permeability changes with doses as low as 100r. Gilmore et al., 1967 studied the histopathological changes in blood vessels in an irradiated area of neonatal rat spinal cord in which there was inhibition of myelination with a decrease in neuroglia. Evidence for blood vessel damage was present in the form of hemorrhages. She suggested that irradiation early in the postnatal period could impair vascular development resulting in decreased number of blood vessels, vasodilations and hemorrhages seen later. It seems then that there is a possibility that the observed neuroglial damage by radiations, resulting in myelination inhibition could be an indirect effect due to

impairment of the blood supply affecting all nerve tissue cells, some at an earlier time than others.

Several investigators (Quastel et al., 1963; Goldring, 1956, Masurovsky et al. 1967a, 1967b, 1967c) studied the radiobiological response of mammalian and chick embryo nerve tissue in vitro which is free from blood vessels. The damage produced, which is the result of direct effects of radiations on the cells, occurred at high doses. Pomerat et al., 1964 irradiated newborn rat dorsal root ganglia in vivo and immediately cultured them. Comparing their results with work done by Quastel et al., 1963, in which the irradiation was done in vitro, they concluded that the lower doses needed to cause nerve cell damage in their system is probably due to blood vessel and/or glial damage in vivo.

None of these investigators, however studied the radiation effects on myelination in vitro. Several conditions beside ionizing radiations are associated with decreased myelin formation in the central nervous system. These include undernutrition (Chase et al., 1967; Dobbing, 1968), phenylketonuria (Crome et al., 1968), Border disease in the lamb (Davison et al., 1966), maple syrup urine disease (Prensky, 1968) and hypothyroidism (Balazs et al., 1969). Healthy animals were used to make the nerve tissue cultures used in the present work, and as discussed earlier, the culture conditions adopted resulted in healthy myelinated cultures from the majority of cultured explants.

Since CNS myelination in vitro, as well as in vivo, is produced by collaboration between the nerve cell, its axon and neuroglia (oligodendrocytes) it was important to investigate whether the radiation damage to myelination was due to nerve cell damage in addition to neuroglial damage. The present findings show that in most cultures irradiated with the highest

dose (4000 rads) of either x-rays or particle radiations many apparently healthy nerve cells were present at 15 days in vitro and remained that way for an observation period lasting one month. These irradiated nerve cells had apparently normal Nissl substance, neurofibrils, axons and synaptic boutons at 15 days post irradiation. In addition spontaneous bioelectric activity was manifested by irradiated nerve cells and seemed to be not different from activity of the controls qualitatively. Thus it appears that nerve cells are not contributing to the observed inhibition of myelination. Neuroglia cells occur inner to and in the peripheral part (outgrowth zone). According to Murray, 1965 oligoglia predominate in the outgrowth area early after culturing. Our results show that there is a remarkable reduction in the density of small cells (glia, and granule cells in case of cerebellar cultures and small nerve cells and glia in case of midbrain cultures) in the culture. The observed reduction in the culture area could be due to oligoglia disappearance since the greater part of area reduction occurred in the outgrowth zone which still had many mesenchymal cells and small nerve cells remain mostly inner to the outgrowth zone (Lumsden, 1968). In addition, obvious increase in debris and macrophages in irradiated cultures (especially at the 4000 rad dose level) are signs of cell damage.

These results then seem to implicate direct oligodendroglial destruction as responsible for inhibition of myelination in vitro by irradiation. This interpretation of the present findings agrees with in vivo observations by Gilmore, 1965; Rodgers 1965, Schjeide et al., 1966 where radiations resulted in absence or decrease of oligoglia in the irradiated area. Our results however do not exclude the possibility of neuroglial damage in vivo being intensified by an additional damage of the blood vessels.

Brownson et al., 1963a among others observed the dose dependency of oligoglial necrosis. Although relatively few cells are damaged by 500r, the number increased with the dose. This agrees with our in vitro observations in which a dose of 4000 rads markedly reduced the density of small cells that include oligoglia. Since the irradiated explants are a multicellular interdependent heterogeneous cell population an exponential dose-effect curve is not to be expected. The culture myelination curves obtained with the different kinds of irradiations were of the sigmoid type. A dose above 400 rads had to be used before any decrease in per cent myelination at 15 days in vitro could be observed. This probably means that at doses upward of 400 rads, the density of glia is reduced enough in some cultures to result in their amyelination. As the dose is further increased, the chance becomes greater for more cultures to be amyelinated. The relatively small differences between the doses for 50% myelination irrespective of the type of irradiation used, (different LET) is probably due to the multicellular nature of the explant in contrast with single cell systems (cell cultures) where LET effects are best manifested. In addition the radiations used did not differ greatly in LET. The used x-rays had an LET approximately 3 KEV/ μ and Hilac helium ions about 20-25 Kev/ μ . Cyclotron deuterium ions were about 5-6 Kev/ μ . The different dose rates associated with the different types of radiation used, did not appear to influence the results. Although it is known that there is a dose rate effect in neuroglial response (Bailey, 1962; Brownson et al., 1963b) yet their findings were made very early after irradiation, while the present findings are based on observations made at 15 days post-irradiation.

Gilmore, 1965 observed that a dose above 1500 rads of 185 Mev protons was needed to produce effects on neuroglia and myelination in the irradiated

area of neonatal rat spinal cord. This observation is undoubtedly due to, not failure of neuroglial cell destruction by the dose used, but to migration of unirradiated glial cells from the adjacent areas and repopulation and eventual myelination of the irradiated area. Our experiments, in which isolated and totally irradiated (in vitro) pieces are cultured, show presence of amyelination in some of the cultures irradiated with doses above 400 rads and 50% effect on myelination incidence at a mean dose of 1727 ± 115 rads for all types of radiations used.

C. Age Dependent Radiation Effects

Results:

Doses of 4000 rads resulted in inhibition of myelination in all cerebellar cultures at 15 days in vitro irrespective of age of animal used. Again as with dose dependent experiments, 50 explants in five groups (each had ten explants) were used for the control group and at each of the dose levels used. No observable damage was seen unless the x-ray dose used, exceeded 400 rads. Inbetween 400-4000 rads the percent incidence of myelination in the cultures depended on, in addition to the dose, the age of animal used in preparation of the explants. Most cultures, irrespective of age of animal used, had many apparently healthy nerve cells even after a dose of 4000 rads. Nissl substance, neurofibrils, axons and synaptic boutons in the irradiated cultures appeared similar to those in control cultures.

The density of small cells (includes oligoglia) was markedly lowered at 15 days in vitro in cultures that received 4000 rads. The reduction in small cell density appeared to be relatively less so in cultures from 8 day old rats than those from 1 day old rats. The small cell mean density in such cultures varied between 0.2 ± 0.1 to 0.5 ± 0.2 cells/400 μ^2 . This is significantly different from mean small cell density for 1 and 8 day control cultures (2 ± 0.5 and 3.1 ± 0.3 cells/400 μ^2) respectively.

Fig. 54 Effects of different doses of X-irradiation on the percentage of myelinated explants at 15 div. Cerebellar explants from 1 day old rats were used. Controls were sham irradiated. Above 400 rads the percentage of myelinated explants at 15 div decreased with the dose received. Fifty explants (in five groups each containing ten explants) were used as controls and at each dose level used. The mean percent myelination of the five groups for each case is plotted against the dose used. The standard deviation was about 10% of the mean value in each case. The dose that resulted in only 50% of the exposed explants becoming myelinated (controls considered as 100% myelinated) was 1750 ± 120 rads.

DBL 6911 5155

Fig. 54

Fig. 55 Effects of different doses of X-irradiation on the percentage of myelinated explants at 15 div. Cerebellar explants from 5 day old rats were used. Controls were sham irradiated. Above 400 rads the percentage of myelinated explants at 15 div decreased with the dose received. Fifty explants (in five groups each containing ten explants) were used as controls and at each dose level used. The mean percent myelination of the five groups for each case is plotted against the dose used. The standard deviation was about 10% of the mean value in each case. The dose that resulted in only 50% of the exposed explants becoming myelinated (controls considered as 100% myelinated) was 1900 ± 145 rads.

Fig. 55

DBL 6911 5156

Fig. 56 Effects of different doses of X-irradiation on the percentage of myelinated explants at 15 div. Cerebellar explants from 8 day old rats were used. Controls were sham irradiated. Above 400 rads the percentage of myelinated explants at 15 div decreased with the dose received. Fifty explants (in five groups each containing ten explants) were used as controls and at each dose level used. The mean percent myelination of the five groups for each case is plotted against the dose used. The standard deviation was about 10% of the mean value in each case. The dose that resulted in only 50% of the exposed explants becoming myelinated (controls considered as 100% myelinated) was 2100 ± 160 .

DBL 6911 5157

Fig. 56

The dose that caused 50% myelination occurred at approximately 1750 ± 120 rads for cultures from 1-day old animals, it occurred at 1900 ± 145 rads and 2100 ± 160 rads approximately for cultures from 5-day and 8-day old animals respectively, (fig.54-56). The difference between mean doses for 50% myelination in cultures from 1-day old animal and those from 5-day old animals was not statistically significant. However the corresponding difference between the mean doses for cultures from 1-day old animal and those from 8-day old animals was statistically significant at the 1% level. No recovery of myelination was observed in the amyelinated cultures even after 1-month in vitro.

Discussion:

Radiation damage to nerve tissue (spinal cord and brain) varies in its severity according to the age of animal at time of its irradiation (Yamazaki et al., 1960; Clemente et al., 1960; Gilmore, 1963b, 1964b, 1965; Schjeide et al., 1966). Neurological and histopathological damage were well correlated. Histopathological damage included inhibition of myelination, decrease or absence of neuroglia in the irradiated area as well as blood vessel damage. Since blood vessels are still developing during the early post natal days (Gilmore et al., 1967) the observed age dependent damage to myelination in vivo could be influenced by different degrees of vascular damage occurring at the different postnatal days. Because nerve tissue cultures lack a vascular system, an age dependent myelination damage in such a system will then be due to age dependent variations in nerve cells and/or glia.

Myelin formation in the rat brain commences about ten days postnatally (McIlwain, 1966). Myelination is preceded by a gradual increase in the density of glia (myelination gliosis) and their enzymatic differentiation, during the early post-natal days. Schonbach et al., 1968 estimated changes

in glial cell density and in the number of cells distinguishable by NAD diaphorase activity in the rat pyramidal tract at different postnatal ages and reported the following results:

Glial Cell Density		Cells With NAD-Diaphorase Activity	
newborn	8.7 ± 2.5 cells/ $10^5 \mu^3$	newborn	0.9 ± 0.9 cells/ $3 \times 10^5 \mu^3$
2 days	11.5 ± 1.4	4 days	6.2 ± 1.1 "
6 days	11.6 ± 2.4	8 days	13.2 ± 2.1 "
8 days	12.5 ± 3.7	12 days	17.3 ± 1.8 "

In addition to glial proliferation and its enzymatic differentiation, maturation of the nerve cell and its axon is required before myelination begins (Murray, 1965; Hild, 1960). Myelination in cultures from neonatal rat brain was found in the present study, as well as by others (Hild, 1960; Murray, 1965, Lumsden, 1968) to be very similar in its structural and temporal development to myelination in vivo.

The observed radioresistance of myelination with increased age of used animal is apparently not due to a preferential preservation of nerve cells and their axons in cultures from older animals since normal nerve cells and axons were present in irradiated cultures irrespective of age of animal used. The other possible cause for the observed age dependent increase in radioresistance of myelination could be related to the increased density of enzymatically active glia in older animals. Oligodendrocyte are known to be radiosensitive (Brownson, et al., 1963a), and (DeVellis, 1968) found that radiation inhibits enzymes involved in synthesis of phospholipids which are needed for myelin formation. Explants taken from 1 day, 5 day and 8-day old animals are then in different stages of myelination gliosis, i.e. with different densities of oligodendrocytes, the tissue from 8-day olds having the highest glial cell density. Thus higher doses will be needed to eliminate and or metabolically damage enough oligoglia to inhibit myelination in explants from 8 day old animals than from 1-day olds. No statisti-

cally significant change in the radiosensitivity of myelination in vitro was found when cultures from 1-day old rat cerebella were compared with those from 5-day old animals. However, a statistically significant decrease in radiosensitivity of myelination in vitro was observed in cultures from 8-day old animals when compared to those from 1-day old animals. Associated with inhibition of myelination, a reduction in the density of small cells (includes oligoglia) was noticed in our cultures particularly those that received 4000 rads, and more markedly in cultures from 1-day old than in those from 8-day old animals. Besides, (Diller, et al. 1964) has shown that oligoglia radiosensitivity decreases with age. If this change in oligoglial radiosensitivity is operative in vitro then it may also contribute to the observed age dependent radioresistance of myelination.

IV GENERAL CONCLUSIONS

Nerve tissue cultures maintain structural and functional features that are characteristic of in vivo nerve tissue. Thus most cultures had many apparently healthy nerve cells and glia after about two weeks of incubation. Nissl substance, axons, synaptic boutons, myelin formation and fasciculation of nerve cell axons into bundles appeared to maintain their in vivo characteristics. Explants from newborn rat brain almost always successfully myelinated in vitro at about the same time myelination occurs in vivo. The myelin was apparently healthy and survived in most cases as long as the culture was fed regularly. Myelination was affected by both temperature of incubation and glucose concentration in the feeding medium. Functionally, nerve cells and glia displayed active RNA synthesis from labelled precursors and spontaneous bioelectric activity was exhibited by most nerve cells tested extracellularly. These evidence at the structural and functional level justify the use of nerve tissue cultures in exploring many of the structural and functional properties of in vivo tissue and its responses to the different environmental situations. The limitations of this in vitro model system of nerve tissue should however be realized and caution exercised in extending in vitro results directly to the in vivo situation. It is suggested that many neurobiological problems can benefit in their investigation by a combined use of in vivo and in vitro studies of this tissue.

Nerve tissue cultures offer certain advantages in the study of the effects of ionizing radiations on myelination. Blood vessels are sensitive to ionizing radiations, thus making it difficult for the investigator to assess the direct radiobiological response in vivo of nerve tissue cells involved in myelination. Since blood vessels and any preexisting myelin, irrespective of the age of animal used in preparing the explants, degenerate

upon explantation, nerve tissue cultures can be used to estimate the direct effects of ionizing radiations on myelination. The modification of such direct damage by radiation dose and age of used animal can be explored. The results of the present study suggest that the myelination process in vitro is directly damaged by ionizing radiations at doses comparable to those shown by other investigators to inhibit myelination in vivo. Such direct damage may occur in vivo in addition to indirect damage due to blood vessels injury, if any. Nerve cells seemed to be not involved in myelination damage in vitro. Morphologically, nerve cells and their axons seemed to remain, at the dose levels and ages of animals used, mostly healthy at 15 days postirradiation in cultures where myelination was inhibited by irradiation, (400-4000 rads). Electro-physiologically, most nerve cells tested were still exhibiting their spontaneous bioelectric activity 15 days after a dose of 4000 rads. The degree of damage to myelination (expressed as the number of myelinated explants at 15 days in vitro, compared to the total number of irradiated explants) was influenced by the radiation dose and age of used animal, thus corresponding to similar observations in vivo by other investigators. Our observations suggest, then, that the dose and age modifications of myelination damage in vitro are modifications of the direct injury to glial cells. Blood vessels may or may not be involved in the similar modifications observed in vivo.

Our radiation studies suggest the following explorations as an extension of the present research. If the nerve cells and axons in the radiation amyelinated cultures were found after several months of incubation, to be indeed free of any latent radiation damage, then the possibility of myelinating such cultures with added glial cells should be investigated. Should such remyelination occur, it may open new avenues in the search for treatment of delayed radiation necrosis and demyelinating diseases like multiple

sclerosis, where nerve cells and axons remain apparently undamaged in the fresh lesion, while glia are injured or absent.

Studies of the effects of very high LET radiations on nerve tissue could be made less complicated by using the blood vessel free tissue culture system. Such very high LET radiations occur in cosmic radiations in space and the stratosphere. The Hilac can provide beams of some heavy ions although their energies, fluxes and composition cannot be considered as truly representative of heavy ion cosmic radiations. An attempt was made in the present study to investigate the effects of heavy ion irradiations on myelin formation in nerve tissue cultures. The very short range of Hilac heavy ions in tissue limited this investigation to deuterium and helium ions. Carbon ions at high doses did not produce any apparent effect on myelination by 15 days postirradiation. It was observed, however, that about that time the cultures were essentially a monolayer in the large nerve cells, and hence the net culture thickness can be assumed to be between 30 and 60 μ . Such thickness can be penetrated by most Hilac heavy ions. In addition nerve tissue explants and cultures can be exposed (in a Rose Chamber assembly) in balloon flights to stratospheric cosmic radiations. Both these approaches, Hilac irradiations and balloon flights, may prove to be useful in expanding our understanding about some aspects of the effects of heavy ions in space on the brain.

V SUMMARY

The experimental findings of this work can be listed as follows:

A. Unirradiated Nerve Tissue Cultures

1. Nerve tissue cultures from neonatal rat brain can be maintained for several months under controlled environmental conditions.
2. Oxygen uptake of freshly dissected explants was approximately 1.25 $\mu\text{l/mg}$ wet weight/hour at 37°C.
3. Approximately 10% of the large nerve cells ($> 20 \mu$) originally present in the explant survived in vitro.
4. The density of small cells ranging in diameter between 5-15 μ (includes oligoglia) was approximately 2 ± 0.1 cells/400 μ^2 at 15 days in vitro.
5. The surviving large nerve cells appeared mostly normal at 15 days in vitro. Thus Nissl substance, axons and synaptic boutons were structurally normal. About 5% of surviving nerve cells were chromatolytic at 15 days in vitro.
6. By 15 days in vitro, the culture area has increased about 16 folds from the original explant area.
7. Nerve cell and glial maturation in vitro resulted in apparently normal myelination beginning at about ten days post-incubation, irrespective of age of animal used in preparing the explants. Upwards of 90% of all cultured explants showed abundant myelination by 15 days in vitro. The myelin survived as long as the culture was fed regularly. Myelination was usually observed in culture areas that had a high density of small cells.
8. Optimal myelination occurred at an incubation temperature of 36°C. A temperature of 30°C resulted in absence of myelination at 15 days in vitro. An incubation temperature of 32°C resulted in 20% of the incubated explants myelinating at 15 days in vitro, although after several more weeks

near optimal myelination was achieved.

9. Percent incidence of myelination at 15 days in vitro increased with glucose enrichment of the feeding medium and optimal myelination occurred at about 6.5 mg glucose/ml. of medium. Further increase of glucose concentration till 11.5 mg/ml. while it did not increase further the mean percentage of myelinated cultures compared to the total explanted it did not appear to inhibit myelination, at least during a one month period of observation.

10. Scanning electron microscopy of the cultures showed that nerve cells and glia and their processes retained much of their three dimensional shape inspite of the essentially two dimensional support used. Nerve fibres appeared to fasciculate into fibre bundles as in vivo.

11. Active synthesis of RNA from Uridine-¹⁴C was observed by whole mount autoradiography in many nerve cells, small cells (includes glia) and mesenchymal cells at 15 days in vitro. The grains, in all cases, were mostly over the nucleus.

12. Extracellular spontaneous bioelectric activity recorded in the vicinity of the soma was exhibited by most nerve cells tested in vitro. Spike frequencies of 50-500 per minute were most often found.

B. Irradiated Nerve Tissue Cultures

1. Exposure of freshly dissected explants (about 1mm. in thickness) to 4000 rads of any of these radiations: 145 Kvp x-rays, 36 Mev He ions, 54 Mev ions and 30 Mev deuterium ions inhibited myelination in all cultures at 15 days post irradiation. A marked reduction in the density of small cells (0.2 ± 0.1 cells/400 μ^2) was concomittantly observed in the whole culture area at 15 div. The irradiated culture area was only about 6 folds the original explant area. No recovery of myelination in such amyelinated

cultures was observed up to one month in vitro.

2. At the highest radiation doses used (4000 rads of either x-rays or particulate radiations) many nerve cells and axons in most cultures were still apparently structurally normal at 15 days post-irradiation.

3. Extracellular spontaneous bioelectric activity was retained by most nerve cells tested at 15 days in vitro after a dose of 4000 rads. Spike frequencies and pulse shape appeared to be not different from those of the controls.

4. A dose of more than 400 rads was needed to produce observable effect on per cent myelination incidence at 15 days in vitro.

5. Inbetween 400-4000 rads, the percent incidence of myelination varied according to the dose used. This presumably reflects the dose dependent radiation necrosis of oligoglia as observed in vivo by other investigators. Fifty percent myelination at 15 days in vitro was obtained by nearly similar doses of x-rays, deuterium ions, helium ions inspite of the difference in LET of these radiations and inspite of different dose rates used. The mean dose of ionizing radiations (x-rays and particulate radiations) that produced 50% myelination was 1727 ± 115 rads.

6. Radiation damage to myelination in vitro seemed to be age dependent. Thus the mean does that resulted in 50% of exposed explants to have myelin by 15 days in vitro was 1750 ± 120 rads and 2100 ± 160 rads for cultures from 1-day old and 8-day old animals respectively. The difference is statistically highly significant. It is suggested that the higher dose needed to depress the incidence of myelinated cultures from the older animals to the 50% level is due to the relatively higher density of glia in explants from such animals. Thus a relatively smaller dose will be required in the case of explants from 1 day old animals than in the case of explants from 8-day old animals in order to depress glial cell density (by metabolic

cell damage and or cell death) sufficiently to inhibit myelination. It is possible that increase in glial radioresistance with age is also a factor.

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