INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

Xerox University Microfilms
300 North Zeeb Road
Ann Arbor, Michigan 48106

THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

AN INVESTIGATION OF CHEMICALLY INDUCED MICROCEPHALY IN THE RAT

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

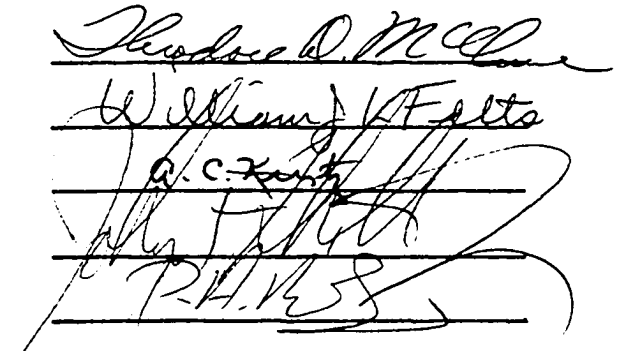
PHILIP MOSCA

Oklahoma City, Oklahoma

1974

AN INVESTIGATION OF CHEMICALLY INDUCED MICROCEPHALY IN THE RAT

APPROVED BY



DISSERTATION COMMITTEE

DEDICATED TO MY PARENTS:

Pasquale and Carmen Mosca

ACKNOWLEDGEMENTS

I am deeply indebted to Professor Theodore D. McClure, who directed this problem. His cooperation, criticisms, many suggestions, and ever helping hand during its planning and preparation, were essential for its successful completion.

I wish to express my appreciation to Drs. P. A. Roberts, J. F. Lhotka, A. C. Kurtz, and W. J. L. Felts for serving on my examination and reading committees.

I also express my gratitude to Dr. Thomas Briggs for his help and equipment for the thin layer chromatography, and to Dr. Jacqueline Coalson and Zelma Proctor for their preparation of experimental material for electron microscopic examination and photography. I also thank them for the use of their equipment without which the electron microscopic studies could not have been accomplished. In addition, special thanks are due to Peter Mosca, Dr. Robert Coalson and Dr. John Allison for their help and suggestions.

I wish to express my grateful appreciation to Dr. Alton C. Kurtz with whom I spent many hours discussing the biochemical aspects of this problem and to Dr. Fay Myers and Dr. Walter Joel for their review and comments concerning

pathological lesions seen in this study.

I would also like to especially express my deep appreciation to Elaine Rosinski for the many tiring hours she spent typing and proofreading this manuscript.

Special thanks are also due to Dr. W. J. L. Felts and the Department of Anatomical Sciences for making available the facilities, equipment, and supplies used in this study.

TABLE OF CONTENTS

LIST OF TABLES

AN INVESTIGATION OF CHEMICALLY INDUCED MICROCEPHALY IN THE RAT

CHAPTER I

INTRODUCTION

Microcephaly is a condition whose major characteristic is a disproportionately small cranium. It is clinically standardized by a cranial circumference less than three standard deviations below the normal for a particular age and sex (Farmer, 1964).

The etiologies of microcephaly, although numerous and varied, may generally be divided into three major categories: 1) genetic origin, currently believed to be due to one or more autosomal recessive genes (Brook, Schut and Reed, 1953; Davies and Kirman, 1962; Komai, Kisimoto and Ozaki, 1955; McKusic, Stauffer, Knox and Clark, 1966); 2) intra-uterine injury or disease, i.e., hypoxia, radiation, rubella, drugs, etc. (Brandon, Kirman and Williams, 1959; Farmer, 1964; Plummer, 1952); and 3) perinatal or postnatal disorders, e.g., cytomegalo virus (Farmer, 1964).

The histo-pathologic description of microcephaly varies according to the etiologic agents (Farmer, 1964);

however, the prototype description of Sachs (1887) has been relied upon heavily to this day (Farmer, 1964; Zeman, 1968).

Although known for the last few decades that ionizing radiation and nitrosourea compounds produce microcephaly in experimental animals, only with the discovery of methyl-azoxylmethanol (MAM), the aglycone of cycasin (Kobayashi and Matsumoto, 1965), could this condition be produced uniformly among litter mates (Spatz and Laqueur, 1968).

Spatz and Laqueur (1968) and Valsamis and Laqueur (1969) demonstrated that MAM injected intraperitoneally (IP) into female rats, at 15 days after conception, crossed the placental barrier, and produced a highly uniform microcephaly in all of the offspring. These microcephalic animals lived to an old age and did not display other malformations. Fertility of brother-sister matings of microcephalic rats was not impaired, and their offspring were indistinguishable from control animals. In 14 month old microcephalic rats the reduction of brain weight, approximately 30%, was essentially due to diminution of both cerebral hemispheres resulting in broad exposure of the corpora quadrigemina.

The aim of this research was to 1) define some of the factors of MAM induced microcephaly using anatomical (physical, radiological, histological and cytological) and biochemical (chromatographic and spectrophotometric) methods, 2) compare MAM induced microcephaly with some of those previously reported microcephalies induced by other

experimental means, and 3) propose a mechanism of action to account for the various structural and biochemical findings in MAM induced microcephaly.

CHAPTER II

MATERIALS AND METHODS

Care and Mating of Animals

The King-Holtzman hybrid strain of rats obtained from the Stanley-Gumbreck Colony (Introgene Foundation) were used in this study. The animals were routinely fed Purina rat chow and water ad. lib. and received standard animal house care.

Timed matings were used to determine gestational age of the specimens. The estrus cycle was determined in healthy female rats by daily examination of vaginal smears (Long and Evans, 1920). Females in late proestrus and early estrus were placed with males in the evening and examined the next morning for the presence of sperm in the vaginal canal. If sperm were present, the animal was assumed to have conceived and the age of the embryo was calculated from this time. No attempt was made to determine either the exact time of copulation or the exact time of fertilization. A total of 84 animals, 56 experimental and 28 controls, were used for this study.

Injection of the Pregnant Female and Care of the Offspring

The pregnant female was weighed and given a single

IP injection of MAM-acetate (20 mg/kg) on the fifteenth day of gestation. The pups were allowed to remain with the mother until killed. The litter size was kept at eight pups or less. Comparable saline injected litters were used for control animals.

Removal and Fixation of the Brains and Skulls

When the animal reached the desired age it was weighed and then killed by decapitation. In one group of animals the brain was immediately removed, weighed and either frozen or immersed in 10% neutral buffered formalin (NBF). In a second series, the removed head was hemisectioned to enable later histological study of the skull sutures. In these instances, the brain was removed from each hemisection, weighed and fixed in NBF along with the remaining hard tissue. In all cases the obtained brain weights were compared to predicted brain weights and the relative percentage recorded. The predicted brain weights were computed from the body weights according to the formula of Donaldson (1909) as modified on Page 10.

Radiologic Studies

At seven day intervals from birth through 56 days, and again at nine months, two experimental and two control animals, randomly selected, were weighed and x-rayed. The animals were anesthetized with ether and placed directly on the film cassette. A standard dental x-ray machine (Ritter)

was employed with the cone six inches from the plate with the exposure set at 63 KV for 0.8 seconds. Both posteroanterior and lateral skull films were thus obtained. Both serial and longitudinal studies were performed. All animals were then weighed, killed and processed according to the prescribed method.

Measurements on the lateral films were done according to Spence (1969), with the following measurements recorded: the occipital-nasal length (ON), the crown-maxillary height (CM), the crown-mesial cusp of first molar height (CD), and the occipital-mesial cusp of first molar length (OD). On the posteroanterior films the maximal width of the parietal bones (MPW) was recorded (Fig. 1).

Histological and Cytological Studies

Formalin fixed brains were prepared in the conventional manner for paraffin embedding. The material was sectioned at 11 μ and mounted from a water bath to albuminized slides. The tissues were stained using Hematoxylin and Eosin (H & E) as well as Periodic Acid Schiff (PAS) (Davenport, 1960), Cresyl Violet Acetate (CVA), Weil (Clark and Clark, 1971), Luxol Fast Blue (LFB) and Holtzer's Fibrous Glial stain (Drury and Wallington, 1967).

Sutures from skulls were removed along with the immediate surrounding bone. After hard tissue treatment with Cal-Ex (Fisher), they were processed for routine paraffin sections. These paraffin sectioned tissues (10 μ) were

stained with H & E and with Azure A according to the method of Kramer and Windrum (1955). All photomicrographs were taken on an Olympus KH super wide microscope with the PM-10 35 mm camera.

Portions of tissue from the body of the corpus callosum were removed from one experimental and one control brain under the dissecting microscope and prepared for electron microscopic (EM) examination. These sections were examined and photographed using an RCA model III electron microscope.

Biochemical Analyses

Water and Lipid Determinations

A series of control and experimental brains, which had previously been frozen, were hemisectioned along the midsaggital plane. Each hemisection was placed above Dririte in a dessicator at 56°C. These hemisections were weighed twice daily until a constant weight was obtained.

For other analyses each portion of brain was then extracted with 2:1 chloroform-methanol (Cl-Me) in a Vir Tiss tissue homogenizer. The Cl-Me mixture was subsequently diluted to 20:1 (volume-weight). The homogenate was filtered through tared fat free filter paper and the insoluble tissue was again placed into closed containers above Dririte at 56°C until they reached a constant weight.

Thin Layer Chromatography

One complete lipid extracted hemisection (cerebrum

and brain stem) was evaporated to approximately one milliliter. Portions of this milliliter of lipid extract were then separated by thin layer chromatography according to Freeman and West (1966). The spots were visualized using iodine vapor and photographed. Drawings were made from the photographed plates.

Spectrophotometric Analysis

The tube containing the lipid extract of the second hemisection, contained 0.05 gm lipid tissue in one milliliter of extract. One tenth of a milliliter of extract was then removed and total cholesterol was determined by the modified Zlatkis method (Frankel, Rectman and Sonnewirth, 1963).

The remaining portion of the tube of extracted total lipid was then partitioned using a 0.2 volume of 0.9% NaCl, hence, Folch conditions were established (Folch, Lees and Stanley, 1956). After centrifugation, upper and lower phases were separated. The upper phases were discarded and the lower phase was diluted by 10:1 (V:V) with prepared lower phase. Cerebrosides were determined in the following manner based on the micromethod of Hess and Lewin (1965). Only relative spectrophotometric absorbtion of cerebrosides was determined, so, standard galactose solution was not required. The orcinol-sulfuric acid reagent was prepared according to Hess and Lewin (1965) just before use. Two milliliters of lower phase extract were placed in a clean

test tube into which 5 milliliters of orcinol-sulfuric acid reagent was added. The solutions were mixed well and samples and blanks (two milliliters lower phase and five milliliters orcinol-sulfuric acid) were heated together in a boiling water bath. After exactly 25 minutes the reaction was stopped by transfering to an ice-water bath. The sample was then centrifuged at 2,400 rpm for three minutes. The supernatant was poured into matched cuvettes and read in a Beckman Jr. Spectrophotometer at 425 μ, with the blank set at 100% transmission. The biochemical procedures are summarized in Figure 2.

CHAPTER III

RESULTS

Body and Brain Weight Studies

The body weights of the experimental animals were 74% of the control animals (2-3 gms) at seven days of age. This difference declined to 65% of control values at 28 days. After 28 days the difference in body weights became progressively less until approximately 60 days when both sets of animals had the same average body weights (Fig. 3).

The brain weights, which are a function of body weights, may be predicted using the formula of Donaldson (1909). Donaldson's formula

$$\text{Brain weight} = 0.567 \log (\text{Body weight} - 8.7) + 0.55$$

$$(\text{Body weight} \geq 15 \text{ gms})$$

was consistently 10% high for our control animals. Therefore, the formula was modified as follows:

$$\text{Brain weight} = 0.9 \left[0.567 \log (\text{Body weight} - 8.7) + 0.55\right].$$

Using the modified formula, the observed brain weight equals the predicted brain weight for any control animal whose body weight is 15 grams or greater.

At fourteen days the brain weight of the experimental

animals was 93% of the value predicted using the modified formula. This difference (observed-predicted) decreases to approximately 86% and remained at that level through 56 days (Figs. 4 and 5).

Radiological Studies

The radiological data obtained paralleled that of the physical findings. The average OD length at seven days of age was 81% of that of the control. Similarly the CD and MPW measurements were 70% and 85% respectively of the control values. By 35 days the OD length was 93% of the control value; the CD and MPW measurements were 94% and 90% respectively. At nine months of age the OD and CD lengths were equal to or greater than that of the controls. However, the MPW measurement never rose beyond 96% of the control value. The skull measurements are shown in Table 1.

Histological and Cytological Studies

The most striking difference between the control and experimental brains was the generalized immature appearance of the experimental brains, regardless of their postnatal age. This immaturity was reflected in assorted anatomical configurations. There was a relative and absolute decrease in the outer molecular layer of the cerebral cortex. This decrease in the depth of the molecular layer was accompanied by a relative disorganization in the underlying cell layers (Figs. 6 and 7). Other distinct morphological abnormalities

TABLE 1

RADIOLOGIC CEPHALOMETRIC MEASUREMENTS

Age	ON		OD		CD		CM		MPW	
	C	E	C	E	C	E	C	E	C	E
7	24.0	19.0	16.5	13.5	8.5	5.0	15.5	7.0	14.0	12.0
14	27.5	26.0	19.0	17.5	9.0	8.0	15.5	14.0	14.0	13.0
21	31.0	29.0	20.5	20.5	9.0	8.0	15.5	14.5	14.5	13.5
28	33.0	31.0	22.0	21.0	9.5	9.0	15.5	15.5	15.0	14.0
35	36.5	33.0	23.5	22.0	9.5	9.0	17.5	16.0	15.0	14.5
42	36.5	35.5	24.5	23.5	10.0	9.0	17.5	17.5	16.0	15.5
49	37.0	36.5	25.0	24.5	10.0	9.5	18.0	18.0	16.0	15.5
56	37.5	-	25.0	-	10.5	-	18.5	-	16.5	-
Adult	45.0	47.0	28.0	29.5	11.5	11.5	21.5	22.5	17.0	15.5

C - Control (18 animals)

E - Experimental (18 animals)

were seen. There was marked clumping of neurons. This clumping was an abnormal "disorganized" histological pattern (Fig. 8). In the hippocampus there was a similar degree of disorganization and the overall neuronal population was reduced (Figs. 9 and 10).

In addition to the previously described cerebral cortical changes, one striking histological phenomenon was identified, however, not consistently. "Rosettes" scattered throughout the cortex were found in two experimental brains and are histologically similar to those described by Hicks, et. al., in 1959 (Fig. 11).

In most aspects the cerebellum appeared to be differentiating normally. The disappearance of the external granular layer occurred between the fourteenth and the twenty-first day postnatally (Fig. 12). However, a definite cerebellar abnormality was evident in older animals. It appeared that Bergman's astrocytic layer was severely depleted or completely absent. Furthermore, the Purkinje cells in the experimental brains appeared smaller, slightly disorganized, more hyperchromatic, and more immature than controls of the same age (Figs. 13 and 14).

In summary, there was: 1) disorganization and neuronal clumping in the cerebral cortex, 2) reduced numbers of neurons in the cerebral cortex, 3) "rosettes" in the cerebral cortex, 4) morphological changes in the cerebellum, and 5) a generalized immature-looking histological pattern.

The preliminary electron microscopic study, although limited, has yielded some interesting data. Electron micrographs show large amounts of artifactual shrinkage; however, this was to be expected using non-perfused, formalin material. The experimental plates show considerably fewer numbers of myelinated axons (Figs. 15 and 16). In addition, Type C myelin sheaths (Hirano and Dembitzer, 1967), with islands of cytoplasm sandwiched between the myelin lamellae, were evident in the experimental sections but not in the control sections (Fig. 17). One additional myelin pattern of unknown significance was noted only in the experimental material. This configuration is seen as the surrounding of two axis cylinders by what appears to be a single set of myelin lamellae in a figure-eight arrangement (Figs. 18 and 19). Whether this alteration was artifact or induced structural change needs further investigation.

Histological examination of the saggital and lambdoidal sutures of the cranial vault shows no evidence of premature closure. The histological findings confirmed the radiological impression that premature craniosynostosis did not exist.

Biochemical Analyses

Water determinations on experimental and control brains showed some slight differences. The water percentage wet weight decreased continuously from 7 to 59 days in both experimental and control animals. However, the percentage

of water was greater in experimental animals than in control animals by 4-8% regardless of age. The percentage difference in the water values increased between 21 and 42 days, and by 57 days this percentage decreased to previous levels. The values are summarized in Table 2.

Total lipid percentage of dry weight, showed a steady rise as expected from 7-56 days. Again, however, the experimental animals showed a "time lag" behind the control animals. The percent of lipid in the experimental brain ultimately rose to within 2% of the control values at 57 days. The values are summarized in Table 2.

Thin Layer Chromatography

Experimental brain extract, control brain extract, and control lipids were chromatographed. Various quantities ranging from 50 µl to 250 µl of concentrated lipid were used. The results of each run appeared qualitatively identical. Rough quantitative measurements (diameter of the visualized spots) yielded data suggestive of no gross lipid disturbances (Fig. 20). The inference here was that even though there was less lipid in the experimental brain, the lipid composition was normal.

Spectrophotometric Methods

Cholesterol and cerebroside determinations between experimental and control animals were nearly identical at all postnatal ages. The values for cholesterol never varied

TABLE 2

WATER AND LIPID DETERMINATIONS

Number of Animals	Age in Days	% of Water (Wet Weight)		% of Lipid (Dry Weight)	
		C	E	C	E
4	7	89%	89%	28%	27%
4	14	84%	88%	30%	27%
4	21	80%	86%	31%	28%
2	32	-	86%	-	30%
2	35	78%	-	31%	-
4	42	77%	84%	33%	31%
4	56	76%	80%	35%	33%

C - Control

E - Experimental

further than 2-4 mg%. In view of the experimental method this small difference cannot be considered significant. Similarly the absorbtion spectra for cerebroside determinations were almost identical and, therefore, cannot be considered significant.

CHAPTER IV

DISCUSSION

Transplacentally induced uniform microcephaly in the rat, using a single intraperitoneal injection of MAM, has been demonstrated by a number of authors (Spatz and Laqueur, 1968; Valsamis and Laqueur, 1969; and Haddad, Rabe and Dumas, 1972). Transplacental microcephaly has also been demonstrated with MAM in other species of animals (Haddad, Rabe and Dumas, 1972). A variety of other agents, including ionizing radiation, methyl nitrosourea, hypoxia, rubella and ethyl nitrosourea, have also been shown to be etiologic agents of microcephaly (Brandon, Kirman and Williams, 1959; Farmer, 1964; Plumber, 1952; Haddad, Rabe and Dumas, 1972). Among etiologic agents of microcephaly it has been claimed that MAM produced microcephaly, although not qualitatively unique (similar brain aberrations have been produced using a wide variety of etiologic agents), seemed to be quantitatively quite specific in that it did not produce a generalized inhibition of body growth (Haddad, Rabe and Dumas, 1972).

Although transplacental induction of microcephaly with MAM produced a uniform lesion, according to this study

part of the observed diminished brain weight ostensibly was due to the early generalized inhibition of body growth. As Donaldson (1908, 1909, 1911) discovered over 60 years ago, the relationship of brain size to body size was almost unalterable throughout postnatal life, but that any early influence which lead to a small mature body also lead to a small mature brain. In addition, the optimal time for producing lasting effects on growing rats was during the period of fastest brain growth. The period of fastest brain growth in the rat is during the suckling period (Davison and Dobbing, 1968). The MAM insult to the developing fetus was, therefore, not at the optimum time for lasting brain growth retardation. However, due to the early somatic growth retardation, the MAM induced microcephaly was not as severe as it first appeared. In nutrition studies undernutrition of the adult organism "spares" the adult brain (Dobbing, 1968). In addition, experimental undernutrition studies showed both body and/or brain size can be effected to a lesser or greater extent depending on the time and severity of the insult. Furthermore, in these same studies (Dobbing, 1968; Dobbing and Sands, 1971), the amount of whole body "catch-up" and/or brain "catch-up was also dependent upon the time and severity of the insult.

In this experiment whole body "catch-up" was complete by 60 days of age but total brain "catch-up was never fully effected. This disparity between body and brain "catch-up"

might be explained by the actions of the MAM itself. MAM is soluble in aqueous solutions and reactive by methylating bases of ribonucleic acid (RNA) and deoxyribonucelic acid (DNA) both *in vitro* (Matsumoto and Higa, 1966) and *in vivo* (Shank and Magee, 1967). It has been shown to be a mutagen (Smith, 1966; Teas and Dyson, 1967), and a teratogen (Spatz, Dougherty and Smith, 1967), and its general carcinogenic properties have been established (Laqueur and Matsumoto, 1966; Laqueur, McDaniel and Matsumoto, 1967). Due to the varied effects of this very active biological compound it seems reasonable to assume that MAM might methylate either the structural and/or regulator genes of a variety of developing and/or mature cells in the rat central nervous system (CNS) at the time of injection. Assuming this, the effects on the coding properties of the methylated bases might be expressed any time during the subsequent development of the CNS. Furthermore, the effects might be either mild (e.g., resulting in a lowered activity for any particular enzyme) or severe (i.e., resulting in a cellular death). Transplacental methylation of guanine in both DNA and RNA in fetal brain, does occur with a single IP injection of radioactively labeled MAM. However, the protein reaction products of these methylated nucleic acids were not determined (Nagata and Matsumoto, 1969). It is interesting to note that the coding properties of 7-methylguanine have been studied and that due to the steric effects of the 7-methyl

groups, contrary to earlier expectations, these polymers do not permit the incorporation of amino acids whose codons contain the bases uracil and adenine (Whilhelm and Ludlum, 1966).

If one examines the data produced in this study, in light of the previously discussed possible mutagenic mechanisms, bearing in mind the concepts of experimental embryology and our current knowledge of central nervous system embryo-histogenesis, a foreseeable pattern manifests itself. Since the time of Spemann, one of the major axioms of embryogenesis has been the concept based on the "sequential development in time" of any particular organ or organ part. That is to say, in order for "B" to develop it must follow the development of "A". Furthermore, not only must "B" follow "A", it must do so within a specific time period. It has also been known for the last few decades that cells undergoing active differentiation, migration, and/or mitosis are particularly vulnerable to noxious substances. Hicks, D'Amato, and Lowe (1959) showed that on neonatal day 15 in the rat, those structures most effected by 200 rads of irradiation were the cerebral cortex and to a lesser extent, the retina. In this and other studies, (Hicks, 1954, 1958; Hicks, et. al., 1957) defects of the skull, viscera, brain stem and spinal cord caused by irradiation were all produced at times earlier in neonatal development than 15 days.

In this series of experiments the MAM insult was

administered to the embryos at 15 days. The resulting gross appearance of the brain with its small cerebral hemispheres was almost indistinguishable from brains irradiated on the same developmental day. In addition, the neuronal decrease seen in this MAM study was comparable with irradiation studies. Furthermore, disorganization of the hippocampal neurons as well as the cerebral "rosette" formation have their structural counterparts in irradiation microcephaly. As stated earlier, the retina is susceptable to irradiation at neonatal day 15 (Hicks, et. al., 1959). Haddad, Rabe, Laqueur, Spatz and Valsamis showed in 1969 that MAM administration at developmental day 15, in fact, did cause retinal damage. Therefore, one of the actions of MAM appears to be on those cells forming the anlage of the cerebral cortex and retinal blastema.

In this study it was shown that MAM produces an early generalized somatic growth retardation and that generalized whole body "catch-up" was effected by 60 days (Fig. 3). In light of the fact that most somatic components have passed their embryonic "critical periods" by developmental day 15, this was not surprising. In light of the proposed actions of MAM, the radiologic data presented in this study was consistent. The neurocranial development in the rat proceeds by two important processes designated as transformative growth processes and translative growth processes. Transformative growth is defined as the

deposition and resorbtion of calvarial bones in response to periosteal functional matricies (i.e., genetically predetermined). Translative growth is that process in which the spatial relocation of calvarial bones is a secondary response to the primary volumetric changes of the neural mass (Moss, Meeham and Salentijn, 1972; Vilmann, 1972; Young, 1959). The fact that MAM treated animals shows no gross cranial skeletal abnormalities at birth has been explained previously (in essence it was because the transformative growth processes were relatively uneffected). However, the data as presented in this study indicated that although the linear and vertical dimensions of the skull eventually effected a "complete catch-up", the maximal parietal width never achieved control values. This parietal narrowing was a direct result of the translative cranial growth processes in response to the reduced cerebral size.

In regard to the biochemical data presented in this study concerning the effects of MAM on lipid metabolism and myelinization, the following general concepts of myelinization in the rat brain must first be taken into consideration. Active myelinization in the rat is a postnatal event occuring primarily between 14-23 days. During myelinization the percent of water in the brain decreases while the percent of lipids increases. During myelinization the percent of cholesterol also shows a decrease while cerebroside percentage increases (Norton and Poduslo, 1973; Davison and Dobbing,

1968; Dobbing, 1968; Dobbing and Sands, 1971; Benton, Moser, Dodge and Carr, 1966; Cuzner and Davison, 1968). Due to the fact that myelinization is primarily a postnatal phenomenon, any substance which would interfere with this process would be expected to exert its effects at this time. If MAM were to alter either structural and/or regulator genes at the time of its administration, the effects on myelinization per se might be masked until approximately 14 days postnatally. The increased percentage of water and the decreased percentage of lipid, which first became noticeable at 14 days, seemed to substantiate this fact. In addition, close scrutiny of the observed-predicted brain weight curves (Fig. 4) also indicated the greatest disparity during the "post-myelinization onset" period. Precisely why the cerebroside and cholesterol determinations did not substantiate this data is unknown. Explanations for this disparity would have to include discrepancies too small for these methods to detect. The fact that chromatographic separations of control and experimental brain lipids showed essentially identical composition was not unexpected. The fact that MAM effect on the enzyme systems might be quantitative rather than qualitative is wholly within reason. It must also be remembered that neuronal axons are the structures which are myelinated and since there appears to be fewer neurons, there should, therefore, be fewer myelin sheaths. Although preliminary and scant, the electron microscopic aberrations

substantiated the hypothesis that myelinization was affected by prenatal MAM administration.

Cerebellar damage by prenatal MAM administration has not been previously reported. The significance of finding reduced numbers of Bergman's cells in the experimental animals needs further investigation. The only assumption that could be made at this time is that Bergman's cells are either undergoing active differentiation or migration at the time of MAM administration. However, the cell reduction was consistent, marked and apparently caused by MAM administration.

CHAPTER V

SUMMARY AND CONCLUSIONS

In a series of experiments MAM induced microcephalic rats were examined by anatomical and biochemical procedures. A series of specimens was obtained from animals ranging in age from birth to nine months. These tissue samples were examined using radiologic, histologic, cytologic, chromatographic, and spectrophotometric techniques.

The following are the most significant points of this study.

(1) MAM induced microcephaly was accompanied by early somatic growth retardation.

(2) There appears to be fewer neurons and clumping of neurons in the cerebral cortex of MAM treated animals.

(3) MAM induced microcephaly also exhibits previously unreported suppression of Bergman's cells in the cerebellum.

(4) Lipid and water disturbances appear to be associated with MAM induced microcephaly but do not appear to be related to cholesterol and cerebrosides.

(5) MAM induced microcephaly most closely resembled microcephaly produced by irradiation rather than any other

etiologic agent.

(6) A hypothesis was presented to account for some of the structural and biochemical aberrations seen in MAM induced microcephaly.

BIBLIOGRAPHY

Benton, J. W., Moser, H. W., Dodge, P. R. and Carr, S. 1966 Modification of the schedule of myelination in the rat by early nutritional deprivation. Pediatrics, 38:801-807.

Brandon, M. G. W., Kirman, B. H. and Williams, C. E. 1959 Microcephaly in one of monozygous twins. Arch. Dis. Childhood, 34:56-59.

Brook, J. A., Schut, J. W. and Reed, S. C. 1953 A clinical and genetical study of microcephaly. Am. J. Ment. Deficiency, 57:637-660.

Clark, G. and Clark, M. P. 1971 A Primer in Neurological Staining Procedures. Charles C. Thomas, Springfield, Illinois, pp. 28-37.

Cuzner, M. L. and Davison, A. N. 1968 The lipid composition of rat brain myelin and subcellular fractions during development. Biochem. J., 106:29-34.

Davenport, H. A. 1960 Histological and Histochemical Technics. W. B. Saunders Company, Philadelphia, pp. 229-230, 373-374.

Davies, H. and Kirman, B. H. 1962 Microcephaly. Arch. Dis. Childhood, 37:623-627.

Davison, A. N. and Dobbing, J. 1968 The developing brain. In: Contemporary Neurology Series. Applied Neurochemistry, (eds.) Davison, A. N. and Dobbing, J. F. A. Davis Company, Philadelphia, pp. 253-286.

Dobbing, J. 1968 Volnerable periods in the developing brain. In: Contemporary Neurology Series. Applied Neurochemistry, (eds.) Davison, A. N. and Dobbing, J. F. A. Davis Company, Philadelphia, pp. 287-315.

Dobbing, J. and Sands J. 1971 Vulnerability of developing brain. Biol. Neonate, 19:363-378.

Donaldson, H. H. 1908 A comparison of the albino rat with man in respect to the growth of the brain and of the spinal cord. J. Comp. Neurol. Psychol., 18:345-393.

Donaldson, H. H. 1909 On the relation of the body length to the body length to the body weight and to the weight of the brain and of the spinal cord in the albino rat. J. Comp. Neurol. Psychol., 19:155-167.

Donaldson, H. H. 1911 President's address. J. Nerv. and Ment. Dis., 38:257-266.

Drury, R. A. B. and Wallington, E. A. 1967 Carlton's Histological Technique. Oxford University Press, New York, pp. 266-267 and 274-275.

Farmer, Thomas W. 1964 Pediatric Neurology. Hoeber Medical Division, Harper and Roe, New York, pp. 176-183.

Folch, J., Lees, M. and Stanley, G. H. S. 1957 A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem., 226: 497-509.

Frankel, S., Rectman, S. and Sonnewirth, A. C. 1963 Clinical Laboratory Methods and Diagnosis. A Textbook on Laboratory Procedures and Their Interpretation, Vol. 1, C. V. Mosby, St. Louis, pp. 257.

Freeman, C. P. and West, D. 1966 Complete separation of lipid classes on a single thin-layer plate. J. Lipid Res., 7:324-327.

Haddad, R. K., Rabe, A. and Dumas, R. 1972 Comparison of effects of methylazoxymethanol acetate on brain development in different species. Fed. Proc., 31:1520-1523.

Haddad, R. K., Rabe, A., Laqueur, G. L., Spatz, M. and Valsamis, M. P. 1969 Intellectual deficit associated with transplacentally induced microcephaly in the rat. Science, 163:88-90.

Hess, H. H. and Lewin, E. 1965 Microassay of biochemical structural components in nervous tissues - II. J. Neurochem., 12:205-211.

Hicks, S. P. 1954 Effects of ionizing radiation, certain hormones and radiomimetic drugs on the developing central nervous system. J. Cell and Comp. Physiol., 43:151-178.

Hicks, S. P. 1958 Radiation as an experimental tool in developmental neurology. Physiol. Rev., 38:337-356.

Hicks, S. P., Brown, B. L. and D'Amato, C. J. 1957 Regeneration and malformation in the nervous system, eye and mesenchyme of the mammalian embryo after radiation injury. Am. J. Path., 33:459-481.

Hicks, S. P., D'Amato, C. J. and Lowe, M. J. 1959 The development of the mammalian nervous system. J. Comp. Neurol., 113:435-469.

Hirano, A. and Dembitzer, H. 1967 A structural analysis of the central nervous system. J. Cell Biol., 34:555-567.

Kobayashi, A. and Matsumoto, H. 1965 Studies on methylazoxymethanol, the aglycone of cycasin. Isolation, biological, and chemical properties. Arch. Biochem. Biophys., 110:373-380.

Komai, T. Kishimoto, K. and Ozaki, Y. 1955 Genetic study of microcephaly based on Japanese material. Am. J. Human Genet., 7:51-65.

Kramer, H. and Windrum, G. M. 1955 The metachromatic staining reaction. J. Histochem., 3:227-237.

Laqueur, G. L. and Matsumoto, H. 1966 Neoplasms in female Fischer rats following intraperitoneal injection of methylazoxymethanol. J. Nat. Cancer Inst., 37: 217-232.

Laqueur, G. L., McDaniel, E. G. and Matsumoto, H. 1967 Tumor induction in germfree rats with methylazoxymethanol (MAM) and synthetic MAM acetate. J. Nat. Cancer Inst., 39:355-371.

Long, J. A. and Evans, H. M. 1920 The oestrus cycle in the rat. (Abstract) Anat. Rec., 18:241-244.

Matsumoto, H. and Higa, H. H. 1966 Studies on methylazoxymethanol, the aglycone of cycasin: Methylation of nucleic acids *in vitro*. Biochem. J., 98:20c-22c.

McKusic, V. A., Stauffer, M., Knox, D. L. and Clark, D. B. 1966 Chorionopathy with hereditary microcephaly. Arch. Oph., 75-597-600.

Moss, M. L., Meehan, M. A. and Salentijn, L. 1972 Transformative and translative growth processes in neurocranial development of the rat. Acta Anat., 81:161-182.

Nagata, Y. and Matsumoto, H. 1969 Studies on methylazoxymethanol: Methylation of nucleic acids in the fetal rat brain. Proc. Soc. Exper. Biol. and Med., 132: 383-385.

Norton, W. T. and Podulso, S. E. 1973 Myelination in rat brain: changes in myelin composition during brain maturation. J. Neurochem., 21:759-773.

Plummer, G. 1952 Anomalies occurring in children exposed in utero to the atomic bomb in Hiroshima. Pediatrics, 10:687-693.

Sachs, B. 1887 On arrested cerebral development with special reference to its cortical pathology. J. Nerv. and Ment. Dis., 9 and 10:541-553.

Shank, R. C. and Magee, P. N. 1967 Similarities between the biochemical action of cycasin and dimethylnitrosamine. Biochem. J., 105:521-527.

Smith, D. W. E. 1966 Mutagenicity of cycasin aglycone (methylazoxymethanol), a naturally occurring carcinogen. Science, 152:1273-1274.

Spatz, M., Dougherty, W. J. and Smith, D. W. E. Teratogenic effects of methylazoxymethanol. Proc. Soc. Exper. Biol. and Med., 124-476-478.

Spatz, M. and Laqueur, G. L. 1968 Evidence for transplacental passage of the natural carcinogen cycasin and its aglycone. Proc. Soc. Exper. Biol. and Med., 127:281-286.

Spence, J. M. 1940 Method of studying the skull development of the living rat by serial cephalometric roentgenograms. Angle Orthodontist, 10:127-139.

Teas, H. J. and Dyson, J. G. 1967 Mutation in Drosophila by methylazoxymethanol, the aglycone of cycasin. Proc. Soc. Exper. Biol. and Med., 125:988-990.

Valsamis, M. P. and Laqueur, G. 1969 Neuropathologic findings in methylazoxymethanol induced microcephaly in rats. J. Neuropath. and Exper. Neurol., 28:177.

Vilmann, H. 1972 The growth of the cranial vault in the albino rat. Arch. Oral Biol., 17:399-414.

Wilhelm, R. C. and Ludlum, D. B. 1966 Coding properties of 7-methylguanine. Science, 153:1403-1405.

Young, R. W. 1959 The influence of cranial contents on postnatal growth of the skull in the rat. Am. J. Anat., 105:383-415.

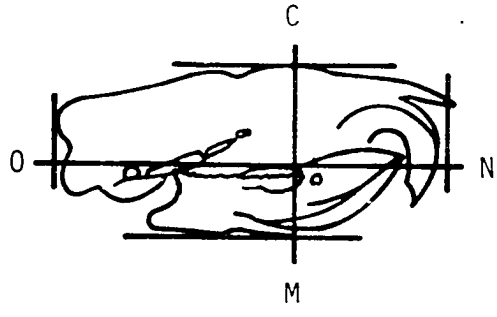
Zeman, W. 1968 The effects of atomic radiations. In: Pathology of the Nervous System, Vol. 1, (ed.) J. Minckler. McGraw-Hill Book Company, New York, pp. 864-939.

APPENDIX

PLATE I

Figure 1. Schematic radiographic tracings showing coordinates for cephalometric measurements. (A) lateral view; (B) posteroanterior view.

PLATE I



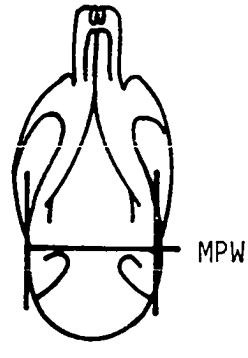


PLATE II

Figure 2. Schematic representation summarizing the major steps in the biochemical analyses.

PLATE II

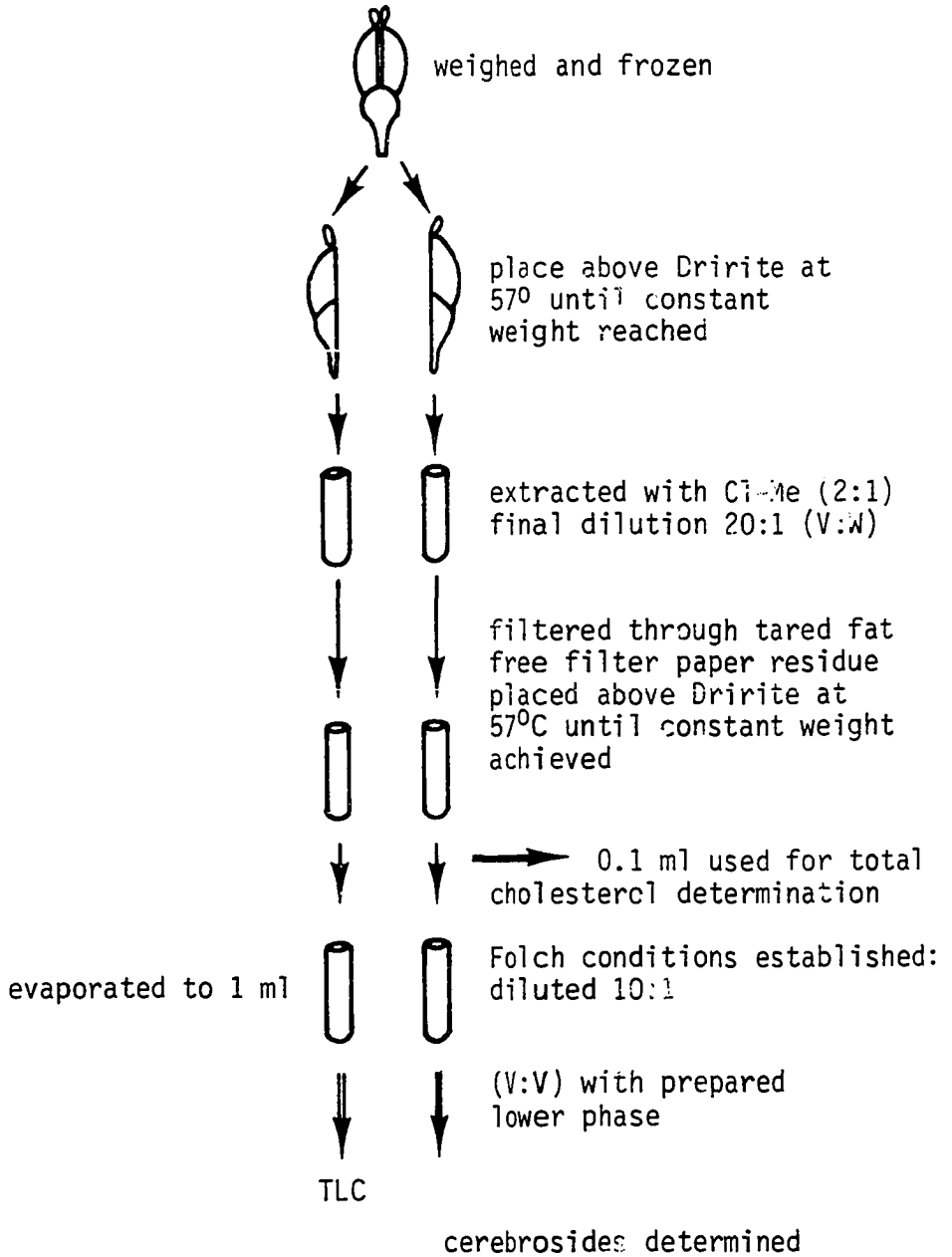

weighed and frozen
place above Drierite at 57° until constant weight reached
extracted with Cl-Me (2:1) final dilution 20:1 (V:W)
filtered through tared fat free filter paper residue placed above Drierite at 57°C until constant weight achieved
0.1 ml used for total cholesterol determination
Folch conditions established: diluted 10:1
(V:V) with prepared lower phase
evaporated to 1 ml
TLC
cerebrosides determined

PLATE III

Figure 3. Graph depicting the average body weights for control and experimental animals according to postnatal age.

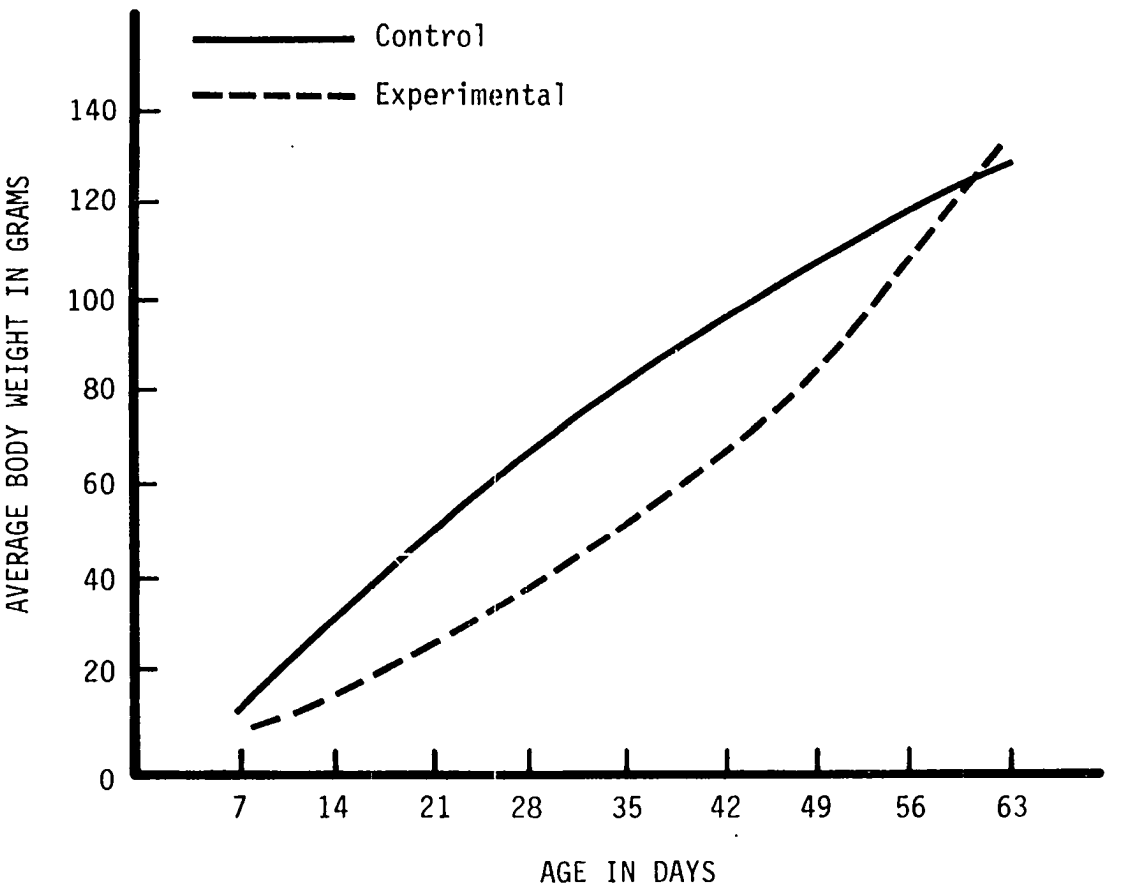

Control
Experimental
AVERAGE BODY WEIGHT IN GRAMS
0
20
40
60
80
100
120
140
7
14
21
28
35
42
49
56
63
AGE IN DAYS

PLATE III

PLATE IV

Figure 4. Graph representing observed-predicted brain weights in control and experimental animals according to age.

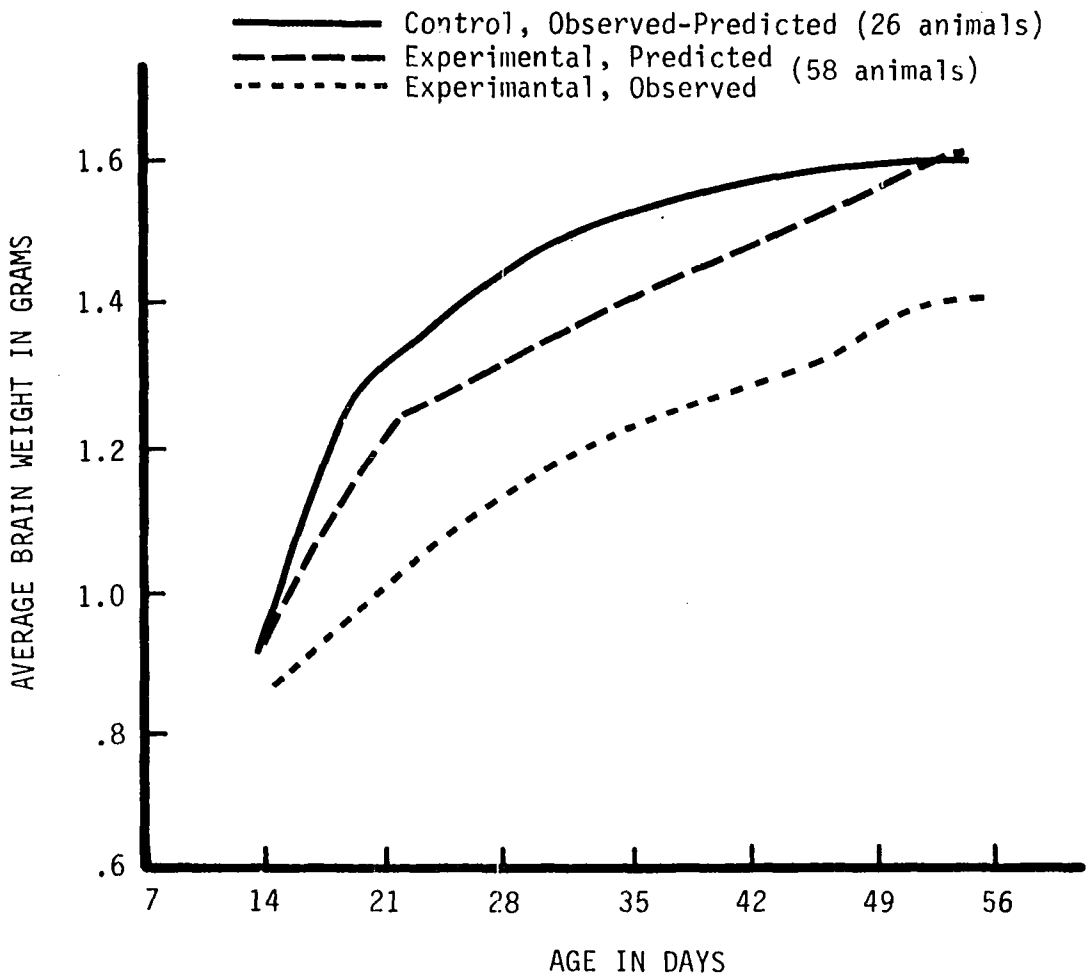

Control, Observed-Predicted (26 animals)
Experimental, Predicted (58 animals)
Experimantal, Observed
AVERAGE BRAIN WEIGHT IN GRAMS
1.6
1.4
1.2
1.0
.8
.6
7
14
21
28
35
42
49
56
AGE IN DAYS

PLATE IV

PLATE V

Figure 5. Photograph showing removed brains of control (C) and experimental (E) animals at 35 days of age. 3.8X

PLATE V

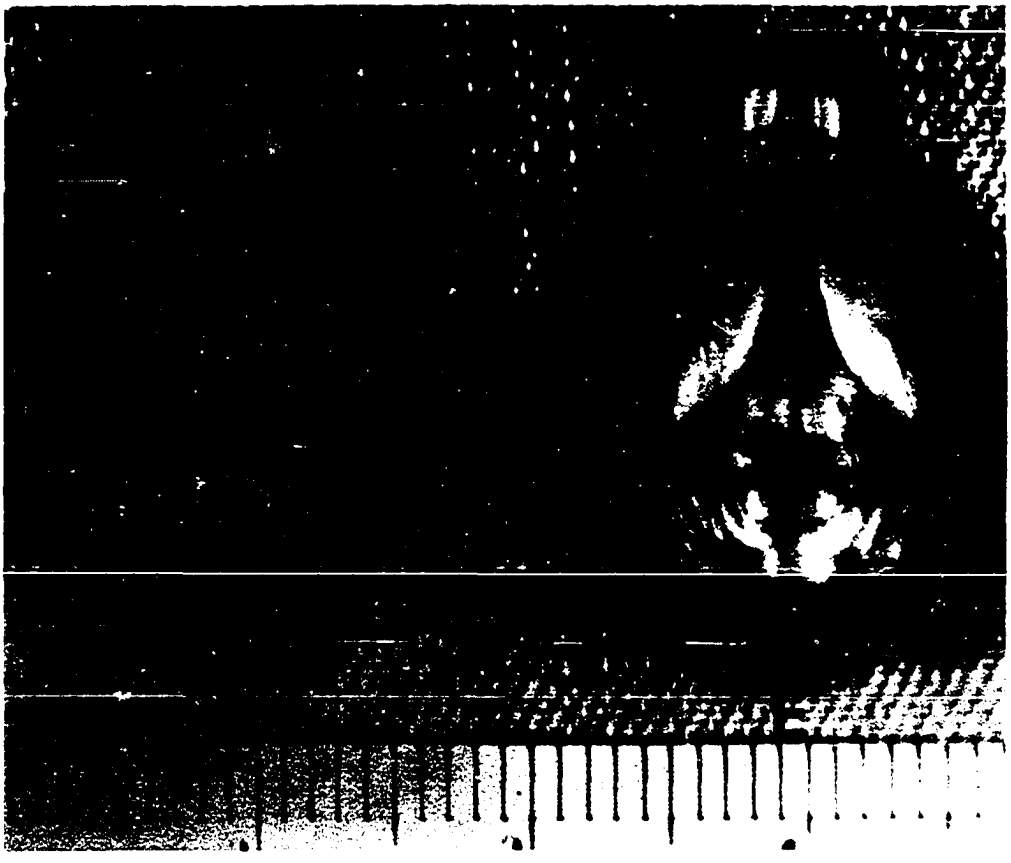
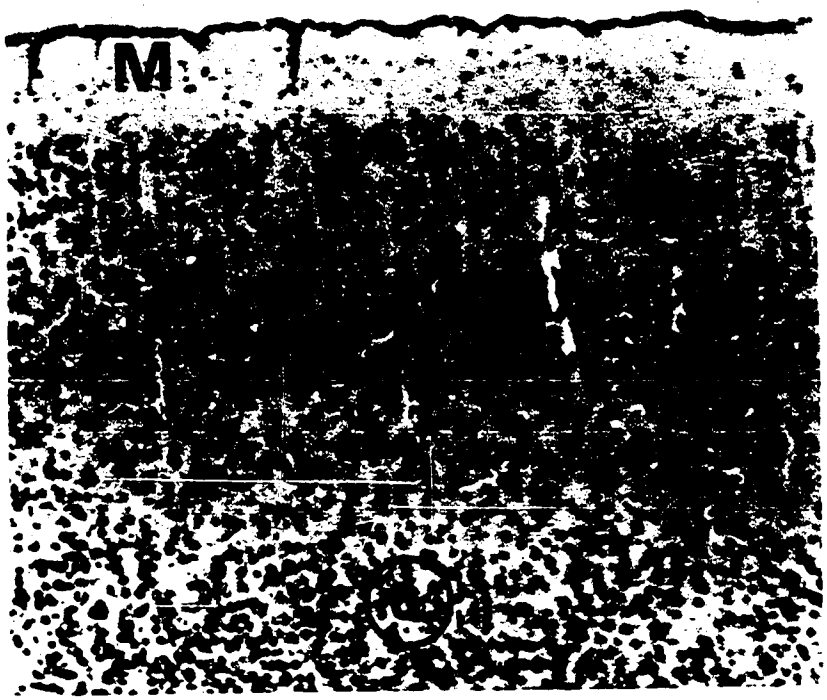


PLATE VI

Figure 6. Photomicrograph. 14 day old control animal. Parietal cortex showing normal depth of the outer molecular layer (M). (H & E, 60X).

Figure 7. Photomicrograph. 14 day old experimental animal. Parietal cortex showing decreased depth of the outer molecular layer (M) and disorganization of underlying cortical neurons. (H & E, 60X).

PLATE VI



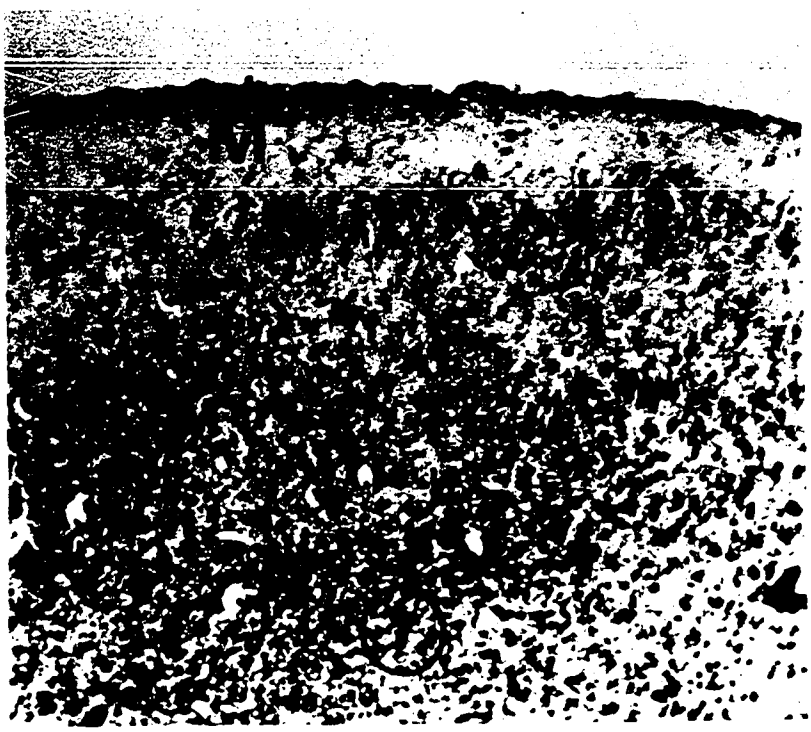


PLATE VII

Figure 8. Photomicrograph. 28 day old experimental animal. Parietal cortex showing abnormally clumped neurons (NC). (CVA, 150X).

Figure 9. Photomicrograph. 28 day old control animal. Hippocampal gyrus showing normal neuronal (HN) configuration. (CVA, 150X)

PLATE VII

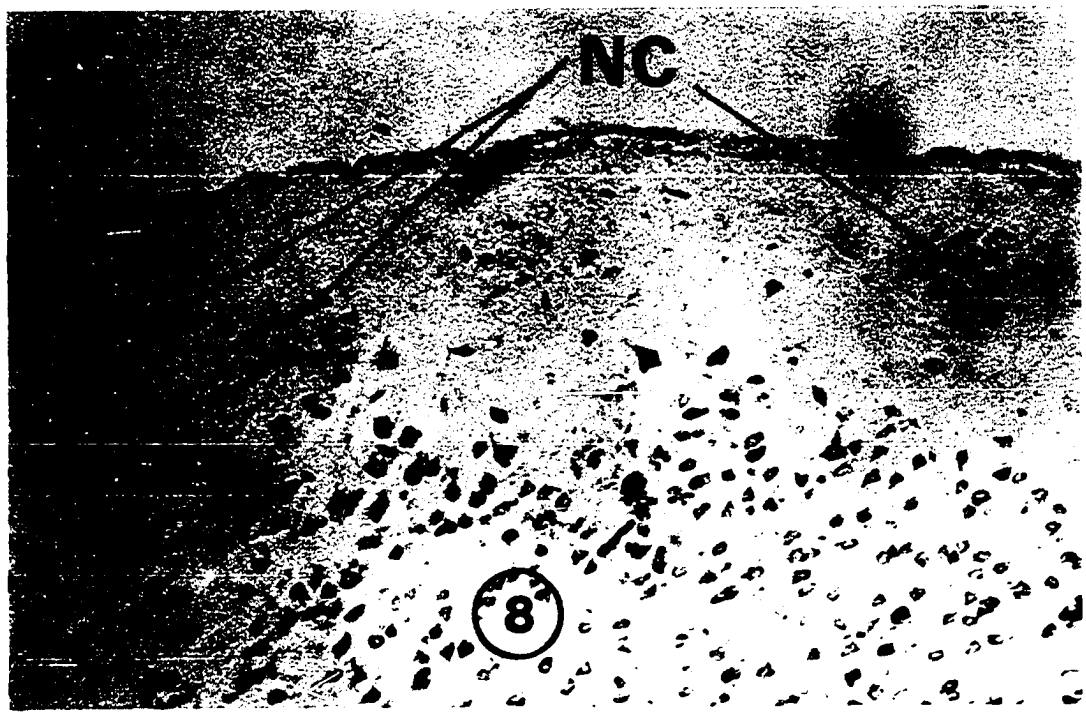


PLATE VIII

Figure 10. Photomicrograph. 28 day old experimental animal. Hippocampal gyrus showing fewer than normal numbers of neurons and disorganization (HN). (CVA, 150X).

Figure 11. Photomicrograph. 51 day old experimental animal. Occipital cortex showing "rosette". (H & E, 300X).

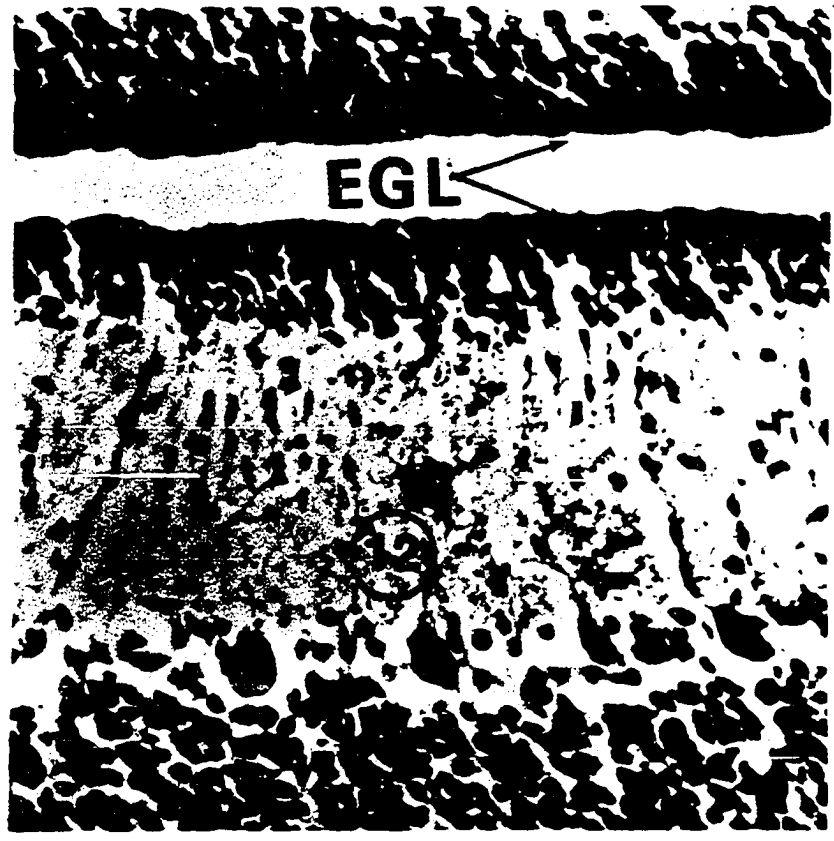
PLATE VIII

PLATE IX

Figure 12. Photomicrograph. 14 day old experimental animal. Cerebellum showing the external granular layer (EGL). (H & E, 300X).

Figure 13. Photomicrograph. 28 day old control animal. Cerebellum showing Purkinje cells and Bergman's cells (BC). (H & E, 600X).

PLATE IX



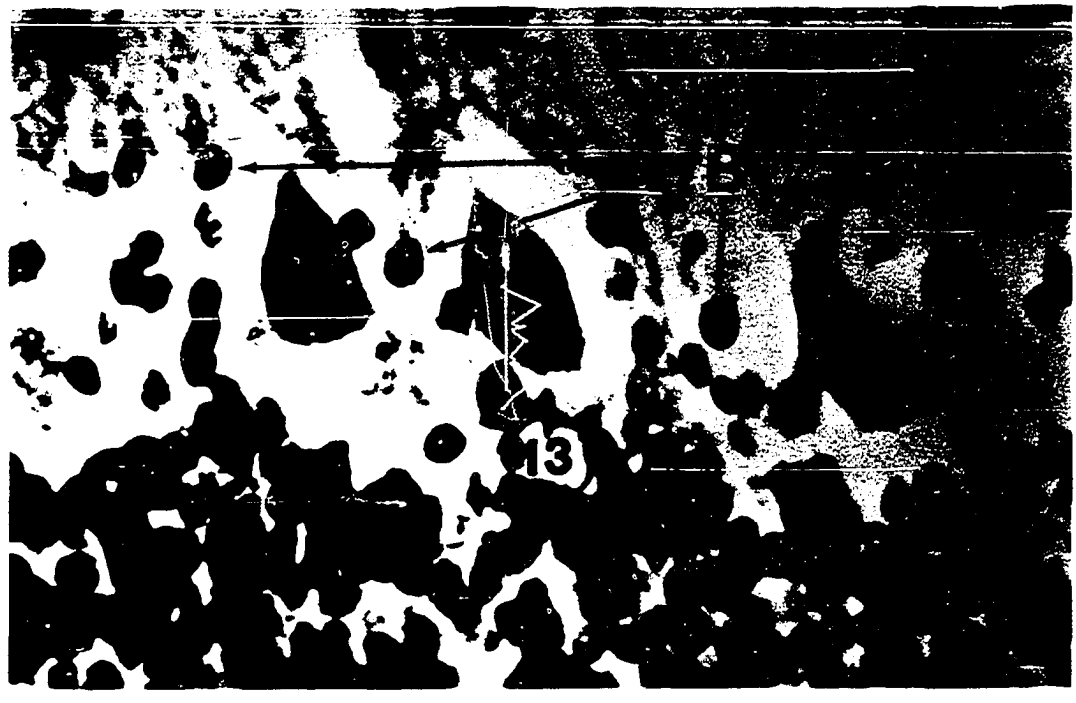


PLATE X

Figure 14. Photomicrograph. 28 day old experimental animal. Cerebellum showing Purkinje cells and lack of Bergman's cells. (H & E, 600X).

Figure 15. Electronmicrograph. 40 day old control animal. Corpus callosum showing numerous myelin sheaths. Stained with uranyl acetate. (11,600X)

PLATE X

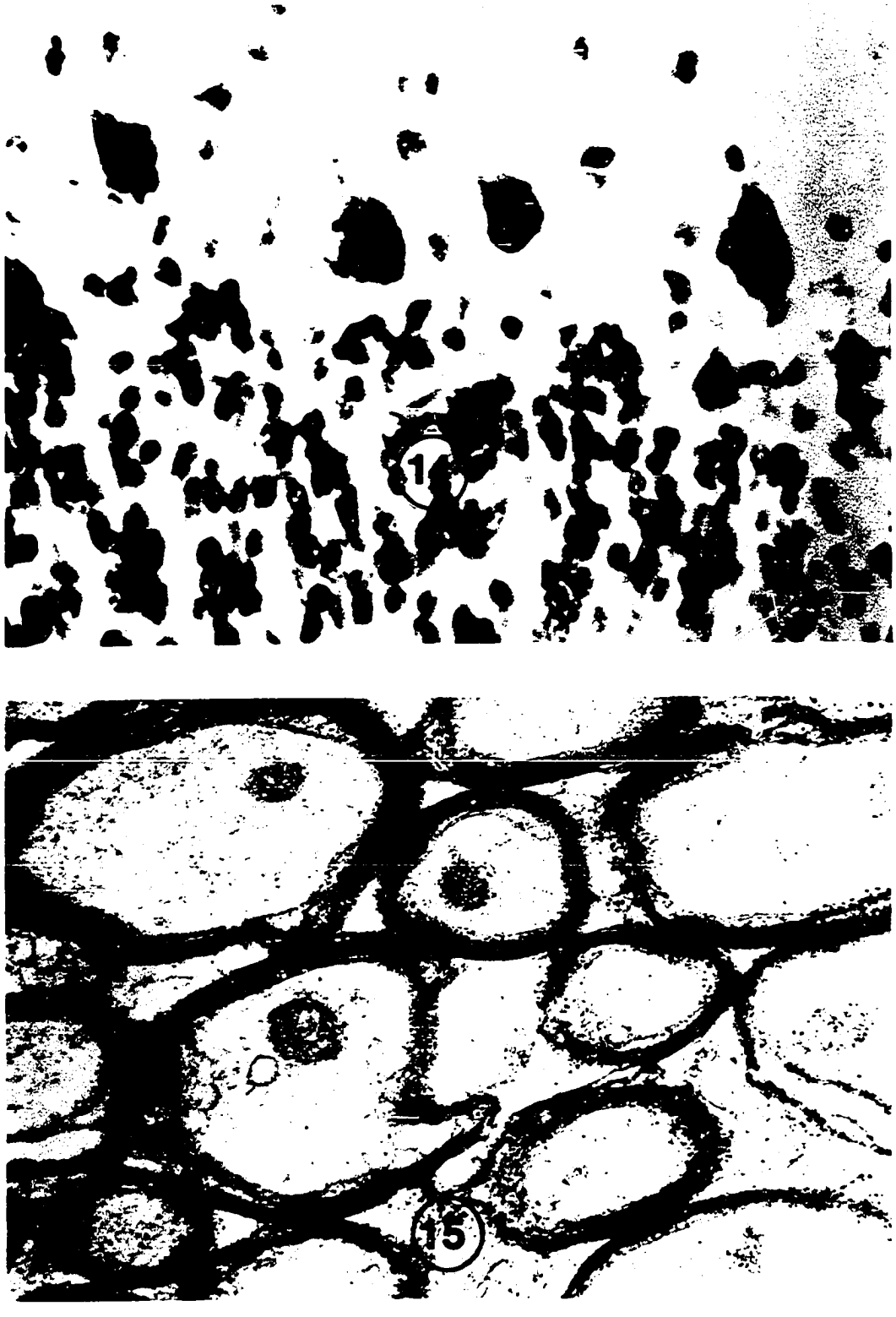


PLATE XI

Figure 16. Electronmicrograph. 40 day old experimental animal. Corpus callosum showing sparse myelin sheaths. Stained with uranyl acetate. (11,600X).

Figure 17. Electronmicrograph. 40 day old experimental animal. Corpus callosum showing axon with Type C myelin sheath defect (arrow). Note the cytoplasmic organelle between myelin lamelle. Stained with uranyl acetate. (11,600X).

PLATE XI

PLATE XII

Figure 18. Electronmicrograph. 40 day old experimental animal. Corpus callosum showing figure 8 myelin sheath configuration (arrow). Stained with uranyl acetate. (11,600X).

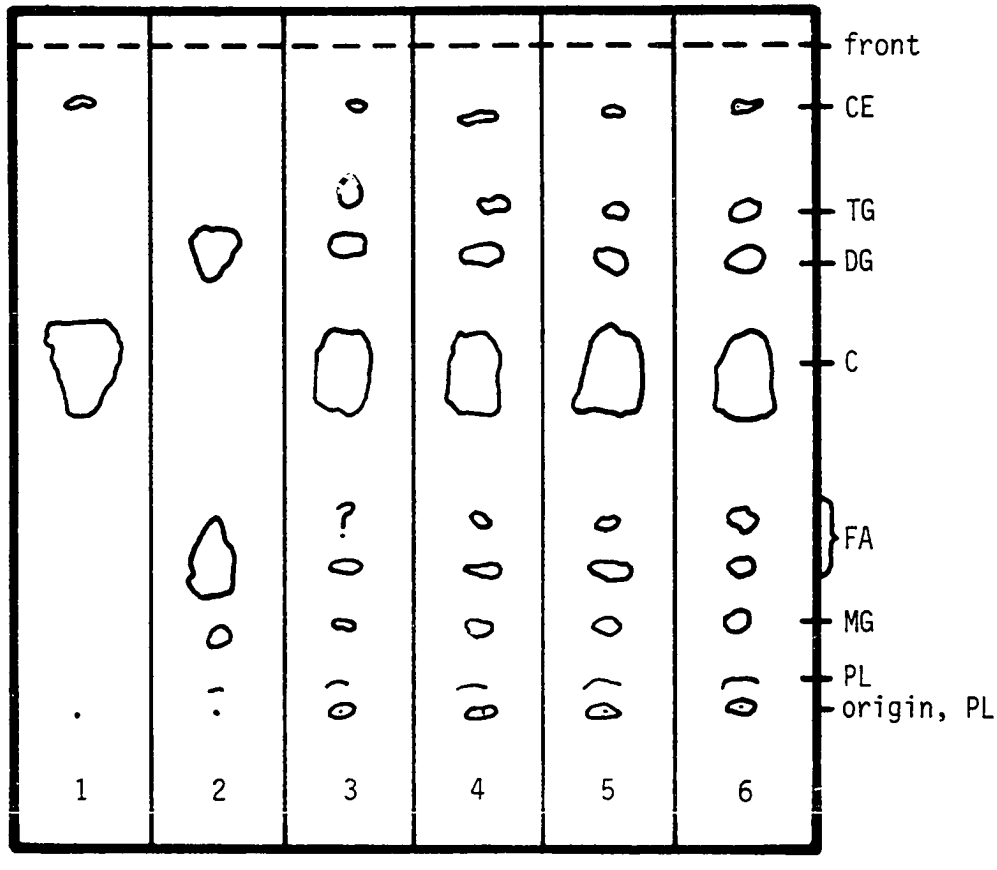
Figure 19. Electronmicrograph. 40 day old. Experimental animal. Corpus callosum showing figure 8 myelin sheath configuration (arrow). Stained with uranyl acetate. (11,600X).

PLATE XII

PLATE XIII

Figure 20. Composite drawing of thin layer chromatographic plates.

PLATE XIII



1 - cholesterol and cholesterol oleate

2 - monopalmitin, 1-3 dipalmitin, fatty acids

3 - Exp. 50 µl - 150 µl

4 - Cont. 50 µl - 150 µl

5 - Exp. 150 µl - 250 µl

6 - Cont. 150 µl - 250 µl

FA - fatty acids

CE - cholesterol ester

TG - triglycerid

DG - diglyceride

C - cholesterol

MG - monoglyceride

PL - phospholipid