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Considerations on the Development of Experimental Lead Encephalopathy*

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Summary. Experimental lead encephalopathy was produced in developing rats. The cerebellar changes that developed were studied by light and electron microscopy. Although edema was observed in all phases of the encephalopathy, changes in the blood vessels, formation of platelet thrombi, and alterations of the Purkinje cells and their dendrites warranted special attention. The lead and calcium content of the brain and of the blood in the lead poisoned and control groups were determined chemically. The results are discussed from a morphological point of view and a hypothesis of their pathogenesis is presented.

Zusammenfassung. Wir haben bei neugeborenen Ratten eine experimentelle Blei-Encephalopathie hervorgerufen und die cerebellaren Veränderungen licht- und elektronenmikroskopisch untersucht. In allen Stadien der Encephalopathie war ein Ödem vorhanden, darüber hinaus fanden sich Anzeichen für Capillarschäden, Bildung von Plättchenthromben und Veränderungen der Purkinje-Zellen mit ihren Dendriten. Blei- und Calciumgehalt des Gehirnes wie des Blutes von bleivergifteten Ratten und von Kontrolltieren wurden chemisch untersucht. Die Ergebnisse werden aus der Sicht des Morphologen besprochen, die Pathogenese der Veränderungen diskutiert.

The effects of lead poisoning in humans have been known from ancient times. Ramazzini in 1739 and Tanqueral des Planches in 1839 were among the first to note the effects of this metal on the nervous system. Although many studies since then have been made on human material, attempts to reproduce comparable nervous disease in animals for the purpose of studying the sequence of events that gave rise to cerebellar lesions generally have failed. Pentschew and Garro (1966), however, introduced a new experimental model of lead poisoning in which they utilized young rats. They took advantage of the fact that lead fed the mother rat is transmitted to the young by the maternal milk. In concluding their studies they postulated that a certain parallel existed between lead encephalopathy and Wernicke's encephalopathy, and that both these maladies belonged to what they called "system bound dysoric encephalopathies".

Recent studies by Ule and Kolkmann (1967) have proven, however, that the primary lesion of experimental Wernicke's encephalopathy is a hydropic swelling of the neuropil that develops independent of vascular involvement. To learn

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whether that pertains also for experimental lead encephalopathy we instituted the present study.

The methods that we employed in this study of the development of cerebellar changes in lead encephalopathy were based on Pentschew's work, but were modified to suit our experimental needs. It was our aim to determine the possible cause and sequence of events that led up to the peak of the disease processes which were marked, clinically, by paralysis in the animal, and microscopically, by severe cerebellar edema. For that purpose the animals were studied in the acute or preparalytic and paralytic phase, whereas a study of the postparalytic period revealed the reparative changes that occurred after lead poisoning.

Material and Methods

Thirty seven pregnant Wistar rats were utilized in our series of experiments.

The diet used to produce lead poisoning was made as follows: To normal laboratory chow[1], which was first softened in distilled water, we mixed laboratory reagent Lead Carbonate to give a 4.5% concentration. The mixture was then allowed to harden in an oven. The drinking water given to the mothers was a 1% solution of laboratory reagent Lead Acetate and distilled water. As soon as each female gave birth to her young, she was started on the lead diet and lead drinking water. Comparable litters that received normal laboratory chow and distilled water were used as controls and studied concurrently.

From preliminary studies we learned how many days of lead poisoning it generally took before the infant rats revealed paralysis (31 days). For studying the acute changes we selected and killed newborn rats from different litters, starting ten days before the expected onset of their paralysis, and thereafter killed litter-mates every alternate day until the day of paralysis. The animal utilized for the study of the reparative processes were changed from a lead diet to a normal diet immediately after the paralysis manifested itself, and killed 5, 10, 15, 20, 30 and 60 days after the onset of their paralysis. Only animals that had exhibited a florid paralysis were used for these studies of the reparative processes. To ensure optimal fixation of the brain for electron microscopy, we injected the animals intraperitoneally with Liquemin (a heparin anticoagulant) anaesthetized them with Thiogenal (a short acting barbiturate) and perfused them by way of the left cardiac ventricle with Haemaccel (a plasma substitute) followed by 3.5% gluteraldehyde at a pressure of one meter of water. The brains were removed 30 min after completion of the perfusion. Since it was felt the contents and size of blood vessels could best be studied in unperfused brains, a parallel group of poisoned newborns were killed under anaesthesia by quick decapitation and the brains removed, carefully sectioned and immersed immediately in 3.5% gluteraldehyde, the whole procedure never lasting more than two minutes. For these nonperfusion studies we used animals only up to the expected day of paralysis. The tissue for the light and electron microscopic studies were selected from the cerebellum since that part of the brain proved to be the most severely involved in lead encephalopathy. For light microscopic studies the hematoxylin-eosin, Nissl, Klüver-Barrera, Masson-Goldner and the PAS stains were used. When study of the nature of deposits on the Purkinje cells and their dendritic processes required it, Turnbull's reaction for iron and von Kossa's reaction for calcium were done. The tissues for electron microscopy were processed and embedded in Araldite, sectioned by the Reichert Om U 2 ultramicrotome and examined with the Zeiss EM 9A electron microscope.

Since it is well known that lead displaces calcium in the body, the amounts of these substances in pooled rat brain and blood were determined chemically by the atom absorption technique. Ten rats in the paralytic phase were used for this purpose and the results obtained from them were compared with those from five control rats of similar age.

The results of the chemical analysis of the lead and calcium in the brains and blood of the lead poisoned and control animals are shown in the Table.

1 Altromin.

Table. *The determinations of lead and calcium in the brain and blood of the experimental and control animals by the atom absorption method*

	Brain (in μg/G fresh weight)		Blood (in μg/G)	
	Experimental	Control	Experimental	Control
Lead	11.5	Nil	3.0	Nil
Calcium	137.0	24.0	25.0	25.0

Observations

The newborn rats in both the experimental and control groups appeared to develop normally. This was judged by way of assessing certain cardinal milestones, such as the first appearance of hair, the opening of the eyes and the ability to eat. The differences in size between the experimental and control groups however were remarkable, as revealed by comparing the weights of lead poisoned rats with the control rats born on the same day. Those of the lead poisoned group weighed on an average 15 g per animal 15 days after birth whereas the control group of similar age weighed on an average 44.2 g.

The processes that brought about lead encephalopathy eventually caused paralysis, a sign we accepted as indicating severe internal pathology. The paralysis in the experimental group developed about 31 days after birth and manifested itself as weakness of the rear limbs and incontinence. These manifestations progressed over the next 12 h to frank paraplegia with lethargy and extreme generalized weakness. Some animals developed intraocular hemorrhages. When the lead diet was replaced by a normal diet, most of the animals survived and recovered from their paralysis within a period of 24 to 48 h. It was remarkable how much more susceptible the animals belonging to the experimental groups were to the barbiturate, Thiogenal, than were the controls.

The longer the period of poisoning the more extensive were the hemorrhages in the cerebellar cortex; they became maximal on the day of paralysis (Fig. 1). In spite of the good fixation of the rest of the brain, the cerebellum usually was soft and friable. The cerebella of animals killed after the day of paralysis were discolored. The longer the animals survived in the postparalytic period, the less the cerebellum showed this discoloration. Vacuoles in the folia of the cerebellum of these animals became evident and were always larger in the older animals.

Microscopic Studies

Light Microscope: *Acute Phase*. The most striking changes in the cerebellum of infant rats killed ten days before the expected date of paralysis were the small fresh hemorrhages scattered here and there throughout the molecular and granular layers and in the pial membranes. Although most were located about small capillaries, many of which were collapsed, other extravasations of red blood cells could not be related to blood vessels. Most red blood cells exhibited distinct variation in size and shape. Clearing about the capillaries by accumulation of edema fluid made most capillaries stand out prominently.

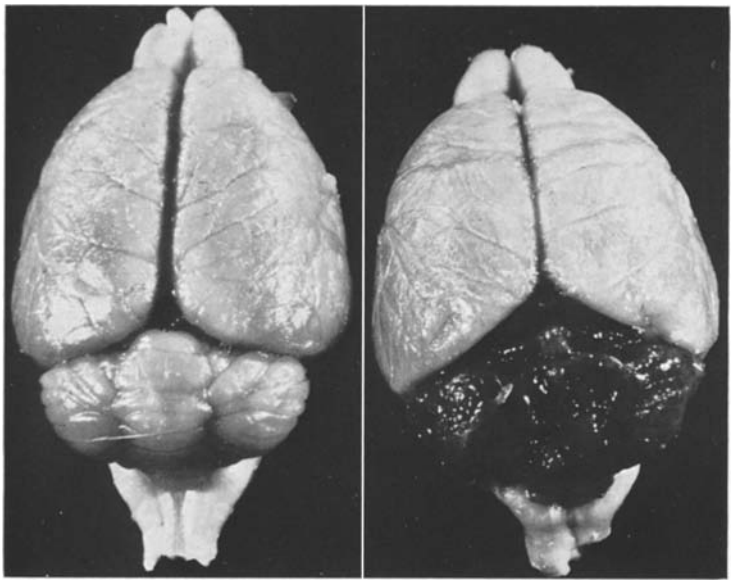

Fig. 1. On the right a brain removed on day of paralysis. Note the appearance of the cerebellum in comparison to that of the brain on the left, which was taken from a control animal of similar age (29th day after paralysis)

Moderate numbers of Purkinje cells were found shrunken, their nuclei pyknotic, their cytoplasm condensed and darkly stained but sometimes vacuolated, with their borders irregularly scalloped, separated from adjacent structures by clear spaces, and with their dendritic processes thickened.

Although all of these changes were seen in the non-perfused brains, they were generally much more conspicuous in those perfused.

In the brains of the animals killed eight days before the estimated date of paralysis, the changes described above had become more numerous and more extensive. In addition, many capillaries were found to be lined by degenerating endothelial cells, the nuclei of which were often either swollen and vacuolated, or pyknotic. Most striking at this time, however, were the occasional thrombi found in larger capillaries. These were formed of agglutinated red blood cells, brightly acidophilic proteinaceous material and finely granular deposits of platelets. The contiguous wall of the vessel often was thickened and hyalinized. Adjacent capillaries were bloodless and collapsed, their lining cells appearing enlarged and conspicuous.

Fig. 2. a Note the large lakes of edema fluid in the white matter and the hemorrhages. Cerebellum. Klüver-Barrera. (On the day of paralysis. Perfused specimen). Magnification $\times 56$. b Notable in this picture are the dark-stained vacuolated Purkinje cells with swollen glial processes, Cerebellum. Azur II-Methylene Blue. Semi-thin 1 μ section, Araldite embedded. (6 days before paralysis. Perfused specimen). Magnification $\times 600$. c "Inkrustationen" in the cerebellar cortex in relationship to the Purkinje cell dendrites. Some Purkinje cells have disappeared. Cerebellum. Nissl. (15 days after paralysis. Perfused specimen). Magnification $\times 500$

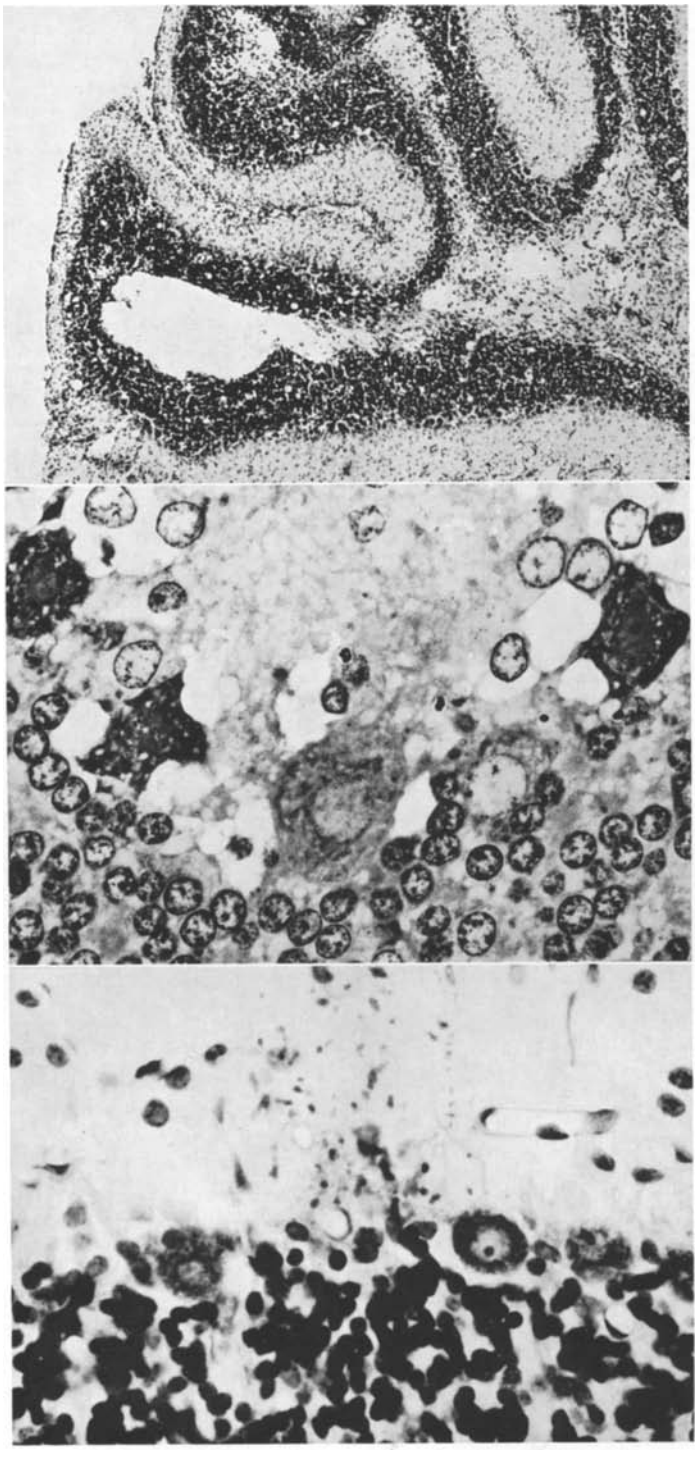

Fig. 2 a–c

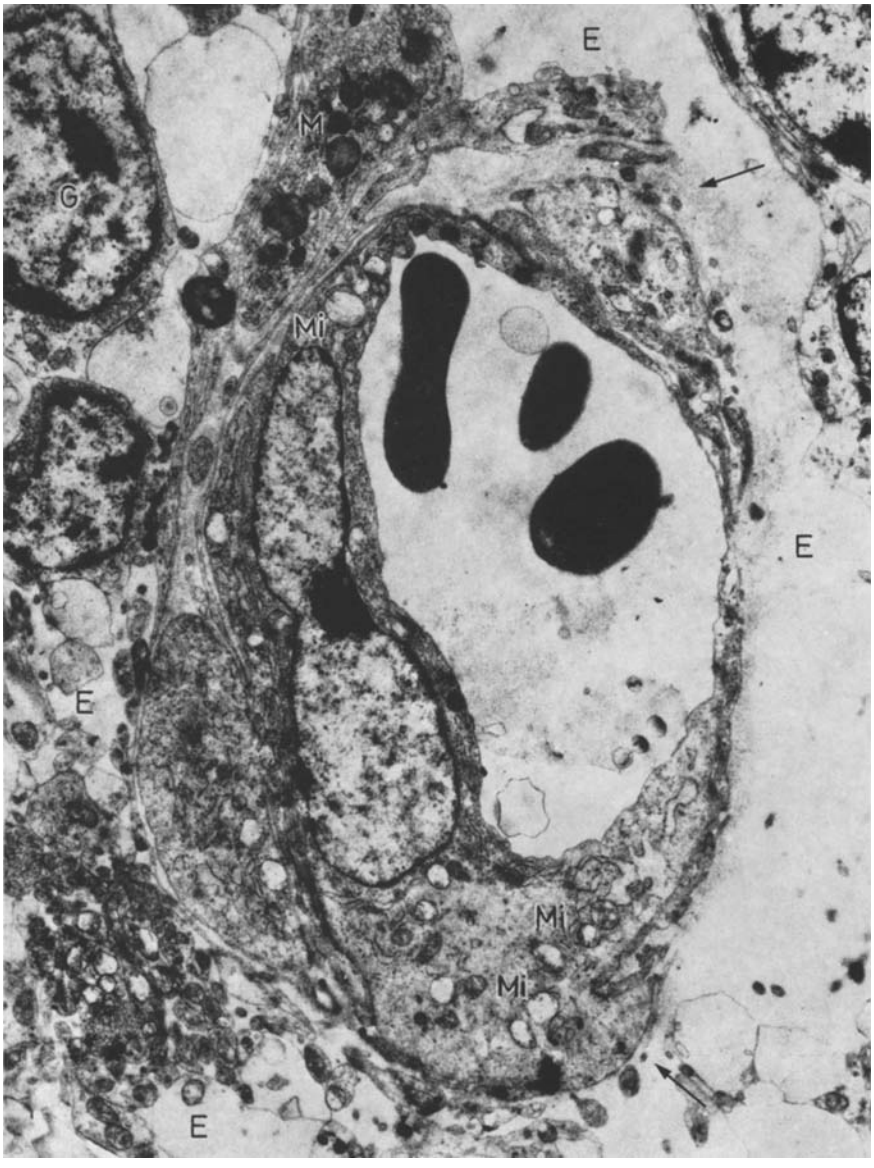

Fig. 3 a. A capillary showing thickening of the endothelial cytoplasm with numerous vacuolated mitochondria containing remnants of cristae mitochondriales within. A part of the basement membrane appears to be dissolved (arrows) and there is much edema around the capillary. (On the day of paralysis. Perfused specimen). Magnification ×7200. *E* Edema, *G* Granular cell, *M* Macrophage, *Mi* Mitochondria

The longer the period of lead poisoning, that is, the closer to the expected date of paralysis that the animals were killed, the more severe the vascular changes and hemorrhages. The lesions, initially focal, extended to coalesce and

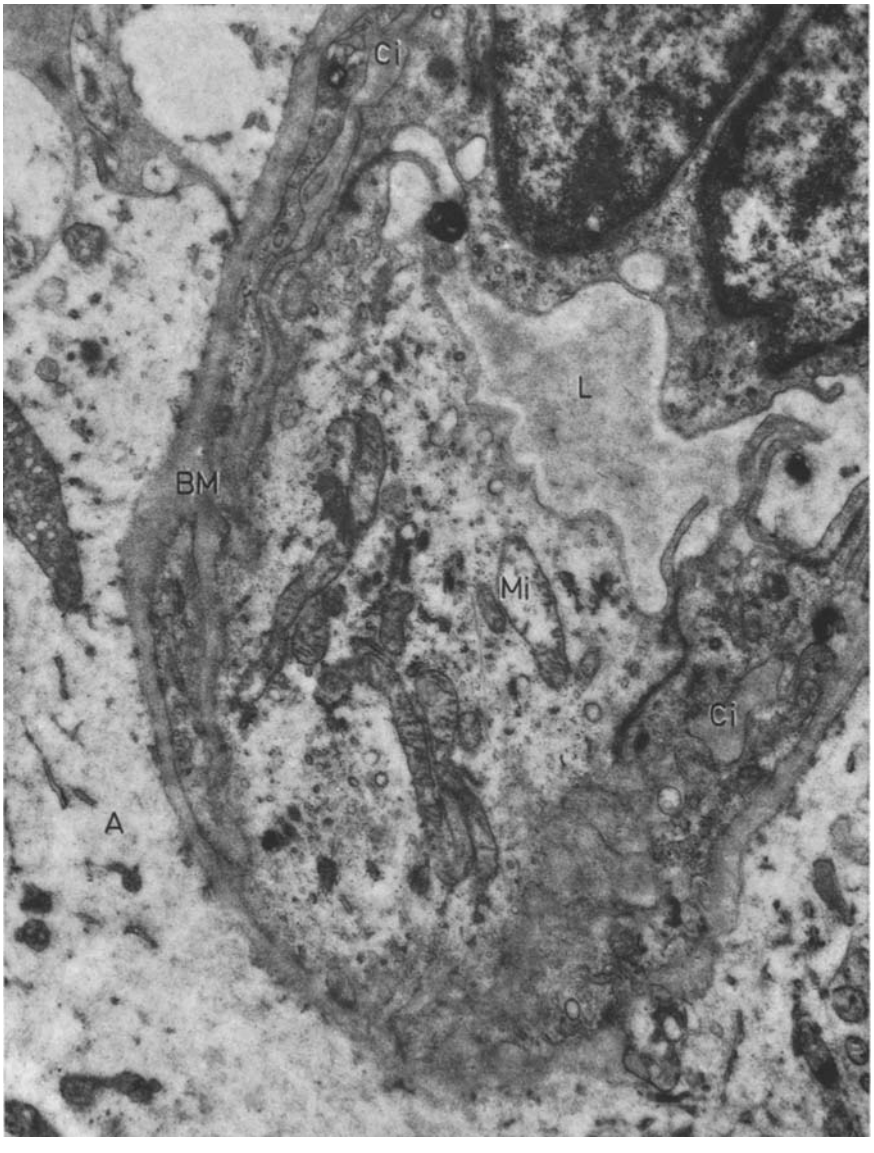

Fig. 3 b. Part of another capillary showing some swelling with rarefaction of cytoplasm of the endothelial cell. The swelling of the mitochondria, widening of the endoplasmic cisternae and increase in the number of pinocytic vesicles are noteworthy. Note also the decrease in luminal size and the edema of the astrocytic foot processes. (On the day of paralysis. No perfusion). Magnification ×18000. *A* Astrocytic foot processes, *BM* Basement membrane, *Ci* Endoplasmic cisternae, *L* Lumen, *Mi* Mitochondria

become widespread, showing no predilection however for any part of the cerebellum. The edema fluid, accumulated at first in the perivascular end processes of the astroglia, extended to become confluent, forming shortly before the date of

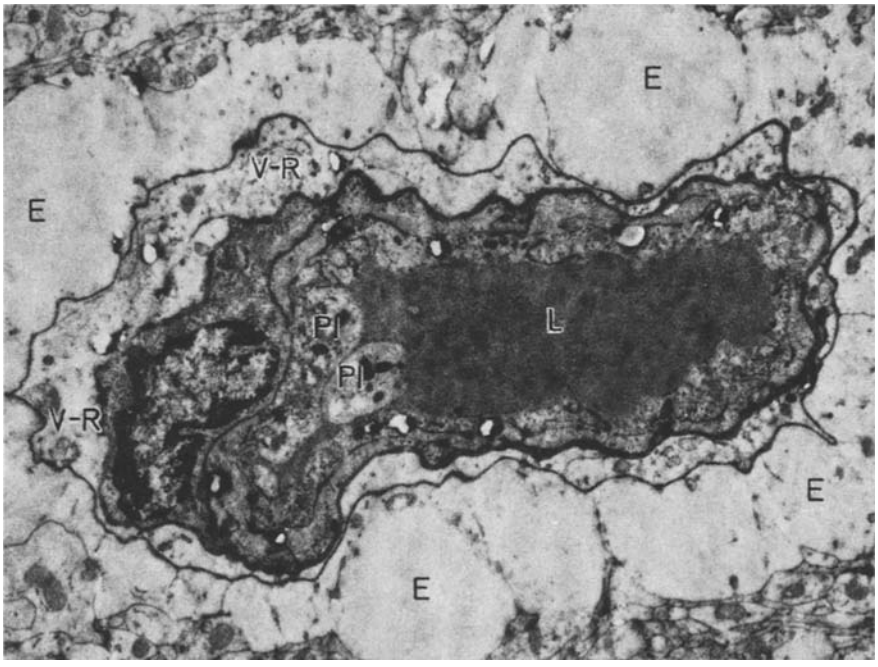

Fig. 3 c. Note the platelet thrombi in the unperfused capillary that appear to be degranulating and adhering to the endothelial cell. This vessel also shows a prominent Virchow-Robin space and edema in the astrocytic foot processes. (10 days before paralysis. No perfusion). Magnification ×7200. *E* Edema, *L* Lumen, *Pl* Platelet, *V-R* Virchow-Robin space

paralysis large lakes within the white matter (Fig. 2a). Here, dispersed small necroses of neuroglial elements became more conspicuous, especially by the day of paralysis. Swollen macrophages, located about larger vessels and near older hemorrhages and focal regions of necroses, contained yellow pigment that gave a positive stain for iron. Although the scattered numbers of pyknotic Purkinje cells were found in the brains of all animals (Fig. 2b), they did become numerous at later stages of the lead poisoning but were not localized in any special region of the cerebellum.

Reparative Phase. During the postparalytic phase the hemorrhages, which had been such a prominent feature of the acute phase, were less apparent by the 5th postparalytic day, and by the 10th postparalytic day were inconspicuous. The capillary prominence that had been so marked during the acute phases disappeared between the 10th and 15th postparalytic days. The lakes of edema fluid in the white matter reached their maximal size by the 30th postparalytic day, apparently by coalescing. By the 60th postparalytic day very little edema remained.

The degenerative changes in the Purkinje cells became less frequent and were last found 15 days after the day of paralysis. At the same time irregular „Inkrustationen" (in the sense used by Colmant, 1965) first appeared and were related to remnants of Purkinje cells and their dendrites (Fig. 2c). These

"Inkrustationen" increased in number up to the 30th postparalytic day. They gave a negative reaction for iron (Turnbull's blue reaction) and a negative von Kossa's test for calcium.

Electron-Microscopic Studies. In these studies the earliest and most prominent changes were found in the capillaries and Purkinje cells. As early as the 10th day before the expected date of paralysis most of the endothelial cells of the capillaries were swollen, in some so greatly that the lumen of the vessels was almost obliterated. In the cytoplasm of these swollen cells three types of vacuoles could be distinguished. First, there were the double-walled rounded vacuoles that contained remnants of cristae mitochondriales and were situated near mitochondria. A second type of vacuole had a clear interior and a rim stippled with granules of RNA; they resembled a swollen segment of endoplasmic reticulum. The third and smallest type of vacuole was characteristic of pinocytosis. Although most endothelial cells exhibited these changes, some instead had a rarefied cytoplasm and few granules of RNA (Fig. 3a and b), or their endoplasmic cisternae were greatly widened. The endothelial cells of venules or arterioles rarely disclosed these pathologic changes.

Also on the 10th day before the excepted day of paralysis, small thrombi of platelets, red blood cells, and plasma proteins were found adherent to an occasional altered endothelial cell of a capillary in the cerebella of the non-perfused animals (Fig. 3c). Such thrombi were rarely encountered in the perfused brains, although these revealed more extensive pericapillary hemorrhage and hydrops of the astroglial footprocesses. In later stages of the acute phase, shortly before the day of paralysis when virtually all endothelial cells of the capillaries showed pathologic change and the thromboses were much more common, the basement membranes of the capillaries were still normal. Only in a few vessels was the membrane thickened or partially destroyed by dissolution.

In the earliest phases (the 10th preparalytic day) the footprocesses of the astroglial cells were generally swollen by edema fluid and dispersed amongst them were variable numbers of red blood cells of all shapes and sizes, and occasional macrophages. Later the edema had extended to dissect between neural structures of the white matter, often widely separating them. Few of these structures, however, disclosed pathologic change (Fig. 4a). In later stages in the regions of the white matter where the edema seemed especially severe, cellular debris and macrophages were distinct. Many of the macrophages contained prominent vacuoles and rounded dense bodies. Myelin bodies, first evident in the brains of animals killed two days before excepted paralysis, were numerous by the day of paralysis.

From the very earliest phase (10 days before excepted paralysis) some Purkinje cells were pyknotic with loss of the nuclear-cytoplasmic demarcation. In their cytoplasm there were many double-walled vacuoles which contained remnants of mitochondrial cristae (Fig. 4b). These vacuoles corresponded to those occasionally apparent in the Purkinje cells with the light microscope. The cytoplasmic borders of the affected cells appeared frayed and were surrounded by profound hydrops of the Bergmann astroglia.

Reparative Phase. In some respects it was surprising how rapidly some of the pathologic changes resolved. By the 5th postparalytic day the capillaries appeared generally normal, lined by healthy endothelial cells. Intravascular thrombi were

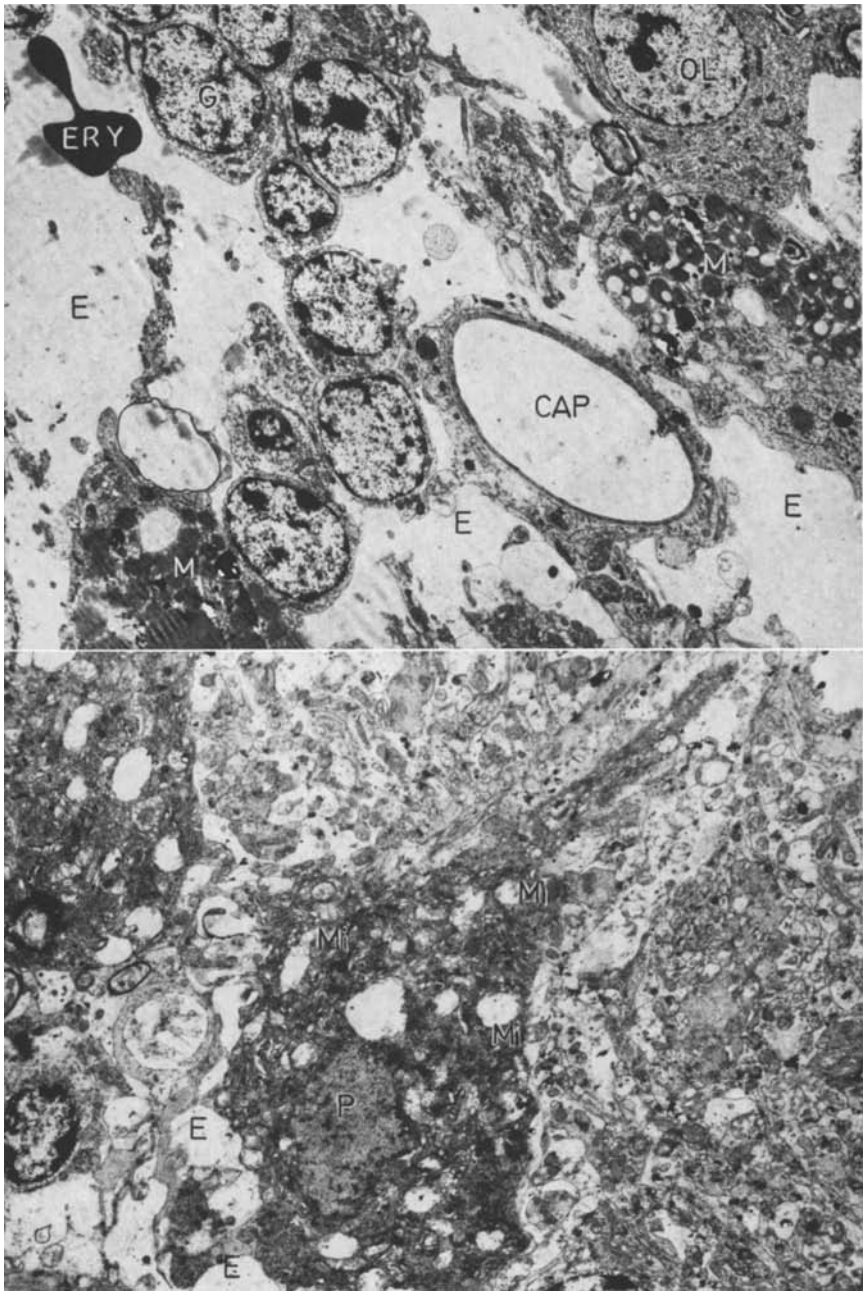

Fig. 4. a Note the extreme extracellular edema, with wide separation of the cells which in general appear unaffected. (On the day of paralysis. Perfused specimen). Magnification ×3600. *CAP* Capillary, *E* Edema, *ERY* Erythrocyte, *G* Granular cell, *M* Macrophage, *OL* Oligodendrocyte. b A Purkinje cell showing condensation of nuclear and cytoplasmic structures, with loss of detail. Some pericellular edema is notable in the glial processes. (15 days after paralysis. Perfused specimen). Magnification ×4000. *E* Edema, *Mi* Mitochondria, *P* Purkinje cell

gone. By the fifteenth day the Purkinje cells were normal, a finding that correlated well with the light microcopic studies. Occasional axons, however, disclosed dystrophy and degenerative changes between the 10th and 15th day after paralysis. The number of macrophages and microglial cells increased up to about the 15th postparalytic day, then diminished slowly thereafter but were still evident by the 60th postparalytic day. The lakes of edema in the white substance disappeared slowly. and traces of them were still evident by the 60th postparalytic day.

Discussion

In our preliminary studies we confirmed the observations of Pentschew *et al.* that newborn rats developed an encephalopathy at about 31 days of age if their mothers were maintained on a diet contaminated with lead. We assumed, as did Pentschew, that the newborn rats were poisoned by lead in the mother's milk. To learn what the sequence of pathologic changes was in the development of the encephalopathy we killed the newborn rats at various times before the expected date of paralysis at 31 days of age. Our light and electron microscopic studies of the cerebellum indicated clearly that the capillaries and the Purkinje cells exhibited the first pathologic changes, and that the changes that followed merely represented progression of the capillary injury and its consequences.

At ten days before the expected date of paralysis, long before the animals manifested any signs or symptoms of cerebellar disease, we noted degenerative changes in endothelial cells of numerous capillaries in all parts of the cerebellum. Vacuoles were detected in these altered endothelial cells with the electron microscope, and could be classified into three groups. One of these represented degenerated mitochondria, another alterations in the endoplasmic reticulum. Similar changes were recorded by Hill (1964) in anoxic-ischemic lesions of the brain, but since the mitochondria were not involved he believed the changes were of pinocytic nature. Altered mitochondria were noted by Watrach (1964) in the liver cells of lead-intoxicated swine. Totović (1964) however found no primary alterations of mitochondria in the epithelial cells of the kidney of lead poisoned rats.

Invariably associated with these degenerative changes were hemorrhages, perivascular edema, and occasional thromboses, all of which became more extensive as the animals grew older. The capillary thrombi were especially distinct in the non-perfused brains, whereas the pericapillary extravasations of red blood cells and the edema of the perivascular footprocesses of astroglia were much more apparent in the perfused brains, no doubt an expression of the capillary fragility and the effect of the pressure of injection.

Electron-microscopically, the aggregation of platelets in the lumina of the capillaries was striking; it progressed as the day of paralysis was approached. Platelet degranulation and fibrin production became increasingly apparent, and resembled those changes described by Schulz und Rabanus (1965). Hirsch *et al.* (1965) found similar aggregates of platelets and fibrin in the ischemic brains of dogs; they thought these aggregates were capable of slowing or stopping the flow of blood and were even capable of producing changes in the vascular wall of the occluded blood vessels. Whether alterations of the red blood cells, as evidenced by the anisocytosis and poikilocytosis, promoted thrombosis we do not

know. The red blood cells of these lead poisoned animals did display marked variation in their size and shape. Since we did not study the coagulation of the blood or perform counts on its cellular elements we cannot say whether anemia or thrombocytosis developed in the animals to complicate the cerebellar disease.

It was interesting to note that the first accumulation of edema fluid found was in the footprocesses of astroglia, indicating that the capillary walls had become abnormally permeable. In later phases the edema fluid spread throughout the white matter, separating its components. When the edema persisted for a long time then microglial cells appeared and the neural parenchyma underwent degeneration and focal necroses developed. The peculiar character of that observation suggested two etiological possibilities. The site of action of lead has been variously thought to be either on the nerve and glial tissue (Hassin, 1921; Aub *et al.*, 1925; MacLaurin and Nichols, 1957) or on the capillaries (Okazaki *et al.*, 1963; Pentschew and Garro, 1966). In our experimental model it seemed unlikely that since morphological evidence of neural damage was lacking in the initial stages of the disease the expansion of the edema was caused by the loss of fluid from injured degenerating neural cells.

On the contrary, it seemed most probable from the changes in the blood vessels and astroglial footprocesses early in the disease that the primary change in lead intoxication was capillary damage.

Bauer (1970) in his studies of the capillaries of the developing fetal brain states that the basement membrane at this time of life may vary in thickness between 37 and 500 Å. He maintains the membrane performs no mechanical supporting function for the endothelial cell and its function in ultrafilter is poorly developed. Torack (1961) studied the ultrastructure of the capillaries of brain tumors and the edema that invariably developed; he commented that the capillary changes varied depending upon their involvement in the pathological process.

The alterations in the Purkinje cells were seen both with the light and electron microscopes as early as the 10th day before the expected date of paralysis. The cellular shrinkage, with scalloping of the cytoplasmic borders, loss of nuclear-cytoplasmic detail, and the appearance of vacuolated mitochondria suggested an anoxic etiology. Bakay and Lee (1965), in reviewing the findings of other authors in cerebral edema, noted that such acute degenerative changes as vacuolations, tigrolysis, pyknosis and necrosis of neurons with neuronophagia may occur as a result of cerebral edema. They further stated that in some types of cerebral edema the large nerve cells and the Purkinje cells were usually affected. The slight edema we noted around the altered Purkinje cells suggested that the fluid had been extruded from the Purkinje cells themselves and was not the cause of the degenerative changes in these cells. We know from other studies that Purkinje cells are more resistant to edema than are the granular cells.

The "Inkrustationen" (in the meaning of Colmant, 1965) were closely related to the altered Purkinje cells and their dendritic systems. Although he always saw the "Inkrustationen" in relation to ganglion cells with well vacuolated cytoplasm, Colmant emphasized that these changes were by no means obligatory. He stated further that the basophilic material that gave the "Inkrustationen" their granular appearance was a deposit on the Golgi network and was possibly made up of RNA.

Környey (as quoted by Colmant, 1965) postulated these "Inkrustationen" might represent a synaptic or membrane disturbance or ruptured endings of nerve cells. Pentschew and Garro (1966) mentioned the appearance of what they called "bushy formations" in the molecular layer of the cerebella of their lead-poisoned rats. They found these formations only in the reparative stages where the dendritic systems of the Purkinje cells had been located. In electron microscopic studies of experimental lead encephalopathy Lampert *et al.* (1967) were unable to demonstrate injury of Purkinje cells or dendritic "Inkrustationen".

Himwich and Fazekas (1941) experimenting with the brains of developing dogs, and Tyler and van Harreveld (1942) working on the developing rat brain, found the highest levels of oxygen and glucose utilization in these animals between the 4th and 7th week after birth. They observed that this high utilization corresponded roughly with the phylogenetic development of the brain. When their observations were correlated with ours it was noted that our experimental lesions achieved their maximal intensity between the mid-third and fourth week after birth. From such a correlation and with the morphological endothelial cell changes, it is our hypothesis that in lead poisoning the respiratory enzymes of the endothelial cells are interfered with, preventing them in some way from taking part in the increased metabolic demand of the developing cerebellum. The patchy nature of the lesions in the early stages of the disease suggested that not all of the microcirculation was affected at the same time, but rather that certain cells of the capillaries were more sensitive than others. Therefore, we concur with the theory put forward by Pentschew and Garro (1966) in that these vascular lesions with the resultant edema are caused in part by a chronic metabolic dysoxidosis.

Although several explanations of the changes of the Purkinje cells are possible, their early focal nature best correlates with the patchy occlusion of capillaries by swollen endothelial cells and platelet thrombi, suggesting that the Purkinje cells were injured by anoxia. As the day of paralysis approached the vascular damage and platelet thrombi increased, more Purkinje cells were affected and they exhibited greater degenerative change.

Whether capillary damage represents direct toxic action of lead, or indirect injury mediated by deficiency of a vitamin or other substance induced by the poisoning with lead, we do not know. Lead competes with calcium ion in cellular metabolic processes and binds readily to -SH radicals of enzyme systems and components of cells. The chemical estimations of lead and calcium in the brain and blood of the lead-poisoned animals and of the control animals indicated that lead had reached the brain in large amounts. Whether it had become bound, and if so where, we do not know. The results of our chemical analyses suggest that lead had interfered with the metabolism of the calcium ion. Perhaps the increased concentrations of calcium found in the brains of the lead poisoned animals represented calcium needed for and incorporated into the thrombi found in the capillaries.

In conclusion, from the findings we have presented and discussed we believe that the changes of experimental lead encephalopathy, although primarily the result of chronic metabolic dysoxidosis from injury to the capillary endothelial cells, are in part due to anoxia caused by disturbances in the microcirculation.

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