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Regressive or Lethal Lead Encephalopathy in the Suckling Rat

Correlation of Lead Levels and Morphological Findings

J. M. LEFAUCONNIER, M.D., PH.D., J. J. HAUW, M.D., AND G. BERNARD

Abstract. Lead encephalopathy was produced in immature Sprague-Dawley rats with an intraperitoneal (IP) injection of 60 μg/g body weight of lead acetate administered daily from the fifth day after birth. Microscopic and light microscopic study of the nervous system, estimations of the blood-brain barrier permeability to proteins and brain water content were performed every two days thereafter. Lead levels in total blood, plasma, and several brain areas were measured at the same intervals by flameless atomic absorption spectrometry. Electron microscopic study of the cerebellum was done 2, 6, and 12 days after beginning lead administration. After two days of lead administration and before any pathological change occurred the increase in lead level was greater in the cerebellum than in other brain areas. After four to six days, hemorrhagic lead encephalopathy developed and was most prominent in regions with higher lead levels. From day 11 to 14, there were two possible courses: a) improvement of the clinical status and morphological findings in 25% of the animals, or b) progression of abnormal clinical signs and death. Cerebral edema, both intra- and extracellular, may have contributed to the fatal evolution. The mechanism of this edema appeared complex and may have involved resorption failure. Good correlations were observed among progression of the clinical signs, high water content in the brain, morphological evidence of cerebral edema, and a high cerebellar lead level. In contrast, high blood lead levels could be associated with clinical improvement, normal brain water content, and regression of the pathological findings. These data suggest that differences in evolution are more likely related to differences in the development of resistance of the cerebral capillary to lead, or in the efflux of lead, rather than to the blood lead concentrations.

Key Words: Brain edema, chemically induced; Lead poisoning; Microscopy, electron; Spectrophotometry, atomic absorption; Tissue distribution.

INTRODUCTION

Pentschew and Garro described hemorrhagic lead encephalopathy in immature rats (1). Since that time, several reports (2–13) have contributed to a better knowledge of this animal model and its relationship to human lead encephalopathy (4). Pentschew and Garro noted that in animals intoxicated orally since birth, there was a spontaneous regression in the clinical signs (paraplegia) after about 15 days in 10 to 15% of the animals, even though the lead administration was carried on after the appearance of the hemorrhagic lead encephalopathy. In the same animals, with signs of clinical regression, the nervous system appeared nearly normal. Spontaneous clinical improvement and a regression in the morphological abnormalities of capillaries were also observed by Toews (12) in 60% of five-day-old animals treated with lead acetate administered by gastric gavage.

While detailed electron microscopic descriptions have been reported of the nervous system capillary lesions observed during the acute stage (4, 6, 10, 11, 13) or during regression following the discontinuation of lead intoxication (11), there is no study of spontaneous regression. In addition, no assay of lead levels in blood and brain has been done to compare animals which spontaneously improved with those which worsened.

From the Institut National de la Santé et de la Recherche Médicale (INSERM), Unité 26, Hôpital Fernand Widal, 75475 Paris Cedex 10, France and Laboratoire de Neuropathologie Charles Foix, Hôpital de la Salpêtrière, 75651 Paris Cedex 13, France.

Address correspondence to: Dr. J. M. Lefauconnier, INSERM U 26, Hôpital Fernand Widal, 200, rue du Faubourg St Denis, 75475 Paris Cedex 10, France.

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Our aims in the present study were: 1) to compare the clinical and morphological evolution in animals whose intoxication was stopped after the onset of the hemorrhages and in animals whose intoxication was continued, 2) among the latter group, to compare the clinical and morphological evolution in animals which spontaneously improved with those which worsened, 3) to try to correlate the regression or absence of regression with the total blood, plasma, and brain lead content. The latter results could help in understanding the mechanism of the difference between the two possible evolutions: difference in plasma lead concentration in some animals, in the blood-brain barrier permeability to lead, in the blood-brain barrier sensitivity to lead, or in the rate of efflux of lead from the central nervous system.

We chose a model of intoxication by the intraperitoneal route which we have previously described (14). In this model, a reproducible hemorrhagic encephalopathy, similar to that already described (1, 4, 6, 10, 11) develops in a few days. This model uses an unphysiological route for the lead administration, but it has the advantage that, unlike the others, the brain lesions appear before the retardation in body growth caused by lead. This allows a study of the direct effect of lead rather than a secondary effect due to undernutrition.

MATERIALS AND METHODS

Animals and Treatments

Pregnant female rats of the Sprague-Dawley strain were obtained from Iffa Credo (3 allée des Platanes, Fresnes 94260, France) on the fourteenth day of pregnancy. On arrival, they were individually housed in plastic cages. They were fed a commercial solid diet and unlimited amounts of tap water. The day following parturition was designated as day one, when the offspring were distributed at random among the nursing mothers (eight rats per nursing mother). Nursing mothers and offspring were weighed every day between 10 and 12 A.M. Animals were maintained in a temperature-controlled room (23°C) with a constant cycle of 12 hours (h) of light and 12 h of darkness and a constant humidity.

From the fifth day after birth, the experimental animals received an intraperitoneal (IP) daily injection of 60 μg/g body weight of lead acetate, $Pb(CH_3COO)_2, 3H_2O$. The lead acetate was prepared every day in freshly boiled distilled water in order to prevent the precipitation of lead as lead carbonate. Control litters were injected with the same volume of a solution containing the same weight of sodium acetate. In some experiments, the lead was administered continuously from day five to day 19 after birth. In other experiments, lead administration was discontinued at day 11 after birth (day six after the beginning of the intoxication).

For light microscopy and lead assay, one or more animals of each litter was killed at day 2, 4, 6, 8, 11, and 14 after the beginning of the intoxication.

For electron microscopy, one control and two test animals were killed at day 2, 6, and 12 after the beginning of the intoxication.

A total of 48 litters were used. For comparison, 12 adult animals were treated with the same weight of lead acetate per gram body weight. They were killed four or eight days after the beginning of the lead administration.

Morphology

All animals, including those used for lead assay, were macroscopically examined for the following features: a) separation of the skull sutures; b) presence of cerebral edema; c) occurrence of hemorrhages. The intensity of hemorrhages was graded as: 0 if none, 1 if only dispersed petechial hemorrhages were seen, 2 if a great number of petechial hemorrhages was observed, 3 if the hemorrhages appeared confluent.

At each time-period, the brains of two or three animals were fixed by immersion in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for light microscopy.

For electron microscopic examination, a solution of 1% glutaraldehyde and 4% paraformaldehyde in 0.2 *M* Sörensen buffer, pH 7.4 was perfused in one control and two or three test animals. Cerebellar blocks were embedded in Epon 812. One μm semi-thin sections were stained with paraphenylene diamine. Thin sections were stained with uranyl acetate and lead citrate.

At each time-period, six animals were tested for endothelial permeability to proteins. This was evaluated by the intensity of blue staining of the brain after injection of Evans blue. A solution of 2% Evans blue in isotonic saline was filtered and given at 4 μl/g body weight. The injected pups were returned to their mother and killed 12 h later.

The extent of cerebral edema was estimated from the ratio dry/fresh weight in a number of animals.

Lead Analysis

Rats were anesthetized with ether and a blood sample was collected by jugular or cardiac puncture and transferred into a heparinized nitric acid-washed tube. Fifty μl were taken for the cell count and 200 μl for lead analysis of whole blood; the rest was centrifuged at 3,000 g and 100 μl of supernatant were taken for plasma lead analysis. All samples were transferred into heparinized nitric acid-washed plastic tubes. After decapitation, the brain was quickly excised and dissected out into: pons-medulla, cerebellum, midbrain, and hemisphere + striatum. Each region was placed in a tightly capped, preweighed, nitric acid-washed Eppendorf tube. After weighing, it was stored at −20°C till assay. For assay, blood, plasma, and brain samples were placed in nitric acid-washed quartz tubes and digested in concentrated nitric acid at 100°C in a multi-block heater (Lab-Line Instruments).

The samples were then analyzed by flameless atomic absorption spectrometry (Varian-Techtron AA5 atomic absorption spectrophotometer, fitted with a CRA-90 graphite furnace). The lead concentration was determined by adding aliquots of lead solutions of known concentration to each sample.

RESULTS

Morphology

At day two, after beginning the intoxication (seven days of age), there was no significant difference in the mean body weight or the behavior of control and test animals, except for abdominal distension of the latter due to a peritoneal inflammatory reaction to injected lead; this reaction persisted throughout the experiment. Macroscopic, light, and electron microscopic examination of the test animals was unremarkable. There was no extravasation of Evans blue and the ratio of dry/fresh weight was identical to that of the control animals.

At day four, after beginning the intoxication, fresh hemorrhages could be seen macroscopically on the surface of the cerebellum of a number of animals (23/36). Their intensity could easily be graded. By light microscopy, they involved the hemispheres as well as the vermis, but were most prominent in the latter. Most hemorrhages were seen in the deep external granule cell layer, extending through the molecular layer towards the Purkinje cells. However, the white matter of folia and the neighboring internal granule cell layer were also affected. The leptomeningeal space and the deep cerebellar nuclei were spared. A few small hemorrhages and congested vessels could also be found in the pons and cerebral hemispheres where they involved the subventricular regions. A blue staining of the cerebellum after injection of Evans blue could be observed in all hemorrhagic cerebella. In a few cases, a diffuse light staining was observed in a nonhemorrhagic cerebrum.

Between day six and eight, after beginning the intoxication, most animals were less active, although they had no weight retardation. None had neurological signs. In contrast, all of them had macroscopically obvious cerebellar hemorrhages, some of which appeared old. In a few cases, hemorrhages could also be observed in the cerebrum, in the basal ganglia, corpus callosum, hippocampus, and less often in the cerebral neocortex. By light microscopy, the cerebellar hemorrhages tended to coalesce (Fig. 1A and 3A). Marked extracellular edema was extensive in the deep internal granule cell layer and most severe in the white matter of folia with the formation of large cysts (Fig. 1A). By electron microscopy (Fig. 2), the distended extracellular space in the cerebellum sometimes contained lightly floccular material (Fig. 2A, B). Cell processes, some of which could be identified as astrocytic, were swollen and contained vacuoles (Fig. 2A, B). There was no obvious increase in pinocytic vacuoles, and no degenerative change of endothelial cell cytoplasm except for some swelling of the

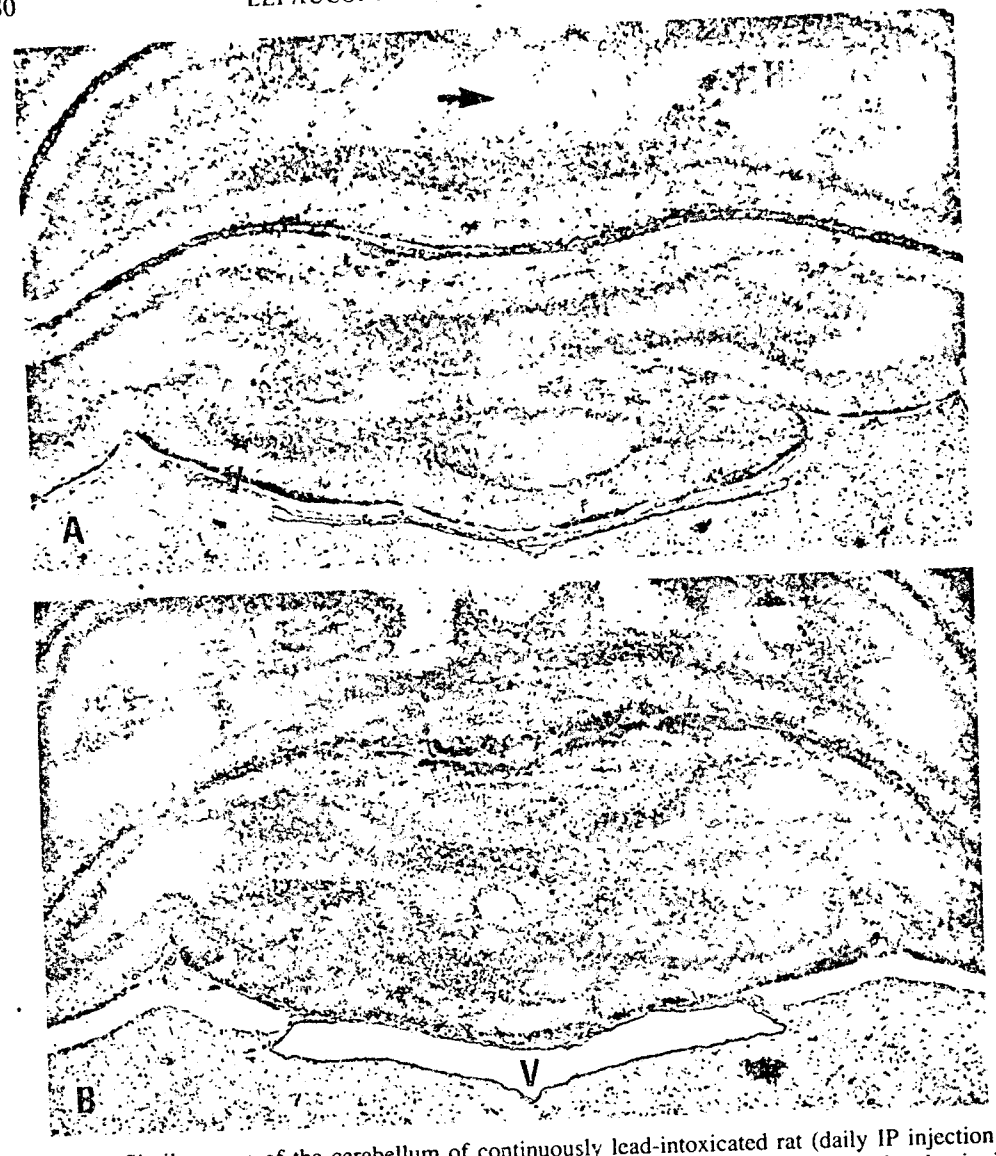
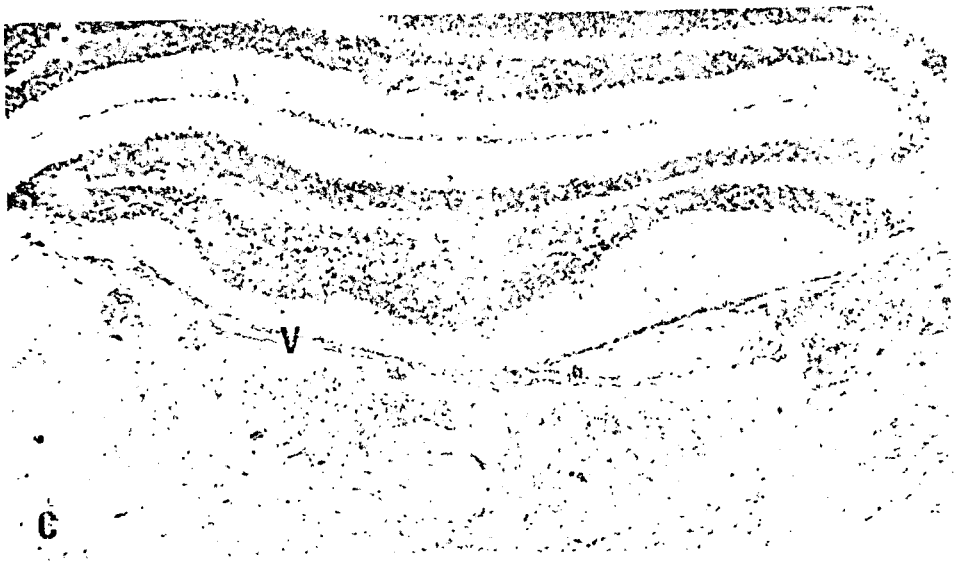


Fig. 1. Similar areas of the cerebellum of continuously lead-intoxicated rat (daily IP injection of lead acetate: 60 μg/g body weight). Hematoxylin and eosin ×120. A. Six days after beginning intoxication (11-day-old animal). Recent hemorrhages (H) in the internal granule cell layer, external granule cell layer, and molecular layer. Large cysts involve the white matter of the folia. Narrow fourth ventricle (V) and leptomeningeal space. B. Fourteen days after beginning intoxication (19-day-old rat); symptomatic animal. Numerous cysts and hemorrhages in the cerebellar white matter, internal, and external granule cell layers. Distended fourth ventricle (V) and leptomeningeal space. C. Fourteen days after beginning intoxication (19-day-old rat); asymptomatic animal. Nearly normal appearance and ventricular size.

parajunctional zones. In contrast, intercellular junctions appeared shortened and focal enlargements of the endothelial clefts were seen (Fig. 2C). Electron-dense inclusions of various appearances were seen in pericytes and astrocytes, and some contained dense granules. A few fat-laden phagocytic cells (Fig. 2C) and erythrocyte remnants were seen (Fig. 2D). An intense blue staining of the cerebellum was constantly observed after Evans blue injection.



Nearly constant blue staining of cerebrum was observed even in animals without cerebral hemorrhages.

At day 11, after beginning the intoxication, a retardation in body weight (−26%) could be observed in test animals which were slightly less active than control animals; none had neurological signs. The test animals were anemic ($3.4 \pm 0.76.10^6$ red cells/ml). The macroscopic and microscopic observations were intermediate between those of day eight and day 14.

Between day 11 and day 14, of 38 animals alive at day 11, 14 died spontaneously or were killed because of coma. Before death, they were sometimes paraplegic. In every case, postmortem examination showed cerebellar and cerebral edema and numerous fresh and old hemorrhages, primarily located in the cerebellum, but nearly constant in the cerebrum also.

At day 14, among the 24 animals killed, eight had severe retardation in body weight (−44%) and neurological signs: lethargy four of eight, paraplegia four of eight; in contrast, 16 looked normal except for a slight retardation in body growth (−22%).

Symptomatic animals always had separation of the skull sutures, an increased volume of hemorrhagic or xanthochromic CSF upon opening the dura mater, and obvious ventricular enlargement. All had numerous hemorrhages, most often fresh, in the cerebellum. In some cases, large cystic cavities were observed in the cerebellar vermis. Obvious cerebral edema was present in every case, corroborated by the increase in cerebellar water content (dry/wet weight: 0.164 ± 0.005) as compared to controls (0.179 ± 0.002). By light microscopy, fresh cerebellar hemorrhages of which some had pigment deposits, large cystic cavities, and ventricular enlargement were observed (Fig. 1B, 3B). A few small hemorrhages were also seen in centrum semi-ovale and the paraventricular hemispheric regions. The epithelium of the enlarged ventricles appeared normal.

Many changes were seen by electron microscopy. There was extracellular edema. The large distended extracellular space was electron-lucent in some regions, contained faintly electron-dense material in others. Enlarged and vacuolated cell processes were also seen. Capillaries exhibited obvious abnormalities: the cytoplasm of endothelial cells and pericytes was frequently enlarged, and contained numerous dense inclusions and 10-nm filaments,

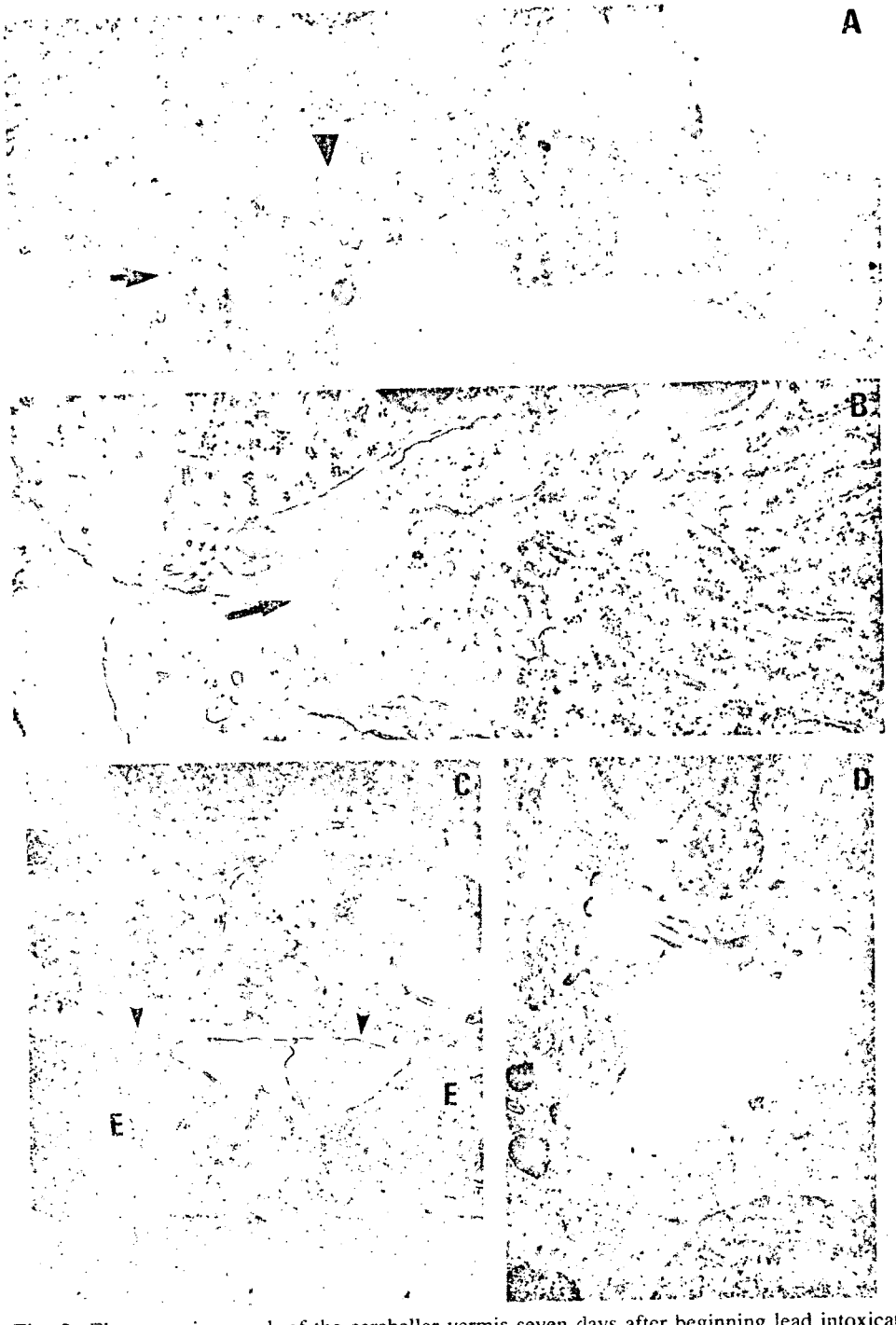


Fig. 2. Electron micrograph of the cerebellar vermis seven days after beginning lead intoxication (daily IP injection of 60 μg/g body weight of lead acetate) (12-day-old rat). A. Large extracellular space with floccular material (arrow) and swollen and vacuolated cell processes (arrowhead), sometimes obvious astrocytes, indicate both extracellular and intracellular edema. No endothelial changes. ×4,600. B. Floccular extracellular material (arrow) and few swollen processes are seen. ×22,000. C. Abnormal distended endothelial junction. Numerous endothelial processes, some labelled (E) are separated by large gaps. Basement membrane is indicated by arrowheads. Note the large extracellular space and a phagocytic cell. ×11,000. D. Erythrocyte remnants and dense bodies in a phagocytic cell. ×6,600.

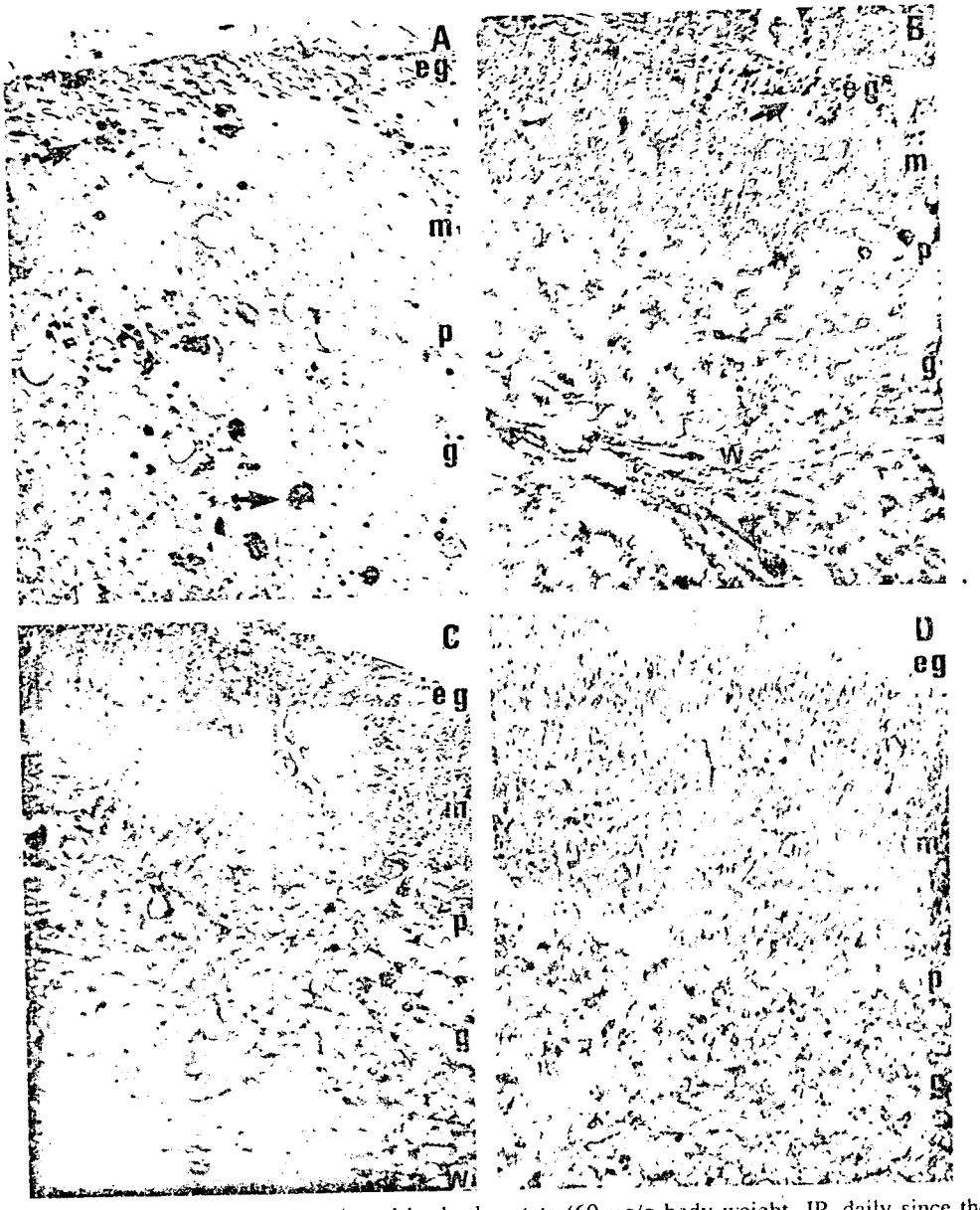


Fig. 3. Cerebellum of rats intoxicated by lead acetate (60 μg/g body weight, IP, daily since the fifth day after birth). Epoxy-embedded specimens. Semi-thin sections. Paraphenylene-diamine. Normarski optics ×250. (eg: external granule cell layer, m: molecular layer, p: Purkinje cell layer, g: internal granule cell layer, w: white matter). A. Seven days after beginning intoxication (12-day-old rat). Numerous hemorrhages (arrows) in the deep external granule cell layer, Purkinje cell layer, and internal granule cell layer. Molecular layer is less affected. B. Twelve days after beginning intoxication (17-day-old rat). Symptomatic animal. Hemorrhages mainly in the external granule cell layer (arrow) and white matter. C. Twelve days after beginning intoxication (17-day-old rat). Asymptomatic animal. This area of cerebellum appears normal. D. Twelve days after beginning intoxication (17-day-old rat). Intoxication discontinued after six days. Normal appearance of cerebellum.

basement membranes were often thickened (Fig. 4A, B). No junctional abnormality was found at this stage. Numerous dense bodies were seen in astrocytes (Fig. 4C). Neuronal perikarya appeared normal, but some degenerative neuronal processes were seen. A blue staining of the cerebellum was constantly observed after injection of Evans blue. It was most intense in the white matter of the cerebellar folia. In some instances, parts of the cerebrum, in particular the striatum, were also stained.

Asymptomatic animals exhibited much less marked changes; 12/16 did not have suture separation or cerebral edema. Macroscopically these animals had a nearly normal cerebellum and cerebrum or old lesions: old cerebellar hemorrhages (five of 12), a small cystic cavity (one of 12), light cerebellar and cerebral yellow discoloration (five of 12); they had no increase in water content. However, four of 16 animals had macroscopically recent changes: separation of the sutures (two of four), fresh cerebellar hemorrhages (two of four), large cystic cavity in cerebellum (one of four). They had also an increase in water content (dry/fresh weight: 0.155 ± 0.002). By light microscopy, a few old hemorrhages, some pigment deposits, and a few large cysts were seen in some cerebellar areas. The ventricular size was normal (Fig. 1C, 3C). No change was found in cerebral hemispheric sections. By electron microscopy, cerebellar changes were mild in areas without cysts, and consisted of swelling and clearing of astrocytic processes in the absence of extracellular edema. Capillaries appeared normal or showed a few electron-dense pericytic or endothelial inclusions. Similar figures were sometimes found in astrocytes; lipid-laden macrophages and erythrophagocytosis were seldom seen. In six of eight animals a blue staining of cerebellum was observed after Evans blue injection. No or very light staining of cerebrum could be observed.

Animals in which the lead administration had been discontinued at day six, had, at day 14, a slight weight retardation (-12%), normal behavior and macroscopically normal cerebrum and cerebellum. Their cerebellar water content was identical to that of the controls. By electron microscopy, numerous electron-dense, sometimes granular, inclusions were more frequently found in pericytes and astrocytes than were present in control animals.

Lead Analysis

At every time-period, lead concentrations in the whole blood of the control animals were less than 0.05 μg/ml; plasma lead as well as cerebellar lead could not be detected (limit of detection for solid tissue with the method used: 0.05 μg/g wet weight).

In one experiment, lead levels in whole blood, plasma, and cerebellum of animals intoxicated between the fifth and sixteenth day postnatal, were measured and related to the intensity of the cerebellar hemorrhages (Fig. 5). Between the first and fourth day of intoxication, the whole blood lead increased to about 5 μg/ml, leveled off for at least four days and increased to about 8 μg/ml on the eleventh day of intoxication. In contrast, plasma lead concentration had increased to about 0.2 μg/ml on the second day of intoxication and did not change much thereafter. The evolution of the cerebellar lead content was parallel to the blood lead concentration during the first eight days of intoxication, but always remained less than one-half the whole blood lead concentration. At the eleventh day of intoxication it had reached the whole blood lead concentration. At this time, the rat population was heterogeneous, including asymptomatic animals with resolving lesions and symptomatic animals with active lesions.

In another experiment, the two clinically different groups were separated. The difference between the evolution of whole blood and cerebellar lead levels in these two groups is shown on Figure 6. On the fourteenth day of intoxication, while the difference in blood lead concentrations was small and not statistically significant, a large difference in the cerebellar lead content was observed between the two groups ($P < 0.01$): in symptomatic animals, the cerebellar lead content in this experiment reached the whole blood lead concentration,

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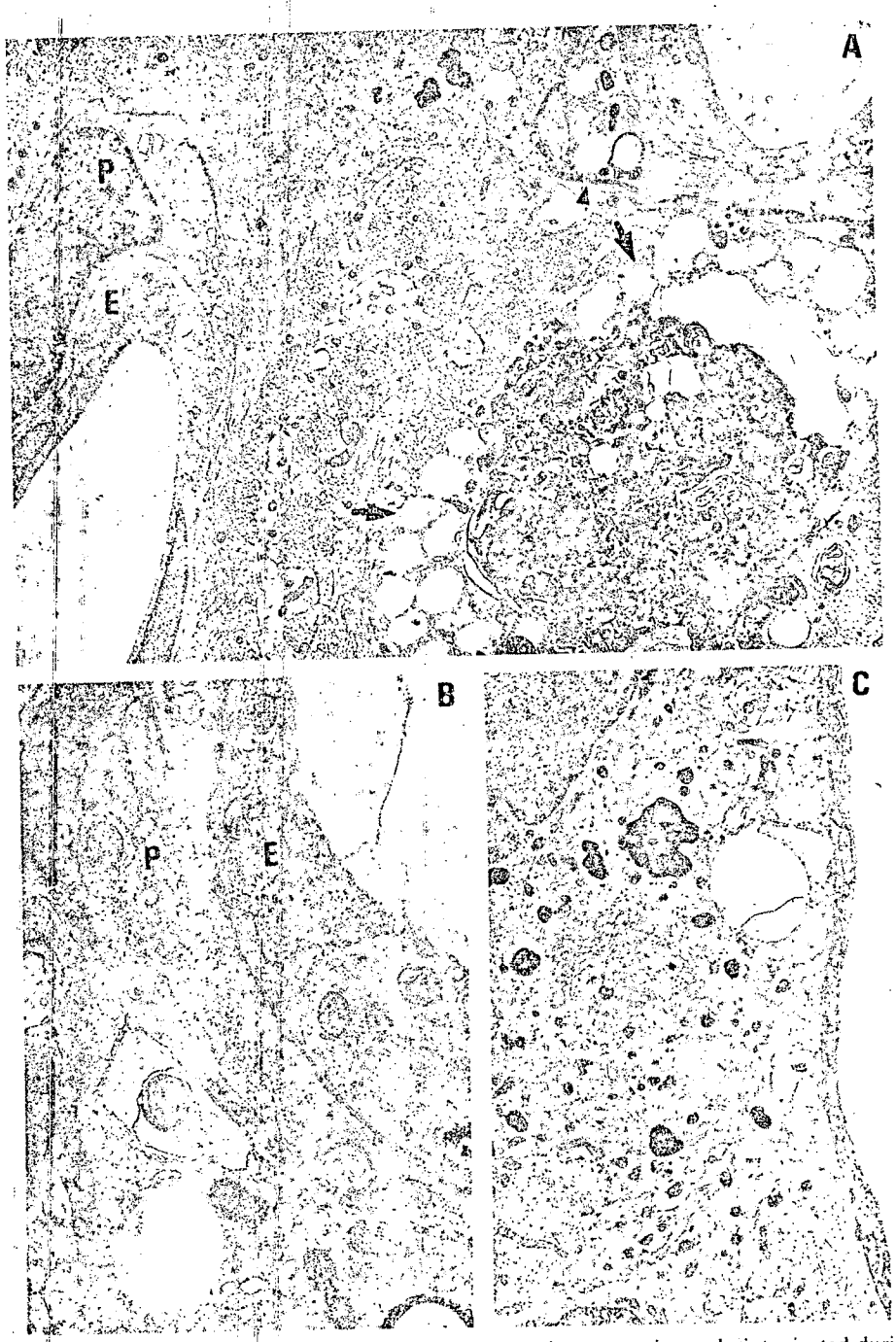

Fig. 4. Electron micrograph of a 17-day-old, symptomatic rat, continuously intoxicated during 12 days by a daily IP injection of lead acetate (60 μg/g body weight). A. Capillaries with large endothelial cells (E) and pericytes (P). Note the dense, wide basement membrane (arrowhead). A phagocyte with numerous dense cell remnants (arrows). ×4,000. B. Endothelial (E) and pericyte (P) cytoplasm with numerous microfilaments. ×11,000. C. Dense erythrocyte remnants in a perivascular astrocyte. Note the enlarged process in the neuropil. ×4,400.

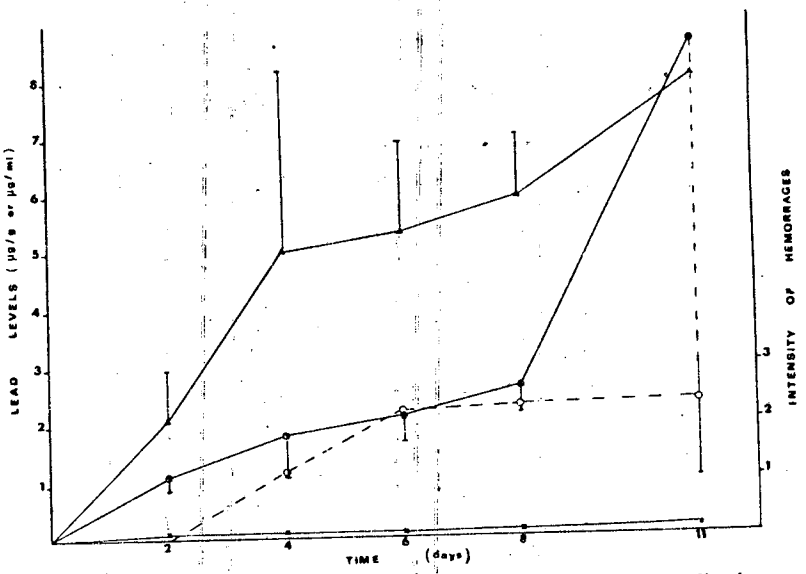

Fig. 5. Whole blood, plasma, and cerebellar lead levels; intensity of cerebellar hemorrhages as a function of time in rats administered lead acetate (60 µg/g body weight, daily, IP since day five after birth). ▲ = whole blood concentration, × = plasma lead concentration, ● = cerebellar lead content, and ○ = intensity of hemorrhage graded from 0 to 3. Time is indicated as days after beginning lead administration. Values are mean ± SD.

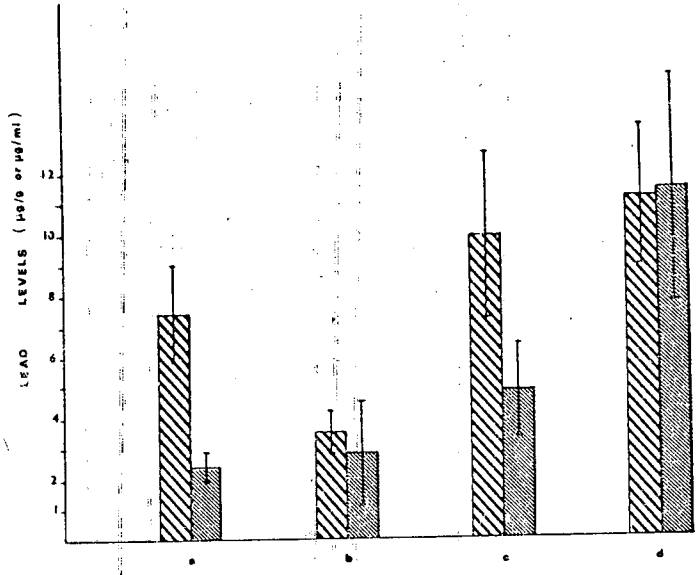

Fig. 6. Whole blood and cerebellar lead levels in experimental rats with regressive and worsening cerebellar lesions, eight and 14 days after beginning lead acetate administration (60 µg/g body weight, daily, ip, since day five after birth). \\\\ = whole blood lead concentration in µg/ml ± SD and ▨ = cerebellar lead content in µg/g wet weight ± SD. A. Blood and cerebellar lead levels, eight days after beginning intoxication. B, C, D. Blood and cerebellar lead levels, 14 days after beginning intoxication: B. In animals in which lead administration was discontinued the sixth day of intoxication. C. In animals in which lead administration was continued and clinically improving. D. In animals in which lead administration was continued and clinically worsening.

while in the asymptomatic group, it remained at about one half the whole blood lead concentration.

Animals in which the lead administration had been stopped on the sixth day of intoxication, were clinically healthy on the fourteenth day with a nearly normal brain morphology. However, there was no significant difference in the cerebellar lead concentration between the day when the intoxication was stopped (3.5 ± 0.7 μg/g wet weight) and eight days later (2.8 ± 0.8). In contrast, blood lead concentrations in these animals significantly decreased over the same period of time (7.4 ± 1.6 to 3.5 ± 0.7 μg/ml).

The lead content in four brain regions on the second and fourteenth day of intoxication is shown on Table 1. Before the occurrence of the first cerebellar hemorrhage, the lead content was significantly higher in the cerebellum than in other assayed brain regions, and it remained so throughout the remainder of the intoxication.

A comparison was made with adult animals receiving the same dose of lead per gram body weight. After four days of lead administration the adults had a blood lead concentration (4.5 ± 2.2 μg/ml) similar to that observed in a few suckling animals which had not yet developed cerebellar hemorrhages by the same period of intoxication (5.0 ± 3.2 μg/ml), while the cerebellar lead level was lower in adult (0.52 ± 0.08 μg/g wet weight) than in suckling rats (1.6 ± 0.5).

DISCUSSION

Since the first description of lead encephalopathy in the suckling rat (1), it has been repeatedly observed that the immature central nervous system (CNS) was more sensitive to lead than that of the adult which was resistant to lead encephalopathy. Two explanations have been suggested: a difference in the maturity of brain capillaries (6, 9, 12), or a difference in the blood lead concentrations (15), a consequence of a higher gastrointestinal absorption in the suckling rat (15, 16).

In our model there was no statistically significant difference between blood lead levels of immature and adult animals. In contrast, a significant difference was observed between the cerebellar lead levels which were much lower in adult animals (−50%). These results do not preclude the suggested role of a different intestinal absorption in the pathogenesis of lead encephalopathy of the immature rat, since our model bypasses intestinal absorption. Nevertheless, a difference in the permeability of the cerebral capillaries to lead, or in binding of lead exists between immature and mature animals in accord with the results obtained by Momčilović and Kostial (17) after a single injection of ^{203}Pb.

TABLE 1
Lead Levels in Selected Brain Areas After Two and Fourteen Days of Continuous Administration of Lead Acetate

	Brain areas			
Time	Pons-medulla	Cerebellum	Midbrain	Cortex + striatum
2 Days	0.51 ± 0.16 (5)	1.09 ± 0.15 (5)	0.49 ± 0.03 (5)	
14 Days Asymptomatic	3.3 ± 1.1 (4)	4.7 ± 2.0 (4)	2.9 ± 1.1 (4)	3.1 ± 0.7 (4)
14 Days Symptomatic	4.5 ± 0.7 (3)	11.7 ± 4.7 (4)	4.5 ± 1.3 (4)	3.8 ± 0.7 (4)

Lead levels in μg/g wet weight.
Values are mean ± SD. The number of animals in parentheses.
At day two: midbrain and cortex + striatum were analyzed together. Macroscopically the brain was similar to that of controls.

During the first two days of intoxication, when cerebellar hemorrhages had not yet developed, the CNS lead content (0.5 to 1.1 μg/g wet weight according to the area) increased well above the plasma lead concentration (0.138 ± 0.034 μg/ml), but remained less than the whole blood lead concentration (2.1 ± 0.9 μg/ml). This high cerebellar lead level could not be due to a contamination by blood left in the vascular compartment after decapitation, as the proportion of blood left in adult rats, under such conditions, is known to be about 2% of the brain wet weight (18). Even if this proportion were higher in immature animals, it seems unlikely that it could explain the high level found in our experiments. This observation of a CNS lead content higher than the plasma lead content suggests that binding sites with affinity for lead are present in brain or at least at the blood-brain interface, the endothelium of capillaries. It has been shown by Toews et al (12) that cerebral capillaries isolated from brains of immature intoxicated animals had a higher content of lead than the brain homogenate. Autoradiographic studies have also shown that radioactive lead was concentrated in the cells of brain capillaries (19, 20). We observed that this uptake of lead was not uniform, since the lead content of cerebellum was twice that of medulla or the cerebral hemispheres. Since these results were obtained before the appearance of the hemorrhages, the higher level in cerebellum at this time could not be due to the presence of extravasated erythrocytes as has been suggested for a later stage of intoxication (10). The higher level in cerebellum could perhaps have been due to a greater permeability, or greater binding of lead to cerebellar capillaries (less mature?). Greater permeability or greater lead-binding to capillaries could have contributed to the prevalence of the hemorrhagic lesions in this area of the nervous system, or it could also be a consequence of an early lesion of the cerebellar endothelium.

Between the fourth and eighth day of intoxication, the morphological features observed in intoxicated animals were very similar to those reported by others (1, 4–6, 11), in particular, by Press (10). At this time since the hemorrhages had already developed, lead bound to erythrocytes (about 95% of the total blood lead) obviously contributed to the increase in the lead cerebellar content which remained nevertheless well below the total blood lead concentration. Our results for test animals at this stage are of the same order of magnitude as those published by others (5, 6, 9–12).

As the lead administration was carried on, two types of evolution could be observed: some animals (25%) improved, while others developed neurological deficits and died. These two possible evolutions have been previously reported (1, 12). In the group of animals which became worse, the clinical status (paraplegia or coma) was correlated with morphological changes of brain edema. This was obvious to the unaided eye and corroborated by an increase in cerebellar water content. At the electron microscopic level, both extracellular and intracellular edema appeared. The precise mechanism of this edema was difficult to assess. The hemorrhages and occasional electron-dense fluid indicated a "vasogenic" component. In addition, large distended electron-lucent processes were characteristic of intracellular edema (21, 22). Such intracellular edema could be linked to defective sodium pumps, since it has been shown that brain Na^+ K^+ ATPases can be inhibited by lead (23). A superimposed "hydrocephalic" edema due to posthemorrhagic resorption failure could also be involved. The increased volume of xanthochromic cerebrospinal fluid (CSF) when the dura mater was opened, the ventricular enlargement, and the electron-lucency of extracellular edema in some regions, favor this hypothesis. However, there is no definite morphological proof.

In the group of animals which improved, the clinical status was well correlated with the microscopic findings. However, these animals had not been spared the pathological process; cerebral edema and hemorrhages had been present, since several animals had macroscopically obvious old cerebellar hemorrhages and cystic cerebellar cavitations. Light and electron microscopy indicated moderate intracellular edema associated with some pigment deposits,

but it was absent at the stage of overt extracellular edema. In spite of these pathological changes, the animals had improved and had no more severe edema, since their brain water content was similar to that of control animals. This improvement could not have been due to a mechanism which would decrease the concentration of lead in blood, since the blood level continued to increase and was not statistically different in animals which improved and in those which became worse. In contrast, a statistically significant difference was observed in the cerebellar lead levels of the two groups.

The evolution toward improvement in a number of animals could be due to the fact that they were initially less sensitive to the deleterious effect of lead, and thus had fewer initial hemorrhages and consequently a lower cerebellar lead level. However, this explanation probably does not hold true for all these animals since by day six or eight, all the animals killed had had cerebellar hemorrhages and their cerebellar lead contents were of the same order of magnitude. In addition, at day 14 some animals in the group which improved still had sequellae indicating previous severe pathological changes. A resistance to the effect of lead on the CNS thus developed in the group which improved. The development of such a resistance to lead intoxication was also demonstrated by the experiments of Toews et al (12) who observed obvious abnormalities in the capillaries of five-day-old treated animals; these abnormalities disappeared when the animals were treated for a longer period of time.

In conclusion, our results do not provide a clear answer to the question raised in the introduction concerning the mechanism of the variability of lead effect on the nervous system of immature animals. However, they provide evidence against some of the possible mechanisms suggested and support for some others. The difference in evolution: 1) could not be due to a difference in blood lead concentrations since they did not differ in either the animals that became worse or those that improved; 2) might be due to a difference in the blood-brain barrier permeability to lead; this hypothesis seems unlikely because by day four to six, at the beginning of the hemorrhagic period, it was not possible to differentiate two populations by means of their cerebellar lead content; such a difference could only be shown later at day 14; 3) might also be due to variability in the development of resistance to the deleterious effect of lead; 4) might be due to a difference in the efflux of lead from the CNS. We have already mentioned the possibility of a CSF resorption defect and it may be that in the group of animals that became worse, an impediment to the circulation of CSF prevented the elimination of lead from the CNS, resulting in a progressive increase in cerebellar lead. The capillary could thereby have been exposed to a higher content of lead at its abluminal membrane, which might hinder the development of a resistance to lead.

ACKNOWLEDGMENTS

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Pathogenesis of Lead Encephalopathy

Uptake of Lead and Reaction of Brain Capillaries

Gary W. Goldstein, MD; Arthur K. Asbury, MD; Ivan Diamond, MD, PhD

Lead entry into the central nervous system and effects on brain metabolism were studied in immature rats. Lead 210 readily enters the brain after intravenous injection; uptake is proportional to dose. Although blood levels fall rapidly after injection, brain lead retention is prolonged and unaffected by edetic acid therapy. In individuals who ingest lead episodically, progressive accumulation in the brain can occur without detection of changes in blood.

Excessive lead deposition in the brain of nursing rat pups provokes severe hemorrhagic reactions limited to the cerebellum although lead concentrations are the same as in other brain regions. A prehemorrhagic stage of lead encephalopathy could be identified in the cerebellum by monitoring brain edema. This appears to be due to capillary dysfunction and suggests that cerebellar capillaries are more vulnerable to lead than capillaries elsewhere in the brain.

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Acute lead poisoning can produce a devastating hemorrhagic and edematous encephalopathy that is more frequently encountered in children than adults.[1,2] However, despite widespread experience with measurements of lead in the blood, there is no information about the relationship between lead in the blood, the amount of lead deposited in the brain, and the acute effects of lead on metabolism in the nervous system.

Lead is readily measured in the blood after episodic ingestion or respiratory exposure to polluted air.[3,4] The purpose of this study is to define the relationship of lead in the blood to lead in the brain and to identify the metabolic consequences of lead deposition in the central nervous system (CNS). The results show conclusively that the entry of lead into the brain is proportional to both the dose of lead received and the circulating blood levels of lead. Moreover, lead is retained in the brain for long periods of time. After lead accumulates in the brain of young rats, a prominent vasogenic edema precedes the development of a cerebellar hemorrhagic encephalopathy. These findings suggest that acute lead encephalopathy results from a disorder of capillary function in the immature nervous system and that capillaries of the cerebellum may be more vulnerable to lead than capillaries elsewhere in the brain.

MATERIALS AND METHODS

Animals

A modification of the model of lead encephalopathy in suckling rats described by Pentshew and Garro[5] was used in this study. Sprague-Dawley nursing mother rats with litters of eight pups, 12 days old, were started on a diet containing 4% lead carbonate that was eaten ad lib. Control mothers and litters did not receive the lead supplement. Under these conditions a fatal hemorrhagic encephalopathy developed when the pups reached 4 weeks of age. If the diet was started when the pups were older than 3 weeks of age, the rats failed to develop neurological symptoms or neuropathologic changes despite receiving the lead diet for as long as eight weeks.

Tracer Experiments

Lead 210 was used to study the deposition and disappearance of lead from the blood and CNS. Saline solution, 0.5 ml, containing 16 microcuries of ^{210}Pb and 1.0μg, 50μg or 200μg of nonradioactive lead nitrate was injected intravenously into 4-week-old rats. Brain and blood samples were collected from rats killed sequentially. Radioactivity was analyzed in triplicate in a gamma well spectrometer with a ± 5% range of replication. The concentration of radioactive lead in the brain was corrected for lead contained in a 2% vascular compartment of the brain.[6]

Edetic Acid Experiments

Edetic acid, 1 millimol/kg, was administered intramuscularly daily for four days to 4-week-old rats after intraperitoneal administration of 10 microcuries of radioactive lead nitrate [$^{210}Pb(NO_3)_2$, specific activity: 14.8 curies/gm]. Control rats were treated with saline. The concentration of ^{210}Pb in the brain was determined as described above.

Mitochondria were prepared as described previously.[7] A 10% homogenate of brain in 0.32M sucrose was centrifuged at 1,000 g for ten minutes. The supernatant was recentrifuged at 18,000 g for ten minutes, and the mitochondrial pellet was washed in 0.32M sucrose and suspended in the incubation medium at a final protein concentration of 1 mg/ml. The incubation medium contained 80 millimols sodium chloride, 10 millimols magnesium chloride, 5 millimols adenosine triphosphate (ATP), 10 millimols sodium succinate, 2 millimols disodium acid phosphate, 2 millimols monosodium acid phosphate, 10 millimols Tris (pH 7.4), 1.5 millimols nitrolotriacetic acid, 0.1 millimols $Pb(NO_3)_2$ and 1 microcurie/ml of ^{210}Pb. Liver mitochondria have been shown to accumulate lead in a similar preparation.[8] The mitochondria were incubated in triplicate at 37 C in a rotating water bath for two 30-minute periods. Edetic acid (1.0 millimol) was added before the addition of lead or after an initial 30-minute incubation with lead. Control suspensions did not contain edetic acid. The mitochondria were collected and washed by centrifugation at

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From the departments of neurology and pediatrics (Drs. Goldstein and Diamond) and the Neurology Research Laboratory, Veterans Administration Hospital, San Francisco (Dr. Asbury).

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Reprint requests to Department of Neurology, University of California, School of Medicine, San Francisco, CA 94143 (Dr. Goldstein).

4,000 g for ten minutes and the concentration of ^{210}Pb in the mitochondrial pellet was determined by liquid scintillation spectrometry.[9]

Sodium-Potassium-Magnesium Ion Adenosine Triphosphatase

Enzyme activity was measured by modification of the methods of Samson and Hinn[10] and Bonting.[11] The brain was homogenized in nine volumes of distilled deionized water and centrifuged for 30 minutes at 48,000 g. After discarding the supernatant, the pellet was resuspended in the same volume of water and frozen at −15 C for 18 hours. To determine adenosine triphosphatase (ATPase) activity, 0.25 ml of the thawed suspension was incubated in triplicate in a solution containing 0.15 mg of protein, 4 millimols ATP, 4 millimols magnesium chloride, 100 millimols sodium chloride, 30 millimols potassium chloride, 50 millimols Tris (pH 7.4) for ten minutes at 37 C in a final volume of 1.0 ml. Controls were measured at zero time. Protein was measured by the method of Lowry et al with crystalline bovine albumin as a standard.[12] Inorganic phosphate was measured by the method of Bonting.[11] Total ATPase activity was calculated from the production of inorganic phosphate and results were expressed as micromols of phosphate per milligram of protein per hour. The assay was linear with increasing protein concentration and time under these conditions of study. Sodium-potassium-magnesium ion-ATPase activity was considered to be inhibited by 1 millimolar ouabain.

Analytical Procedures

Mothers and litters were decapitated at different intervals after starting the diet, and blood was collected from the severed neck. The brain from mature and immature rats was removed quickly and cleaned of superficial blood with ashless filter paper. In some experiments the cerebrum, pons, and cerebellum were analyzed separately. Brain dry weight was determined in tared bottles after drying 24 hours to a constant weight in a 100 C oven. Brain sodium and potassium concentrations were measured in a flame photometer after suspending the dried brain in five volumes of nitric acid overnight and diluting the sample with water.[13] Lead concentrations in brain and blood were determined by suspending the brain overnight in three volumes of concentrated nitric acid and by diluting the blood with four volumes of distilled water; 1μ was injected into the carbon rod element of a flameless atomic absorption spectrophotometer. The sensitivity of the apparatus permitted analysis of as little as 0.25μg of lead per gram of tissue.[14] Measurements were linear with increasing lead concentrations under the conditions of analysis.

Pathologic Analysis

One litter of eight rats was prepared for electron microscopy. Lead carbonate, 4% by weight, was added to the maternal diet when the pups were 12 days old. All eight rats were killed on the 28th day of life by ether anesthesia followed by perfusion-fixation. A catheter was introduced into the left ventricle of the heart, and blood was flushed from the circulatory system for one minute with Ringer solution followed by 3.6% glutaraldehyde in 0.1M phosphate buffer at pH 7.5 for 15 minutes at a rate of 5 ml/min. Blocks of cerebrum and cerebellum were postfixed in 2% buffered osmium for two hours and embedded in epoxy plastic. All blocks were surveyed in 2μ sections by light microscopy, and selected blocks were ultrasectioned for electron microscopy. Both unstained grids and grids stained with uranyl acetate and lead citrate were examined in an electron microscope. Two unperfused brains were dissected fresh, fixed in 4% formaldehyde solution, and both frozen, and paraffin sections were stained with ammonium sulfide for lead deposition.

RESULTS

Studies With ^{210}Pb

Twenty-four hours after a single intravenous administration of ^{210}Pb, the concentration of radioactive lead in both the blood and brain was directly proportional to the dose of lead (Fig 1). This indicates that there was a linear relationship between the amount of lead in the blood and the amount of lead in the brain. Thus, there was no apparent threshold for entry of lead into brain at low blood

Fig 1.—Effect of dose on uptake of lead by blood and brain. Lead 210 was administered intravenously to 1-month-old rats. Blood and brain lead concentrations were determined 24 hours later. Each point is the average of three animals ± 1 SD. Brain lead was corrected for radioactivity in the vascular space.

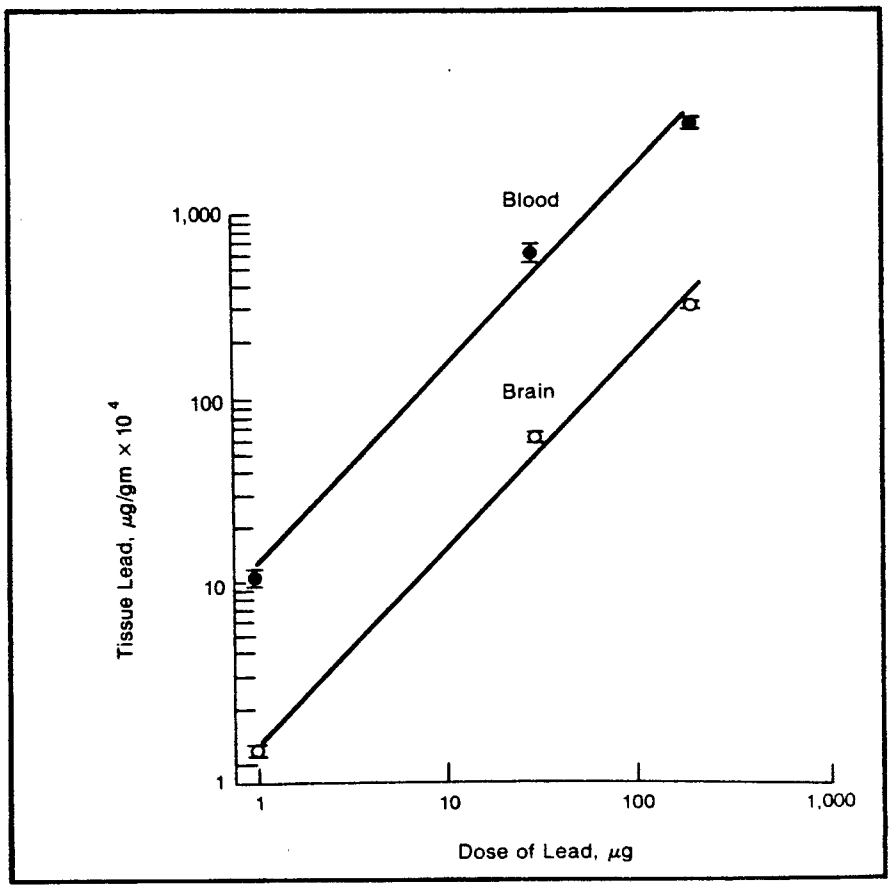
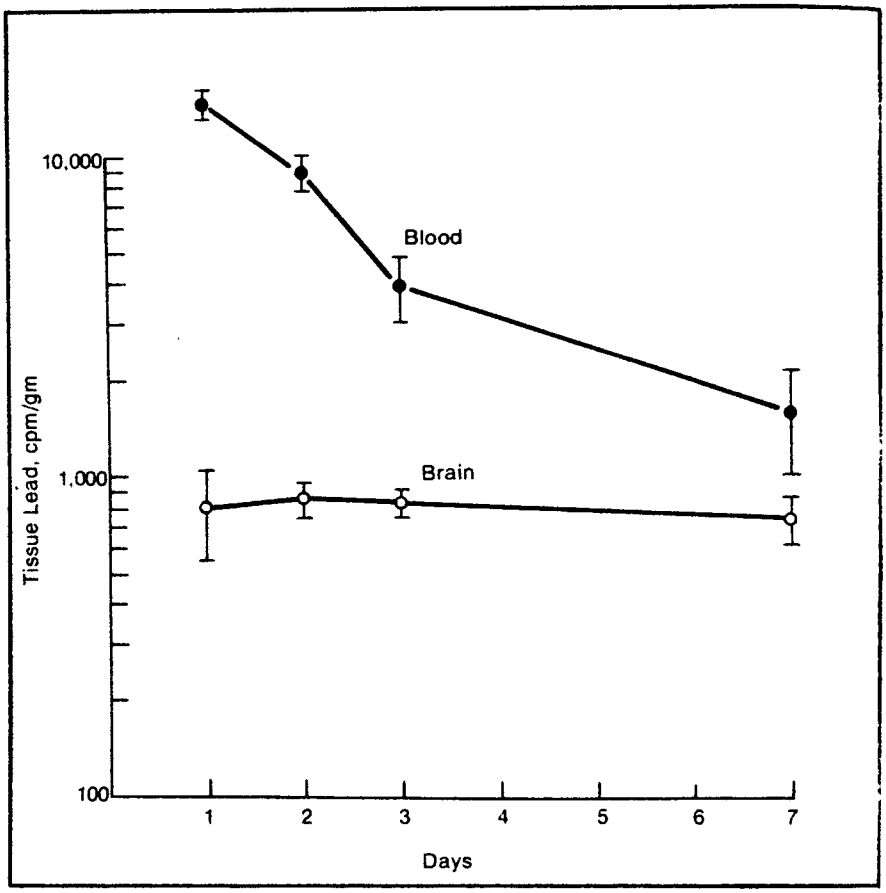


Fig 2.—Effect of time on the retention of lead by blood and brain. Analyses are as described in Fig 1.



levels, and uptake of lead was not saturable at high blood levels. After injection of ^{210}Pb, retention of radioactive lead in the brain was much longer than that in the blood (Fig 2). The half-life for retention of lead in the brain could not be determined accurately even though the half-life for retention in the blood appeared to be less than one week. Subsequent treatment with edetic acid was not effective in removing lead from the brain of animals given a single dose of ^{210}Pb (Fig 3). A similar effect was also observed in vitro where subsequent addition of edetic acid did not remove lead already bound to mitochondria. However, edetic acid could prevent the uptake of lead by brain mitochondria if the chelating agent was added before the lead (Fig 3).

Studies With Nonradioactive Lead

Since lead can also be measured in the blood after dietary intake, the evolution of the clinical and pathophysiologic changes of experimental lead encephalopathy could be followed over long periods of time with the use of nonradioactive lead. When the animals were fed a lead-containing diet, three distinct stages of acute lead encephalopathy were evident.

Stage 1.—Lead was added to the maternal diet when the rat pups were 12 days old. No neurological abnormalities were seen during the first ten days of the diet despite a progressive accumulation of lead in the brain of the mothers and the pups (Fig 4). The concentration of water, sodium, and potassium in brain taken from

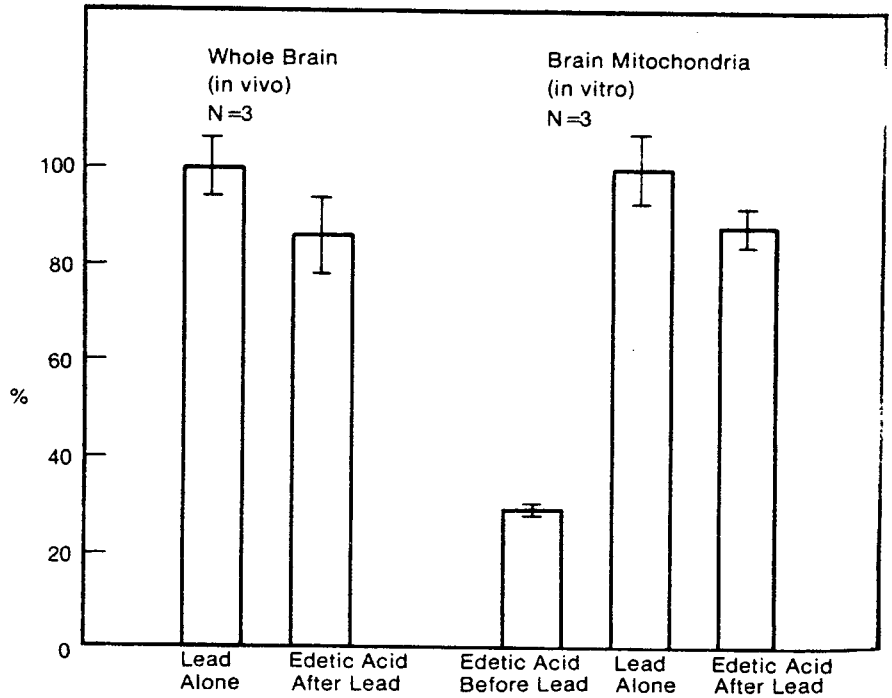


Fig 3.—Effect of edetic acid on uptake and retention of lead. Lead 210 was administered intraperitoneally to 1-month-old rats. Conditions are as described in "Methods"; 100% refers to concentration of lead in untreated preparations. Edetic acid did not remove lead from the brain in vivo or from brain mitochondria when lead was added in vitro ($P = .1$). However, edetic acid in vitro greatly reduced uptake of lead by brain mitochondria ($P < .001$).

lead poisoned animals did not differ from that of untreated controls of the same age. The weight of pups fed the lead diet averaged 50% of untreated controls.

Stage 2: "Prehemorrhagic".—After the animals had been fed a diet containing lead for more than 12 days, mild ataxia developed in the immature rats while the mothers did not exhibit neurological difficulties. In the young animals lead continued to accumulate in the brain and reached 6μg/gm at 25 days of age (Fig 4). By contrast, brain taken from their mothers failed to show a continued increase in the concentration of lead after reaching a plateau of 3μg/gm (Fig 4). Findings on gross examination of the immature brain during this stage were unremarkable. On detailed analysis, however, the dry weight sodium concentration of the immature cerebellum increased by 33%. ($P < .001$) from 240 ± 15 to 320 ± 40 mEq/kg while the potassium concentration remained normal (Fig 5). By contrast, the cerebral hemispheres and brain stem taken from the brain of the immature rats failed to show a similar edematous reaction and had normal water, sodium, and potassium concentrations. Maternal brain also had normal water, sodium, and potassium concentrations.[13]

Stage 3: "Hemorrhagic".—After the animals had been on the lead diet for 14 to 16 days severe ataxia and hind limb paralysis occurred when the young rats reached 26 to 28 days of age. At this time the mothers remained well. Gross examination of the immature brain revealed diffuse hemorrhage throughout the cerebellum while the cerebral hemispheres and brain stem were not similarly affected. The dry weight sodium concentration increased 88% ($P < .001$) from 240 ± 15 to 450 ± 40 mEq/kg in the abnormal cerebellum and the potassium content decreased by 15% ($P < .001$) from 506 ± 18 to 430 ± 15 mEq/kg (Fig 5). By contrast the cerebral hemispheres and brain stem from the young rats and all regions from the maternal brain did not differ from age matched controls. Despite the fact that the immature cerebellum suffered severe edematous and

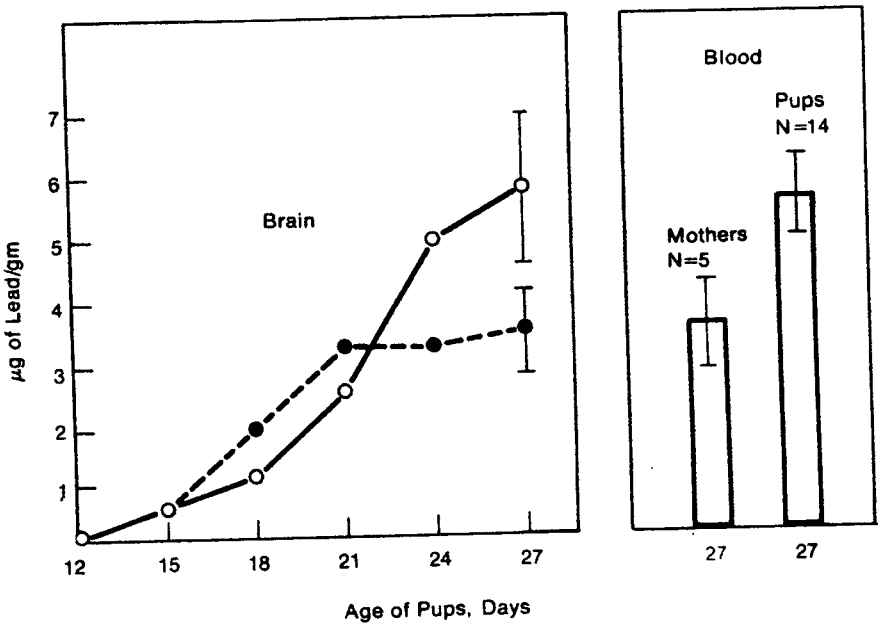
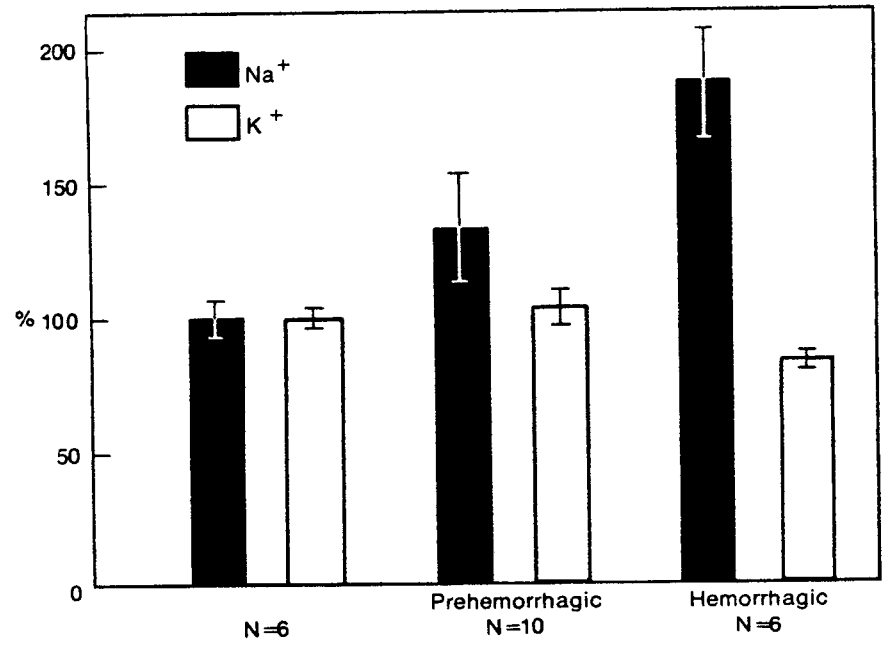


Fig 4.—Concentration of lead in the brain and blood of mothers (solid circles) and pups (open circles) fed a lead-enriched diet for two weeks. Only the pups showed signs of encephalopathy. Each point is the average of three separate animals. At day 27 the vertical bars refer to concentration of lead in blood ± 1 SD.

Fig 5.—Relative concentration ± 1 SD of Na^+ and K^+ in brains of pups developing lead encephalopathy; 100% refers to values obtained in age-matched controls. Three sets of bars refer to three stages of lead encephalopathy.



hemorrhagic changes, the concentration of lead here (6μg/gm) was no different from that in the unaffected regions of the brain (Fig 6).

In order to avoid artifacts due to hemorrhagic necrosis, Na^+-K^+-Mg^{++}-ATPase was measured in the cerebellum at a time when edema was evident but hemorrhage had not yet developed. Enzyme activity in samples of cerebellum taken from animals with stage 2 (prehemorrhagic) encephalopathy was 11 ± 2μmols/mg of protein per hour and not significantly different from age matched controls (13 ± 2μmols/mg of protein per hour).

Pathologic Analysis

Cerebella taken from rats with early stage 3 (hemorrhagic) encephalopathy were prepared for morphologic study (Fig 7). Striking interstitial edema of white matter with wide separation of relatively normal appearing myelinated fibers was the outstanding feature, with lesser degrees of edema present in the overlying granule cell and molecular layers (Fig 8 to 10). Extravasated red blood cells (RBC) were prominent in foci equally distributed in gray and white matter. Numerous macrophages filled with lipid debris and disintegrating erythrocytes were observed primarily in cerebellar white matter (Fig 8). Most blood vessels appeared normal except for surrounding edema and occasional mononuclear cells migrating through vessel walls (Fig 10). Endothelial vacuolation as described by Lampert et al[15] and occlusion of vessels as described by Thomas et al[16] could not be demonstrated, although occasional mild proliferative changes of capillary walls were observed. Sections of cerebrum were normal by light and electron microscopy except for minor swelling of perivascular astrocytic processes. This may represent either minimal edema or artifact of perfusion-fixation.

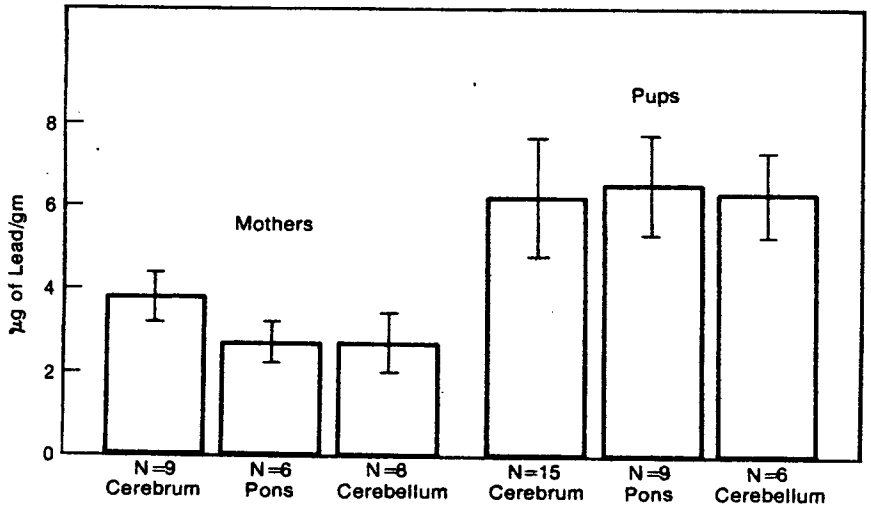
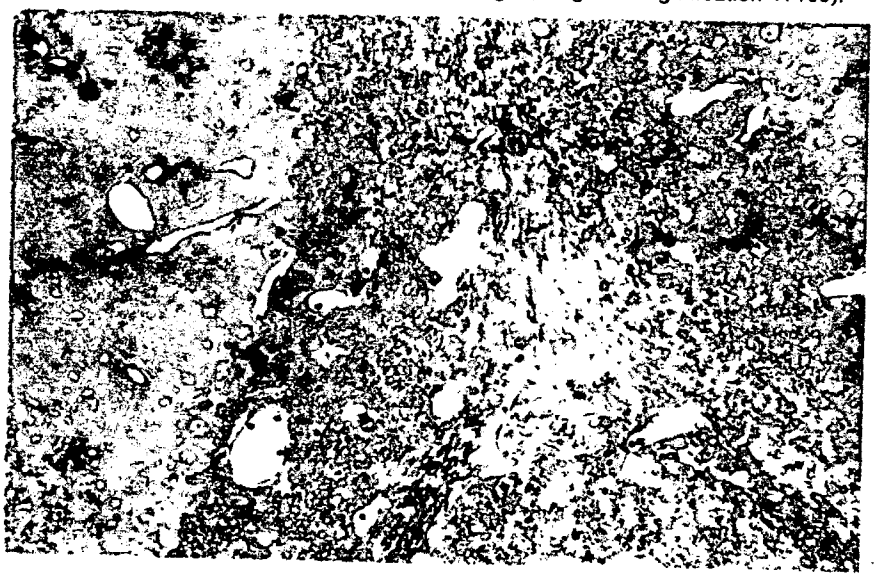


Fig 6.—Concentration of lead (± 1 SD) in regions of brain taken from mothers and pups receiving a lead carbonate diet for two weeks.

Fig 7.—Cerebellum from early stage 3 lead encephalopathy. Parts of molecular layer are visible on the sides. Central portion of field contains the granule cell layer and a small area of edematous white matter. Note foci of extravasated RBC both in molecular and granule cell layers (2μ plastic section, Paragon, original magnification ×100).

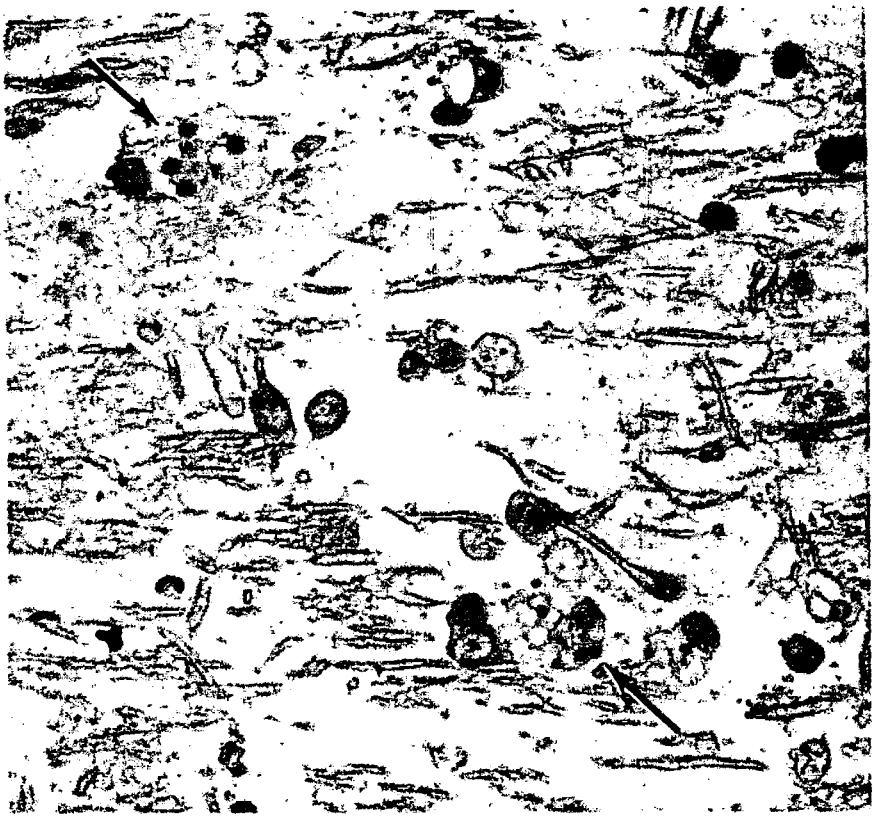


Attempts to demonstrate the distribution of lead histochemically were unsuccessful. In addition, an effort to locate lead deposition by study of unstained ultrathin sections electron microscopically was unrewarding. Intranuclear stellate inclusion bodies of the type seen frequently in renal tubular cells and rarely in astrocytes were not discovered in our material.

COMMENT

An increasing burden of lead in the environment is reflected in rising blood lead levels of children and adults throughout the world.[1,3,18] Moreover, 20% of children in Philadelphia have increased lead in deciduous teeth,[19] and recent surveys in several American cities indicate that more than 15% of preschool children have blood lead concentrations that exceed 40μg/100 ml.[20,21] This is the upper limit thought to be safe and is the level above which abnormalities in erythrocyte porphyrin metabolism become evident.[4] However, we do not know whether these children are at risk for neurological damage.

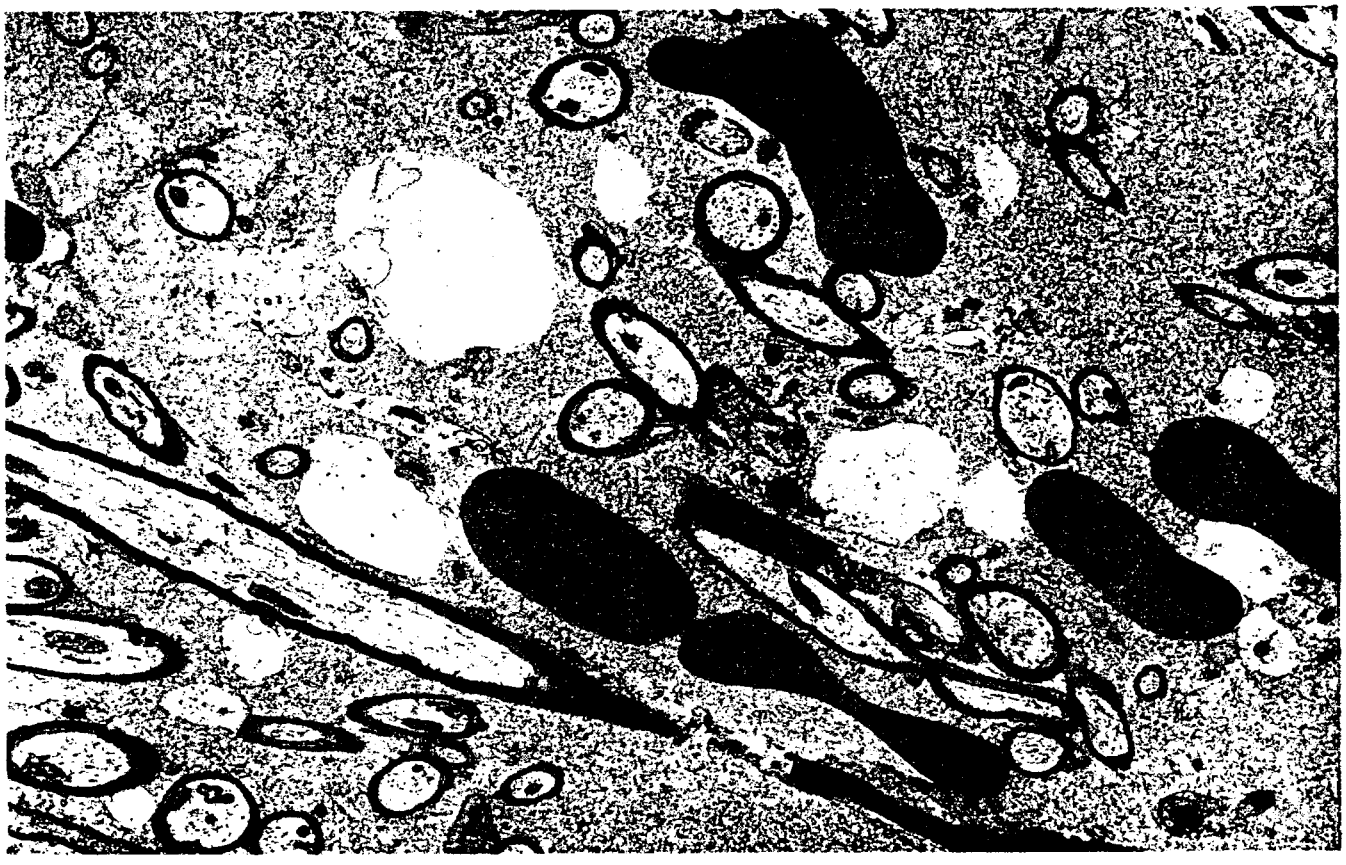
In this experimental model of lead



encephalopathy in young animals, there is a linear relationship between the lead concentration in the blood and the amount of lead that is deposited in the brain. Indeed, lead is taken up by the brain when blood lead levels are quite low, which suggests that there is no threshold to protect the brain against the deposition of lead. These results are consistent with

Fig 8.—Cerebellum from early stage 3 lead encephalopathy, higher power. Note striking separation of myelinated axons by interstitial edema. Macrophages filled with lipid debris and disintegrating RBC are seen (arrows) (2μ plastic section, Paragon, ×600).

Fig 9.—Cerebellar white matter from early stage 3 lead encephalopathy. Normal-appearing myelinated axons are widely separated by edema. Granular, electron-dense appearance suggests that the fluid is rich in protein. Several extravasated RBC occupy the right central portion of the field (original magnification ×10,500).



other findings that lead is deposited in many organs outside the CNS in proportion to the concentration of lead in the circulation.[22]

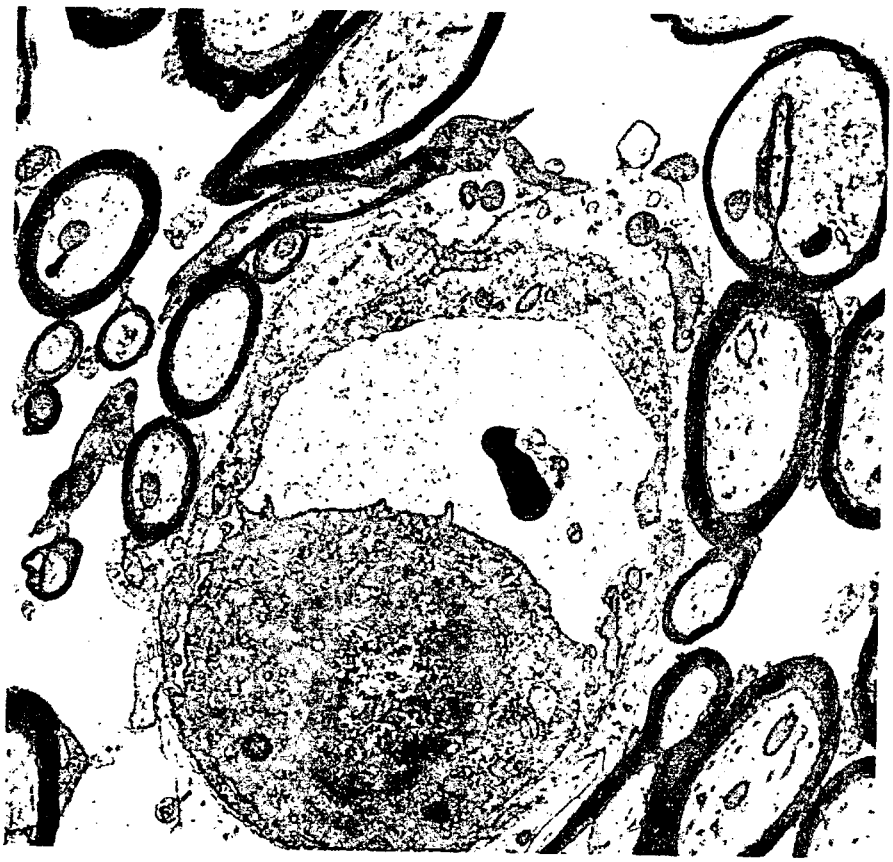
Although the deposition of lead in the brain increases directly with increasing blood concentration (Fig 1), retention of lead by the brain persists after blood levels fall to acceptable values (Fig 2). Therefore, if lead intake is episodic, the concentration of lead in the blood cannot be used as a measure of the amount of lead in the brain. Unfortunately, intermittent exposure to lead often occurs in patients with lead poisoning and transient increases in blood lead levels probably result in significant deposition of lead in the brain of affected individuals. Indeed, it seems likely that progressive accumulation of lead can occur in the brain without detection of substantial changes in blood in individuals who take in lead sporadically. This might explain the poor correlation of single blood lead measurements with symptoms of encephalopathy in children with lead poisoning.[3,4]

Accumulation of lead in the brain in amounts that do not produce an acute encephalopathy may be responsible for a chronic neurological disorder. This speculation is consistent with the suggestion that behavioral abnormalities, learning problems, and hyperactivity appear to be commoner in children[23,24] and animals[25] with elevated blood lead levels. Moreover, recent studies have shown that chronic lead intoxication in young animals also causes abnormalities in brain cerebroside content[26] and catecholamine metabolism.[27] It is uncertain whether or not chelation therapy will be useful in treating children with mild elevation of blood lead levels since our findings suggest that edetic acid does not remove lead once it is bound in the nervous system (Fig 3). This result is in agreement with other studies that demonstrate that edetic acid and penicillamine chelate and remove lead from bone rather than from soft tissues.[28,29] Clearly it will be necessary to learn more about the consequences of chronic deposition of lead in the brain in order to develop new guidelines for clinical therapy.

The acute encephalopathy produced by feeding lead to immature rats is similar to that encountered in children suffering from lead poisoning. The main pathologic findings in both conditions consist of interstitial edema and hemorrhage that is more prominent in the cerebellum than in other regions of the brain.[5] While rats usually succumb to this disorder, children who survive acute lead encephalopathy are often afflicted with recurrent convulsions and mental retardation.[1-4] We have found that suckling rats in litters fed lead carbonate show a steady increase in brain lead concentrations until they develop a fatal lead encephalopathy at 4 weeks of age (Fig 4). At this time the concentration of lead in the brain of the young rats (6μg/gm) is twice that in the brain of their mothers. These results suggest that a critical concentration of lead in the brain may be required for the development of lead encephalopathy. Thus, the failure of adult animals to develop lead encephalopathy after chronic lead ingestion is probably due to the fact that the highest concentration of lead in the adult brain is achieved in one week and further increases do not occur (Fig 4).

Despite a uniform deposition of lead in the CNS (Fig 6), only the cerebellum exhibited severe pathologic changes (Fig 7 to 10). The reason that focal edema and hemorrhage are restricted primarily to the cerebellum in lead encephalopathy is not clear, but it is possible that this region of

Fig 10.—Cerebellar white matter from early stage 3 lead encephalopathy. A relatively normal appearing capillary is at center of photograph, with some attached glial footplates. Severe extracellular edema separates axons in the area surrounding the capillary. A white blood cell is attached to the capillary endothelial surface (original magnification ×11,400).



the brain may be more vulnerable to several toxic[6,30] and viral agents.[31,32]

The cerebellum taken from animals dying of lead encephalopathy is unsuitable for metabolic study because of hemorrhagic necrosis. However, the results in this study have made it possible to identify a prehemorrhagic stage that precedes the appearance of striking neurological signs during the development of lead encephalopathy. This period was characterized by a notable elevation of water and sodium in the cerebellum while potassium concentration and Na^+-K^+-Mg^{++}-ATPase activity were normal. These findings are considered to be characteristic of "vasogenic" edema in which a primary disturbance of capillary permeability leads to an increase in the levels of brain water and sodium.[33] Taken together with the histologic study results, these changes suggest that the earliest functional defect in the pathogenesis of lead encephalopathy is related to altered permeability of the capillaries in the cerebellum. Such a focal edematous reaction is most remarkable and is not ordinarily seen in toxic or metabolic encephalopathies. This suggests that the capillaries of the cerebellum may be different from those elsewhere in the nervous system. Other laboratories have also obtained evidence that suggests that the capillary may be the vulnerable site in acute lead encephalopathy. Thomas et al[34] have demonstrated by autoradiography with ^{210}Pb that 62% of brain lead is associated with brain endothelial cells, and Lampert et al[15] have demonstrated in the cerebellum that such capillaries became leaky to thorium dioxide (Thoratrast). Thus, it seems likely that capillary dysfunction underlies the pathogenesis of acute lead encephalopathy.

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