Virchows Arch. A Path. Anat. and Histol. 387, 147–164 (1980)

Virchows Archiv A

The Pathogenesis of Lead Encephalopathy

Effects of Lead Carbonate Feedings on Morphology, Lead Content, and Mitochondrial Respiration in Brains of Immature and Adult Rats

D. Holtzman, M.M. Herman, J. Shen Hsu, and P. Mortell

Departments of Neurology, Pediatrics, and Pathology (Neuropathology), Stanford University School of Medicine, Stanford, California 94305, USA

Summary. The toxic effects of inorganic lead feedings on the immature brain were studied in the rat pup. Beginning when litters were two weeks old, $PbCO_3$ was fed to nursing mothers and then to pups directly after weaning. Results in lead-fed pups were compared to age-matched controls and to lead-fed young adult males (60 days old). Anaemia and growth failure developed in both pups and adults. In the second week, more than half the pups developed an encephalopathy, with haemorrhage and oedema predominately in the cerebellum and lead-containing densities in the cerebellar molecular layer. The latter were confirmed by X-ray microanalysis. No lead-fed adults showed signs of an encephalopathy.

Cerebellar mitochondria from lead-fed pups, studied polarographically, showed a very early loss of respiratory control and a subsequent inhibition of phosphorylation-coupled respiration with NAD-linked substrates but not with succinate. Compared to the pup cerebellum, these changes were much less marked in immature cerebral mitochondria and were not found in adult cerebral or cerebellar mitochondria.

Cerebral and cerebellar homogenates from immature and mature lead-fed animals showed large increases in lead content measured by atomic absorption spectrophotometry. Immature cerebellar mitochondrial lead contents were increased to the same extent as in the homogenates. Mitochondria from immature cerebrum and from both regions in the mature brain showed less immediate and smaller increases in lead content.

In conclusion, altered mitochondrial respiration occurs early in regional and age-dependent association with lead encephalopathy in the rat pup. The development of lead encephalopathy also is associated with increased mitochondrial lead concentrations.

Key words: Lead encephalopathy – Mitochondria – Respiration – Elemental microanalysis.

Offprint requests to: David Holtzman, M.D., Ph. D., Division of Pediatric Neurology, Stanford University School of Medicine, Stanford, California 94305, USA

0340-1227/80/0387/0147/$03.60

Introduction

The rat has been studied extensively as a model for the toxic effects of inorganic lead in the brain (e.g., Pentschew and Garro, 1966; Clasen et al., 1974; Thomas and Thomas, 1974; Goldstein et al., 1974; Krigman et al., 1974a, b; Michaelson and Sauerhoff, 1974; Holtzman and Hsu, 1976; Ahrens and Vistica, 1977; Press, 1977a, b; Toews et al., 1978; Cavanagh, 1979). In the rat, as in man, acute lead encephalopathy occurs readily in the immature brain and only rarely in the adult, resulting in oedema and haemorrhage most marked in the cerebellum and basal ganglia (Pentschew and Garro, 1966; Goldstein et al., 1974; Clasen et al., 1974; Press, 1977b). It has been postulated that lead may produce an abnormality of energy metabolism in parenchymal cells of susceptible brain regions, based on the similarity to morphological changes seen in the cytotoxic oedematous encephalopathy of thiamine deficiency and by analogy with its effects on renal mitochondria (Pentschew and Garro, 1966; Cavanagh, 1979). In recent studies, we have presented data which support the proposal that lead alters cellular energy metabolism. In pups begun on lead-feedings at 14 days of age, there is an initial uncoupling and later inhibition of cerebellar, but not cerebral, mitochondrial respiration (Holtzman and Hsu, 1976). The changes in mitochondrial respiration appear within two days of beginning lead feedings, which is very early compared to the time of appearance of morphological and chemical evidence for cerebellar oedema reported by others (Goldstein et al., 1974; Clasen et al., 1974).

In this report, the age of appearance and distribution of morphological changes in the cerebellum and cerebrum are compared directly to changes in mitochondrial respiration in pups fed lead for up to two weeks beginning at 14 days of age and in young adult males fed lead for up to four weeks beginning at 60 days of age. Polarographic studies of brain mitochondrial respiration are extended in order to compare results with the substrate-dependent pattern of lead effects on mitochondrial respiration seen in vitro (Holtzman et al., 1978). We also report the concentrations of lead in regional homogenates and mitochondrial isolates from brains of lead-fed and control rat pups and adults.

Materials and Methods

Materials. Mannitol, EDTA, tris (hydroxymethyl) aminomethane (Tris), ADP, Rotenone, defatted bovine serum albumin, tetramethylammonium hydroxide, citric acid cycle substrates, and cacodylic acid were obtained from Sigma Chemical Company (St. Louis, Missouri). Sucrose and $PbCO_3$ were obtained from J.T. Baker Chemical Company (Phillipsburg, New Jersey), Bacillus proteinase from Nagase and Co., Ltd. (Osaka, Japan), and Shell Epon Resin 812 from E.V. Roberts Company (Culver City, California). All inorganic chemicals and solvents were reagent grade.

Animals. Sprague-Dawley albino rats were used. In studies of rat pups, pregnant females arrived before the end of gestation to allow accurate age-matching of control and experimental litters before beginning lead feedings. Litters were reduced to eight pups on the first day of life and maintained in the same room with one litter and mother per cage. When the pups were 14 days old, experimental mothers were begun on ad libitum feedings of powdered laboratory chow containing 1% calcium, 0.74% phosphorous, 3.3 IU/g vitamin D, and 4% $PbCO_3$. Control litters received the same chow without $PbCO_3$. Pups had free access to all feedings. Adult animals were males

begun on feedings at 60 days of age. Experimental and control adults were matched by weight and age and then fed the powdered chows as described for the rat pups. All $PbCO_3$-containing chows were prepared by the laboratory staff.

Morphology. Lead-treated animals were examined by light and electron microscopy as follows: 7 animals treated for 2 days (day 14–16) and killed on day 16; 3 animals treated for 1 week (day 14–21) and killed on day 21; 3 animals treated for 2 weeks (day 14–28) and killed at 4 weeks. Two animals, treated for one week (day 14–21) and killed at 21 days, were examined only by light microscopy. As controls, one normal animal was examined at 14 days of age, 6 at 16 days, 2 at 21 days, and 2 at 4 weeks.

Brains were prepared for microscopy with methods used previously (Ralston and Herman, 1969). Control and experimental animals were sacrificed by cervical dislocation and perfusion begun as rapidly as possible. A phosphate-buffered saline solution (pH 7.2) was infused via a cannula into the left cardiac ventricle for 15 s. Using the same cannula, a phosphate-buffered 3% glutaraldehyde-1% paraformaldehyde fixative (pH 7.0–7.2) then was infused for 10 min. Both solutions were at 35–37° C. The animals were stored at 4° C for 1 or 2 h before the skulls were opened and tissue sections removed from the frontal cortex and cerebellum. The sections were immediately trimmed into 1 mm^3 blocks and stored at 4° C in cacodylate-buffered, 3.5% glutaraldehyde fixative for 1 h to several days (usually less than 24 h). They were then washed briefly in a cacodylate-buffered wash solution and fixed for 1 h in veronal acetate-buffered 1% OsO_4. The tissue blocks were further processed, sectioned and examined with methods in use in our laboratory (Sipe et al., 1973).

After small tissue blocks were obtained for electron microscopy, the remainder of the brain was removed, coronally sectioned, transferred to phosphate-buffered formalin for further fixation for several days, and then processed and embedded in paraffin. Five micron sections were stained with Nissl (cresyl violet), von Kossa (for calcium), and Prussian blue (for iron).

In animals used for *electron microscopy* the frontal cortex and cerebellum were examined. In the two-week treated animals showing pathological changes in paraffin sections, about twenty blocks were studied in one micron thick sections and four to six blocks were selected for thin sections and photomicroscopy. Fifteen to fifty photographs were taken from each block which was thin-sectioned. In blocks showing densities in the molecular layer, both stained (uranyl acetate and lead citrate) and unstained sections were examined. Numerous photographs were also taken of brains from control animals and from animals fed lead for 2 and 7 days.

For *X-ray microanalysis*, a block of osmicated epon-embeded cerebellum from an animal receiving lead feedings from day 14 to day 28 was examined. Gold and gold-light blue sections were collected on copper and titanium grids; the sections were not stained with metallic salts. They were analyzed using a computerized energy dispersive X-ray analysis system (EDAX 707A with EPIC module 609A) attached to a Philips EM 400 operated in the transmission mode. An accelerating voltage of 80 KV was used and the beam diameter was approximately 200 ηm. The dense bodies were analyzed for approximately 700 s and the normal-appearing adjacent tissue for about 360 s.

Mitochondrial Isolation. All isolation steps were carried out simultaneously in matched experimental and control tissues. The rats were weighed prior to decapitation. After decapitation, blood samples were collected in heparinized capillary microhematocrit tubes for measurement of packed cell volumes (PVC). The brain was removed immediately. The cerebrum was separated by cutting above the colliculi. The cerebellum was cut from the underlying brain-stem. These tissues were immersed in separate beakers of ice-cold isolation medium (225 mM mannitol, 75 mM sucrose, 0.2 mM EDTA, and 5.0 mM Tris HCl pH 7.4) and cleaned of overlying blood vessels and pia mater. Mitochondria were isolated from cerebrums and cerebellums pooled from two to four rat pups or from individual adult rat cerebrums and cerebellums. The isolation steps are described in detail in previous publications (Holtzman and Hsu, 1976; Holtzman et al., 1978). All steps were carried out at 0–5° C. The tissues were homogenized with three to five strokes in a Ten Broeck glass homogenizer. An aliquot of this initial homogenate was frozen for lead and protein determinations (Lowry et al., 1951). After addition of Bacillus proteinase (0.1 mg/g brain tissue), $KHCO_3$ (0.1 mg/g), and bovine serum albumin (0.05 mg/g) for 2 min, the mitochondria were separated with the usual low ($1{,}085 \times g$ for 2 min) and high ($17{,}000 \times g$ for 5 min) speed centrifugations. The fluffy white

layer overlying the final mitochondrial pellet was removed by hand and the mitochondria were resuspended in a small volume of the isolation medium without EDTA. Aliquots of this suspension were frozen for mitochondrial lead and protein determinations (Lowry et al., 1951).

Polarography. Oxygen consumption was measured polarographically in freshly isolated mitochondria using the Gilson Oxygraph with 2 ml reaction chamber and Clarke platinum cathode assembly polarized to −0.8 V (Estabrook, 1967). The order of study of experimental and control mitochondrial samples was varied from day to day. The system was calibrated for O_2 content using frozen and thawed liver mitochondria and small quantities of NADH (Estabrook, 1967; Holtzman and Moore, 1971). Respiration was measured at 22° C in a medium containing 225 mM mannitol, 75 mM sucrose, 0.2 mM EDTA, 5 mM Tris Hcl pH 7.4, 10 mM Tris PO_4 pH 7.4, 5 mM KCl, and 0.25 mg fresh bovine serum albumin. NAD-linked respiration was measured with the substrate pair, glutamate plus malate (1.25 mM each). Rotenone (5 μg/ml), an inhibitor of NAD-linked substrate oxidation (Slater, 1967), was added before measuring respiratory rates with succinate (10 mM) as substrate. With each substrate, respiratory rates were measured in the presence of ADP (0.25 μmol), defined as State 3, and after consumption of the added ADP, defined as State 4 (Chance and Williams, 1955). The respiratory control ratio (RCR) is defined as the ratio of State 3 to State 4 rates (Chance, 1959).

Lead Analysis. Brain homogenate and mitochondrial lead contents were analyzed by graphite furnace atomic absorption spectrophotometry (Gross and Parkinson, 1974). 250 μl of the suspended brain homogenate or isolated mitochondria (about 1 mg protein/ml) was dissolved in 0.5 ml of 25% tetramethylammonium hydroxide in EtOH and diluted with 0.5–2 ml of double distilled water. 50–100 μl of this solution was aspirated into the graphite furnace attachment (Perkin Elmer Model 2000) to the atomic absorption spectrophotometer (Perkin Elmer Model 206). Control and experimental samples were analyzed sequentially in random order. All solutions and water samples were tested separately for lead contamination. Final lead values are expressed as a fraction of homogenate or mitochondrial protein.

Statistics. The results in lead-fed animals were compared with age-matched control animals as non-independent samples. Significance values were calculated for the arithmetic differences between the matched groups by the standard Student t test (Croxton, 1958).

Results

Lead feedings to rat pups, beginning at 14 days of age, produced an almost immediate failure of weight gain and an anaemia when compared to age matched controls (Table 1). Lead-fed pups failed to show the increase in PCV from 2–4 weeks of age seen in control animals. In the first week of lead feedings, there was a 7% mortality but no pups showed the hindleg paresis characteristic of lead encephalopathy. Only three pups in one of the eight lead-fed litters sacrificed after one week demonstrated haemorrhagic cerebellums. After 2 weeks of feedings, 70% of all experimental animals showed both hindleg paresis and haemorrhagic cerebellums. Young adult rats, begun on lead feedings at 60 days of age, also gained weight less rapidly than controls, resulting in a weight deficiency of about 15% compared to age-matched controls after 2 and 4 weeks. After 4 weeks of lead feedings, the adults showed a moderate anaemia comparable to that seen in pups fed lead for 2 weeks. None of the adult animals fed lead for two or four weeks showed hindleg paresis or haemorrhagic cerebellums.

Table 1. Body weights and packed cell volumes (PCV, mean ±S.E.M.) of controls and of rats fed $PbCO_3$ from 2 weeks of age (pups) or from 60 days of age (adults)

	Duration of feedings (days)	Body wts.		PCV	
		Control (g)	Lead (g)	Control (%)	Lead (%)
Pups	2 ($n=128$)	41.5±1.7	35.6±1.1 $P<0.02$[a]	31.7±1.6	33.3±0.9
	7 ($n=125$)	56.7±2.7	39.0±1.1 $P<0.001$	32.4±1.1	27.1±0.9 $P<0.025$
	14 ($n=128$)	84.2±3.0	39.2±2.9 $P<0.001$	40.8±0.3	29.1±0.9 $P<0.001$
Adults	14 ($n=28$)	339.8±7.8	292.9±8.0 $P<0.001$	50.9±0.5	47.2±1.2 $P<0.01$
	28 ($N=30$)	408.5±15.1	341.8±11.8 $P<0.001$	50.7±1.2	40.4±0.8 $P<0.001$

[a] P values (<0.05) are given for differences between experimental and matched control animals

Morphology

The brains of controls and of pups fed lead for 2 and 7 days were macroscopically unremarkable. In two of six animals fed lead for 14 days, the cerebrum was swollen and the cerebellum haemorrhagic.

By *light microscopy* (Fig. 1), three animals treated for 2 weeks (including the two with macroscopic findings) and two animals treated for 1 week showed pathological changes, while all two-day treated animals were similar to the controls. In the brains of the two-week treated animals, there was oedema (vacuolation) in the corpus callosum, mid-brain, white tracks in the pons and dorsal medulla, cerebellar molecular layer (Fig. 1 a), deep white matter of the cerebellum, and around the dentate nucleus. This was associated with proliferation and swelling of astrocytes and numerous regions showing similar changes in endothelial cells (Fig. 1 a). Fig. 1 b shows capillary proliferation in the cerebellar white matter of a one-week treated animal; this was only found in two or three foci in the one-week animals.

Also in the two-week animals, there were focal perivascular haemorrhages in the corpus callosum, corona radiata, cerebellar molecular layer and internal granular layer. (Similar haemorrhages were noted in the cerebellums of two of the one-week treated animals.) In the cerebellar granular layer, there were foci of oedema and necrosis (Figs. 1 c and d), while in the molecular layer there was mineralization around blood vessels (Fig. 1 e) and along cell processes (Fig. 1 f). These mineral deposits were positive for iron.

The frontal cortex and cerebellum in each animal were examined by *electron microscopy*. The findings were similar to those reported by others (see Discus-

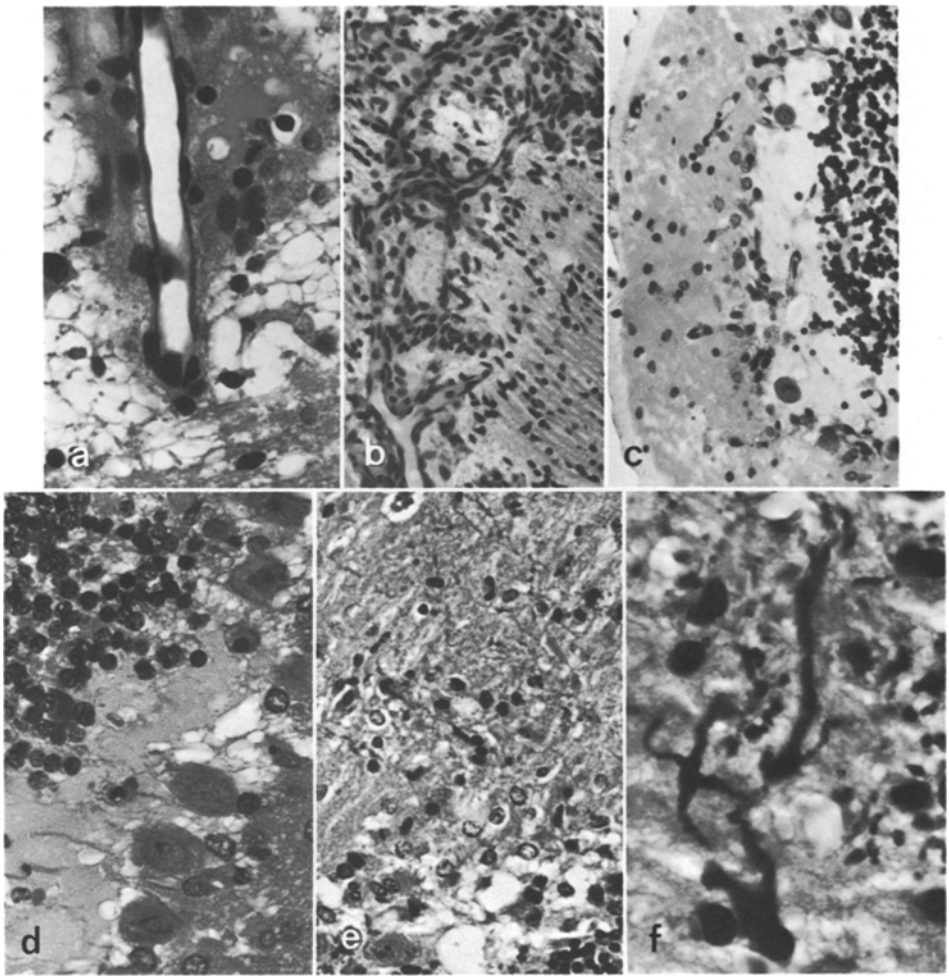

Fig. 1a–f. Cerebellum, treated with lead carbonate. Hematoxylin-eosin (**a**, **c**–**e** in four week old rats treated for two weeks; **b** in three week old rat, treated for one week.) **a** Pericapillary edema fluid and vacuolation ×550. **b** Vascular endothelial proliferation ×200. **c** Marked edema in lamina dissecans (Purkinje cell layer) ×230. **d** Edema fluid in lamina dissecans. Note preservation of Purkinje cells ×480. **e** Focal mineralization in molecular layer (center field) ×340. **f** High power view of mineral deposits in molecular layer ×970. Original mag. reduced 15%

sion) and will be summarized only briefly. Control animals showed no changes. Animals treated for less than two weeks also showed no changes except for occasional small dense bodies (as in Fig. 6d) in cell bodies and processes in the cerebellum at one week.

Three animals fed lead for two weeks showed the following pathological changes. In the *frontal cortex* about one-third to one-half of the capillaries and small blood vessels had hypertrophied endothelial cells (Fig. 2). This was sometimes associated with thickening of the adjacent basement membrane (Fig. 2). In the *cerebellar molecular layer* the majority of small vessels showed

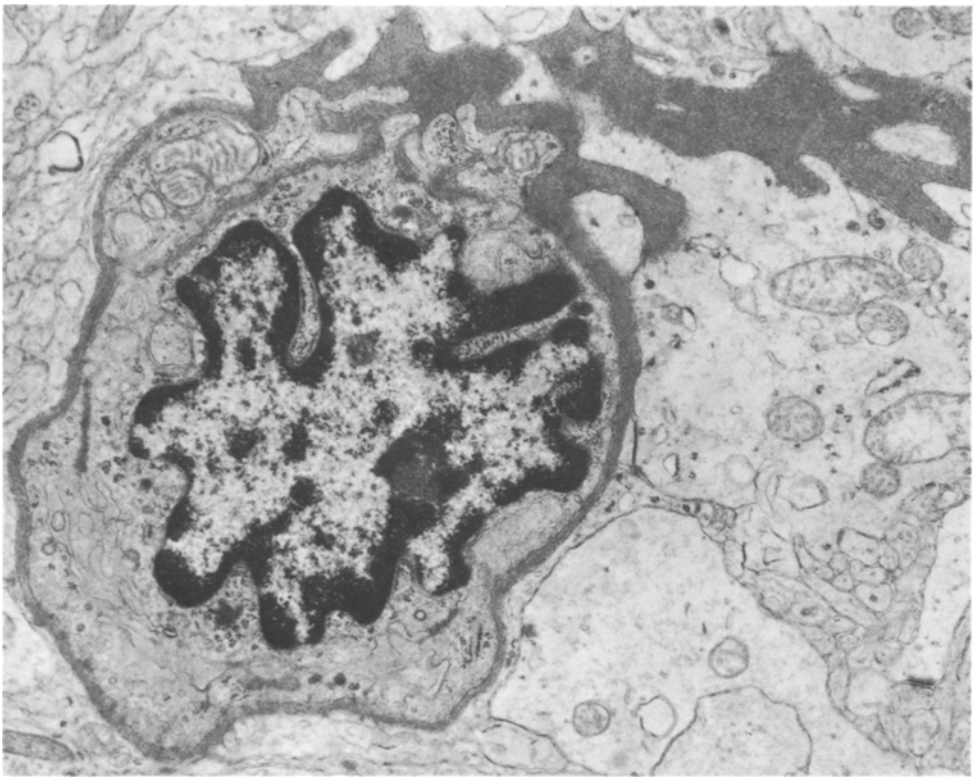

Fig. 2. Frontal cortex, four week old rat, treated for two weeks with lead carbonate. Capillary blood vessel with hypertrophied endothelial cell. Note focal increase in width of pericapillary basal lamina and increased basal lamina material in right upper field × 13,300

these changes (Fig. 3a and b), as well as hypertrophy of pericytes. Striking fields of extracellular oedema (Fig. 4a) in the molecular layer and adjacent to granular neurons were found, as were granular cell swelling and necrosis (Fig. 4b). In addition, there were degenerating myelinated and non-myelinated axons, degenerating synapses, occasional axonal swellings, and, in areas of maximal damage, markedly swollen mitochondria and extravasated red blood cells.

The most interesting finding was that of numerous oval, round or dumbbell-shaped dense bodies measuring 0.8 to 7.8 μm in longest dimension in the cerebellar molecular layer (Figs. 5 and 6). They occurred in processes which could sometimes be identified as dendritic spines and which may be part of Purkinje or Golgi neurons (Fig. 6a). The densities often completely filled cell processes and frequently had a spicular configuration (Fig. 5b). They sometimes contained one or several foci of compactly-arranged dense particles which measured about 20 to 60 Å diameter (Figs. 5c and d). The dense particles and spicules were also present in osmicated tissue which had not been stained with lead or uranyl salts. Mitochondria were sometimes identified in dense processes (Fig. 6b) and also often contained dense particles and spicules. We believe the dense bodies correspond to the mineralized processes noted by light microscopy.

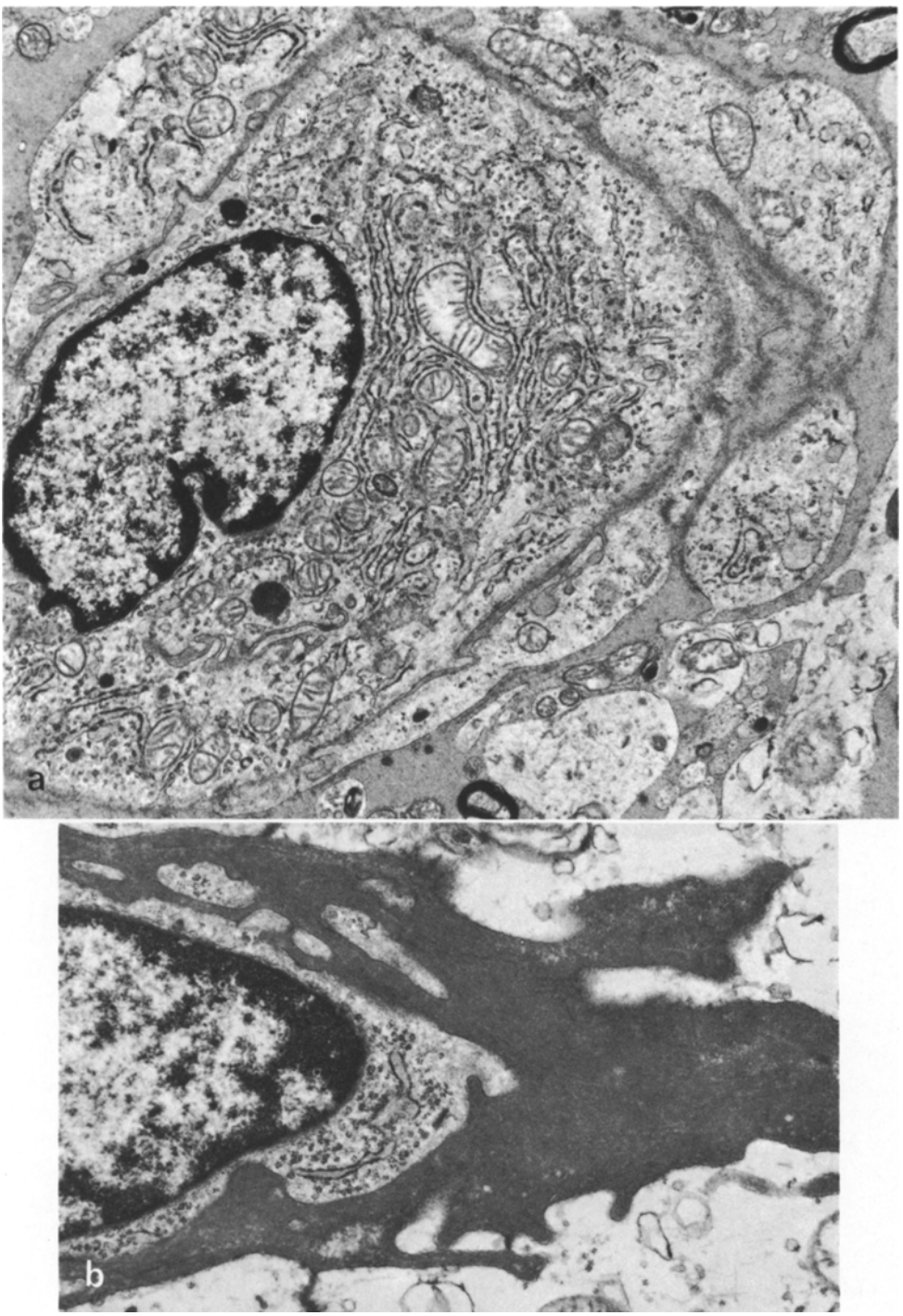

Fig. 3a and b. Cerebellum in four week old rat, treated for two weeks with lead carbonate. **a** Hypertrophic endothelial cell ×11,300. **b** Marked irregular thickening of pericapillary basal lamina ×13,200

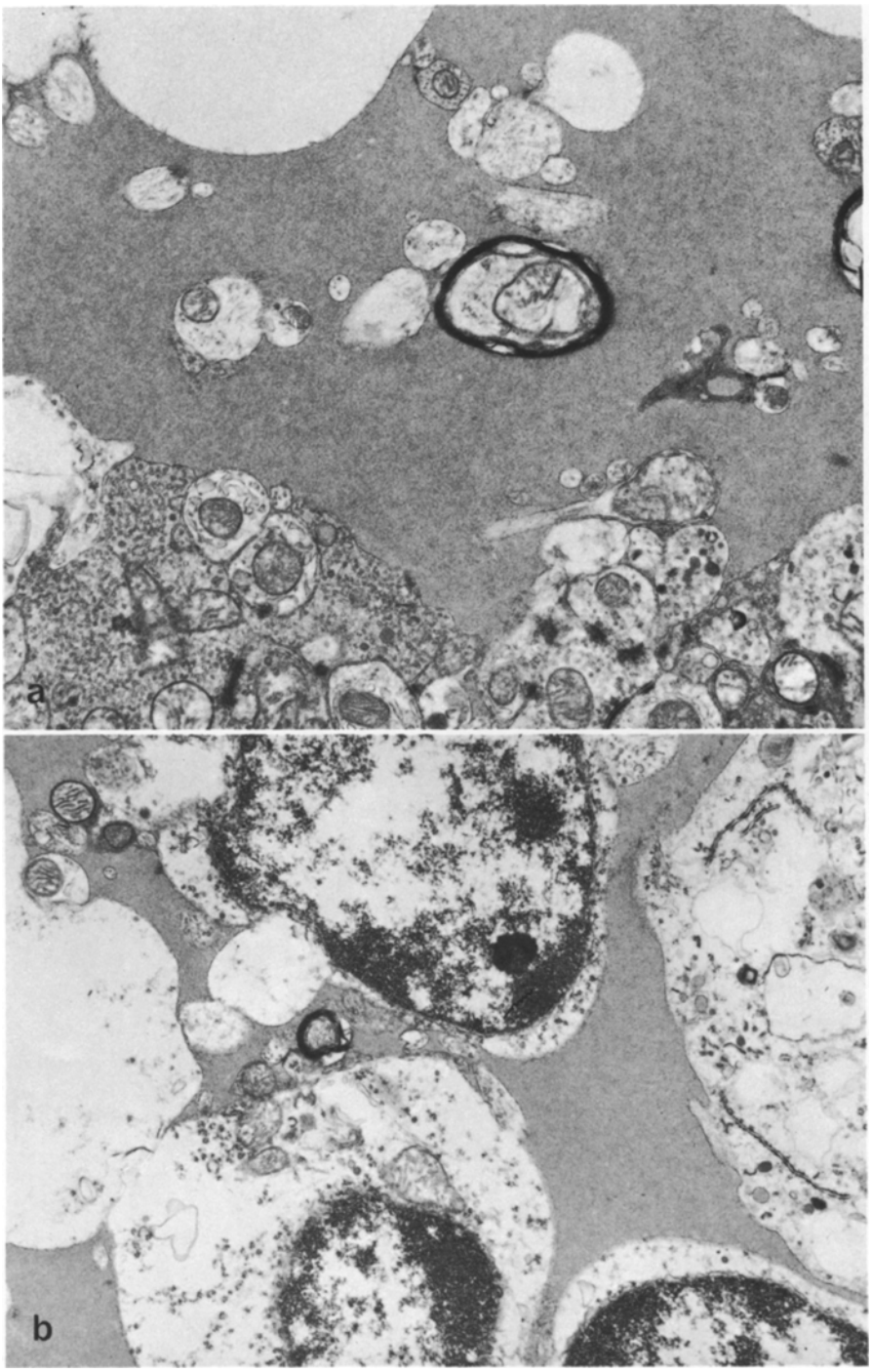

Fig. 4a and b. Cerebellum in four week old rat, treated for two weeks with lead carbonate. **a** Marked increase of extracellular edema fluid. Note preservation of myelin sheaths and of synapses $\times 12{,}000$. **b** Extracellular edema fluid separating swollen granular neurons $\times 10{,}000$

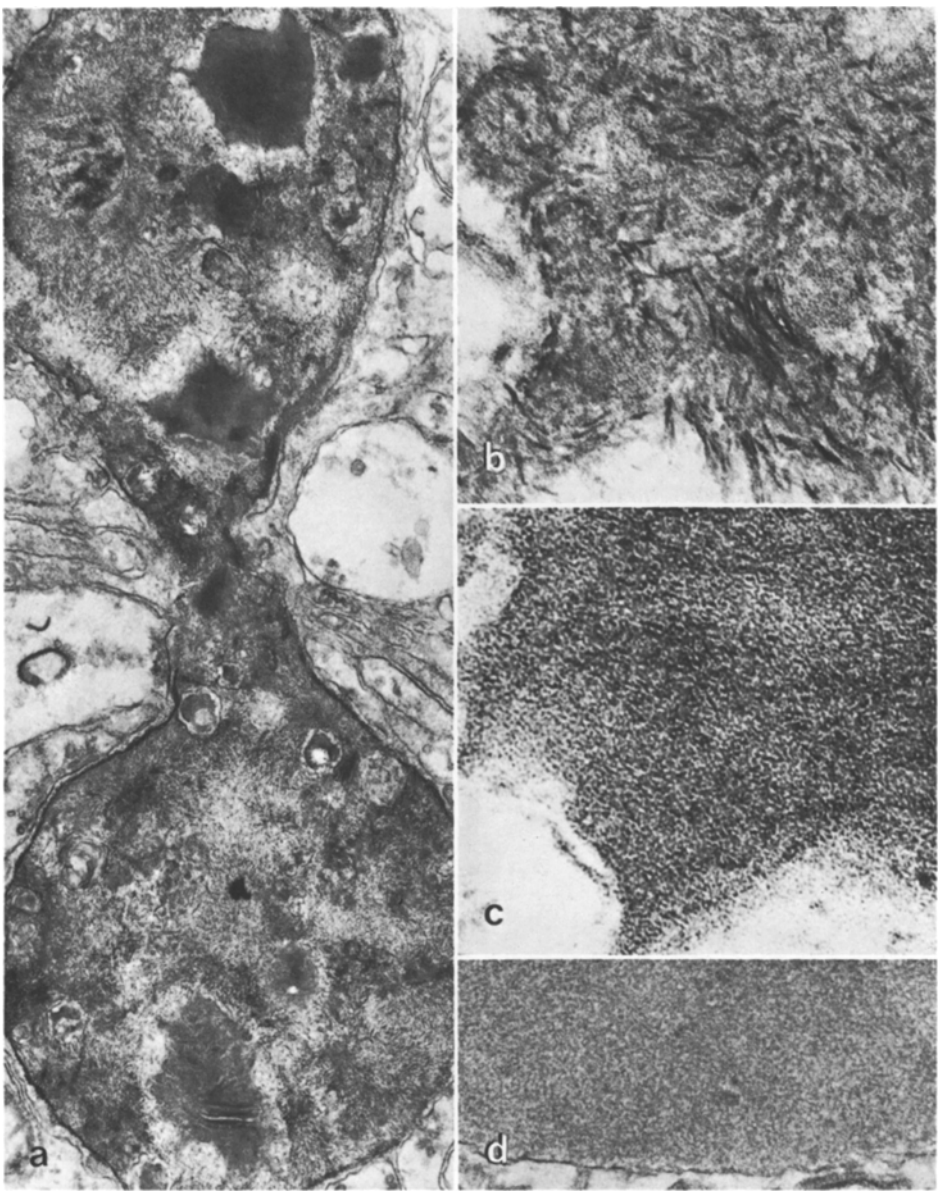

Fig. 5a–d. Cerebellum in four week old rat, treated for two weeks with lead carbonate. **a** Intracytoplasmic dumbbell-shaped density in a post-synaptic cell process, presumably a dendrite ×23,000. **b** Detail of spicules in cytoplasmic density ×155,200. **c** Compact discrete particles (as in the upper field in Fig. 5a) forming part of another cytoplasmic density ×250,000. **d** Particulate densities in a large membrane-bound autophagic vacuole which in another field also contained numerous membrane fragments ×52,500

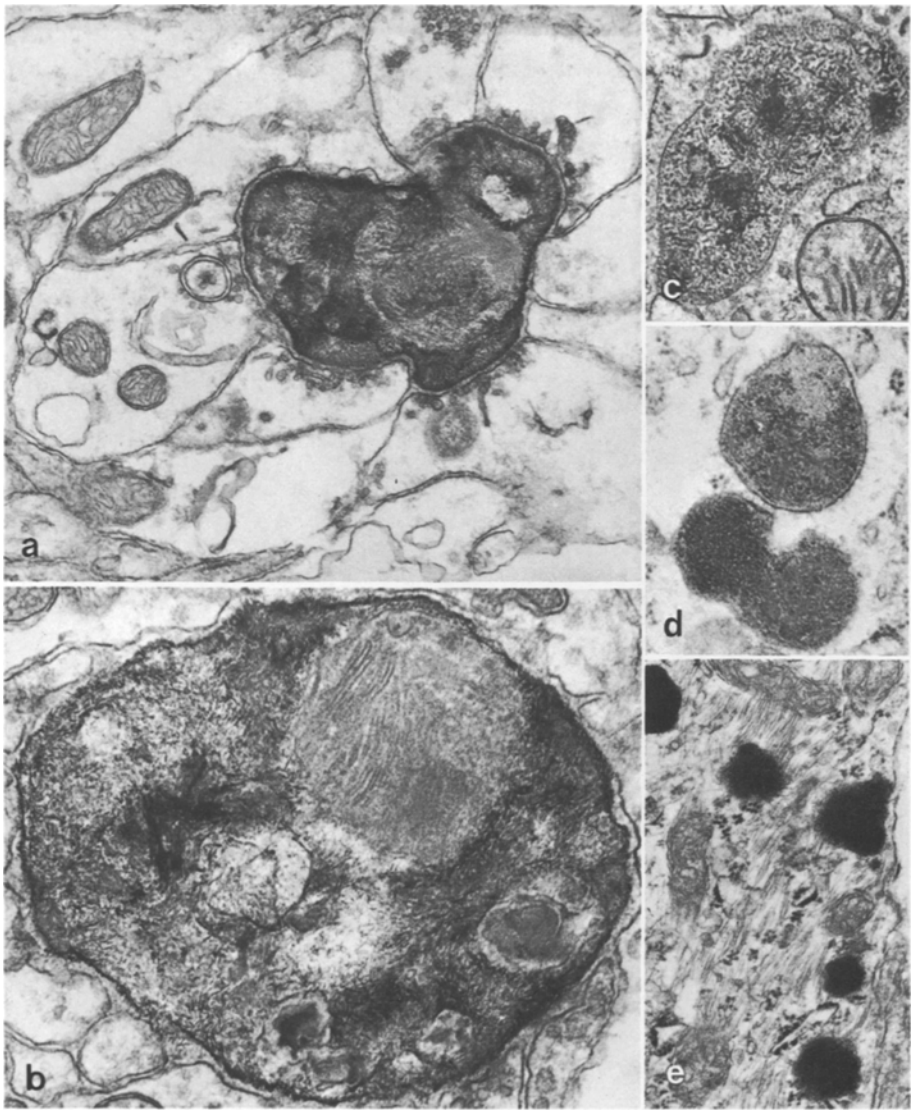

Fig. 6a–e. Cerebellum in four week old rats, treated for two weeks with lead carbonate. **a** Postsynaptic density, presumably in a dendrite ×29,000. **b** Membrane-bound cytoplasmic dense body containing a remnant of a mitochondrion ×47,300. **c** and **d** Membrane-bound autophagic vacuoles in neuronal cytoplasm containing discrete densities ×30,400 and 42,000. **e** Intracytoplasmic dense bodies in an astrocyte ×31,000. Original mag. reduced 20%

Small round or oval dense bodies were found in the cell body and processes of neurons (Figs. 6c and d), astrocytes (Fig. 6e), and macrophages. They measured 0.15 to 2 μm in longest dimension and probably represent lysosomes and autophagic vacuoles which had ingested particulate material. They contained numerous compact dense particles measuring 100 to 250 Å in diameter. Intranuclear inclusions were not found.

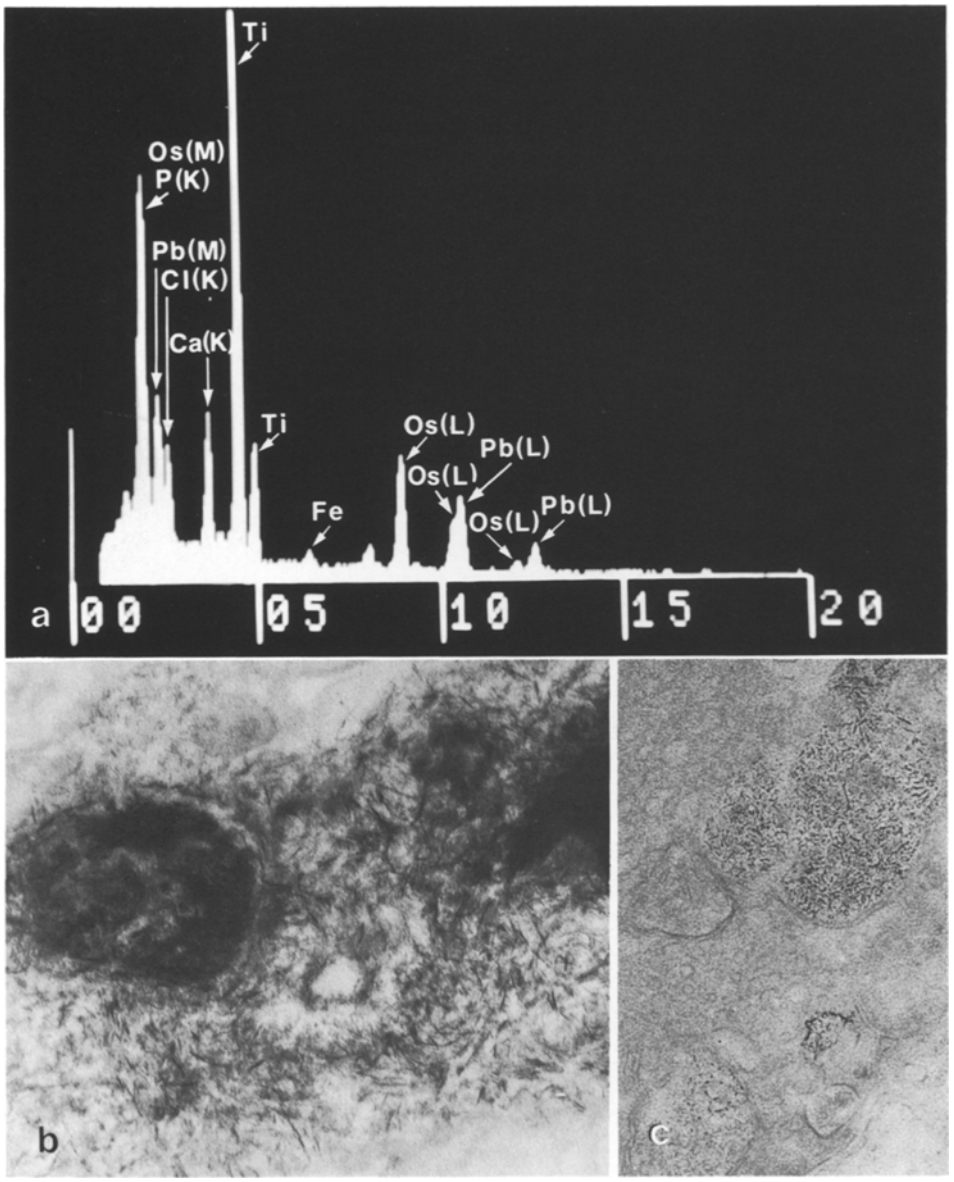

Fig. 7a–c. Cerebellum in four week old rat, treated for two weeks with lead carbonate. **a** X-ray dispersive elemental microanalysis of a thin-sectioned unstained osmicated dense body on a titanium grid. Note the lead, calcium and iron peaks. **b** Unstained osmicated dense body on a copper grid which gave elemental peaks similar to those in 7a. ×119,000. **c** Unstained osmicated dense body on a titanium grid which gave the spectral analysis in Fig. 7a. ×41,000

Table 2. Respiratory control ratios (mean ±S.E.M.), with glutamate plus malate (g+m) or with succinate as substrate, in cerebral and cerebellar mitochondria isolated from rats fed $PbCO_3$ from 14 days of age (pups) or from 60 days of age (adults)

	Duration of lead feedings (days)	Respiratory control ratios[a]			
		Cerebellum		Cerebrum	
		g+m %	succinate %	g+m %	succinate %
Pups	2	65.8±7.9 (11)[b] $P<0.005$[c]	95.5±5.6 (4)	99.9±7.7 (13)	100.6±9.1 (4)
	7	72.4±8.3 (20) $P<0.005$	105.5±5.4 (9)	91.1±5.0 (20)	103.8±10.4 (10)
	14	77.6±8.7 (16) $P<0.025$	98.7±5.5 (9)	93.9±6.6 (21)	111.8±9.4 (14)
Adults	14	84.7±15.6 (10)	102.6±3.9 (10)	111.2±8.2 (10)	111.8±5.3 (10)
	28	107.0±11.3 (9)	109.1±5.2 (8)	113.5±7.3 (8)	111.1±5.6 (8)

[a] Respiratory control ratio is defined in METHODS. RCRs are expressed as a percentage of RCRs in mitochondria isolated from age-matched control animals

[b] Number of experiments given in parentheses

[c] P values (<0.05) are given for differences between results in mitochondria from experimental and matched control animals

X-ray dispersive elemental microanalysis (Fig. 7a) was performed on several dense bodies and on tissue immediately adjacent to one of the densities. It showed peaks of lead, calcium and iron in the dense bodies while the adjacent tissue was negative for these elements. In addition, osmium, phosphorous, and chlorine were found both in the densities and in the adjacent tissue, while copper was present in the sample mounted on a copper grid and titanium in the titanium-mounted specimen (Fig. 7a). Lead was detected as an M and L peak in the densities on both types of grids. The phosphorous (k peak) and osmium (M peak) were sometimes indistinguishable. Figures 7b and c are electron micrographs of two different dense bodies which were assayed, one on a copper grid (Fig. 7b) and one on a titanium grid (Fig. 7c).

Mitochondrial Respiration

The effects of lead feedings on respiratory control in mitochondria from brains of rat pups and adults are shown in Table 2. In brain mitochondria from control animals of all ages, RCRs with the NAD-linked substrate pair, glutamate plus malate, ranged from 4–9. With the NAD-linked substrates, RCRs were significantly decreased in cerebellar mitochondria within two days of beginning lead feedings to 14 day old pups. This early decrease in RCRs was due to a large increase in State 4 respiratory rates (130–140% of controls). After 1

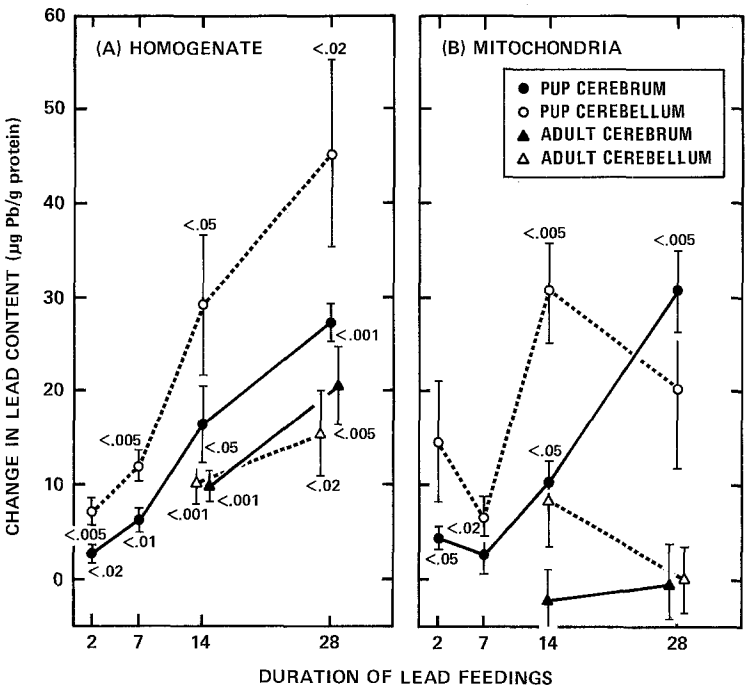

Fig. 8A and B. Changes in lead content of cerebellar and cerebral homogenates (**A**) and mitochondrial isolates (**B**) from lead-fed rat pups and adults. Each value is the mean (±S.E.M.) difference between results in 5–9 lead-fed and matched control animals. p values less than .05 are shown for the significance of the differences between these matched groups. Quantities of lead were measured by atomic absorption spectroscopy

and 2 weeks of lead feedings, RCRs were decreased because of decreased State 3 rates (70–75% of controls). With succinate as substrate, RCRs in lead-fed pup cerebellar mitochondria were not different from controls. In lead-fed pup cerebral mitochondria, there were no significant changes in RCRs or respiratory rates with either glutamate plus malate or with succinate as substrate. In adult rats after 2 and 4 weeks of lead feedings, there also were no changes in RCRs or respiratory rates in mitochondria isolated from either the cerebrum or cerebellum.

Lead Content

The changes in lead contents of pup and adult brain homogenates during the course of lead feedings are shown in Fig. 8A. In both pups and adults, the normal cerebellum contained more lead than the cerebrum (pup cerebellum 10.62 ± 0.64 µg Pb/g protein, cerebrum 7.89 ± 0.47; adult cerebellum 18.95 ± 2.85, cerebrum 8.10 ± 1.27). The differences between normal lead contents were statistically significant in the cerebellum of the adult compared to the pup ($P < 0.01$) and in the cerebellum compared to the cerebrum of the adult ($P < 0.005$). After

2 and 4 weeks of lead feedings, there were similar increases in the lead contents of the pup cerebrum and the adult cerebellum and cerebrum. In the pup, the larger increases in cerebellar than cerebral lead content, occurring with lead feedings, were probably due to the presence of red blood cell-bound lead in the haemorrhagic tissue (Kochen and Greener, 1977). The increases in adult brain lead levels did not change further between 28 and 56 days of lead feedings. During the period of lead feedings, there were similar increases in blood lead levels in the pups and adults (e.g., 5.0 ± 0.48 µg/ml whole blood in pups and 6.26 ± 2.07 in adults after two weeks of lead feedings).

The effects of lead feedings on cerebral and cerebellar mitochondrial lead contents in the rat pup are shown in Fig. 8B. As in the homogenates, lead content was higher in normal cerebellar mitochondria than cerebral mitochondria in both pups and adults (pup cerebellum 20.67 ± 1.59 µg Pb/g protein, cerebrum 13.59 ± 0.96; adult cerebellum 29.03 ± 2.54, cerebrum 20.05 ± 1.77). Cerebellar mitochondrial lead levels were significantly higher than cerebral levels at each age ($P < 0.001$). With lead feedings, mitochondrial lead contents increased much more in the pup compared to the adult brain (pup cerebellum versus adult cerebellum $P < 0.001$ after 14 days and < 0.05 after 28 days of lead feedings; pup cerebrum versus adult cerebrum $P < 0.05$ after 14 days and < 0.001 after 28 days of lead feedings). After 56 days of feedings, adult cerebellar and cerebral mitochondrial lead contents were the same as those found after 28 days of feedings. After 2 weeks of lead feedings in the pups, coincident with the appearance of the encephalopathy, there was a three-fold increase in cerebellar mitochondrial lead contents compared to controls. This increase was much greater than that seen concurrently in the cerebrum (cerebellum versus cerebrum, $P < 0.05$). However, pup cerebral mitochondrial lead contents did show a similar increase after four weeks of lead feedings.

Discussion

The morphological and X-ray analytical studies confirm features reported by others in lead-induced encephalopathy in suckling rats or following lead implantation in adult rat brains. Recent reviews of the morphological literature are provided by McConnell and Berry (1979) and Cavanagh (1979). Light microscopic findings in our study are most similar to those reported by Pentschew and Garro (1966), Lampert et al. (1967), Thomas et al. (1971), Thomas and Thomas (1974), and Press (1977a), while the fine structural features resemble some of those reported by Lampert et al. (1967), Clasen et al. (1974), Goldstein et al. (1974), and Press (1977a), and in the forebrain by Hirano and Kochen (1976). Unlike the latter study we did not find extracellular spicular densities nor identifiable intranuclear inclusions. The X-ray analytical studies are similar to those reported by Shirabe and Hirano (1977) using lead implants in the mature brain. Osmium and chlorine peaks are most likely derived from elements in the fixative and epon. The association of calcium and iron with the lead deposits is of interest. Iron is probably related to hemorrhage, which is a well-known feature of the experimental encephalopathy, while elevated levels of

calcium (Thomas et al., 1971) and phosphorus have been reported in lead-treated tissues (see Shirabe and Hirano, 1977).

The finding of increased mitochondrial lead content in age and regional association with lead encephalopathy is new and potentially of importance in the pathogenesis of lead toxicity in the immature cerebellum. The relatively small changes in mitochondrial lead contents with lead feedings in the encephalopathy-resistant adult brain, in spite of large increases in tissue lead contents, suggest a compartmentalization of brain lead which appears with maturation. Our values for lead contents of normal brain homogenates are comparable to those reported on a dry weight basis by Danscher et al. (1973). Similar increases in tissue lead contents of cerebrum and cerebellum in both immature and adult lead-fed rats also have been reported by others (Michaelson, 1973; Goldstein et al., 1974). Only in the immature cerebellum do the increases in mitochondrial lead concentrations per mg protein parallel the increased tissue contents throughout the period of lead feedings, reaching significantly higher levels than found at any time in the lead-fed adult. However, there is no clear "encephalopathic level" of brain mitochondrial lead since high levels in pup cerebral and cerebellar mitochondrial lead contents after 28 days of feedings (42 days of age) are not associated with behavioral or gross encephalopathic changes.

The findings of greater mitochondrial lead contents with altered mitochondrial respiration in the pre-encephalopathic immature cerebellum, and not in adult non-encephalopathic regions, provides further support for the hypothesis that altered cellular energy metabolism is involved in the pathogenesis of lead encephalopathy (Pentschew and Garro, 1966; Holtzman and Hsu, 1976; Cavanagh, 1979). The substrate-dependence of the inhibitory effects of lead feedings on cerebellar mitochondrial respiration, similar to that seen with in vitro exposure of normal brain mitochondria to lead, suggests that these respiratory changes are due directly to the presence of inorganic lead in the mitochondria (Holtzman et al., 1978). From our studies comparing pups and adults, we cannot say whether variables such as malnutrition, anemia, or the rate of lead accumulation also may contribute to lead toxicity in the immature brain (Cavanagh, 1979). We also cannot say whether the metabolic effects of lead toxicity are greater or functionally more critical in particular cell types showing pathological changes, such as the granular neurons or the endothelial cells of capillaries. Observing swollen cerebral capillary endothelial cells without associated oedema or neuronal necrosis, suggests that these changes may be an early lead effect (Goldstein et al., 1974; Press, 1977a; Toews et al., 1978). The recent finding, that high capillary lead contents are associated with both immature morphologically-altered and more mature normal appearing capillary endothelium (Toews et al., 1978), is consistent with the hypothesis that, coincident with the maturation of resistance to lead toxicity, cellular lead is sequestered in a non-mitochondrial compartment where it is relatively non-toxic.

Acknowledgements. This work was supported by research grants to D.H. from the Research Development Fund of Stanford University and from the National Institute of Environmental Health Sciences, Department of Health, Education, and Welfare and the Environmental Protection Agency

(ES 01197), and to M.M.H. from the National Institute on Alcohol Abuse and Alcoholism (AA 00322). D.H. was supported, in part, by a Mellon Foundation fellowship during the period of this research. Mr. Erich Meier (Microanalytical Laboratory, Department of Chemistry, Stanford University) performed atomic absorption analyses of lead contents. Drs. Ann Marshall and Robert Sinclair (Center for Materials Research, Stanford University) performed the X-ray dispersive elemental microanalysis. Ms. Sharon Graf, Ms. Elizabeth Juhasz, Mr. Robert McGowan, Mr. Edmund Rubinstein, and Ms. Maureen Whitlock provided technical assistance in the morphological studies.

These results were presented, in part, at the 10th Annual Meeting of the American Society for Neurochemistry, March, 1979.

References

Ahrens, F.A., Vistica, D.T.: Microvascular effects of lead in the neonatal rat. I. Histochemical and light microscopic studies. Exp. Mol. Pathol. **26**, 129–153 (1977)

Cavanagh, J.B.: Metallic toxicity and the nervous system. In: Recent advances in neuropathology, W.T. Smith and J.B. Cavanagh (eds.), No. 1, pp. 247–275. Edinburgh: Churchill Livingstone 1979

Chance, B.: Quantitative aspects of the control of oxygen utilization. In: Ciba Foundation Symposium on the regulation of cell metabolism, G.E.W. Walstenholme and C.M. O'Connor (eds.), pp. 91–129. Boston: Little, Brown 1959

Chance, B., Williams, G.R.: A simple and rapid assay of oxidative phosphorylation. Nature (Lond.) **175**, 1120–1121 (1955)

Clasen, R.A., Hartmann, J.F., Starr, A.J., Coogan, P.S., Pandolfi, S., Laing, I., Becker, R., Hass, G.M.: Electron microscopic and chemical studies of the vascular changes and edema of lead encephalopathy. A comparative study of the human and experimental disease. Am. J. Pathol. **74**, 215–239 (1974)

Croxton, F.E.: Elementary statistics with applications in medicine and the biological sciences, pp. 240–242. New York: Dover 1953

Danscher, G., Hall, E., Fredens, K., Fjerdingstad, E., Fjerdingstad, E.J.: Heavy metals in the amygdala of the rat: zinc, lead, and copper. Brain Res. **94**, 167–172 (1975)

Estabrook, R.W.: Mitochondrial respiratory control and the polarographic measurement of ADP/O ratios. In: Methods in enzymology, R.W. Estabrook and M.D. Pullman (eds.), pp. 41–47. New York: Academic Press 1967

Goldstein, G.W., Asbury, A.K., Diamond I: Pathogenesis of lead encephalopathy. Uptake of lead and reaction of brain capillaries. Arch. Neurol. **31**, 382–389 (1974)

Gross, S.B., Parkinson, E.S.: Analysis of metals in human tissues using base TMAH digests and graphite furnace atomic absorption spectrophotometry. Atomic Absorp. News **13**, 107–108 (1974)

Hirano, A., Kochen, J.A.: Experimental lead encephalopathy. Morphological studies. In: Progress in neuropathology, H.M. Zimmerman (ed.), Vol. 3, pp. 319–342. New York: Grune and Stratton 1976

Holtzman, D., Hsu, J.S., Mortell, P.: In vitro effects of inorganic lead on isolated rat brain mitochondrial respiration. Neurochem. Res. **3**, 195–206 (1978)

Holtzman, D., Hsu, J.S.: Early effects of inorganic lead on immature rat brain mitochondrial respiration. Pediatr. Res. **10**, 70–75 (1976)

Holtzman, D., Moore, C.L.: A micro-method for the study of oxidative phosphorylation. Biochim. Biophys. Acta. **234**, 1–8 (1971)

Kochen, J.A., Greener, Y.: Brain lead levels in hemorrhagic lead encephalopathy. Pediatr. Res. **11**, 563 (1977)

Krigman, M.R., Druse, M.J., Traylor, T.D., Wilson, M.H., Newell, L.R., Hogan, E.L.: Lead encephalopathy in the developing rat: Effect upon myelination. J. Neuropathol. Exp. Neurol. **33**, 58–73 (1974a)

Krigman, M.R., Druse, M.J., Traylor, T.D., Wilson, M.H., Newell, L.R., Hogan, E.L.: Lead encephalopathy in the developing rat: effect on cortical ontogenesis. J. Neuropathol. Exp. Neurol. **33**, 671–686 (1974b)

Lampert, P., Garro, F., Pentschew, A.: Lead encephalopathy in suckling rats. An electron microscopic study. In: Brain edema, I. Klatzko and F. Seitelberger (eds.), pp. 207–222. New York: Springer 1967

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with Folin phenol reagent, J. Biol. Chem. **193**, 265–275 (1951)
McConnell, P., Berry, M.: The effects of postnatal lead exposure on Purkinje cell dendritic development in the rat. Neuropathol. Appl. Neurobiol. **5**, 115–132 (1979)
Michaelson, I.A.: Effects of inorganic lead on RNA, DNA, and protein content in the developing neonatal rat brain. Toxicol. Appl. Pharmacol. **26**, 539–548 (1973)
Michaelson, I.A., Sauerhoff, M.W.: An improved model of lead-induced dysfunction in the suckling rat. Toxicol. Appl. Pharmacol. **28**, 88–96 (1974)
Pentschew, A., Garro, F.: Lead encephalo-myelopathy of the suckling rat and its implications on the porphyrinopathic nervous diseases. Acta Neuropathol. (Berl.) **6**, 266–278 (1966)
Press, M.F.: Lead encephalopathy in neonatal Long-Evans rats: Morphologic studies. J. Neuropathol. Exp. Neurol. **34**, 169–193 (1977a)
Press, M.F.: Lead encephalopathy in children. Am. J. Pathol. **84**, 485–488 (1977b)
Ralston, H.J., Herman, M.M.: The fine structure of neurons and synapses in the ventrobasal thalamus of the cat. Brain Res. **14**, 77–97 (1969)
Shirabe, T., Hirano, A.: X-ray microanalytical studies of lead-implanted rat brains. Acta Neuropathol. (Berl.) **40**, 189–192 (1977)
Sipe, J.C., Herman, M.M., Rubinstein, L.J.: Electron microscopic observations on human glioblastomas and astrocytomas maintained in organ culture systems. Am. J. Pathol. **73**, 589–606 (1973)
Slater, E.C.: Applications of inhibitors and uncouplers for a study of oxidative phosphorylation. In: Methods in enzymology, R.W. Estabrook and M.D. Pullman (eds.), X, pp. 48–57. New York: Academic Press 1967
Thomas, J.A., Dallenbach, F.D., Thomas, M.: Considerations on the development of experimental lead encephalopathy. Virchows Arch. Abt. A Path. Anat. **352**, 61–74 (1971)
Thomas, J.A., Thomas, I.M.: The pathogenesis of lead encephalopathy. Ind. J. Med. Res. **62**, 36–45 (1974)
Toews, A.D., Kolber, A., Hayward, J., Krigman, M.R., Morrell, P.: Experimental lead encephalopathy in the suckling rat: concentration of lead in cellular fractions enriched in brain capillaries. Brain Res. **147**, 131–138 (1978)

Accepted February 26, 1980