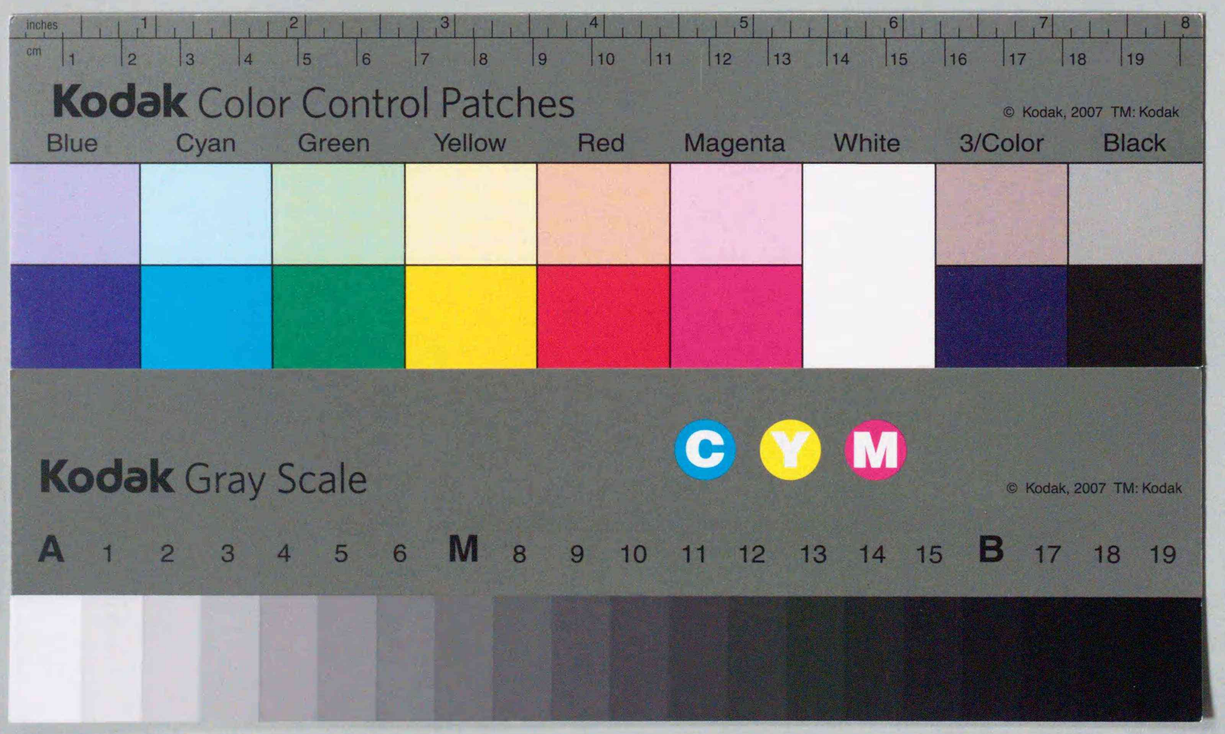
PATHOLOGIC STUDIES OF LEAD POISONING IN BIRDS

（鳥類の鉛中毒症に関する病理学的研究）

KENJI OCHIAI



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CONTENTS

PREFACE

Lead poisoning due to ingestion and retention of spent lead gunshot has been recognized as a common disease of wild waterfowl in heavily hunted areas in the United States of America.[6,105] In this country, lead poisoning of waterfowl from ingestion of lead shot was recognized as early as 1874.[25] The annual loss due to this disease was estimated to be between 2% and 3% of the population.[6] Each year more than 1300 metric tons of lead shot were deposited by hunters in the waterfowl habitat of the country in 1970's.[101] The mallard duck (Anas platyrynchos) and Canada goose (Branta canadensis) are the most commonly affected, although many species of waterfowl are victims of this poisoning.[6,99] The use of lead shot has been banned for all waterfowl and coot hunting nationwide since the 1991/1992 hunting season in this country.[71] Lead poisoning in waterfowl, however, has now been recorded in at least 21 countries, and is a serious problem in waterfowl the world over.[71] In addition, this poisoning was also seen in 31 species of free-ranging birds other than waterfowl.[56]

The birds in search of grid or feed particles pick up spent lead shot from the sediments of shallow waters. The ingested shots are retained in the gizzard until they are solubilized by a combination of the grinding action and low pH

(2.0 to 3.5)[93] and ultimately lead is absorbed from the intestine into the blood stream or eliminated in the feces. Experimental studies with wild mallards have shown the average residence time of one No. 6 sized shot pellet in gizzards to be 18-21 days.[6]

Acute lead poisoning usually follows the ingestion of a large number of shots and birds die within 6 days of exposure with very little loss of weight and few pathologic lesions of poisoning.[6,18] In chronic poisoning, waterfowl die more than 2 or 3 weeks after the ingestion of a small number of shot, and exhibit classical signs including severe weight loss, green watery feces and a green stained vent, general weakness and an inability to hold up the wings or tail.[6,18]

Blood changes are fairly early and characteristic. Lead interferes with aminolevulinic acid dehydratase[20] and heme synthetase, an enzyme responsible for incorporating iron into protoporphyrin IX.[58,105] Affected birds reveal anemia, described as a fluctuating anemia with anisocytosis and poikilocytosis,[17] microcytic anemia due to erythroid maturation arrest,[5] hemolytic anemia in acute poisoning and myelotoxic when chronic.[19]

The typical necropsy findings of lead-poisoned mallards have been described by Jordan and Bellrose.[44] Necropsy of affected mallards reveals an emaciated specimen with muscular atrophy, a greenish to gray discoloration and atrophy of the

liver and kidney, distended gall bladder, flaccid heart with increase of cardiac fluid, impacted proventriculus, reduction in size of gizzard, bile-stained lining of gizzard with ulcer, moderate to severe enteritis, greenish to blue-gray slate discoloration of the intestinal tract, and greenish diarrhea which tends to stain the feathers surrounding the vent. Presence of ingested shots in the lumen of the proventriculus and gizzard is a highly suggestive finding. An atypical lead poisoning syndrome, which is characterized by cephalic edema consisting of the accumulation of serous fluids in the submandibular subcutaneous connective tissue and in the periorbital tissues, has also been reported in Canada geese.[3,99]

The histopathologic lesions have been described in various body systems, reflecting the widespread impact of lead on basic biochemical pathways. Liver lesions in acute lead-poisoned domestic ducks and mallards are characterized by necrosis and fatty degeneration of hepatocytes.[19] Subsequently, accumulation of bile pigments, hemosiderosis of Kupffer cells, and edema of connective tissue are found.[19,45] Spleen reveals marked hemosiderosis, marked increase and activation of the reticuloendothelial system,[19,39] and loss of follicular activity.[39] In kidney there occurs nephrosis consisting of degeneration, necrosis, and sloughing of tubular epithelium with an accumulation of brownish pigment in tubular

epithelial cells.[19] Acid-fast intranuclear inclusion bodies may be recognized in the epithelial cells of proximal tubules.[19,54,55] Foci of myocardial degeneration are scattered in association with fibrinoid necrosis of arterioles.[18,46] Pressure atrophy of both proventricular plica and gland is seen in birds with severe proventricular impaction, and atrophy and degeneration of smooth muscle in gizzard.[18,105] Hyaline degeneration and atrophy of skeletal muscle are common findings, especially in the pectoral muscles.[16,46] Lead-poisoned young birds may have focal microhemorrhages, hyaline thrombi in the capillaries, vacuolation between the granular and molecular layers, and necrosis of Purkinje cells in the cerebellum.[40,46] Peripheral neuropathy in vagus, brachial, and sciatic nerves has been described in a lead-poisoned guinea fowl[39] and mallards.[40] As the vagus nerve was most consistently affected, this change is considered to account for the frequent macroscopic lesion of proventricular dilation.[40]

Several investigators have reported other toxicities of lead for various organs such as arrest of mitotic activity in proventricular epithelium with a decrease in height of the plica,[16] testicular atrophy and reduction or absence of spermatogenesis,[60] necrosis of both acinar and islet tissues in the pancreas,[18] atrophy of the cortex of the thymus and an increase in the number of Hassall's corpuscles and myoid cells

in the medulla.[45] Osteonecrosis of medullary bone is recognized in lead-poisoned female ducks.[16] Spinal motor neuron degeneration, motor axonal loss and atrophy of muscle are experimentally produced in chickens by long-term exposure to this metal.[61]

These pathologic results have been obtained mainly through the studies of the spontaneous and/or experimental cases of mallards,[16,19,40,54,55] domestic ducks,[19] Canada geese,[3,18,99] and chickens.[45,60,61] However, pathologic lesions associated with this disease of waterfowl vary considerably because of the variation in degree of toxicity present, and cases can range from acute to chronic with all of the intermediate stages represented.[99] In addition, lead poisoning appears to depend upon species, sex, age, and a number of ecological and environmental factors including the weather, water level, hunting pressure and diet.[43,82,99] The species differences in susceptibility to lead toxicity have been reported to depend on feeding and food habits.[83] It has been experimentally demonstrated that dietary deficiencies or excesses of certain essential metals alter the absorption, elimination, or dose response of lead.[63,76] Low dietary Ca enhances the pathologic effects of lead in birds[13] as well as mammals.[59,91] Despite numerous reports of lead poisoning among wild ducks and Canada geese, there is only little information on the pathologic findings of this disease in whooper swans

(Cygnus cygnus) and white-fronted geese (Anser albifrons).

On the other hand, lead is well known primarily as a neurotoxin in mammals including human. Most pathologic studies on acute lead encephalopathy in mammals suggested that there is a lead-induced defect in the developing blood-brain barrier (BBB) by the early appearance of hemorrhages of central nervous system (CNS) and marked brain edema.[14,15,24,73,77-79,96,98] In contrast, the CNS of birds has apparent resistance to high circulating concentrations of lead in comparison with that of mammals.[40,58] Only a few examinations of the nervous system, however, have been performed in birds, and the neurotoxic effects of lead and details of the mechanism of lead-resistance in birds are still poorly understood.

In Japan, although lead poisoning in whooper swans and whistling swans (Cygnus columbianus) has occurred sporadically during the winters of 1984-7,[38] mortality of wild waterfowl caused by this poisoning had not been officially recorded until 1988. In all Japan, it is estimated that the annual weight of lead fired into habitats is 300 tons, of which 75 tons are fired into wetlands.[71] We encountered mortalities of whooper swans and white-fronted geese at one small lake in Hokkaido in 1989-1990, and pathologically studied these cases to conclude that the main cause of the mortalities was lead poisoning.

The objectives of this treatise were to observe and discuss pathologic findings in the affected whooper swans and white-fronted geese to clarify the pathologic conditions of lead poisoning of waterfowl. Besides, I experimentally examined the influence of dietary calcium on the development and the severity of experimental acute lead encephalopathy in chicks to elucidate the neurotoxity of lead in birds.

CHAPTER I

PATHOMORPHOLOGIC FINDINGS OF SPONTANEOUS LEAD POISONING IN WHOOPER SWANS

INTRODUCTION

In the United States of America, extensive losses of swans caused by lead poisoning and/or fowl cholera were reported between 1948 and 1954 in California,[80] and deaths of whistling swans (Cygnus columbianus) due to lead poisoning have occurred in Wisconsin since 1944.[99,100] Likewise, the ingestion of anglers' split lead weights is a major cause of mortality in mute swans (Cygnus olor) in Great Britain.[8,85,90] However, there are limited data on the pathologic manifestations of this disease in swans.

In Japan, this problem has not been officially observed. Mortality of whooper swans (Cygnus cygnus) due to this disease occurred between April and May of 1989 at Lake Miyajima in Hokkaido, Japan. When the whooper swans return to Siberia, they usually stay for 3 to 4 weeks at the lake during the migrating season. The dead swans in the present study were found during this short period.

Lake Miyajima is a small lake, 0.36 km^2 in area, with a maximum depth of 2.4 m. Because wild waterfowl usually feed on grains grown on nearby farmland, hunting has been allowed since the 19th century. It can be assumed that a large amount of spent lead shot has accumulated at the bottom of the lake. Pathologic findings in the affected swans are described and discussed in this chapter.

MATERIALS AND METHODS

Birds and environment

Wild waterfowl including whooper swans usually migrate between Japan and Siberia. Lake Miyajima in Hokkaido is the northernmost point in Japan where wild ducks, geese, and swans arrive in spring and fall every year.

Clinical and hematological examination

Thirty-three young or adult whooper swans were found dead or in weakened condition between April and May 1989 at Lake Miyajima by bird-watchers. That year, up to 500 whooper swans arrived each day. Six live-trapped swans with general weakness and prostration were submitted to the Department of Veterinary Surgery. They were clinically treated, but died 1 to 7 days after hospitalization. Fifteen dead swans, inclusive of the 6 treated birds, were submitted to the Department of Comparative Pathology for pathologic studies. Blood from the live birds was collected into heparinized tubes by brachial vein puncture. This blood was used for preparing Giemsa-stained blood smears and determining erythrocyte counts and hematocrit. Immature erythrocytes were classified according to Hawkey and Dennett.[26] Erythrocyte count was determined by preparing an appropriate dilution and counting in a Neubauer hemocytometer, and hematocrit was determined by

the microhematocrit method for each sample. Five of the six live-trapped birds were examined by radiography.

Macroscopic and histopathologic examination

Fifteen dead swans, eight males and seven females including 9 immature birds, were necropsied. For histopathology, tissues fixed in 10% buffered neutral formalin were processed for paraffin embedding, sectioned, and stained or treated with hematoxylin and eosin (HE), acid-fast, and Prussian blue reaction. The latter two stains were served as special stains for intranuclear inclusion bodies and iron, respectively. The severity of histological lesions of each organ or tissue was assessed and graded on the following scale of - = normal, + = mild, ++ = moderate, and +++ = severe lesion.

Teased-fiber preparation of sciatic nerve

Teased-fibers were prepared according to Dyck.[21] The method is a histologic procedure to evaluate the morphology of nerve fiber degeneration like segmental demyelination. However, abnormalities of internode length and diameter for peripheral nerve tissue cannot be evaluated by this method. I studied whether lead-induced peripheral neuropathy occurred in our cases. The sciatic nerves from three cases were fixed in 10% neutral buffered formalin, after which they were divided

longitudinally and post-fixed in 1% osmium tetroxide for 3 to 4 hours. The fascicles were then placed in 66% glycerin for 48 hours and in 100% glycerin for 24 hours for teased-fiber preparation. One hundred single fibers per nerve sample were teased randomly from all the fascicles.

Electron microscopic examination

Samples of kidney from two cases were immersed in 10% buffered neutral formalin. Tissues were postfixed in 1% osmium tetroxide and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi HU12 transmission electron microscope.

Lead concentration in the tissues

Lead concentrations in the livers of 14 swans were determined by atomic absorption spectroscopy (Hitachi 180-50) after digestion with nitric acid and perchloric acid. Similarly, the concentration of lead in whole blood, kidney, or bone marrow from some of the birds also was determined. Blood lead levels in excess of 40 μg/dl are considered diagnostic of lead poisoning in mute swans.[9,90] The median lead level for normal mute swans is 4 mg/kg dry-matter liver,[8] and liver lead concentration in excess of 6 mg/kg wet weight is considered diagnostic of acute lead poisoning in waterfowl.[70]

RESULTS

Clinical findings (Table 1)

All six swans (one male and five females) examined clinically showed signs of general weakness, inappetence, green watery feces, and pale conjunctiva. Body weights varied from 3.6 to 7.0 kg, with some birds being emaciated. They finally died after showing signs of dysstasia, lethargy, and hypothermia. By hematology, two swans (swan 1 and 4 in Table 1) were apparently anemic, and all six swans revealed an increase of polychromatic erythroblasts with mild to moderate anisocytosis and/or poikilocytosis (Fig. 1). Radiographically, lead shot (six to 27 pieces per bird) was found in the proventriculus and gizzard (Fig. 2).

Necropsy findings (Table 2)

Fifteen swans (eight males and seven females) were necropsied. There was no apparent correlation between body weight and the amount of lead shot per bird. Greenish diarrhea was indicated by staining of the feathers around the vent. Common gross findings were greenish, bile-stained livers, distention of the gall bladder, impaction of the proventriculus with feed (Fig. 3), green staining and focal hyperkeratosis of the gizzard lining, pale or greenish kidneys, and edema or gelatinization of bone marrow in the diaphysis of the femur. The dilated proventriculus was

Table 1. Clinical and hematological features of six whooper swans with lead toxicosis.

Swan	Sex	Age[A]	Body weight (kg)	Erythrocyte count (x $10^4/mm^3$)	Hematocrit (%)	Number of lead pellets[B]
1	F	A	3.6	131	25.5	8
2	F	A	5.0	520	37.0	15
3	F	I	6.0	241	39.0	16
4	M	I	7.0	165	26.0	6
5	F	A	5.3	238	42.0	27
15	F	I	4.2	240	32.5	NE[C]

[A]A = adult, I = immature.

[B]Number recognized by radiography.

[C]Not examined.

Table 2. Gross necropsy findings in 15 whooper swans with lead toxicosis.

Organ	Necropsy finding	Incidence[A]
Liver	Greenish change	10/15
	Swelling	3/15
Gall bladder	Distention	14/15
Kidney	Paleness	7/15
	Greenish change	4/15
Proventriculus	Impaction	11/15
Gizzard	Bile-stained lining	15/15
	Hyperkeratosis	14/15
Bone marrow	Edema or gelatinization	7/14

[A]No. birds with lesions/total examined.

impacted with water plants, mud, and pebbles. Lead shot (five to 30 pieces per bird) up to 3 mm in diameter was recovered from these contents. Most pellets were irregular in shape and in various stages of erosion (Fig. 4).

The entire liver parenchyma was uniformly colored dark green. Bile-colored viscous fluid oozed from the cut surface of the liver. There was moderate to severe distention of the gall bladder. Biliary atresia was not seen in any of the birds.

The lining of the gizzard was generally stained green by bile, and in the center there was a pair of mild to severe hyperkeratotic foci, 4.5 x 4.5 cm in size, in all but one case. These paired foci were adjacent to each other on the inner surface. In severe cases, the foci were so hard that they could not be easily cut by necropsy knives. Mild ulcers with mild hemorrhage were seen around the foci.

In addition, Aspergillus infection in the respiratory organs was seen in three birds and gout was seen in the kidney of one bird.

Histopathologic findings (Table 3)

The main lesions were hemosiderosis in the liver and spleen, hemolytic jaundice in the liver, tubulonephrosis in the kidney, hypoplasia of the bone marrow with an increase in the number of polychromatic erythroblasts, follicular atrophy and lymphoid depletion in the spleen, and focal myocardial

Table 3. Histopathologic lesions in whooper swans with lead toxicosis.

Organ	Lesion	Severity of lesions[A] -	+	++	+++
Liver	Hemosiderosis	0/15	5/15	5/15	5/15
	Granular degeneration	1/15	14/15	0/15	0/15
	Focal necrosis	8/15	7/15	0/15	0/15
	Bile stasis in bile capillaries	7/15	4/15	3/15	1/15
Spleen	Hemosiderosis	0/15	9/15	6/15	0/15
	Atrophy of lymphoid follicles	0/15	6/15	9/15	0/15
Kidney	Tubulonephrosis	0/15	9/15	6/15	0/15
	Intranuclear inclusion body	8/15	6/15	1/15	0/15
Heart	Multifocal myocardial necrosis	0/15	10/15	4/15	1/15
	Interstitial fibrosis	2/15	6/15	5/15	2/15
	Anitschkow cells	5/15	7/15	3/15	0/15
Gizzard	Accumulation of degenerated epithelium in keratinoid layer	0/13	5/13	6/13	2/13
	Degeneration of muscle layer	6/13	5/13	2/13	0/13
Pectoral muscle	Hyaline degeneration	0/11	5/11	6/11	0/11
Bone marrow	Hypoplasia	0/13	10/13	3/13	0/13
	Increase of erythroblasts	3/13	5/13	5/13	0/13
Cerebrum	Perivascular hemorrhage	8/13	4/13	1/13	0/13
Cerebellum	Perivascular hemorrhage	11/13	1/13	1/13	0/13

[A] - = normal, + = mild, ++ = moderate, +++ = severe. Ratio shows no. birds with lesions/total examined.

necrosis with fibrosis.

Liver

Severe hemosiderosis was observed diffusely in both the hepatocytes and Kupffer cells. Kupffer cells were activated, and large amounts of hemosiderin and bile pigments were seen in the swollen cytoplasm (Fig. 5). In hepatocytes, hemosiderin was deposited in the cytoplasm adjacent to the bile pole. The central line between the hepatocellular rows was evident by the hemosiderin deposition. Dilation of biliary capillaries were seen, frequently with bile stasis. There was mild granular or fatty degeneration of the hepatocytes, while some cases showed small necrotic foci. Bile plugs were not recognized in any of the interlobular bile ducts. From these findings, the bile-stained liver was diagnosed as hemolytic jaundice.

Spleen

The cells of the mononuclear phagocyte system in the red pulp were activated and often contained hemosiderin (Fig. 6). In the white pulp, lymphoid follicles were atrophied or revealed lymphocytic depletion. Some lymphocytes were pyknotic in the remaining lymphoid follicles.

Kidney

Mild to moderate granular and fatty degeneration and

focal necrosis were seen in the proximal tubules. Pigmentation with hemosiderin was mildly and diffusely recognized in the proximal tubules by Prussian blue reaction. Acid-fast intranuclear inclusion bodies were recognized in the epithelium of the proximal tubules of seven cases by HE and acid-fast stains. However, the inclusion bodies in all seven birds were detected with difficulty, even on the sections stained with acid-fast. There were generally one to three small granular inclusion bodies in each of the cells. Larger inclusions that filled the nucleus were not seen.

Heart

Various stages of focal myocardial degeneration and necrosis were scattered in the heart. These lesions were commonly associated with focal fibrosis and mild interstitial edema and fibrosis in the other birds (Fig. 7), although fresh necrosis without fibrosis was seen in two birds. The lesions were progressive, except in one bird that had small scars. The fibrotic changes were predominantly seen in the subendocardium in 10 cases, whereas interstitial edema was prevailed in the perivascular spaces. Anitschkow cells with caterpillar nuclei occasionally were found in the edematous interstitium. In some arterioles, there was slight edematous thickening of the media. Most of the myocardial fibers in the other areas were atrophic.

Alimentary tracts

The proventriculus revealed mild mucinous catarrhal changes with bile pigments in the mucosa. The hyperkeratinized foci of the gizzards consisted of normal vertical striations, although there was mild to severe accumulation of necrotic epithelium and deposition of bile pigments in the horizontal striations (Fig. 8). Some foci had a trellis-like appearance and contained many desquamated surface cells. The glands under the foci were sometimes dilated, although necrotic changes of the glandular epithelium were not seen.

Other findings were degeneration and necrosis of the muscle layer of the gizzards, bile pigmentation on the mucosa of the small and large intestines, and mild hemosiderosis in the tunica propria of the small and large intestines.

Skeletal muscle

The musculus pectoralis superficialis in all cases that were examined revealed granular degeneration, mild to moderate hyaline degeneration, and myolysis. There was moderate macrophage infiltration and mild heterophilic infiltration in the necrotic foci of one case.

Bone marrow

Hypoplasia of the bone marrow in the diaphysis of the femur was seen in all the cases examined. Granuloblasts and

myeloid cells declined in number from the extravascular spaces, which were replaced by adipose tissue. There was a moderate to severe decline in the number of mature erythrocytes, and in almost all cases early and late polychromatic erythroblasts increased in number in the sinuses of the marrows (Fig. 9). Hemosiderin-laden macrophages were seen in the extravascular spaces.

Central nervous system

Mild congestion and hemorrhage around capillaries were recognized in the cerebrum and cerebellum, although they were not common. A few hemosiderin-laden macrophages infiltrated into the hemorrhagic foci of two cases. Vacuolization of the Purkinje cell layer with mild disappearance of Purkinje cells was found in the cerebellum of one bird.

Teased-fiber studies of sciatic nerve

Most teased fibers of sciatic nerves showed normal appearance or excessive irregularity, wrinkling, and folding of myelin. These changes were equivalent to conditions A and B according to Dyck.[21] Two of three swans examined revealed paranodal segmental demyelination without myelin ovoids or balls in the cytoplasm of the associated Schwann cells in 4.4% and 4.7% of the nerve fibers (Fig. 10). This change was comparable to condition C.[21]

Electron microscopic findings

The kidneys in two of seven cases with intranuclear inclusion bodies were examined by electron microscopy. The inclusion bodies could be easily distinguished from nucleoli by density and structure (Fig. 11). These were of higher electron density than the nucleoli and could be detected even without staining with uranyl acetate and lead citrate. They had frayed contours and consisted of fine, high-electron-dense granules (Fig. 12). The larger inclusion bodies were more homogeneous than the smaller ones.

Lead concentration in the tissues (Table 4)

Lead concentration was determined in the livers of 14 cases and in the blood and/or bone marrow of eight cases. Lead levels in the livers reached 5.5 to 44.3 mg/kg wet weight, 1.2 to 12 times higher than levels in blood samples and 4.8 to 16 times higher than levels in the marrow. The lead level in the kidney of one swan (swan 6 in Table 4) was determined to be 16.7 mg/kg wet weight, about 1.3 times higher than the level in the liver and about 5.8 times higher than the level in the blood. Results of the chemical analysis did not correlate with the number of lead pellets in the gizzard.

DISCUSSION

From the results, I diagnosed these swans as having

Table 4. Lead concentrations in liver, blood, and bone marrow of 14 whooper swans with lead toxicosis.

	Lead concentrations			
Swan	Liver (mg/kg)[A]	Kidney (mg/kg)[A]	Blood (μg/dl)	Bone marrow (mg/kg)[A]
1	44.3	NE[B]	390	2.8
2	29.0	NE	340	4.8
3	19.4	NE	630	NE
4	24.0	NE	370	NE
5	5.5	NE	490	NE
6	13.0	16.7	300	NE
7	20.5	NE	NE	2.5
8	24.0	NE	NE	4.9
9	14.4	NE	NE	NE
10	7.9	NE	NE	NE
11	16.7	NE	NE	NE
12	13.8	NE	NE	NE
13	15.1	NE	NE	NE
14	24.1	NE	NE	NE

[A]Wet weight.

[B]Not examined.

subacute lead poisoning resulting from ingestion of spent lead shot. The examined swans were all considered to be at almost the same stage of lead poisoning. Pathogenetically, the birds were anemic, and successively toxic lesions occurred in the liver, spleen, and bone marrow.

Radiography to detect lead shot in the gizzard and proventriculus, and blood lead estimation can be used for the initial diagnosis of this condition.[41,75] A blood lead level of more than 40 μg/dl is generally regarded as an undesirable threshold level of lead exposure in waterfowl[70] including Canada geese[18] and mute swans.[9,90] The highest reported whole-blood lead concentration in swans was 3,290 μg/dl in a mute swan.[90] The whole-blood lead concentrations in the live-trapped whooper swans examined were 7.5 to 16 times higher than the undesirable threshold level for waterfowl referred above.

Iron-containing brown pigment is frequently observed in the livers of birds of several different orders and families, including adult swans and geese. Lowenstine and Petrak[57] studied iron overload of livers in mynah birds. All the livers that were examined contained large amounts of parenchymal cell iron, whereas pigment in Kupffer cells was not prominent. In the present study, iron was seen prominently in Kupffer cells, spleen, and bone marrow, as well as in hepatic parenchymal cells. These findings suggest

increased hemolysis, which could be considered to be the result of lead toxicosis.

Anemia in lead poisoning is considered to result from shortening of erythrocyte survival times;[27] inhibition of heme synthesis, including the interference with delta aminolevulinic acid dehydratase and heme synthetase;[58,105] and defective erythrocyte production and impaired release of these cells from the bone marrow.[5] O'Halloran et al.[68] reported that mute swans with acute lead poisoning showed hypochromic anemia biochemically. The present study did not include examination of various biochemical and hematological values. However, accumulation of hemosiderin in the liver and spleen and reduction in erythrocyte counts in birds in the present study suggest an increase in erythrocyte destruction due to a shortened erythrocyte life span, whereas hypoplasia of bone marrow with increased numbers of polychromatic erythroblasts indicates impaired erythropoiesis. The former changes were more prominent in the present study.

Bile-stained liver, a remarkable gross finding in the present study, has not been emphasized in previous reports of lead-poisoned ducks and swans, including experimental studies. Some investigators have described gray or bile-stained liver as a characteristic lesion of lead intoxication in whistling swans[80,100] and Canada geese.[18,99] Swans in the present study had anisocytosis and poikilocytosis, severe hemosiderosis in

Kupffer cells, distension without biliary obstruction in the gall bladder, hypercholia in alimentary tracts, and green watery feces. These are related to lead-hemolytic jaundice of the liver. The high lead concentration in the liver might suggest an excess biochemical disintegration of degenerated erythrocytes.

Acid-fast intranuclear inclusion bodies in the proximal tubules of the kidney are regarded as presumptive, but strong, evidence for lead poisoning in birds.[54] The inclusion bodies cannot always be detected in lead-poisoned birds, however.[41,47,55] This may be due to species variation, dosage, length of exposure, and diet.

Del Bono and Braca[19] discussed the lesions of different stages in experimental lead poisoning in mallards and domestic ducks. They observed hemolytic anemia in acute lead toxicosis and myelotoxic anemia in chronic toxicosis. Del Bono and Braca[19] classified the intranuclear inclusions into essentially two categories: the first type, seen in acute lead toxicosis, is characterized by small granular inclusions. The second type, seen in chronic toxicosis, is characterized by large round inclusions. In the present study, the inclusions in the tubules in seven of the 15 swans examined resembled the first type. In addition, the ultrastructure of the inclusions was similar to that of lead-induced inclusion bodies in domestic chicks.[89]

Frequent gross lesions in lead-poisoned waterfowl, including atrophy of the pectoral muscles and food impaction of the proventriculus, have been attributed to anorexia,[16] direct toxic effects of lead on muscles,[16] inhibition of neuromuscular transmission,[49] and impaired peripheral nerves.[40] Skeletal muscle degeneration in the present study represented myogenic changes, not neurogenic changes, suggesting a direct toxic effect on the muscle cells.

Heart lesions previously reported in lead-poisoned ducks, geese and swans were patchy myocardial necrosis[18] or myocardial infarction.[46] Myocardial infarction, which occurred in 75% of 67 lead-poisoned birds,[46] was associated with fibrinoid necrosis of the media of arterioles and small arteries. Multifocal myocardial necrosis was recognized in all swans in the present study, and most of the foci had progressive fibrosis in the myocardium and interstitium. However, no fibrinoid necrosis was seen in the arterial media. Although I do not have clear evidence that the myocardial lesions were produced by a direct cardiotoxic effect of lead, it should be noted that the changes in the heart were frequent, as seen in human patients with lead poisoning.[48]

Cerebral edema has been observed in lead-poisoned mallard ducks[40] and Canada geese.[3] The pathogenesis of lead encephalopathy is considered to be mediated through primary vascular damage.[73] In general, younger animals are more

susceptible to lead intoxication than adults. According to experimental results,[40] the central nervous system of ducks has an apparent resistance to high circulating lead concentration, compared with the nervous system of mammals, and peripheral nerves are more vulnerable to lead than is the central nervous system. Some of the swans in the present study had mild hemorrhagic foci in the cerebrum and cerebellum. The sciatic nerves of two birds were impaired.

Three primary diseases — encephalomalacia, exudative diathesis, and nutritional muscular dystrophy — are associated with the vitamin E deficiency disease syndrome in poults. In nutritional muscular dystrophy of this syndrome, myopathy of the gizzard is the first symptom to appear in chicks.[84] It is followed by myopathy of the heart resulting in pericardial transudates, which mainly cause death. Myopathy of the pectoral muscle is seen in birds that are deficient in selenium and vitamin E, but this lesion is observed only in the birds that survived for a long time. Histological characteristics of myopathy in the vitamin E deficiency disease syndrome are edema between the muscle fibers and focal necrotic areas that in many instances have undergone calcinosis.[64] Lesions of the gizzard, heart, and pectoral muscle in the present study seemed to be different from myopathy of vitamin E deficiency diseases in poults in order of occurrence, distribution, and histological findings.

However, it is uncertain whether there was a relationship between vitamin E deficiency and mild hemorrhages in the cerebrum and cerebellum in some of the swans in the present study. The brain lesion may possibly be attributed to intercurrent vitamin E deficiency.

Fourteen of the 15 swans examined in the present study had hyperkeratinized foci of the gizzard lining. The foci were formed by a large accumulation of cellular debris trapped within the horizontal striations. Degeneration of the muscle layer of the gizzard was seen in about half of the cases. As the upper layers of the gizzard lining are usually removed by abrasion, the more recently secreted material moves toward the surface.[101] Therefore, these changes may be related to a reduction of the constructive activity of the gizzard, which suppresses shedding of the superficial layer and causes consecutive lamination and thickening of the keratinoid layer.

SUMMARY

During spring 1989, thirty-three whooper swans (Cygnus cygnus) died at Lake Miyajima in Hokkaido, Japan; 15 were examined. The birds were diagnosed as having subacute lead poisoning due to ingestion of spent lead shot. The main gross findings were bile-stained liver, edematous or gelatinous bone marrow, bile-stained lining with hyperkeratosis and lead

pellets in the gizzard, and proventricular impaction. Histopathologically, there were lead-hemolytic jaundice of the liver, hemosiderosis in the liver and spleen, and hypoplasia of the bone marrow with increased numbers of polychromatic erythroblasts. Acid-fast intranuclear inclusion bodies were seen in kidneys of seven swans. Under electron microscopy, inclusion bodies had frayed contours and consisted of high-electron-dense fine granules. The lead concentration of the liver ranged from 5.5 to 44.3 mg/kg wet weight. It was suggested that these changes resulted from excess breakdown of erythrocytes, inhibition of heme synthesis, and impaired erythropoiesis caused by lead shot in the gizzard.

CHAPTER II

PATHOMORPHOLOGIC FINDINGS OF SPONTANEOUS LEAD POISONING IN WHITE-FRONTED GEESE

INTRODUCTION

Lead poisoning in geese was initially noted in 1893 in North Carolina.[25] Five lead-poisoned Canada geese (Branta canadensis) were reported in Michigan in 1933.[74] Thereafter, Canada goose mortality due to this poisoning has been reported from various states in the United States of America,[2,3,6,99,104] and mortality of blue geese (Chen caerulescens), snow geese (Chen hyperborea), and white-fronted geese (Anser albifrons) in Louisiana and Nebraska.[6,106] However, there are limited data on the histopathologic manifestations of this intoxication in geese.

The white-fronted geese are designated as a natural monument of Japan. We encountered a mortality of these geese due to this disease between April and May of 1990 at Lake Miyajima in Hokkaido, Japan. This chapter describes the pathologic findings, with emphasis on liver lesions in the affected geese.

MATERIALS AND METHODS

Birds and environment

Lake Miyajima in Hokkaido is the northernmost point in Japan where wild ducks, geese and swans arrive in spring and fall every year. Eighty white-fronted geese and 18 swans

(Cygnus spp.) were dead or in weakened condition between April and May of 1990 at Lake Miyajima. Out of these, 27 geese and 6 swans were trapped and clinically diagnosed as having lead poisoning.[65] I pathologically and biochemically determined that the cause of death of all the 35 geese and 13 swans which could be examined was lead poisoning. From these results, I concluded that the main cause of the mortality was lead poisoning. In the spring of 1990, up to approximately 22,300 white-fronted geese arrived each day at the lake.

Nineteen white-fronted geese appropriate for systemic pathologic examinations without severe postmortem changes, 13 males and 6 females, including 9 immature birds, were used in the present study. Five of the 19 geese were live-trapped and treated with disodium calcium ethylenediaminetetraacetate every 12 hours,[65] but died within 7 days after trapping. Age, immature or adult, of white-fronted geese was determined by the characteristic external appearance. The immature bird has no white face patch at the base of the beak and neither breast black blotch nor bar, and the age is considered to be approximately from 8 months old to 2 years old.[7] Normal ranges of body weights of adult male, adult female, immature male, and immature female are 2.5-3.3 kg (mean = 2.9 kg), 2.1-3.0 kg (2.5 kg), 2.1-3.0 kg (2.6 kg), and 2.0-2.8 kg (2.3 kg), respectively.[7]

Histopathologic examination

Nineteen white-fronted geese were necropsied. For histopathologic examination, liver, spleen, kidney, heart, gizzard, adrenal gland, pectoral muscle, bone marrow, cerebrum and cerebellum fixed in 10% neutral buffered formalin were processed for paraffin embedding, sectioned, and stained with hematoxylin and eosin (HE). Selected sections were stained with Prussian blue reaction, Fontana-Masson, reticulin silver impregnation, Masson trichrome methods, Gmelin reaction and Stein's iodine test. The severity of histologic lesions of liver was assessed and graded on the following scale; 0 = normal, + = mild, ++ = moderate, and +++ = severe lesion. Immature erythrocytes were classified according to Hawkey and Dennett.[26] The liver and kidney sections from all birds were stained with acid-fast stain with Fite's calbol fuchsin for acid-fast nuclear inclusions.[52] The number of mitosis (meta- and anaphases) of polychromatic erythroblasts in bone marrow of each bird was counted in 10 fields at 600x magnification. Mitotic activity was expressed as the number of mitoses per 100 polychromatic erythroblasts (mitotic index). Liver and spleen of one non-lead poisoned immature, male white-fronted goose, with necrotizing enteritis was compared with the present cases as a pathologic control.

Lead concentration in the tissues

Lead concentrations in the livers of 19 geese were determined by atomic absorption spectroscopy (Hitachi 180-50) after digestion with nitric acid and perchloric acid. Similarly, concentration of lead in the kidney, or bone marrow from some birds was also determined. Liver lead concentration in excess of 6 mg/kg wet weight is considered diagnostic of acute lead poisoning in waterfowl.[70] Pearson's product moment correlation coefficients were calculated for the lead concentration in each organ and the number of lead pellets recovered from proventricular and gizzard contents.

RESULTS

Clinical findings

Clinically, live-trapped geese showed weakness, inappetence, green watery feces, and pale conjunctiva. Greenish diarrhea was indicated by stains on the feathers around the vent. In some birds, severe atrophy of the pectoral muscle and comparison to normal ranges of body weights were suggestive of emaciation (Table 5). A drooping appearance of the wings was seen in a bird.

Necropsy findings

Nineteen geese were necropsied. The liver was atrophic

Table 5. Body weight, number of ingested lead pellets and lead concentrations in liver, kidney, and bone marrow of 19 white-fronted geese with lead toxicosis, subdivided by age classes and sex.

	Body weight (kg)			Lead concentrations (mg/kg)[A]		
Geese	Normal ref.range[7]	Affected	Number of ingested lead pellets	Liver	Kidney	Bone marrow
Adult-male	2.5-3.3	1.9 ± 0.4[B] [n = 7]	9.8 ± 9.1 [n = 8]	25.0 ± 19.4 [n = 8]	47.8 ± 32.7 [n = 7]	4.0 ± 1.4 [n = 3]
Adult-female	2.1-3.0	2.0 [n = 2]	12.0 [n = 2]	24.1 [n = 2]	25.1 [n = 2]	10 [n = 2]
Immature-male	2.1-3.0	2.1 ± 0.2 [n = 4]	7.6 ± 2.4 [n = 5]	33.1 ± 14.3 [n = 5]	48.3 ± 45.7 [n = 5]	7.6 ± 3.5 [n = 3]
Immature-female	2.0-2.8	1.9 ± 0.6 [n = 4]	6.8 ± 5.6 [n = 4]	28.0 ± 18.8 [n = 4]	33.8 ± 24.9 [n = 4]	32.9 [n = 2]
Total	ND[C]	2.0 ± 0.4 [n = 17]	8.8 ± 6.5 [n = 19]	27.7 ± 16.3 [n = 19]	42.3 ± 32.5 [n = 18]	12.1 ± 13.7 [n = 10]

[A] Wet weight.

[B] Data expressed as mean ± standard deviation.

[C] ND = not described.

and brownish in all the cases. However, in 8 (42%) of the cases, focal parts of the liver were discolored dark green either diffusely or mottled by retained bile pigments (Figs. 13, 14). The gall bladder in all the cases was distended with dark green viscous bile. Biliary atresia was not seen in any of the birds.

Eight (42%) birds showed an impaction of the proventriculus with feed including water plants, mud, and pebbles. Lead shots (range = 0 to 30 pieces, mean ± SD = 8.8 ± 6.5 pieces) up to 3 mm in diameter, irregular in shape and in various stages of erosion were recovered from the proventricular and gizzard contents of all but one goose (Table 5).

Other common gross findings were bile-stained lining with focal hyperkeratosis in grinding pads of the gizzard (percent affected = 100%), fatty (63%) or edematous (11%) bone marrow in the diaphysis of the femur, atrophic spleen brownish in color (58%), and enlarged adrenal gland (53%). The intestinal mucosa and contents were dark green in color. Only one male immature goose showed moderate cephalo-cervical cutaneous edema. Two adult male birds and an immature male had mild cerebellar petechia.

Histopathologic findings

The main histopathologic changes were seen in the liver, spleen and bone marrow, although other organs also showed

significant lesions.

Liver

Cholestasis and hemosiderosis were prominent in the liver (Table 6). Although there were various degrees of cholestasis in different parts of the same liver, the severity of hemosiderosis was almost the same throughout the liver. Distended bile canaliculi in 13 cases (68%) contained large, elongated bile plugs. Fifteen geese (79%) had bile extravasation associated with hepatic necrosis. One bird had an accumulation of extravasated yellow bile crystals, and faintly basophilic or colorless crystals surrounded by degenerated or necrotic hepatocytes. The yellow bile crystals were radiating to extend peripherally in the foci (Fig. 15). All crystals, including bile retained in hepatocytes and the bile plugs, were stained black by Fontana-Masson stain corresponding to the degree of green discoloration in the liver (Figs. 16, 17). In other birds, the lesion was hepatocytic necrosis with faintly basophilic or colorless crystals. There was disorganization and fragmentation of hepatic cell plates due to focal hepatocytic rupture and extravasated bile (Fig. 18). On hematoxylin stained sections, hepatocytes and Kupffer cells in the areas with frequent bile plugs appeared green due to biliverdin. The yellow crystals and bile pigments were positive in Gmelin reaction and Stein's iodine test, while faintly basophilic and colorless crystals

Table 6. Occurrence, and severity of lesions in livers of 19 white-fronted geese with lead toxicosis.

Goose	Macroscopic mottled-green liver	Hemosiderosis		Bile extravasation	Bile plug	Atrophy of hepatocytes
		Kupffer cells	Hepatocytes			
Adult-male						
1	++[A]	+	+	+++	++	++
2	+	++	++	++	++	++
3	+	++	+	++	++	++
4	+	+	++	+	0	+
5	0	+++	+++	+	+	+
6	0	+++	+++	+	0	++
7	0	+++	+++	0	+	+
8	0	++	+	0	+	+
Adult-female						
9	++	++	+	+++	+++	++
10	++	+++	+++	+	++	+
Immature-male						
11	++	++	+	+++	+++	++
12	+	+	+	+	+	++
13	0	+++	+++	+	0	++
14	0	++	+++	+	+	+++
15	0	+	+++	++	0	+++
Immature-female						
16	0	+++	+++	+	+	++
17	0	+	+++	+	+	++
18	0	+	+++	0	0	++
19	0	++	+	0	0	+
Total						
percent affected	42	100	100	79	68	100

[A] The severity of lesion: 0 = normal; + = mild; ++ = moderate; +++ = severe.

were negative in both the methods. These lesions had no specific pattern of distribution. There was scattered or diffuse vacuolation of the cytoplasm of hepatocytes. Mild to moderate bile stasis without inflammation in the interlobular bile ducts was found in some cases.

Hemosiderosis in both hepatocytes and Kupffer cells was distributed diffusely; Kupffer cells had large amounts of hemosiderin in the swollen cytoplasm (Fig. 19), while hepatocytes contained the pigments adjacent to the bile pole and plugs. Most hepatocytes were atrophic or showed granular degeneration.

Spleen

In the spleen of 16/18 (89%) geese, lymphoid follicles were either atrophic or had almost disappeared. The mononuclear phagocyte system cells in the red pulp often contained a large amount of hemosiderin.

Bone marrow

There was a moderate to severe decline in the number of mature erythrocytes in the bone marrow in the diaphysis of the femur in all the birds, while early and late polychromatic erythroblasts were increased in number with frequent mitosis (mitotic index ± SD = 4.7 ± 1.8), occupying almost all the sinuses. Hypoplasia of the bone marrow was seen in 16 birds (89%). Granuloblasts and myeloid cells declined in number

from the extravascular spaces, which were replaced by adipose tissue. In 3/18 (17%) birds extravascular spaces were edematous, and one of these cases showed mild replacement of the spaces by adipose tissue.

Other tissues

Histopathologic lesions in the other tissues were tubulonephrosis (affected percent or rate of examined geese = 79% or 15/19), acid-fast intranuclear inclusion bodies in the epithelium of the proximal tubules in kidney as indicated by acid-fast stains (11% or 2/19), atrophy of myocardial fibers (84% or 16/19), myocardial degeneration and fresh necrosis (84% or 16/19), fibrinoid degeneration of small blood vessels associated with fresh necrosis (16% or 3/19), accumulation of degenerated epithelia in keratinoid layer of gizzard (100% or 18/18), degeneration of gizzard muscle layer (61% or 11/18), pectoral muscle atrophy or necrosis (100% or 8/8), adrenal cortical hyperplasia (94% of 16/17), and cerebellar perivascular hemorrhage (42% or 5/12).

No infectious agent was detected from this histopathologic examination with HE stain, although no culture could be taken.

Lead concentration in the tissues

Lead concentration was determined in the livers of 19 cases, in the kidney of 18 cases, and the bone marrow in the

diaphysis of the femurs of 10 cases (Table 5). Lead concentration in the liver reached 6.9 to 67.7 mg/kg wet weight (total mean ± SD = 27.7 ± 16.3 mg/kg wet weight). The kidney showed variable values with a wide range (range = 9.1 to 126 mg/kg wet weight, total mean ± SD = 42.3 ± 32.5 mg/kg wet weight), while the concentration in the bone marrow was the lowest (total mean ± SD = 12.1 ± 13.7 mg/kg wet weight). The liver lead concentration was slightly correlated to the number of lead pellets in the gizzard (correlation coefficient = 0.465), although other results of the chemical analysis did not correlate with the number of lead pellets. There was no correlation between the incidence and severity of the bile extravasation and the liver lead concentration.

DISCUSSION

The findings obtained here showed that the white-fronted geese were affected by subacute lead poisoning resulting from ingestion of spent lead shots, and the pathologic diagnosis was confirmed by biochemistry on liver lead concentrations. The pathologic details of lead-poisoned white-fronted geese have not been described previously. The gross lesions in the geese that were examined conform with the typical pattern described for mallards and Canada geese affected with lead poisoning.[44,99] However, in our cases, the histopathologic

lesions in the liver were characteristic.

Hematopoietic system is one of the principal targets of lead poisoning.[105] I considered that hemosiderosis in our cases is related with lead poisoning. It was distinguishable from iron overload of hepatocytes frequently seen in the livers of several species of wild birds,[57] with respect to the localization of iron pigments. A marked accumulation of hemosiderin in the mononuclear phagocyte system cells of the liver and spleen suggests an increase in erythrocyte destruction by lead, whereas hypoplasia of the bone marrow with increased numbers of polychromatic erythroblasts indicates impaired erythropoiesis and a secondary reaction to erythrocyte destruction.

Fifteen (79%) of the present cases revealed bile extravasation with hepatocytic necrosis. The lesion resembles what is known as bile infarct or bile lake, defined to be necrosis of groups of hepatocytes together with accumulation of extravasated bile in the area.[81] Such lesions associated with lead poisoning have not been reported previously, although extensive coagulative necrosis and fatty degeneration of hepatocytes have been described in experimental acute lead-poisoned domestic ducks and mallards.[19] Generally, groups of hepatocytes in longstanding cholestasis cause bile infarct or feathery degeneration, which is usually associated with extrahepatic biliary obstruction.[81] However, I did not

observe any biliary obstruction macroscopically and histologically. There was no relationship between the frequency of bile extravasation and that of proventricular impaction, which may suggest the possibility of physical biliary obstruction. A number of large bile plugs, biliary pigments within hepatocytes and the nonspecific location of bile extravasation indicate intrahepatic cholestasis in the present cases. These changes functionally may suggest an impairment of canalicular bile flow within the liver. Although the severe hemosiderosis in liver and spleen suggests that the bile-stained liver is considered as a prehepatic jaundice due to overproduction of bile by breakdown of degenerated erythrocytes, the pathogenesis of the liver changes may be more complex. Intrahepatic cholestasis may have been caused by hepatic disorder due to direct lead effects on hepatocytes and/or functional impairment of bile flow due to vagal lead neuropathy.[40]

Bile-stained liver as well as proventricular impaction and the presence of ingested lead pellets in gizzard are supportive macroscopic features in diagnosis of this poisoning in white-fronted geese. Bile-stained discoloration of liver parenchyma has been described as a characteristic lesion of lead intoxication in Canada geese,[1,18,99] whistling swans (Cygnus columbianus)[80,100] and whooper swans referred to in the chapter I. In mammals including human, however, the

pathologic effects of lead are most prominent in three organ systems, namely, the nervous system, hematopoietic system, and kidney. This difference with respect to liver changes between lead-poisoned mammals and fowls may be related to the following characteristics of fowls. Firstly, erythrocytes of fowls are nucleated, and contain mitochondria, and their life span (20 to 35 days) is much shorter than that of many larger mammals.[94] These erythrocytic nuclei are thought to serve as lead storage sites because erythrocytic nuclear inclusion bodies have been demonstrated in lead-poisoned pigeons.[4] This is related to much higher blood lead concentration in clinically normal birds and in birds with symptoms of lead poisoning, in contrast to mammals.[4,40,58] Secondly, in birds, Kupffer cells are the main sites for the destruction of erythrocytes,[94] and the liver rapidly uptakes either bilirubin or biliverdin from the general circulation.[53] In addition, biliverdin formation is suggested to occur within the biliary tree from a rapid oxidation of bilirubin.[53] Because of the foregoing features, when fowls are exposed to considerable amounts of lead, they may develop more rapid and severe destruction of erythrocytes degenerated by lead and marked hypercholia within the liver follows.

Liver was the best single tissue for significant lead poisoning determinations in waterfowl.[1,70] A liver lead concentration of more than 6 mg/kg wet weight is regarded to

indicate an acute lead exposure in waterfowl.[70] The liver lead concentration in our geese had a range from 6.9 to 67.7 mg/kg wet weight and was 1.2 to 11.3 times higher than the threshold level referred above. The threshold level in the liver is considered to be effective for biochemical diagnosis of acute or subacute lead-poisoned white-fronted geese. This range of the liver lead concentrations in the present geese was almost the same or rather slightly higher than that in subacute lead-poisoned whooper swans described in the chapter I. Nevertheless the present geese showed mottled bile-stained liver, whereas the whooper swans revealed diffuse bile-stained parenchyma of the liver. The development of the bile extravasation associated with lead poisoning may be related to bird species, their food habit and diet.[43,82,83]

SUMMARY

Nineteen lead-poisoned white-fronted geese (Anser albifrons), which died at Lake Miyajima during spring 1990, were examined pathologically. Subacute lead poisoning due to ingestion of spent lead shots was diagnosed and confirmed by demonstrating high lead concentration in the liver. The liver lead concentration ranged from 6.9 to 67.7 mg/kg wet weight. The most suggestive gross lesions were mottled bile-stained liver in 8 geese in addition to proventricular impaction

and/or the presence of lead pellets in the gizzard. Histologic lesions of the liver consisted of Kupffer cell hemosiderosis, large bile plugs in dilated canaliculi, bile pigmentation in hepatocytes, and bile extravasation associated with hepatic necrosis. Seven geese of the remaining 11 birds had also similar hepatic necrosis in the liver, whose greenish discoloration was obscure macroscopically. The liver discoloration was considered a jaundice due to both rapid overproduction of bile from increased breakdown of erythrocytes and intrahepatic impaired excretion of bile pathomorphologically. The severity of lesions was not correlated to the liver lead concentration. All examined geese had hemosiderosis of mononuclear phagocytic system cells in the spleen, and hypoplasia or edema of the bone marrow with increased numbers of polychromatic erythroblasts. It was supposed that these prominent changes resulted from excess breakdown of erythrocytes, hypercholia followed by intrahepatic cholestasis, and disrupted erythropoiesis in bone marrow caused by lead.

CHAPTER III

PERIVASCULAR EOSINOPHILIC HYALINE DROPLETS IN EXPERIMENTAL ACUTE LEAD ENCEPHALOPATHY OF CHICKS

INTRODUCITON

The mechanisms of lead neurotoxicity are extremely complex and still poorly understood. This is because the biological aberrations produced by lead appear to be related to the ability of this heavy metal to either inhibit or mimic the action of calcium as a regulator of cell function and the other essential metals at a biochemical level, and to activate protein kinases in the nerve endings.[12]

Experimental acute lead encephalopathy (LE) was first reported in a suckling rat animal model by Pentschew and Garro.[73] After their work, a series of pathologic studies on acute LE suggested that there is a lead-induced defect in the developing blood-brain barrier (BBB) because of the early appearance of hemorrhages of the central nervous system (CNS) and a marked increase in extracellular fluid.[14,15] The brain edema is caused by a functional change in the state of the endothelium rather than cell necrosis.[15,24,73,77-79,96,98]

Dietary deficiencies or excesses of certain essential metals alter the absorption, elimination, or dose response of lead.[63,76] Low dietary calcium enhances the pathologic effects of lead in birds[13] as well as in mammals.[59,91] However, the interaction between lead and this metal has not been considered in these experimental LE studies. In addition, there are limited data on astrocytic changes in acute LE, even

though astrocytes play an important role in induction of BBB properties,[42,23] and the perivascular eosinophilic hyaline droplets of the glia were described as a characteristic pathologic finding in human cases of LE.[14,15,28,73]

Adult birds generally have less sensitivity to lead than adult mammals,[40,58] and some mature waterfowl with lead poisoning due to ingesting spent lead shot showed proventricular impaction and bile-stained liver as pathognomonic findings.[105] I selected chicks as experimental animals for two reasons. First, it is much easier to supply a particular diet to the birds than to give suckling animals milk with a modified calcium concentration. Second, exposure regimens are easy and labor-saving. When the chicks are fed lead pellets, the ingested lead pellets are retained in the gizzard until they are solubilized by a combination of the grinding action and low pH. The released lead is available for absorption so that the birds with the pellets are exposed to lead continuously.

The aims of our study were to examine (1) the influence of dietary calcium on the development of experimental acute LE in chicks, and (2) astrocytic responses, especially production of glial fibrillary acidic protein (GFAP) and the frequency and nature of perivascular eosinophilic hyaline droplets under various experimental conditions.

MATERIALS AND METHODS

Chicks

Commercial, layer DEKALB TX-35 strain, 2-day-old female chicks (30.2 to 43.4 g) were obtained from a hatchery. Birds were housed in cages of our animal breeding facility and were permitted access to food and water ad libitum.

Experimental design

Experiment 1: The effect of dietary calcium on severity of lead encephalopathy

The 186 chicks were randomly separated into 4 lead-dose groups and were identified by wing badge numbers, as shown in Table 7. Chicks of 3 of these 4 groups were intubated and were given 2, 5 or 10 lead pellets (JIS 3.0 mm lead shot) via an esophageal tube at 2 days of age. The average weight of one pellet was 148 mg. One group was not treated and served as controls. The 3 treatment groups were subdivided into 5 diet subgroups of 10 chicks each, and the control group into 5 diet subgroups of 6 birds each. Chicks of each subgroup were fed calcium-supplemented (3.0% or 1.0%) high fiber diets, calcium-deficient (0.5% or 0.1%) high fiber diets, or a nutritionally balanced commercial chick diet including more than 0.7% calcium. The composition of each calcium-controled diet is shown in Table 8. I used the high fiber diets because these were reported to reduce lead pellet excretion

Table 7. Experimental groups.

Group	Subgroup#	Diet constituents	Subgroup abbreviation
I. 2 lead pellets ingested group	1	High fiber[A] + Ca 0.1% diet	2p-Ca0.1
	2	High fiber + Ca 0.5% diet	2p-Ca0.5
	3	High fiber + Ca 1.0% diet	2p-Ca1.0
	4	High fiber + Ca 3.0% diet	2p-Ca3.0
	5	Commercial ration[B]	2p-cml
II. 5 lead pellets ingested group	6	High fiber + Ca 0.1% diet	5p-Ca0.1
	7	High fiber + Ca 0.5% diet	5p-Ca0.5
	8	High fiber + Ca 1.0% diet	5p-Ca1.0
	9	High fiber + Ca 3.0% diet	5p-Ca3.0
	10	Commercial ration	5p-cml
III. 10 lead pellets ingested group	11	High fiber + Ca 0.1% diet	10p-Ca0.1
	12	High fiber + Ca 0.5% diet	10p-Ca0.5
	13	High fiber + Ca 1.0% diet	10p-Ca1.0
	14	High fiber + Ca 3.0% diet	10p-Ca3.0
	15	Commercial ration	10p-cml
IV. No lead pellet ingested group	16	High fiber + Ca 0.1% diet	Cont-Ca0.1
	17	High fiber + Ca 0.5% diet	Cont-Ca0.5
	18	High fiber + Ca 1.0% diet	Cont-Ca1.0
	19	High fiber + Ca 3.0% diet	Cont-Ca3.0
	20	Commercial ration	Cont-cml

[A] High fiber diet contains 18% crude fiber.

[B] Commercial ration contains more than 6.0% crude fiber and more than 0.7% calcium.

Table 8. Composition of calcium deficient and supplementary diets.

Ingredient	Ca 0.1%	Ca 0.5%	Ca 1.0%	Ca 3.0%
Cornstarch	64.1 [A]	62.65	60.95	54.3
Cellulose	17.0	17.0	17.0	17.0
Bean cake	15.3	15.6	15.9	17.1
Soybean oil	3.0	3.0	3.0	3.0
Calcium phosphate	0.1	0.85	0.85	0.9
Calcium carbonate	0	0.4	1.8	7.2
Sodium chloride	0.25	0.25	0.25	0.25
DL-Methionine	0.05	0.05	0.05	0.05
Choline chloride	0.1	0.1	0.1	0.1
Vitamin mix [B]	0.1	0.1	0.1	0.1

[A] Ingredients are in percent.

[B] The vitamin mix (1 kg) consisted of the following components: vitamin A, 1 x 10^7 IU; vitamin D_3, 1.5 x 10^6 IU; vitamin E, 10 g; vitamine K_3, 1 g; vitamin B_1, 0.25 g; vitamin B_2, 3 g; vitamin B_6, 0.5 g; vitamin B_{12}, 0.01 g; nicotinic acid, 20 g; pantothenic acid, 6 g; folic acid, 0.5 g; biotin, 0.01 g; manganese, 50 g; iron, 50 g; cobalt, 0.1 g; copper, 2 g; zinc, 30 g; iodine, 0.5 g.

from the gizzard greatly.[16] The chicks were examined for behavioral changes and the body weight daily until 14 days old. Dead birds were immediately necropsied and their brains were weighed and examined macroscopically and histopathologically. The number of lead pellets in the gizzard was counted at necropsy.

Experiment 2: Dysfunction of blood-brain barrier

To recognize dysfunction of the BBB due to lead, birds diagnosed to have acute LE by clinical signs were intraperitoneally injected with 1% trypan blue (0.5 ml/bird). Then the birds were sacrificed and necropsied 24 hr later. The brains were examined macroscopically and histopathologically.

Experiment 3: Lead concentrations in erythrocyte and plasma fractions

Lead concentrations in the erythrocytes and blood plasma samples of birds in the two selected subgroups were determined before development of the clinical signs. The chicks were fed lead pellets and kept under the same experimental conditions as in experiment 1. The birds were anesthetized with ketamine (0.066 mg/g body weight, intramuscularly; Veterinary Ketalar 50, Sankyo, Tokyo, Japan) at 2.0, 2.5, 4.0 and 5.0 days of age (postingestion; 0, 0.5, 2.0, and 3.0 days), and whole blood

was obtained from the cervical vein using a syringe and a 27 gauge needle washed with heparin.

Blood samples were centrifuged and separated into erythrocytes and plasma. Each sample (wet) was digested with nitric acid and perchloric acid.[102] Lead in the solutions was determined by air-acetylene flame atomic absorption spectrometry (Hitachi Ltd., Tokyo, Japan) at a wavelength of 217 nm. The background absorption was corrected by a D_2 lamp. Other conditions were those recommended by using the manufacturer.

Pathology and immunocytochemistry

Brains from the experimental birds were immersed in 10% neutral buffered formalin and were embedded in paraffin. Sections were stained with hematoxylin and eosin, and periodic acid-Schiff reaction (PAS). The terminology and anatomical descriptions referred to are those of Breazile.[11]

For immunohistochemical studies, 4-μm-thick paraffin sections were deparaffinized and rehydrated, and endogenous peroxidase was blocked with 3% H_2O_2, followed by a wash in phosphate-buffered saline. Sections were incubated with rabbit anti-cow GFAP (Dakopatts, Glostrup, Denmark), and then stained by the avidin-biotin-complex (ABC) method using a Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA). The antigen localization was visualized by incubation of

the sections with a 3,3'-diaminobenzidine-H_2O_2 solution. The sections were then weakly counterstained with hematoxylin. The selected sections were stained with PAS after visualization of the antigen.

Statistical analysis

The significance of associations was determined by the T-test or Cochran-Cox test. Probability values of less than 0.05 were considered significant.

RESULTS

Clinical signs

Clinical signs were classified into two types with regard to the process. First, birds showed depression with ananastasia and coma, and died within about one day after the signs appeared (Fig. 20A). These symptoms were exhibited in chicks which died at the early stage of the experimental period, i. e. at the age of 6-8 days. All birds of the 10p-Ca0.1 subgroup showed such clinical signs and died. Secondarily, birds showed a different form of depression from that of the first type, namely, "drop head" depression with standing for one to 6 days until ananastasia and coma developed (Fig. 20B). Chicks which died at 10-13 days old in 5p-Ca1.0 and 10p-Ca1.0 subgroups exhibited these abnormal

signs.

Body weight of birds

Growth curves for the control and 2 lead-pellet groups are shown in Fig. 21. In the control (no pellets ingested) group, chicks fed the Ca 3.0% or 0.1% diet grew more slowly than birds having the other diets, but only the retardation of growth in the Ca3.0 subgroup showed a significant difference as compared to that in the commercial diet subgroup at 14 days of age ($p < 0.001$). In the 3 treatment groups, all the subgroups except 2p-Ca1.0 and 2p-cml showed stagnation of growth. The degree of retardation became more prominent despite the differences in diet as the number of ingested lead pellets increased. The body weight in 2p-cml and 2p-Ca1.0 subgroups increased slowly through the examination period. There was a significant difference in body weight between 2p-cml and Cont-cml subgroups ($p < 0.05$), and no difference between 2p-Ca1.0 and Cont-cml subgroups, at 14 days of age.

Mortality of birds

The mortality with age in 3 treatment groups is shown in Fig. 22. It was higher as the number of ingested lead pellets increased. The chicks fed the Ca 0.1% diet showed the highest mortality, while the birds fed the Ca 1.0% diet had the lowest mortality among the 2p and 5p groups. In the 10p group, all

chicks except 2 birds of the 10p-cml subgroup died by 14 days of age. Most of the chicks of the 10p-Ca0.1 subgroup died between 6 and 8 days of age (mean ± SD = 7.8 ± 1.0; Table 9). In contrast, the birds of the 10p-Ca1.0 subgroup died a few days later (mean death age ± SD = 10.2 ± 1.5 day-old) than those of the 10p-Ca0.1 subgroup.

Mean brain weight

Mean brain weight was examined in each subgroup of the 10-pellet-ingested group as shown in Fig. 23. Significant differences were not seen among the subgroups of the no-lead-pellet-ingested control group at 7 days of age. The mean brain weight of the 10p-Ca1.0 subgroup increased significantly, as compared to those of 10p-Ca0.1, -Ca0.5, and -Ca3.0 subgroups ($p < 0.05$, $p < 0.05$, and $p < 0.01$, respectively).

Gross morphology of brains

The main macroscopic findings of brains are presented in Table 9. At necropsy, the chicks in the cml subgroups of each group showed mild to severe hemorrhagic discoloration of the cerebellum, and a few petechial spots in the cerebrum (Figs. 24A-D). These changes occurred more often and were severer, as the number of ingested lead pellets increased. Cerebral edema was seen in 3 birds (33%) of the 5p-cml subgroup and 5

Table 9. Mean age at death and main macroscopic changes of the brain in each subgroup.

			Cerebrum											
									Yellowish change			Cerebellar hemorrhagic discoloration		
	Mean age		Petechia		Edema									
Sub-group	at death (day-old ± SD)	n	+[A]	++	+	++	+++		+	++	+++	+	++	+++
2p-Ca0.1	10.4 ± 2.2	8	3[B]	0	4	1	0		2	1	0	4	3	0
Ca0.5	12.1 ± 2.4	8	0	0	1	1	3		4	1	1	2	3	0
Ca1.0	15.0	10	0	0	1	0	0		0	1	1	0	0	0
Ca3.0	11.9 ± 2.9	8	2	0	2	0	2		1	0	2	5	0	0
cml	13.7 ± 2.8	10	2	0	1	0	0		0	0	0	2	0	0
5p-Ca0.1	8.4 ± 1.7	9	1	0	3	3	2		3	0	0	3	5	0
Ca0.5	10.3 ± 3.0	9	1	1	2	3	2		3	0	0	3	2	2
Ca1.0	12.6 ± 2.3	10	3	1	2	1	5		3	2	3	6	4	0
Ca3.0	10.4 ± 3.5	10	2	0	0	2	0		1	0	0	5	2	0
cml	10.3 ± 3.1	9	4	0	2	1	0		1	1	0	4	2	1
10p-Ca0.1	7.8 ± 1.0	10	2	4	7	3	0		2	0	0	1	7	2
Ca0.5	7.7 ± 1.5	9	1	0	2	3	1		0	1	0	6	2	0
Ca1.0	10.2 ± 1.5	9	4	0	2	3	4		2	5	2	3	4	0
Ca3.0	8.5 ± 1.7	10	3	0	2	0	1		2	0	1	6	2	1
cml	9.7 ± 3.0	10	6	0	3	2	0		4	1	0	5	2	1

[A] + = mild; ++ = moderate; +++ = severe.

[B] Frequency is expressed as the number of chick with the lesion in each subgroup.

(50%) of the 10p-cml subgroup, but severe changes were not observed in either groups.

Hemorrhage in the cerebellum, which is preferably affected with lead toxicity, was more frequent and severe in the subgroups fed the Ca 0.1% diet in each group (Figs. 24E-F). Six birds of the 10p-Ca0.1 subgroup also developed petechial hemorrhage with mild to moderate edema in the cerebrum (Figs. 24G-H). Cerebellar hemorrhage among the birds fed Ca 0.5% and Ca 3.0% diets were similar in degree and frequency. In contrast, cerebellar hemorrhage in the Ca 1.0% subgroups was slighter in degree than in the other subgroups of each group, and the cerebral edema in the 5p- and 10p-Ca1.0 subgroups showed the highest incidence in the respective groups (percent affected = 67% and 100%; Figs. 24I-L). The cerebrum with marked edema was sometimes yellowish (Fig. 24K).

Histopathologic findings

The main histopathologic findings of the brain are summarized in Table 10. In general, Ca-deficiency subgroups, which were given Ca 0.1% and Ca 0.5% diets, showed susceptibility to cerebellar hemorrhage. As the lead dose increased, the hemorrhagic changes were more severe. The cerebellum and cerebrum contained a protein-rich fluid focally or diffusely in these birds. The chicks generally exhibited more severe hemorrhage or edema in the brain, as the birds

Table 10. Main histopathologic changes of the brain in each subgroup.

Subgroup	Cerebrum									Cerebellum								
	Perivascular edema			Eosionophilic droplets			Hemorrhage			Perivascular edema			Eosionophilic droplets			Hemorrhage		
	+[A]	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++	+	++	+++
2p-Ca0.1	3[B]	0	0	6	0	0	5	3	0	1	0	0	1	0	0	0	9	0
2p-Ca0.5	4	1	1	4	1	0	4	4	0	2	0	0	1	0	0	0	6	2
2p-Ca1.0	2	1	0	0	1	0	9	0	0	3	0	0	1	0	0	10	0	0
2p-Ca3.0	1	0	2	1	0	2	5	0	1	3	0	0	2	0	0	3	5	0
2p-cml	1	0	0	1	0	0	7	1	0	0	0	0	0	0	0	7	2	0
5p-Ca0.1	5	1	1	5	0	0	4	3	2	2	1	0	1	0	0	1	3	5
5p-Ca0.5	3	3	0	3	2	0	7	2	0	3	0	0	1	0	0	3	4	2
5p-Ca1.0	2	1	1	3	3	0	7	2	0	3	2	0	3	0	0	2	4	3
5p-Ca3.0	5	0	0	4	0	0	6	1	0	2	0	0	0	0	0	2	4	2
5p-cml	4	1	0	1	2	0	7	1	1	5	0	0	1	0	0	1	6	2
10p-Ca0.1	5	0	0	3	0	0	7	2	0	3	0	0	0	0	0	4	3	3
10p-Ca0.5	5	0	0	1	1	0	6	2	0	6	0	0	0	0	0	6	3	0
10p-Ca1.0	5	4	0	5	2	2	9	0	0	2	2	0	4	1	0	7	2	0
10p-Ca3.0	3	2	0	4	0	0	9	0	0	6	1	0	1	0	0	5	3	1
10p-cml	4	1	1	4	1	0	9	1	0	5	0	0	2	0	0	7	3	0

[A] + = mild; ++ = moderate; +++ = severe.

[B] Frequency is expressed as the number of chick with the lesion in each subgroup.

survived longer. As the brain edema became severe, edematous swelling of neuronal cells, astrocytic nuclei and oligodendroglial cytoplasm were prominent. Neuronal cell necrosis was obscure in all experimental subgroups.

All of the birds in the 10p-Ca0.1 subgroup showed extensive perivascular hemorrhage in the cerebellum, and in more than half of them it was moderate to severe in degree. Hemorrhagic change was seen also in the entire cerebrum in all the birds of this subgroup, and a few eosinophilic hyaline droplets were rarely recognized in the perivascular areas of the cerebrum of three birds. However, swelling and proliferation of capillary endothelial cells were mild in all examined birds. The neuropile was mildly edematous.

In contrast, edematous changes, rather than hemorrhagic lesions, were prominent in both cerebrum and cerebellum of the birds in the 10p-Ca1.0 subgroup, although perivascular hemorrhage was seen in the all birds. In addition, a number of perivascular eosinophilic hyaline droplets was often found in the cerebrum of all the birds and in the cerebellum of the five birds in this subgroup (Figs. 25, 26). The hyaline droplets were preferably formed in the hyperstriatum ventralis and neostriatum. The capillary endothelium swelled slightly and the neuropile was moderately spongiotic. Some of the neuronal cells were pyknotic and necrotic.

The 2p-Ca1.0 subgroup had the mildest encephalopathy. In the birds of the other subgroups of each group, less severe

lesions similar to those in the birds of 10p-Ca0.1 and -Ca1.0 subgroup were seen.

These experimental birds, especially those affected with severe LE, showed some lesions in other organs, namely, mild petechial hemorrhage in spinal cord, lymphoid cell depletion and decrease in number of erythrocytes in spleen, and hypoplasia of bone marrow.

Glial fibrillary acidic protein in astrocytes

In the untreated groups, astrocytes in the brains of 14-day-old chicks were GFAP negative except a few of the cells in the granular layer of the cerebellar cortex and brain stem. In eight of 10 chicks in the 10p-Ca0.1 subgroup, the intensity of stainability and distribution of positive cells with GFAP were the same as in the control groups. In contrast, GFAP-positive astrocytes clearly increased in number in all chicks of the 10p-Ca 1.0 subgroup (Fig. 27), compared with those of the control groups. Prominent hypertrophy of the GFAP-positive astrocytes was seen especially in parahippocampalis, hyperstriatum, neostriatum, and paleostriatum of the cerebrum and all the layers in the cerebellum. Double staining with anti-GFAP immunostaining and PAS reaction demonstrated that the hyaline droplets were formed around or in the GFAP-positive glial processes (Fig. 28).

Capillary permeability

Two birds receiving the same experimental treatment as the 10p-Ca1.0 subgroup were intraperitoneally injected with 0.5 ml of a trypan blue solution shortly after the appearance of "head drop" depression. The whole brains of these birds were diffusely stained pale blue with the dye at necropsy 24 hr later (Fig. 29). The brains of the control birds were not stained. The dye macroscopically seen in the brains of the birds with acute LE could not be recognized histologically.

Lead concentration in erythrocyte and plasma fractions

Lead concentration in the erythrocytes and blood plasma from 10p-Ca0.1 and Ca1.0 subgroups was determined at the preexposure to lead (2 days old) and in the initial stages of exposure to the metal (3-5 days old). The lead concentrations in the erythrocytes and plasma of the 10p-Ca0.1 subgroup increased continuously with experimental days, while those of the 10p-Ca1.0 subgroup from 4 to 5 days of age were constant or decreased (Fig. 30). The lead concentrations in both fractions, however, showed no significant difference between the Ca 0.1% and Ca 1.0% subgroups at any age.

DISCUSSION

It is well known that dietary calcium deficiencies

augment the toxic effects of lead resulting from an increased absorbtion and decreased excretion of lead.[59,76,86,91] Therefore, an increase in dietary calcium prevents the toxicity. Above a certain level, dietary calcium has been reported not to influence lead absorption.[86] The present results showed that dietary calcium influenced the pathologic conditions of acute LE. The birds fed the diet (Ca 1.0% diet) containing a slight excess of calcium were less vulnerable to lead toxicity. In pathologic in vivo studies on experimental acute LE, a relationship between lead and calcium has never been evidenced.

The present experimental acute LE could be classified into at least two forms by the pathomorphologic stages at death; peracute hemorrhagic LE in which mainly the cerebellum was involved, and edematous LE associated with GFAP-positive astrocytes and marked perivascular eosinophilic hyaline droplets. It is said that brain swelling is the most characteristic macroscopic feature in children affected with acute LE.[10,69,92] On the other hand, brain swelling and the subsequent herniations are not as frequent as generally believed.[72] Such a macroscopical discrepancy in acute LE might be caused by lesions modified by various factors, including nutrition and the dietary life of each patient as well as brain maturity.

There was no relationship of lead concentrations in the

fractions of erythrocytes and blood plasma between 10p-Ca0.1 and -Ca1.0 subgroups. LE progression might have a relation to differences in the resistance of the cerebral capillaries to lead, or in the efflux of lead, rather than to the blood lead concentrations.[51] It is said that hemorrhagic lesions precede the accumulation of edema fluid in the cerebellum in LE.[77] This finding may be right as one type of pathogenesis in LE, but the pathologic differences between the two different forms in our experiments seem to reflect the degree of dysfunction of the BBB induced by the interaction of lead and calcium at the cytological level.

Recently, attention has been focused on astrocytes in neurotoxicologic processes. Many of the neurotoxic effects of lead appear to be related to the ability of lead to mimic or inhibit the action of calcium as a regulator of cell function.[12] In the pathogenesis of acute LE due to exposure to high levels of lead, it seems that lead primarily alters the brain capillaries, leading to a breakdown in the BBB.[14,15,24,73,77-79,96,98] However, little in the way of overt endothelial injury and necrosis is seen. In addition, the vulunerability of the endothelium to lead is related with tissue maturity.[22,32,51,62,66,73] From this evidence, one interpretation is that the endothelial cells lose their brain-specific differentiation due to lead toxicity and revert to a more systemic type of permeability which no longer

restricts the movement of plasma into the brain.[12] Therefore, injury to astrocytes induced by lead toxicity also may have a relation to this loss of the special barrier property because astrocytes have a role as a source of the differentiation signals of the BBB and induce the barrier properties.[23,42]

Holtzman et al.[34-36] proposed that effects of lead on the cellular aerobic energy metabolism are important in the pathogenesis of LE from the study of the resistance to lead related with maturity. Mitochondria are a critical subcellular target in lead neurotoxicity in at least three processes, including heme synthesis, oxidative phosphorylation, and intracellular calcium metabolism. Mature cerebral astrocytes seem to have the capacity to sequester lead in non-toxic (i.e., extra-mitochondrial) sites as lead-containing inclusions,[33] although astrocytes appear to be particularly vulnerable to the toxicity of lead in vitro.[22,37] These facts led to the hypothesis that the resistance of the brain to LE during maturation is dependent on the capacity of astrocytes to take up and sequester lead in non-toxic sites (lead-sink hypothesis).[37,97]

Astrocytic alterations in LE are morphologically interpreted by separating primary and secondary responses. Diffuse astrocytic proliferation and reactive gliofibrillogenesis are common in LE of the suckling rat.[15,50,73] These astrocytic changes are considered to be

the secondary response because of lack of pronounced gliotic responses by purified astroglia in cell culture.[37] On the other hand, lead-containing dense cytoplasmic and intranuclear inclusions in astrocytes are construed as the primary response of the cells.[30,31,33,87,88] Astrocytes in LE, however, display other morphological characteristics. Clasen et al.[15] insisted that perivascular eosinophilic hyaline, PAS-positive globules or droplets located in astrocytes, are almost pathognomonic for this disorder. Since experimental acute LE in suckling rats was reported by Pentschew and Garro,[73] a number of studies has been carried out using other animals, including chick embryos,[29] hatched chicks,[67] newly weaned rhesus monkeys,[14] young calves,[103] and cats.[95] The significance of such perivascular droplets in LE has not been discussed except by Clasen et al.,[14,15] although they were found frequently in human cases with acute LE.[10,28,69] In the present experiments, the eosinophilic droplets were observed mainly in hyperstriatum of chicks with acute edematous LE, and GFAP-positive astrocytes appeared to form them. The pathogenetic significance, however, is still unclear; it is unknown whether the morphology of astrocytes is a direct result to sequester lead in the non-toxic, extra-mitochondrial sites or a secondary change resulting from the BBB dysfunction. In addition, these astrocytic changes may explain why birds have stronger resistance to high circulating concentration of lead

than mammals.[40] Our experimental acute LE in chicks is useful for studying these subjects.

SUMMARY

Perivascular hyaline droplets are described as a histological finding in acute lead encephalopathy, but their pathologic significance has almost never been discussed. We examined the potential of dietary calcium (Ca) to modify the effects of lead in experimental acute lead encephalopathy (LE) in chicks, and astrocytic reactions in it.

Two-day-old chicks of 4 groups were given 0, 2, 5, or 10 lead pellets (JIS 3 mm) via an esophageal tube. After that, the birds of each group were fed either high fiber diets containing different amounts of Ca (0.1, 0.5, 1.0, or 3.0%) or a commercial chick diet for 12 days. The mortality, body weight, brain weight, and pathologic changes were examined. The mortality and growth retardation in the subgroup fed the Ca 1.0% diet was the lowest among the groups ingesting 2 or 5 pellets. All the birds in the 10 pellet-Ca 0.1% subgroups showed ananastasia and coma, and died of hemorrhagic encephalopathy at 6-8 days of age (mean ± SD = 7.8 ± 1.0). In contrast, birds in the 10 pellet-Ca 1.0% subgroup exhibited "head drop" depression with standing as the characteristic clinical sign and died of severe brain edema at 8-13 days of

age (mean ± SD = 10.2 ± 1.5). Birds in this subgroup showed marked perivascular eosinophilic, periodic acid-Schiff reaction positive hyaline droplets mainly in the hyperstriatum, in addition to diffuse glial fibrillary acidic protein (GFAP) positive gliofibrillogenesis in the cerebrum. The acute LE induced was pathomorphologically classified into two forms; hemorrhagic and edematous. These results indicate that dietary calcium affects the condition of LE in chicks, and that the frequency of perivascular eosinophilic droplet formation depends on lead-calcium interaction in vivo. It is suggested that perivascular astrocytes can form these hyaline droplets in their cytoplasm after the astrocytes react, exhibiting a GFAP-positive reaction to mild lead-induced damage of the capillary endothelium.

CONCLUSION

The results obtained from both naturally occurring cases and experimental cases of lead poisoning of birds by pathologic investigations are summarized as follows:

1) During the spring of 1989 and 1990, mortalities of swans and white-fronted geese (Anser albifrons) occurred at Lake Miyajima in Hokkaido, Japan. Thirty-three whooper swans (Cygnus cygnus) died in 1989, and 87 waterfowls including 69 white-fronted geese died in 1990. Laboratory examination of 63 of these birds established that lead poisoning was responsible for the majority of the mortalities.

2) Fifteen whooper swans were examined pathologically. Remarkable gross findings were diffuse green discoloration of liver by bile and edematous or gelatinous bone marrow besides proventricular impaction and the presence of lead pellets in the gizzard. Histopathologic features were characterized by lead-induced hemolytic jaundice of the liver, hemosiderosis in the liver and spleen, and hypoplasia of the bone marrow with increased numbers of polychromatic erythroblasts. Acid-fast intranuclear inclusion bodies were found in kidneys of seven swans. The ultrastructural features of inclusions were similar to lead-induced inclusions reported previously. High lead concentrations of the livers were detected, and ranged from 5.5 to 44.3 mg/kg wet weight.

3) All the examined whooper swans were pathologically diagnosed as having subacute lead poisoning due to ingestion of spent lead shot. These morphologic changes were suggested to result from excess breakdown of erythrocytes, inhibition of heme synthesis, and impaired erythropoiesis caused by ingestion of lead shot.

4) Nineteen subacute lead poisoned white-fronted geese, which were collected at Lake Miyajima during spring 1990, were examined pathologically. The diagnosis was confirmed by liver lead analysis. The liver lead concentrations ranged from 6.9 to 67.7 mg/kg wet weight.

5) The liver lesions of the white-fronted geese were worthy of notice. The histopathologic features, which were macroscopically recognized as mottled bile-stained liver, were characterized by Kuppfer cell hemosiderosis, large bile plugs in dilated canaliculi, bile pigmentation in hepatocytes, and bile extravasation associated with hepatic necrosis. The liver discoloration was interpreted as a jaundice due to both rapid overproduction of bile from increased breakdown of erythrocytes and intrahepatic impaired excretion of bile pathomorphologically.

6) All examined geese showed splenic hemosiderosis and hypoplasia or edema of the bone marrow with increased numbers of polychromatic erythroblasts. The pathologic features in these geese were suggested to result from excess breakdown of erythrocytes, hypercholia followed by intrahepatic

cholestasis, and disrupted erythropoiesis in bone marrow caused by lead.

7) I investigated the potential of dietary calcium to modify the effects of lead in experimental acute lead encephalopathy in chicks. Two-day-old chicks which were fed a diet containing a slight excess of calcium in comparison with a commercial chick diet were less vulnerable to lead toxicity with respect to mortality, body weight, brain weight, and pathologic changes. These birds exhibited the characteristic clinical sign and died of severe brain edema with marked perivascular eosinophilic hyaline droplets that were periodic acid-Shiff reaction positive. On the other hand, the lowest calcium diet enhanced neurotoxic effects of lead, but hyaline droplets could not be detected.

8) The acute lead encephalopathy induced was pathomorphologically classified into two forms; hemorrhagic and edematous. Dietary calcium affected the condition of lead encephalopathy in chicks and the frequency of perivascular eosinophilic droplet formation. The results of this experiment suggest that perivascular astrocytes can form these hyaline droplets after the astrocytes react, exhibiting a glial fibrillary acidic protein positive reaction to mild lead-induced damage of the capillary endothelium.

9) The acute lead encephalopathy produced in chicks is useful to elucidate astrocytic roles in this disorder because the pathologic significance of perivascular eosinophilic

hyaline droplets in astrocytes are still unknown.

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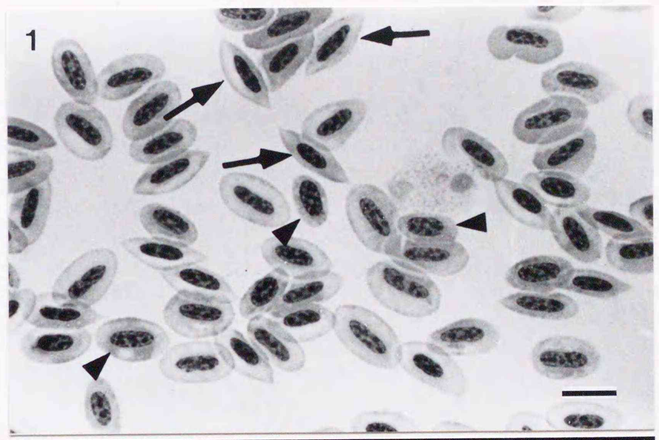
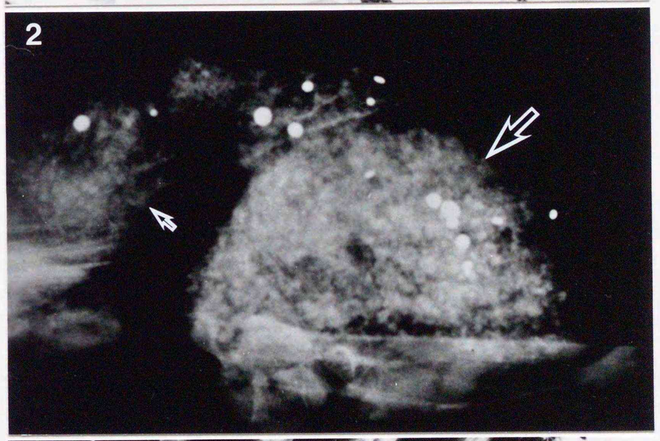
FIGURES AND EXPLANATION OF FIGURES

Fig. 1. Blood; whooper swan. Blood smear showing poikilocytes (arrows) and late polychromatic erythroblasts (arrowheads). Giemsa stain. Bar = 10 μm.

Fig. 2. Radiograph. Proventriculus and gizzard; whooper swan. Numerous lead pellets in the proventriculus (small arrow) and gizzard (large arrow).

Fig. 3. Proventriculus; whooper swan. Dilated, impacted proventriculus.





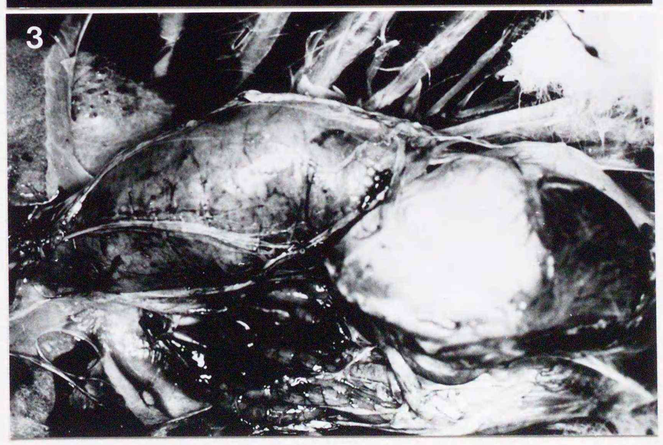
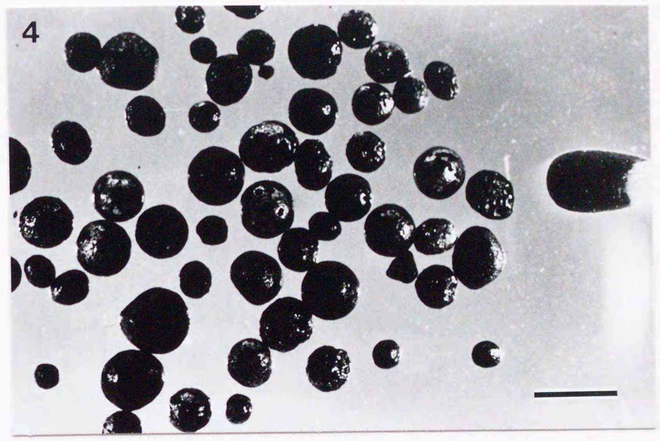


Fig. 4. Spent lead pellets. The head of a matchstick is shown at right center. Bar = 5 mm.

Fig. 5. Liver; whooper swan. Liver stained with Prussian blue method for iron. Large quantities of iron-containing pigments (hemosiderin) are present as dark masses in Kupffer cells. In hepatocytes, there are hemosiderin deposits along bile canaliculi between hepatocellular cords. Bar = 20 μm.



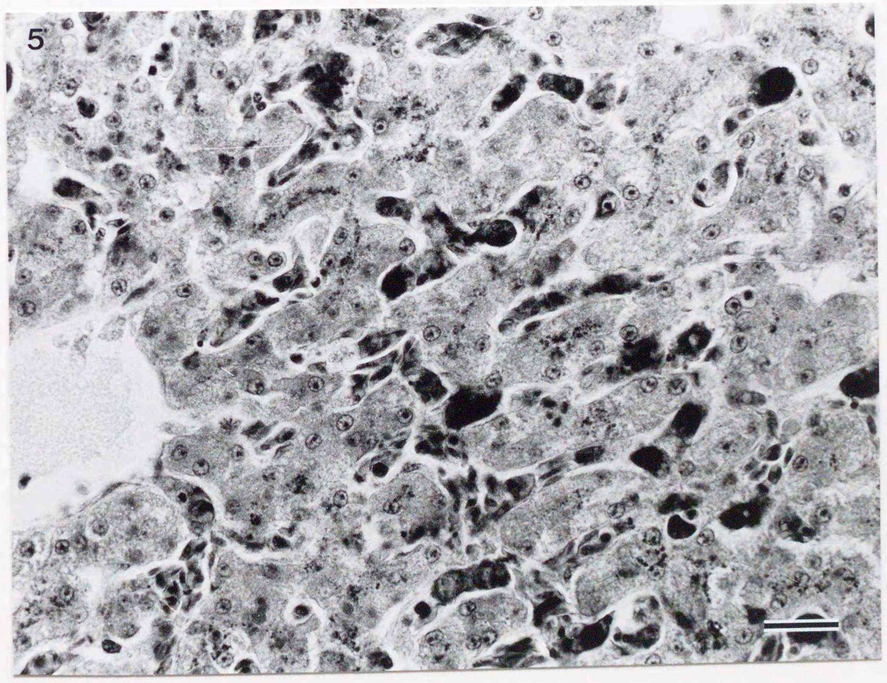
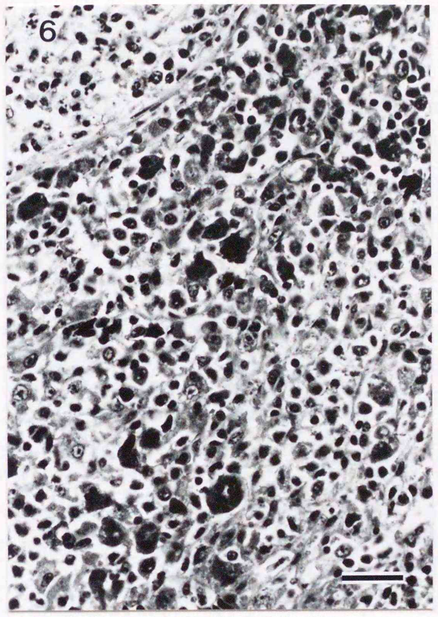


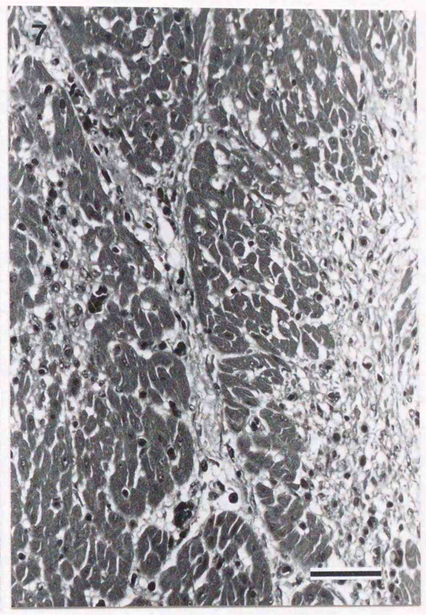
Fig. 6. Spleen; whooper swan. Some lymphocytes are depleted from a lymphoid follicle, and numerous hemosiderin-laden macrophages are seen in the red pulp. HE. Bar = 20 μm.

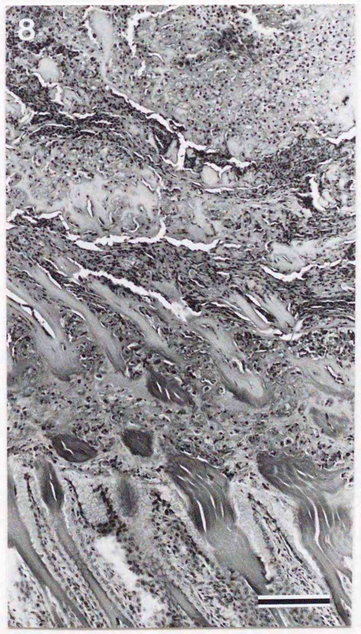
Fig. 7. Heart; whooper swan. Multifocal myocardial fibrosis with mild interstitial edema and fibrosis. HE. Bar = 40 μm.

Fig. 8. Gizzard; whooper swan. Marked accumulation of degenerated and necrotic epithelia is seen in the keratinoid layer of the gizzard. HE. Bar = 100 μm.

Fig. 9. Bone marrow in the diaphysis of the femur; whooper swan. Early and late polychromatic erythroblasts occupy the sinus, at the center of which some mature erythrocytes (arrows) are found. A few proerythroblasts (arrowheads) lie in contact with the sinus wall. HE. Bar = 10 μm.







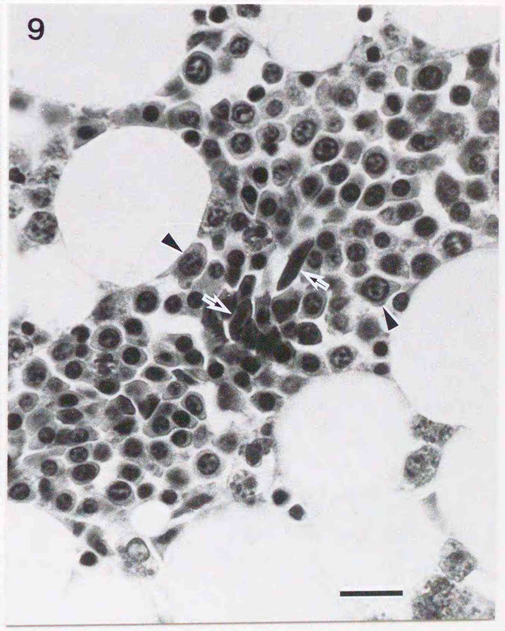
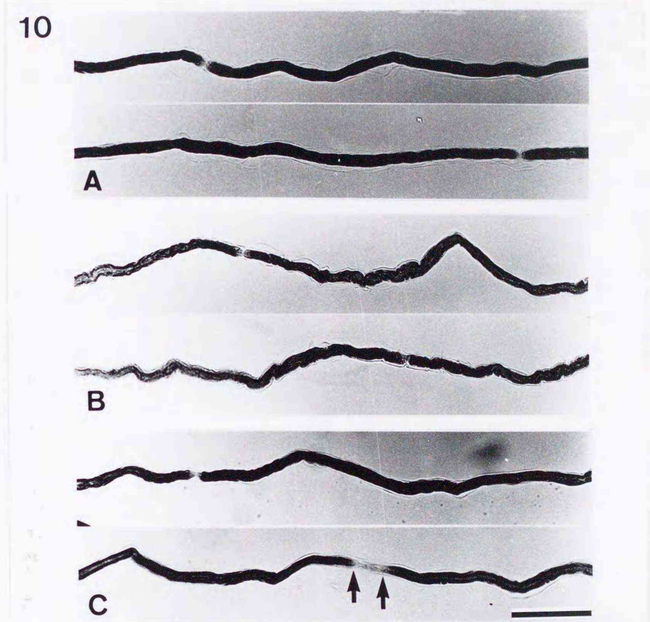
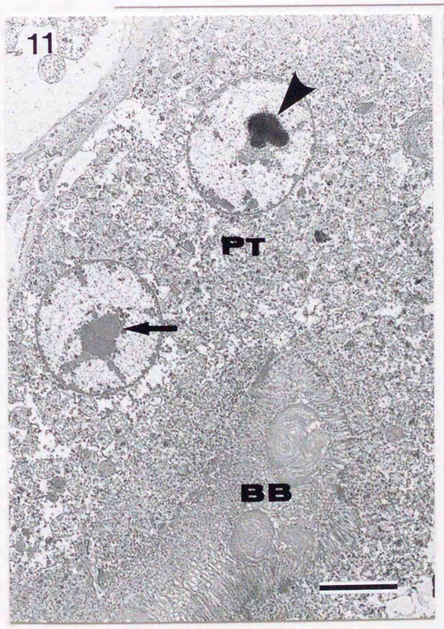


Fig. 10. Sciatic nerve; whooper swan. Teased fibers from the sciatic nerve of a swan with lead poisoning. The changes were typed according to Dyck.[21] A) Normal, condition A. B) Myelin irregularity, condition B. C) Segmental demyelination, condition C (arrows). Bar = 10 μm.

Fig. 11. Transmission electron micrograph. Kidney; whooper swan. Intranuclear inclusion body (arrowhead) with high electron density was recognized in a proximal tubular epithelial cell (PT) with brush border (BB). It can be easily differentiated by electron density from the nucleolus (arrow) in the other nucleus. No electron staining. Bar = 4 μm.

Fig. 12. Transmission electron micrograph. Kidney; whooper swan. Higher magnification of the intranuclear inclusion body in Fig. 11 after staining with uranyl acetate and lead nitrate. It has frayed contours and consists of fine, high-electron-dense granules. Bar = 1 μm.





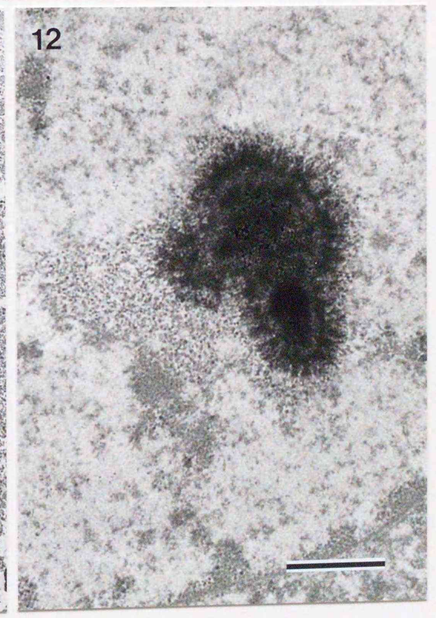
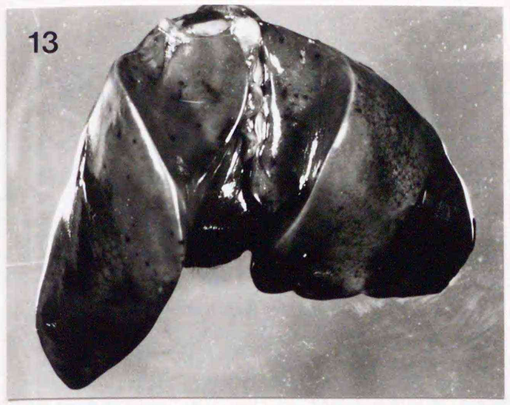


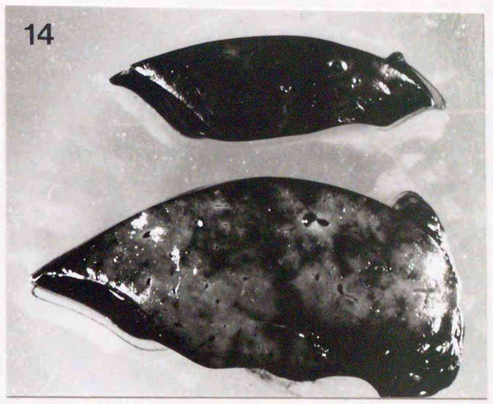
Fig. 13. Liver, uncut; white-fronted goose. Liver parenchyma under facies diaphragmatica of both left and right lobes is stained green by bile showing diffuse or mottled discoloration.

Fig. 14. Liver, cut surface; white-fronted goose. Mottled bile-stained liver parenchyma caused by bile pigmentation in hepatocytes, bile plugs and bile extravasation.

Fig. 15. Liver; white-fronted goose. A bile extravasation lesion (arrow) with yellow bile crystals, which are radiating to extend peripherally. HE. Bar = 150 μm.

Fig. 16. Liver; white-fronted goose. Extravasated bile crystals, which are stained black, are associated with hepatocytic degeneration and necrosis. Fontana-Masson. Bar = 40 μm.





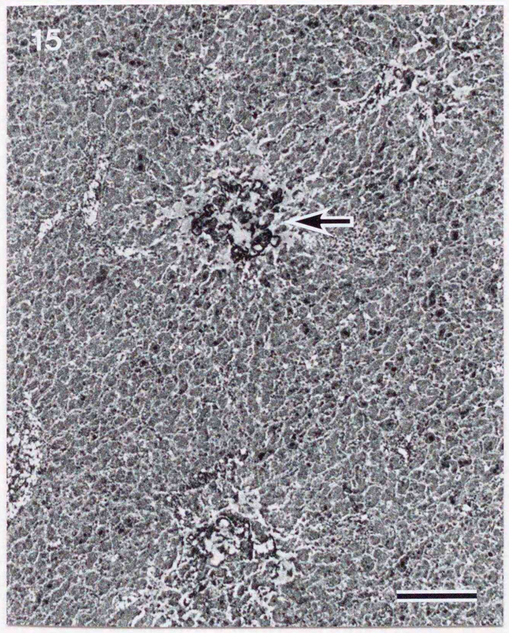
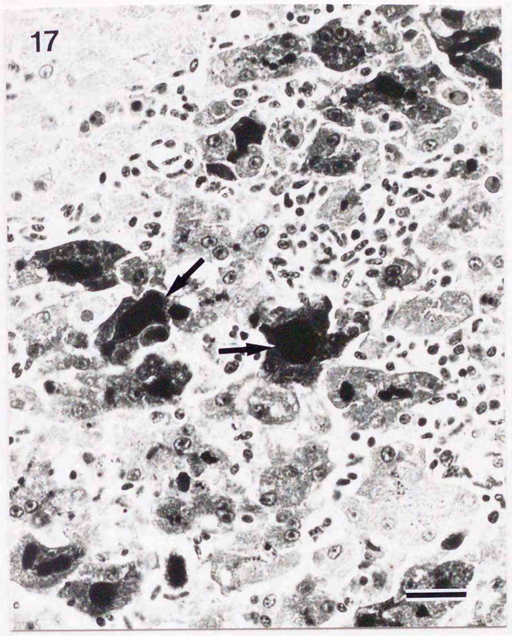
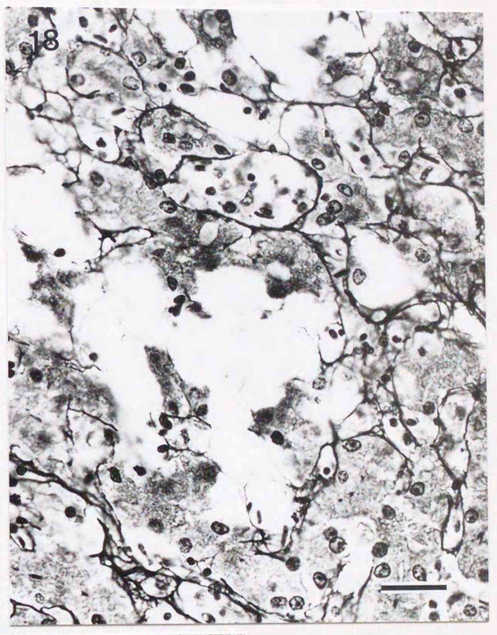


Fig. 17. Liver; white-fronted goose. Both biliverdine retained in hepatocytes and large bile plugs (arrows) are stained black. Ovoid cells in sinuses are erythrocytes. Fontana-Masson. Bar = 20 μm.

Fig. 18. Liver; white-fronted goose. Hepatic cell plates are disorganized and fragmented by hepatocytic rupture. Bile crystal in the foci has been dissolved. Reticulin silver impregnation. Bar = 20 μm.

Fig. 19. Liver; white-fronted goose. Kupffer cells have large amounts of hemosiderin in the swollen cytoplasm. Prussian blue reaction with a nuclear fast red counterstain. Bar = 20 μm.





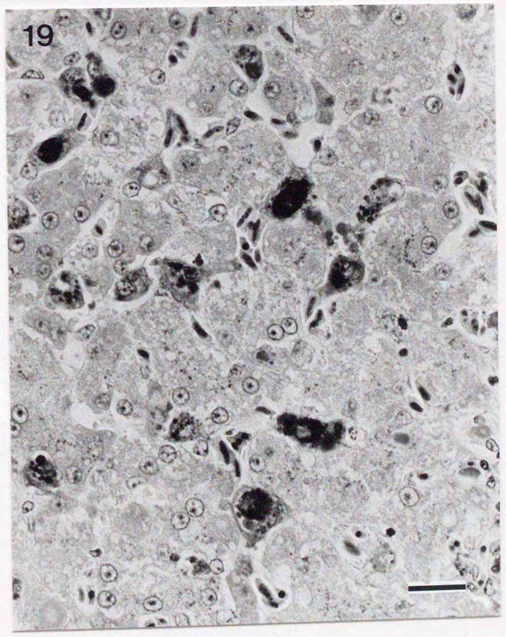


Fig. 20. Two types of clinical signs of chicks with acute lead encephalopathy. Chicks were given lead pellets via an esophageal tube at 2 days of age. A: Chicks of 10p-Ca0.1 subgroup reveal ananastasia and coma immediately after depression. 8 days of age. B: Chicks of 10p-Ca1.0 subgroup show characteristic "drop head" depression while standing. 11 days of age.

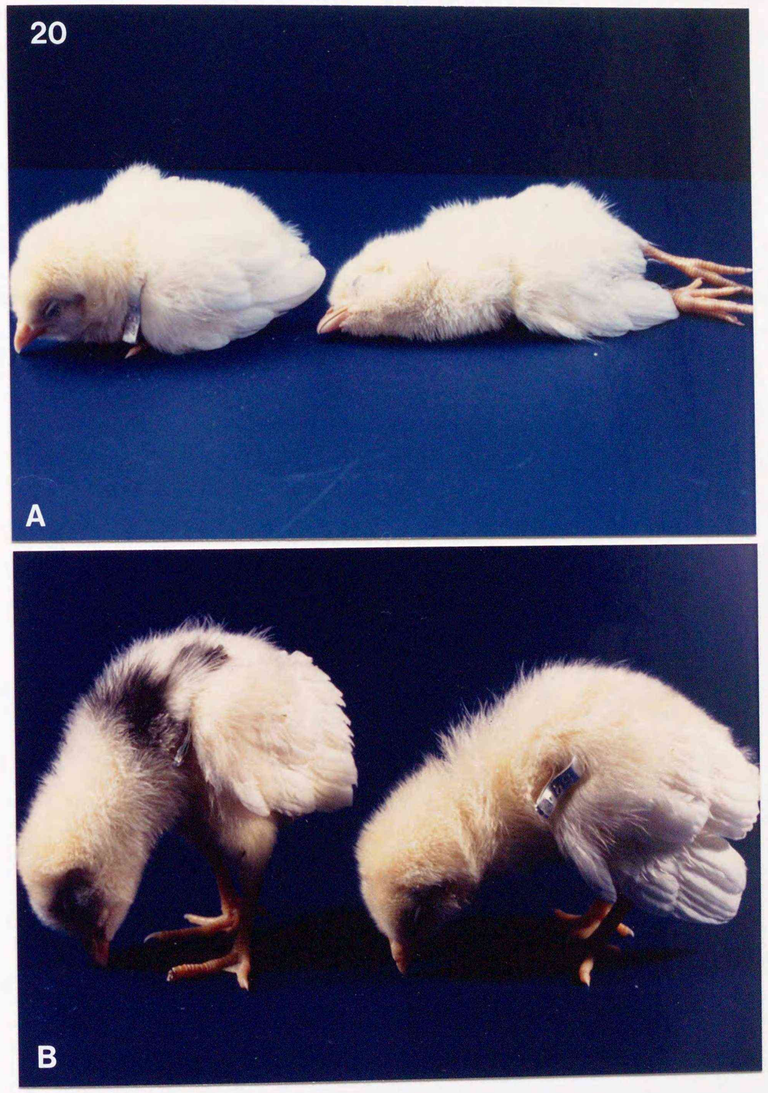


Fig. 21. Mean body weight of each subgroup in the 2 pellets treatment group. A: The values at 14 days of age represent the mean ± SD. Retardation of growth only in Cont-Ca3.0 subgroup shows significant difference, as compared to that in the Cont-cml subgroup at 14 days of age ($p < 0.001$). B: All the subgroups other than 2p-Ca1.0 and -cml subgroups show almost complete stagnation of weight gain.

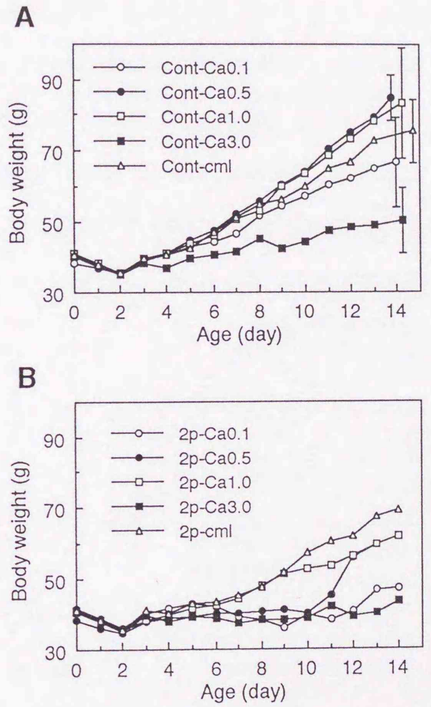


Fig. 22. Mortality in each subgroup of the 3 treatment groups. A: 2 lead pellets treatment group; all the chicks but one in the 2p-Ca0.1 subgroup died by 12 days of age. In contrast, all of the 2p-Ca1.0 subgroup survive during the experimental period. B: 5 lead pellets treatment group; all the chicks of the 5p-Ca0.1 subgroup died by 11 days of age. Some chicks of the 5p-Ca1.0 subgroup died, but the mortality at 14 days of age was the lowest among the subgroups. C: 10 lead pellets treatment group; all the chicks of Ca-modified subgroups died, but the mortality of 10p-Ca1.0 subgroup is the lowest among these 4 subgroups.

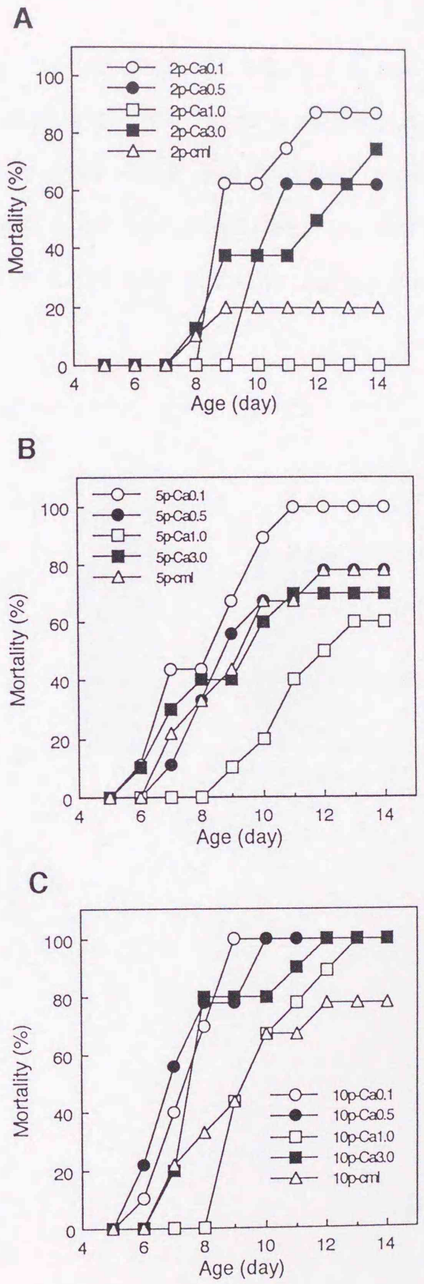


Fig. 23. Mean brain weight in each subgroup (n = 9 or 10 chicks) of the 10 lead pellets treatment group and 7-day-old chicks given no lead pellet but the same diet (n = 5 chicks per subgroup). The values are expressed as a mean ± SD. *p < 0.05, **p < 0.01, when compared with the 10p-Ca1.0 subgroup.

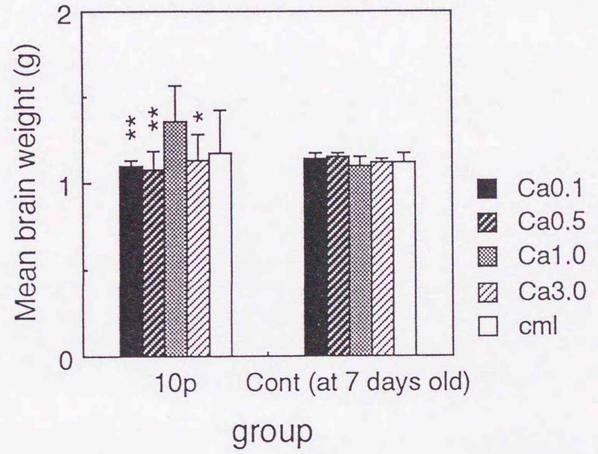


Fig. 24. Brains; chicken. Acute lead encephalopathy in chicks. Brains of birds given lead pellets at 2 days of age. Chicks in the 10p-cml subgroup show severe hemorrhagic discoloration of the whole cerebellum at 7 and 8 days of age (A and B), moderate hemorrhage and edema of the cerebellum at 9 days of age (C), and moderate cerebral and cerebellar edema at 10 days of age (D). Birds in the 2p-Ca0.1 (E-F) and 10p-Ca0.1 (G-H) subgroups have cerebellar discoloration with cerebral petechia at 9 and 7 days of age, respectively. Petechial hemorrhage is seen also in the basal part of these cerebrum (F and H). Chicks in the 10p-Ca1.0 subgroup show moderate to severe cerebral edema with or without yellowish discoloration and mild to moderate cerebellar hemorrhage at 9 (I), 10 (J), 11 (K), and 12 (L) days of age. Bar = 5 mm.

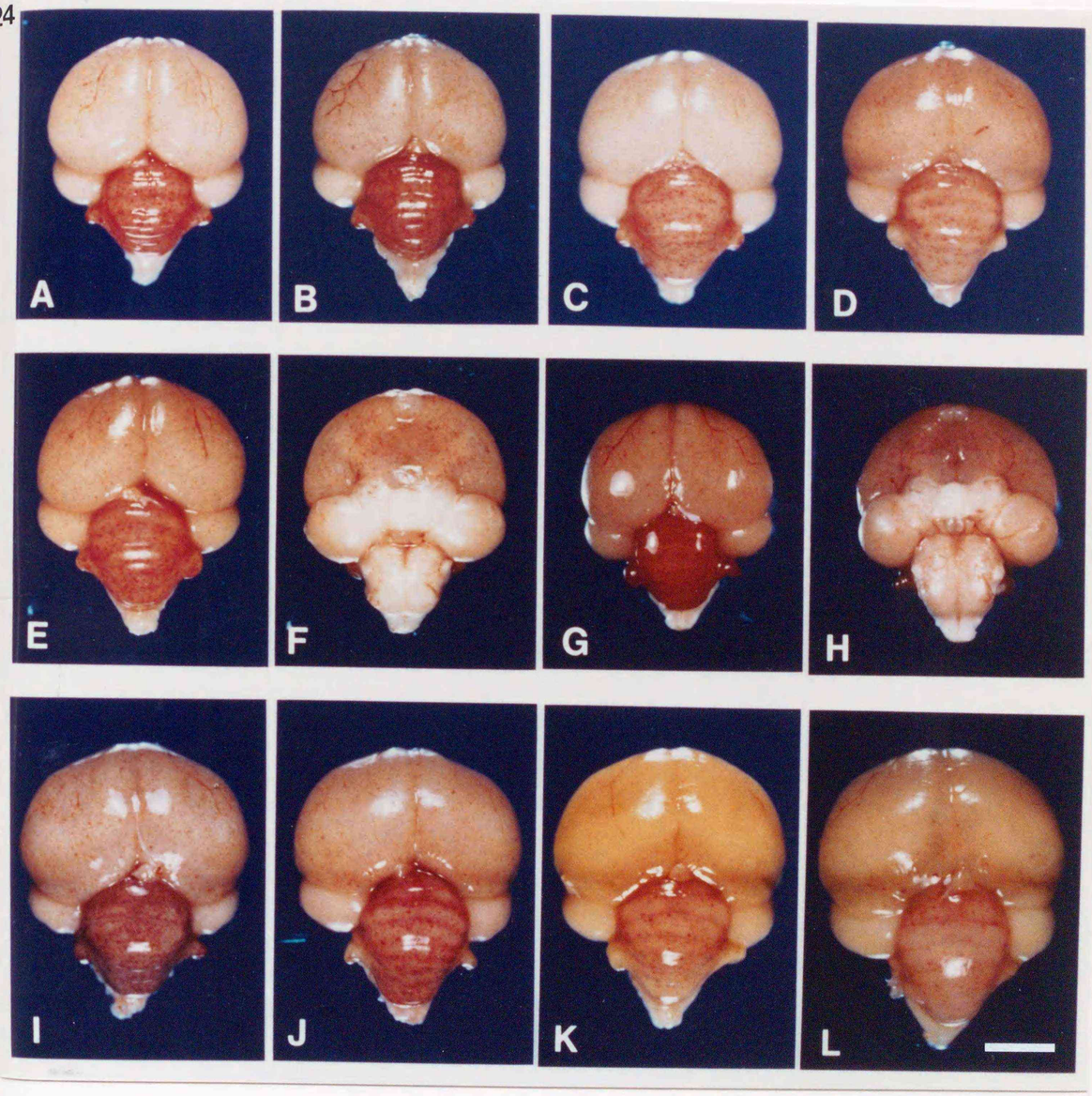
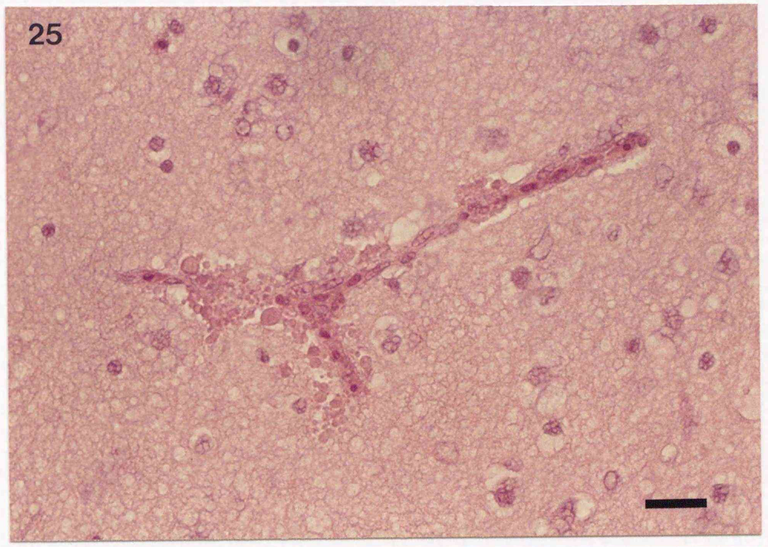


Fig. 25. Cerebrum; chicken. Typical perivascular eosinophilic hyaline droplets in a chick of the 10p-Ca1.0 subgroup. Edematous neuropil and oligodendroglial swelling are also seen. HE. Bar = 20 μm.

Fig. 26. Cerebrum; chicken. PAS-positive hyaline droplets and granules in a chick of the 10p-Ca1.0 subgroup. They formed around swollen astrocytic nuclei. Periodic acid-Schiff reaction. Bar = 20 μm.



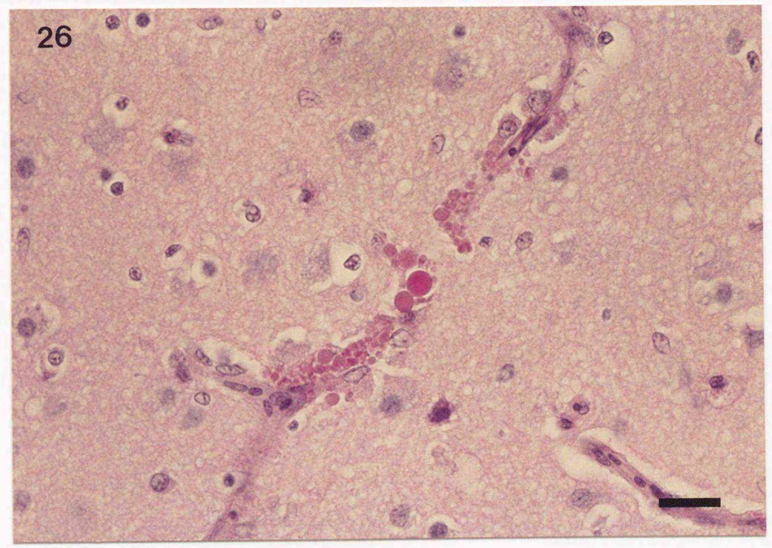
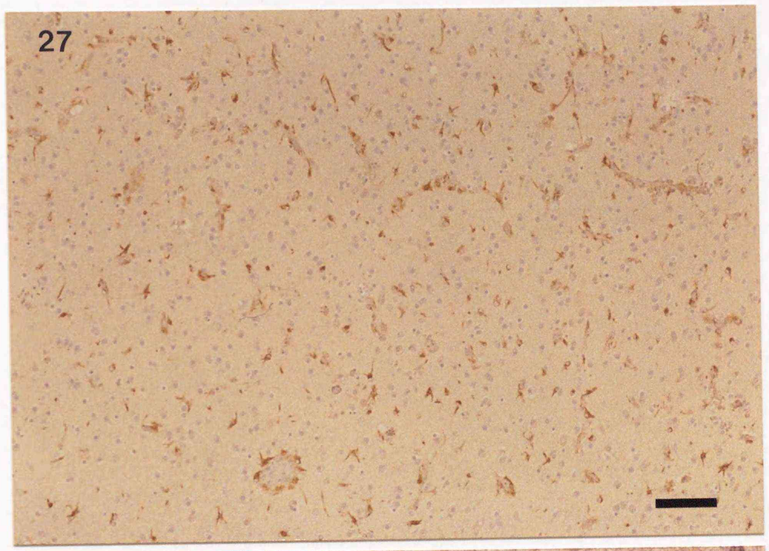


Fig. 27. Cerebrum; chicken. Diffuse GFAP positive fibrillogenesis in the hyperstriatum of a chick in the 10p-Ca1.0 subgroup. Anti-GFAP avidin-biotin-peroxidase complexes. Bar = 100 μm.

Fig. 28. Cerebrum; chicken. GFAP positive gliofibrils and PAS positive droplets are recognized as overlaping one another. Double staining of immunostain with anti-GFAP antibody and periodic acid-Schiff reaction. Bar = 20 μm.



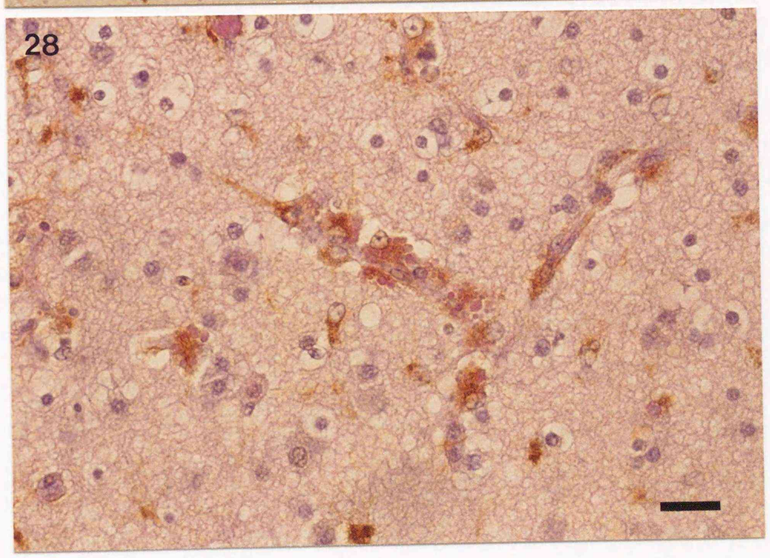


Fig. 29. Brain; chicken. Brain (left) of a 9-day-old chick, which was treated with the same experimental conditions as the 10p-Ca1.0 subgroup. This bird was injected intraperitoneally with trypan blue and sacrificed 24 hr later. The brain is stained blue entirely. Right is the brain of an age matched control that was given no lead pellet and a commercial diet, and similarly treated with trypan blue. Note that the brain is not stained.

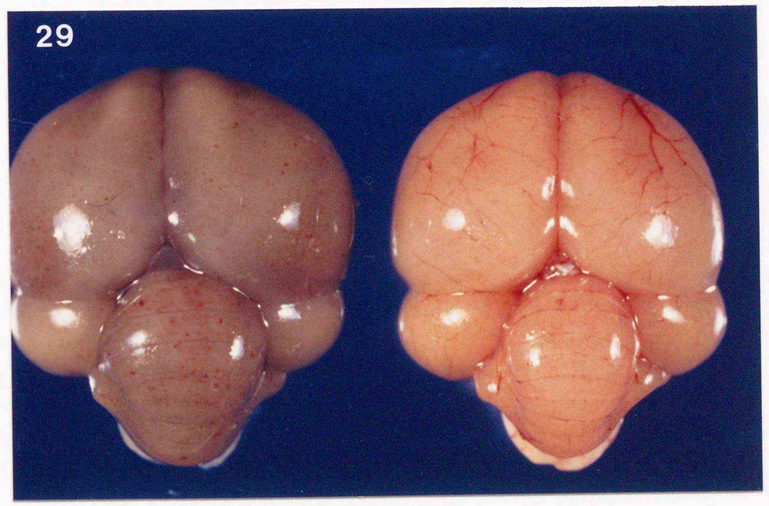


Fig. 30. Blood and blood plasma lead concentrations of chicks in the 10p-Ca0.1 and -Ca1.0 subgroups determined by air-acetylene flame atomic absorption spectrometry at a wavelength of 217 nm. Values are the mean ± SD of 8-10 chicks.