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Effects of lead on collagen synthesis

and renal function in the rat

by

Sonya Agnesi Rodolfo-Sioson

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of

The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

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DEDICATION

To the memory of my late husband

Federico Mariano Sioson,

whose life radiated a light

that still illuminates my path,

this work is humbly dedicated.

INTRODUCTION

Lead poisoning continues to be a significant problem among the children of families of low economic status. These children often dwell in poorly-maintained housing and ingest the leaded paint which flakes off the woodwork. The possibility that low environmental lead levels in food, water and air could cumulatively constitute a health hazard, is causing widespread concern.

In the young, lead encephalopathy is a common manifestation of acute lead exposure. Symptoms may include headaches, convulsions and impaired motor function. Common sequelae among survivors include subnormal intelligence and behavioral aberrations, cerebral palsy and retinal atrophy.

Clinical and experimental lead poisoning in the young has produced evidence that damage to the brain microvasculature leading to a breakdown in the blood-brain barrier may play a primary role in lead encephalopathy. As revealed by light and electron microscopy, edema and extravasation of erythrocytes are among the earliest changes in the brain of lead-exposed animals. Previous work suggested that increased capillary permeability may arise from defective capillary basement membranes in neonatal rats. Since this membrane constitutes part of the blood-brain barrier and is composed, in part, of collagen, this investigation was undertaken to determine the effects of lead on collagen biosynthesis in young rats and on collagen degradation and renal function in older rats.

LITERATURE REVIEW

Lead

Historical background

Many of the symptoms associated with lead toxicity have been known since ancient times. Hippocrates is said to have been familiar with the colic that accompanies the acute form of toxicity, and in the second century B.C. the poet Nikander set the description of a terminal case to verse (Latin tr. 1532, cited in Major 1945). Medical awareness of the toxic properties of lead apparently did not reach the Roman nobility. Water was piped through conduits fashioned of the malleable, corrosion-resistant metal, and food was cooked and beverages stored and drunk out of vessels lined with poorly-fired lead glaze. The resulting neurological and reproductive deficits that plagued many wealthy Romans have been postulated to play a key role in the decline and fall of the Empire (Gilfillan 1965).

An ailment termed the "Devonshire colic" was described by Huxham in England (1738, cited in Major 1945), and 29 years later Baker demonstrated that the drinking of cider contaminated by lead-lined cider presses was the direct cause (1767, cited in Major 1945). The European practice of sweetening alcoholic brews with litharge (lead acetate), and the contemporary custom in this country of distilling illicit whiskey through old automobile radiators and flavoring the distillate with old battery plates have generated a small but steady stream of saturnism cases among the less discriminating imbibers (Hammond 1969). Benjamin Franklin was familiar with the syndrome,

and cited rain water collected off lead roofs under trees with acidic leaves as a lead source (National Academy of Sciences 1972).

With the onset of the Industrial Revolution, a more widespread problem developed among the working population, especially at lead smelters and in battery factories. Until the early 1900's, managerial apathy for the well-being of the common working man resulted in numerous cases of severe adult toxicity. With modern industrial safety regulations, clinical cases of occupational lead poisoning are increasingly rare.

Awareness of toxicity among infants did not become common until about 20 years ago (Hammond 1969). The heaviest incidence of pediatric plumbism occurs among pre-school children in the inner cities. These children dwell in pre-World War II homes with poorly maintained paint and plasterwork, which flake off and may be ingested especially by the child who has pica (Latin: magpie), an appetite for inedible materials. Because of the permanent neurological damage that is sustained by at least 25% of the survivors (Chisolm and Harrison 1956) the problem has received wide attention.

This review will cover the area of the literature dealing with the deleterious effects of lead on the metabolism and function of virtually all body tissues.

Symptomatology

Pediatric plumbism is usually of the chronic type, and three constellations of symptoms appear at various intervals during the course of the affliction (Haley 1971). The hematological picture

shows the first changes: reduced hemoglobin, microcytemia, and basophilic stippling of erythrocytes. The patient's pallor is caused by both anemia and vascular spasm. Iron therapy alone is futile (Berman 1966).

The gastrointestinal syndrome makes itself manifest soon thereafter: thirst, metallic taste in the mouth, anorexia, vomiting, intermittent colic, and constipation. Growth rate declines, and body weight may actually drop. These symptoms may herald the acute form of plumbism with which encephalopathy is associated (Chisolm and Kaplan 1968).

Lead encephalopathy has been defined as any brain disorder caused by lead poisoning, including the subclinical cases characterized only by brain structural changes (Clasen et al. 1974). Cerebral edema, accompanied by intracranial pressure increase, may be indicated by papilledema, increasing clumsiness, frequent loss of balance, ataxia, and lethargy. Convulsive seizures of the grand mal variety frequently portend death within 72 hours (Haley 1971).

Therapy is accomplished with the aid of chelating agents, most commonly calcium disodium ethylenediaminetetraacetate (CaEDTA). Such therapy is indicated when plasma lead levels reach 60 μg/100 ml or urinary delta-aminolevulinic acid (δ-ALA) levels exceed 66 μg/100 ml, especially if any two of the symptoms previously listed are present (Haley 1971). While the severe symptoms of intoxication may be reversed by chelation therapy, neurologic damage may be permanent, especially in the young.

Learning and behavioral manifestations

Learning and psychological deficits, as estimated from standard I.Q. test results in children, have been diagnosed (Byers and Lord 1943, Mellins and Jenkins 1955, Jenkins and Mellins 1957, Byers 1959, Cohen and Ahrens 1959, Smith 1964, Moncrieff et al. 1964, Gibson et al. 1967, Pueschel 1974, Beattie et al. 1975, Landrigan et al. 1975). Behavioral problems, poor discipline, and inability to comprehend have been reported (Nye 1933, Byers and Lord 1943, Lane 1964, Smith 1964, Woods and Walters 1964, Chisolm and Kaplan 1968). Hyperactivity has been seen in children even at "nontoxic" blood lead levels averaging 26 μg/100 ml (David et al. 1972, David 1974). Significantly elevated blood lead levels have been found in autistic children (Cohen et al. 1976). Electroencephalographic abnormalities are seen as long as five years after treatment (Smith et al. 1963).

Impairment in learning has also been reported for suckling rats trained to swim a T-maze (Brown 1975). The lead doses (10 mg Pb/100 ml in maternal drinking water, 25 or 35 mg/kg/day by gavage to the dam, or 5 mg/kg i.p.) were too low to produce overt signs of toxicity. A similar study on rats not exposed to lead until eight days of age revealed no significant effects on learning or memory (Brown et al. 1971). Behavioral and motor retardation is seen in mice whose mothers were receiving 0.8% Pb in the drinking water (Cohen et al. 1976). Hyperactivity is a commonly reported finding in the young rodent, often accompanied by learning and motor deficits if the lead is ingested during the first 10 days of life (Michaelson and Sauerhoff 1974a, 1974b, Sauerhoff and Michaelson 1973, Silbergeld and

Goldberg 1974a, 1974b, Brown 1975). The neurochemical mechanisms underlying the hyperactivity have been postulated to involve defects in aminergic pathways (Arnold et al. 1972, Snyder et al. 1970, Snyder and Meyerhof 1973). Thus, hyperactivity has been attributed to elevation of brain catecholamines and increase in norepinephrine (NE)-dopamine (DA) ratios in 21- to 27-day-old rats (Golter and Michaelson 1975). An alternate theory is that lead acts on cholinergic pathways to reduce acetylcholine (ACh) release (Silbergeld and Goldberg 1974a).

Pathology

Central nervous system (CNS)

Neural tissue Cerebral edema is the most consistent finding in the brain of lead-exposed children (Popoff et al. 1963, Raimondi et al. 1968, Haley 1971, Clasen et al. 1974). The edema is caused by the alteration in the permeability of the microvessels, allowing a protein-rich transudate to leak through the endothelial junctions into the extra-cellular space (Popoff et al. 1963, Clasen et al. 1974). The ensuing tissue hypoxia in turn results in swelling of astrocytes, which take up plasma proteins (Popoff et al. 1963, Clasen et al. 1974) and in chronic edema there may be necrosis of neurons and glia (Clasen et al. 1974). While the cerebral edema is conspicuous, it is the molecular layer of the cerebellum that sustains the greatest damage (Haley 1971). The glial cell nuclei display an exhaustion phenomenon, the myelin sheaths are delaminated, and the neurons in the gray matter develop lamellar disturbances, stacking of

rER granules and diffuse granular material in place of neurofilaments (Raimondi et al. 1968). The reduction in size of the extracellular spaces in the white matter by the swelling of the endothelial cells constitutes the most prominent change (Raimondi et al. 1968, Haley 1971).

When lactating rats are fed 4% lead carbonate, their young receive lead in the milk. As in humans, the brains of the pups contain edemal fluid in the cerebellum (Pentschew and Garro 1966), white matter of the cerebrum, caudate nucleus, septum and Purkinje cell layer (Clasen et al. 1974). There is vacuolation in the cerebellum (Pentschew and Garro 1966) and basal nuclei (Clasen et al. 1974). The molecular layer is replete with capillaries made prominent by the proliferation of endothelial cells with swollen nuclei (Clasen et al. 1974). These changes disappear spontaneously after the first 60 days of life even in rats maintained on lead (Pentschew and Garro 1966). The development of the cerebral cortex is retarded, cerebrum and cerebellum weights are diminished, and myelin formation is delayed (Michaelson and Sauerhoff 1974a). The brains of lead-poisoned dogs show changes similar to the above (Zook 1972). Suckling mice receiving lead through the milk develop a dose-responsive reduction in brain weight (Maker et al. 1975). Intervascular strands are present in the hippocampus and basal nuclei and the staining properties of astrocytes and microglia are altered. No cerebral edema or focal destructive lesions are found (Rosenblum and Johnson 1968). Since there are no ultrastructural changes in adult guinea pigs exposed to lead, perhaps the primary encephalopathic effect is at the neuronal and/or cellular level (Bouldin and Krigman 1975).

Brain microvasculature The microvessels constitute a prominent site of injury in lead encephalopathy in many species. In lead-exposed children the development of the growing blood vessels is arrested, resulting in the formation of numerous intervascular strands (Clasen et al. 1974). The capillaries in the gray matter display the most significant changes (Raimondi et al. 1968). Pericytes are swollen, basement membranes appear homogeneous, there is distension of the rER and increased number of vesicles in the endothelial cytoplasm (Raimondi et al. 1968). The nuclei of the swollen endothelial cells protrude into the capillary lumen, reducing intravascular space and causing the vessels to become engorged with blood (Popoff et al. 1963, Haley 1971). The increased intravascular pressure forces a protein-rich transudate to leak through the endothelial junctions. In many cases ring hemorrhages may form around the capillaries (Popoff et al. 1963). The venous walls may be thickened, and their collagen fibers are in disarray (Popoff et al. 1963).

In the young rat the changes in the microvasculature are very similar to those in children. The capillaries in the molecular layer of the cerebellum are made prominent by proliferation of endothelial cells or nuclear swelling. In the striatum, capillaries are swollen, and there are transudations and hemorrhages both here and in the molecular layer (Pentschew and Garro 1966). The edema associated with these changes is the most commonly reported finding (Pentschew and Garro 1966, Lampert and Schochet 1968, Thomas et al. 1973, Clasen et al. 1974, Goldstein et al. 1974, Michaelson and Sauerhoff 1974a). Again the

primary site of injury is the blood vessel (Thomas et al. 1973). In suckling mice many intervascular strands are seen throughout the brain (Rosenblum and Johnson 1968), but no edema. The guinea pig poisoned in adulthood maintains a normal-appearing microvasculature while developing seizures and death (Bouldin and Krigman 1975). The adult rabbit does not develop a recognizable encephalopathy at all (Hass et al. 1964), demonstrating species-specific differences in response to lead exposure.

Blood-brain barrier Studies of the anatomy of the barrier between the vascular system and the brain tissue reveal that several structures may participate in restricting passage of many compounds from blood to brain. They are, in order, the capillary endothelium, the capillary basement membrane, the astroglial perivascular foot basement membrane, and the astroglial perivascular foot plasma membrane (Jacobson 1972). Functionally, however, the best candidates for the anatomical site of the barrier are the capillary endothelium and basement membrane. Electron microscopic studies of the brains of adult mice receiving horse-radish peroxidase (HRP) via the tail veins indicate exclusion at the level of the endothelium (Reese and Karnovsky 1967). Similar results were seen after intravenous infusion of HRP in 2 to 5 week old untreated rats (Vistica 1975). The endothelial cells of the cerebral capillaries differ from those of other capillaries in the body by possessing zonulae occludentes or tight junctions with no intercellular gaps. This cellular organization provides a very effective barrier to the passage of many potentially toxic substances

into the brain tissue. In the young animal with rapidly proliferating cerebral capillary endothelium bounded by structurally immature membranes, the high metabolic rate and rapid turnover of many substrates could result in increased membrane permeability and susceptibility to insult by lead. Lead has been found in the lysosomes of brain neurons, causing a depression in the activity of alkaline phosphatase in six-week old rats following lead injection (Brun and Brunk 1967). However, no depression in cerebral capillary alkaline phosphatase activity was found in three- to five-week old lead-exposed rats in a later study (Ahrens and Vistica, in press).

Peripheral nervous system The primary site of lead poisoning in the adult nervous system is at the level of the peripheral nerves (Akelaitis 1941). In man the most commonly reported symptom is extensor motor nerve paralysis (Fullerton and Harrison 1969, Haley 1971, Felton et al. 1972) evidenced by wrist drop (Haley 1971). Slowing of conduction has been measured in the lateral popliteal nerve and ascribed to segmental demyelination (Fullerton and Harrison 1969). In the rat, chronic daily ingestion of 0.6 g lead acetate produces Wallerian degeneration, particularly in the larger myelinated fibers of the distal tibial nerve (Schlaepfer 1969). Demyelination, with splitting along the interlamellar lines originating at the nodes of Ranvier, was observed in the sciatic nerve. Myelin sheath damage is ascribed to a metabolic lesion caused by the pressure or ischemia resulting from the edema accompanying increased permeability of the peripheral vascular bed (Lampert and Schochet 1968, Schlaepfer 1969).

<u>Liver and spleen (reticulo-endothelial system, RES)</u> The diagnostic ultrastructural change produced in the cells of liver and kidney by lead is the intranuclear inclusion. These structures appear in liver parenchymal cells of children (Blackman 1936, Popoff et al. 1963), dogs (Zook 1972), and rats (Moore and Goyer 1974) after their appearance in the kidney. Liver weight of rats is increased probably due to edema (Scoppa et al. 1973). Some hemosiderosis is seen in the spleen of rabbits (Hass et al. 1964) and in the liver of dogs, and many parenchymal cells have enlarged nuclei (Zook 1972). In baboons, Kupffer cells are vacuolated, the rER is scanty and disorganized, and the mitochondria are swollen with shrunken cristae. Parenchymal cells of rats injected with 5 mg lead acetate contain scanty rER, increased sER and smooth vesicles containing finely granular, electron dense material after six hours. Spleen RE cells possess vacuolated mitochondria with loss of cristae and smooth vesicles similar to those in the liver parenchymal cells (Hoffmann et al. 1972).

The sensitivity of rats to gram-negative bacterial endotoxin is increased by five orders of magnitude if 10 mg/kg lead acetate is injected i.v. prior to injection of the endotoxin (Selye et al. 1966). When mice are injected with the nontoxic dose of 12.5 mg/kg/d lead nitrate for 30 days before treatment with a dilution of <u>Salmonella typhimurium</u> culture, all mice die. Mice receiving 10 mg/kg/d for the same period prior to the treatment have LD_{50} (dose causing death in 50% of the animals) significantly reduced (Hemphill et al. 1971). Thus, lead may interfere with resistance mechanisms to permit uninhib-

ited bacterial growth by binding antibodies or by inhibiting phagocytic activity of polymorphonuclear leukocytes (Hemphill et al. 1971). Another explanation for impaired hepatic and splenic detoxification is the lead-mediated inactivation of the -SH-containing enzymes (Selye et al. 1966, Bertok 1968, Hoffmann et al. 1972, Trejo et al. 1972). The activity of the drug-metabolizing enzymes is reduced via impairment of the mixed-function oxidase system (Scoppa et al. 1973) and also by reduced availability of cytochrome P-450 (Alvares et al. 1972). Lipid peroxidation by microsomal enzymes is inhibited by the same mechanism (Pani et al. 1975). Nicotinamide adenine dinucleotide phosphate-cytochrome c reductase activities are depressed (Williams and Kamin 1962).

Kidney

Histological changes The kidneys of a wide variety of species of lead-exposed animals have been found to contain intranuclear inclusions. In man they are localized in the epithelium of Henle's loop and the proximal convoluted tubule cells (Blackman 1936, Popoff et al. 1963, Clasen et al. 1974) which may be swollen (Popoff et al. 1963). In the rat the inclusions, which have a dense core and lacy edge (Goyer et al. 1970b), appear in the proximal convoluted tubule after six to eight weeks on a 1% lead diet (Goyer 1971, Tange et al. 1965). Inclusions are also seen in the dog (Zook 1972). Inclusions contain about 90% of the lead in the kidney (Moore and Goyer 1974) sequestered in an insoluble, nondiffusible form until the cell is shed into the urine (Goyer et al. 1970a, 1970b, Goyer 1971). In rats on a low

calcium diet the intranuclear inclusions appear when drinking water lead concentration is 12 μg/ml; with normal calcium intake inclusions do not appear until the lead concentration in the water attains 200 μg/ml (Mahaffey et al. 1973). In the rabbit, brown granular cytoplasmic pigment, giant tubule cells, and intranuclear inclusions appear in the straight, narrow tubules near the cortico-medullary junction at 8 to 12 weeks, and later in the proximal convoluted tubules in rabbits on 500 mg Pb/100 g diet (Hass et al. 1964). Histologic changes in the 30-day old rat on 2 or 4% lead acetate diet include cell vacuolation and necrosis in the distal segment of the proximal convoluted tubule (Hirsch 1973). Ultrastructural changes include mitochondrial swelling with vesiculation of cristae (Mao and Molnar 1967) prior to the appearance of inclusions (Goyer 1968).

Function A feature associated with acute lead nephropathy in children is the Fanconi triad: glucosuria, aminoaciduria, and phosphaturia in the presence of hypophosphatemia (Chisolm 1962). This triad is also present in adults, often accompanied by decreased tubular secretion of urates (Felton et al. 1972). Chronic lead nephropathy in man has been studied only in Queensland, Australia, where the house exteriors were at one time painted with lead oxide-linseed oil paint, which gradually powdered off under the hot sun and was dusted onto clothes and hands of children playing outside. A nephrosclerosis may develop, with mild albuminuria, mild hypertension, hypoparathyroidism and osteodystrophy, and terminally in the adult, renal failure (Henderson 1954, 1958). Tubular cell dysfunction was evidenced by gout

in approximately 50% of these patients (Felton et al. 1972).

Aminoaciduria has been reported for rats (Sun et al. 1966) and rabbits (von Studnitz and Haeger-Aronsen 1962). As in man, the defect resides in the tubular reabsorptive mechanism (Hass et al. 1964, 1967, Hoffmann et al. 1974) and is reversible during recovery (Hass et al. 1964).

In rats fed 1 or 2% lead acetate for 10 to 40 weeks there was an increase in the kidney to body weight ratio but no increase in kidney water content. Accumulation of PAH and tetraethyl ammonium (TEA) by renal cortical slices from treated rats was not altered. The two-hour urinary excretion of PAH was increased. Offspring of dams placed on 2 or 4% lead acetate diet at parturition developed increased kidney to body weight ratios and increased uptake of PAH by renal cortical slices (Hirsch 1973).

Nutritional factors and lead toxicity

Calcium Reduced intakes of either calcium or phosphorus increase lead retention in carcass (Sobel et al. 1940, Shields and Mitchell 1941), and the effects are additive (Quarterman et al. 1974). A reduction in dietary Ca from 0.7 to 0.1% increases Pb retention in tissues of rats receiving 200 μg Pb/ml drinking water. Lead concentration of 12 μg/ml in drinking water given to rats on 20% of normal calcium intake produces the same toxicity as 200 μg/ml in the presence of normal calcium (Mahaffey et al. 1973). Addition of both Ca and P to the milk ration of newborn rats decreases Pb absorption (Kostial et al. 1971).

Calcium-lead interactions may result from similarities in their metabolism. Vitamin D increases blood and bone lead (Sobel et al. 1938, 1940), and parathormone increases urinary Pb excretion (King 1971). Lead may alter intestinal membrane permeability, inhibiting the passive transport of calcium (Gruden 1975). The distribution of Pb and Ca in the body are influenced in a similar manner by P (Potter et al. 1971), and Ca is found with Pb in intranuclear inclusions (Carroll et al. 1970). Lead is taken up by heart mitochondria in apparently the same fashion as calcium, perhaps by the same carrier system (Scott et al. 1971).

Iron Anemia is a common symptom of lead poisoning, primarily via inhibition of heme synthesis. Lead aggravates the impairment of hematopoiesis caused by iron deficiency (Waxman and Rabinowitz 1966, Borsook et al. 1957). Iron-deficient rats dosed with 200 μg Pb/ml drinking water retain 20 times the lead taken up by rats with normal iron intake (Six and Goyer 1972). There is no clear explanation for this dramatic effect, since Pb apparently does not share the absorption pathway of Fe, Zn, Co and Mn (Bremner 1974).

Hemoglobin (Hb) synthesis

The effect of lead on porphyrin metabolism has been well studied (Goldberg 1972). The mechanism by which lead inhibits heme synthesis at several points along the pathway may be by binding -SH groups, although there is no real evidence that these groups are near the active sites of the enzymes or that their blockade would impair activity (White 1975).

The first step in the pathway is the condensation of succinyl CoA and glycine to form δ-ALA, catalyzed by the enzyme δ-ALA synthetase. It is generally believed to be relatively insensitive to lead; however, 10^{-3}M Pb caused 47% reduction in δ-ALA synthetase activity in chicken hemolysates (Morrow et al. 1969).

The second step, the dehydration of δ-ALA to form porphobilinogen, is catalyzed by δ-ALA dehydrase, a -SH containing enzyme dependent on dietary zinc (Finelli et al. 1975). This step is the most sensitive to lead (Haeger-Aronsen 1960), and the accumulating δ-ALA is excreted in the urine. The test for urinary δ-ALA is the most sensitive indication of undue lead exposure, with correlation of 91% between urinary δ-ALA and clinical diagnosis (Davis et al. 1968). The dehydrase has been inhibited in rabbit blood by a concentration of 2×10^{-7}M Pb (Bruin and de Jong-Heisterkamp 1968). Since other heavy metals with high -SH affinities do not exert the same magnitude of inhibition on δ-ALA dehydrase, perhaps it is some feature of the steric configuration of the molecule that makes it bind preferentially to lead (Finelli et al. 1975).

The conversion of coproporphyrinogen III to protoporphyrin, the fifth step in the pathway, is mediated by coproporphyrinogen oxidase. The amount of coproporphyrin that appears in the urine as a result of lead inhibition of the enzyme is of the order of 1/25th to 1/50th the amount of δ-ALA (Fromke et al. 1969). The urinary test for this metabolite is rapid and convenient, and indicates both early exposure and current stage of intoxication. For these reasons

it is routinely used in periodic checks of Russian industrial workers (Lane 1964).

The chelation of one Fe^{++} by the four atoms of nitrogen in the protoporphyrinogen molecule, the sixth step in the pathway, is catalyzed by heme synthetase (ferrochelatase), and results in the formation of heme. This is the third step that is inhibited by lead. Evidence of enzyme inhibition is provided by the presence of ferritin granules around mitochondria in the erythroblasts of lead-exposed animals (Bessis 1958, Bessis and Breton-Gorius 1959).

These lead-mediated defects in the heme biosynthetic pathway also result in inhibition of hepatic synthesis of the cytochromes, whose prosthetic group is provided by heme (Alvares et al. 1972). Cytochrome P-450 is a microsomal hemoprotein which plays a central role in the hepatic detoxification of drugs, hormones and foreign chemicals. When rats were dosed with lead chloride, 5 mg/kg intravenously and killed 24 hours later, the ability of liver microsomal preparations to mediate the N-demethylation of ethylmorphine and hydroxylation of aniline was depressed by 50%. Other rats receiving the same dose of lead chloride also received 100 mg/kg hexobarbital intraperitoneally and the sleeping time measured by righting reflex loss was found to be lengthened approximately 50% over controls (Alvares et al. 1972).

Effects of lead on collagen synthesis

Information on the effects of lead on collagen synthesis is sparse, probably because of the complexity of the interrelationships

between lead, calcium, vitamin D, phosphorus, and collagen metabolism. Weanling rats maintained on low calcium diet develop pica as evidenced by a voluntary increase in ingestion of lead-acetate-containing water. It was postulated that the lead provides sufficient relief from the physiological effects of calcium deficiency to compensate for the aversive taste of the water (Snowdon and Sanderson 1973). If calcium and phosphorus intakes are low, lead toxicity is potentiated by increased retention in tissues (Sobel et al. 1940, Shields and Mitchell 1941), and conversely, if Ca and P intakes are high, Pb absorption is decreased (Kostial et al. 1971). Lead may interfere with the renal hydroxylation of 25-hydroxycholecalciferol by inhibiting the oxidase system, directly diminishing the availability of 1,25-dihydroxycholecalciferol, the active vitamin D metabolite (Mahaffey 1974).

The only studies on a direct relationship between lead poisoning and collagen biosynthesis were conducted on young rabbits. Animals fed a basal diet supplemented with lead sub-acetate displayed retardation in formation of osteoid collagenous matrix and increased osteoclastic bone resorption (Hass et al. 1964). Animals in which hypervitaminosis D was induced resorbed bone and deposited Ca at extraosseous sites, especially in arterial media. Later on, the abnormal calcified deposits were resorbed and there was a florid deposition of intercellular matrix in both bone and the thickening intima of many arteries with previously calcified media. The bone matrix proliferated into the marrow space, acquired Ca, changed into osteoid matrix, and developed into mature bone with dense

osteosclerosis. Animals with hypervitaminosis D which ingested lead subacetate never developed the late increases in bone and arterial intimal intercellular matrix until the lead was withdrawn. Apparently vitamin D favors the development of rich intercellular matrices composed primarily of collagen and its precursors, and this development is inhibited by lead (Hass et al. 1967).

Collagen

Collagen is the most widely distributed protein in the animal kingdom, since it has been identified in the tissues of animals from the primates all the way down to the sponges (Eastoe 1967). As the characteristic extracellular component of the mesodermal tissues, collagen occupies a key position in the molecular architecture of the higher animals which enables it to control the distribution of both externally and internally applied forces within the organism (Eastoe 1967). Since 25 to 30% of the total body protein is collagen, it is probably the most abundant protein in the animal body (Gould 1968).

The α-chains in collagen belong to two general types, α_1 and α_2, each of molecular weight 100,000 (Piez 1964) and containing approximately 1,200 amino acid residues (Hannig and Nordwig 1967). The total sequence of the α_1 chain is known (Fietzek and Kühn 1975). The α_2 chain contains at least two methionine residues more than α_1 (Clark et al. 1975). Apparently there is some resemblance between α_1 and α_2 with respect to amino acid composition and chain conformation (Traub and Piez 1971). A high degree of homology has been found between the α chains of many species. The chick and calf possess 91% identity between the

helical regions of the α chains (Dixit et al. 1975) and the rat and calf show 89% homology (Butler et al. 1974). The homology between collagens of so many species appears to indicate that the amino acid sequence developed eons ago has served so well that very few residue replacements have survived the rigorous selection pressures operating in the evolutionary process.

Tissue distribution of the genetic collagens

Type I collagen, $[\alpha_1(I)]_2\alpha_2$, which is low in hydroxylysine and carbohydrate, is found in skin, bone and tendon. Type II, $[\alpha_1(II)]_3$, with significant hydroxylysine and carbohydrate content, is specific to hyaline cartilage. Type III, $[\alpha_1(III)]_3$, containing cysteine and high amounts of hydroxyproline, glycine and histidine, is found in embryonic tissues, particularly skin, blood vessels, and intestine, and in inflamed adult synovia (Weiss et al. 1975). The high number of disulfide bonds reduces its solubility (Shuttleworth and Forrest 1975) and increases its resistance to cathepsin, thus providing stability in inflammatory conditions (Orkin et al. 1975). Basement membranes contain mostly glycoprotein with an amino acid profile like collagen (Lazarow and Speidel 1964). The collagen was found to be of type IV, with low alanine, high hydroxylysine, and a high molecular weight due to excess hexose. It has been studied most in glomeruli, anterior lens capsules and Descemet's membrane (Kefalides 1971).

Quaternary structure

The requirements that every third residue be glycine and that the imino acids fit naturally into the structure led to the postulation of a triple-helical conformation (Ramachandran and Kartha 1954). This theory was supported by the observed infrared dichroism, indicating that the N-H and C=O bonds are nearly perpendicular to the helix axis. All residues are in the trans configuration, as revealed by infrared spectrum (Badger and Pullin 1954).

Each of the three polypeptide chains that compose the collagen molecule is a tight left-handed helix, with a translation of 0.29 nm and a rotation of approximately 108° (Salem and Traub 1975). The triple helix has a slight twist to the right about a common axis. Every third position along each chain lies in the central groove of the triple helix. Utilization of glycine, the smallest amino acid, in these positions makes possible the tightness of the helical turns in the triple-stranded molecule. The helical pitch of 8.6 nm is produced by the low flexibility of the imino acid residues. The residues in the X and Y positions of the tripeptides gly-X-Y of the neighboring α-chains are dictated by intramolecular interactions. One-third or more of the tripeptide units may achieve additional stabilization in the triple helix conformation via H bonds (Salem and Traub 1975).

Until recent years the special advantage conferred by the presence of hydroxyproline in the collagen structure was something of a mystery. However, it was recently found that when hydroxyproline

was substituted for proline in synthetic tripeptides, the melting temperature was increased by as much as 35°C for $(\text{pro-OHpro-gly})_{10}$ (Sakakibara et al. 1973). When protocollagen (underhydroxylated collagen, Lazarides et al. 1971) is properly hydroxylated, the thermal stability of the protein is increased (Berg and Prockop 1973a). Thus thermal stability varies directly as hydroxyproline content (Rosenbloom et al. 1973, Jimenez et al. 1973). It appears that triple helix formation of procollagen at body temperature requires that at least 35% of the proline residues be hydroxylated (Bornstein 1974), and any underhydroxylated chains are "melted out" (Fessler et al. 1975).

Biosynthesis of collagen

Cells which synthesize collagen   Collagen is elaborated primarily by the fibroblast and its variants, the chondroblast, osteoblast, and odontoblast, all derived from the mesenchymal cell (Ross 1968). However, many other cell types, e.g., neuroblastoma, pituitary tumor, HeLa, melanoma, epithelium, and smooth muscle, all synthesize collagen in vitro (Green and Goldberg 1965, Langness and Udenfriend 1974). Apparently most cells possess the DNA polycistron for collagen synthesis, but this DNA region is normally repressed. The increased collagen synthesis in wound healing and fibrotic processes may not be due solely to fibroblastic activity, but also to derepression caused by trauma in the cells of the tissue involved (Langness and Udenfriend 1974).

<u>Stages of synthesis</u> The following stages in the synthesis of the molecule have been proposed (Bornstein 1974):

1) Peptidyl hydroxylation of proline and lysine on nascent (ribosome-bound chains)
2) Chain alignment
3) Helix formation, stabilized by hydroxyproline
4) Disulfide bond formation between chains
5) Secretion of the procollagen helix into the extracellular space
6) Limited proteolysis by procollagen peptidases
7) Fiber formation and cross-linking

The evidence is that the first four steps occur while the pro-α chains are on the ribosomes.

The above scheme has been substantiated by electron microscopic radioautography studies on incorporation of ^{3}H-proline into the collagen of rat incisor alveolar bone and chick embryo calvaria (Weinstock 1975, Weinstock et al. 1975). Two minutes after the pulse, the label appeared in the rER of the odontoblasts and osteoblasts. At 10 minutes, the label had spread to the "spherical" portions of the Golgi saccules. At 20 minutes, the label was in the "rectangular" portions and secretory granules of the Golgi apparatus. At 30 minutes, the label had reached the secretory granules in the apical cortical cytoplasm. At 90 minutes, the label was in the pre-bone.

Each pro-α chain enters the lumen of the rER while more chain is being synthesized. Since an average of 209 residues are translated per minute, a chain of 1250 amino acids would require six minutes

to produce (Vuust and Piez 1972). Regulation of the synthetic process is accomplished via control of the hydroxylation process (Peterkofsky 1972) or amount of proline available (Rojkind and Diaz De León 1970).

<u>Peptidyl hydroxylation</u> The conversion of peptidyl proline and lysine to their hydroxylated derivatives occurs before the release of the pro-α chains from the ribosomes (Miller and Udenfriend 1970, Lazarides et al. 1971). The hydroxylation is mediated by specific enzymes, proline hydroxylase and lysine hydroxylase respectively, located on the rER (Peterkofsky and Udenfriend 1961, Diegelmann et al. 1973, Olsen et al. 1973) and in the cytoplasm (Rosenbloom et al. 1967). Proline hydroxylase is composed of two unequal subunits (Berg and Prockop 1973a). The cofactors necessary for enzyme activity are ascorbate, Fe^{++}, α-ketoglutarate, and O_2 (Chvapil and Hurych 1968, Barnes and Kodicek 1972, Grant and Prockop 1972, McGee and Udenfriend 1972, Cardinale and Udenfriend 1974). The hydroxylation of each residue is coupled to the stoichiometric oxidative decarboxylation of α-keto-glutarate; one O atom enters the hydroxyl group on the residue and the other enters succinate (Rhoads and Udenfriend 1968). These hydroxylases belong to an unusual group called mixed-function oxygenases, requiring both O_2 and a reducing agent (Mason 1965).

The requirement for Fe^{++} was discovered in the course of studies with chelating agents whose presence inhibited the hydroxylase (Hurych and Chvapil 1965). The addition of heavy metals to the system revealed that only Fe^{++} would reverse the inhibition (Prockop

and Juva 1965, Chvapil et al. 1967, Hutton et al. 1967, Kivirikko and Prockop 1967, Chvapil and Hurych 1968). In the presence of 3.0 mM α,α'-dipyridyl in vivo, proline hydroxylase activity could not be increased by additional ascorbate (Kuttan et al. 1975). The Fe^{++} is believed to couple to the hydroxylase, thus activating it (Barnes 1969). The exact role of ascorbate, tetrahydropteridine, tetrahydrofolate, or dithiothreitol is not clear, but all stimulate proline hydroxylation (Peterkofsky and Udenfriend 1965, Hutton et al. 1967, Priest and Bublitz 1967, Rhoads and Udenfriend 1970, Stassen et al. 1973), with maximum activity at 75 to 100 μg ascorbate/ml medium (Switzer and Sumner 1971) and increased rate of collagen fiber production by fibroblasts (Jeney and Törö 1936). These agents may act by maintaining the Fe in the reduced state, since α,α'-dipyridyl, a Fe^{++}-chelator, inhibits hydroxylation even with saturating ascorbate (Blanck and Peterkofsky 1975). Another possibility is that the ascorbate maintains the free SH group on the enzyme that is essential for activity.

When Fe^{++} or ascorbate is absent or in short supply, an underhydroxylated procollagen, protocollagen, is formed (Lazarides et al. 1971, Peterkofsky 1972). Even when all cofactors are abundant, the extent of hydroxylation and glycosylation of lysine residues varies depending on the type of procollagen (Dehm and Prockop 1973).

The minimum substrate requirement for proline hydroxylase is X-pro-gly, with K_m and V_{max} affected by the nature of the adjacent amino acids (McGee et al. 1971). In mammalian collagens, the hydroxylation that yields 4-hydroxyproline is limited to prolyl residues in position Y

in gly-X-Y (Kang et al. 1967, Fietzek et al. 1973). The substrate effectiveness of $(pro-pro-gly)_n$ increases as n increases from 5 to 20 (Hutton et al. 1968). In general, only about 35% of the proline residues in the random coil are hydroxylated (Rosenbloom et al. 1973). As the hydroxylation exceeds 30%, the melting point of the procollagen is raised above body temperature and the formation of the triple helix is initiated. Once the helix is formed, it becomes resistant to the action of the hydroxylases (Berg and Prockop 1973c) because the residues are no longer accessible to the hydroxylases.

Chain alignment The existence of a pro-collagen molecule was not proposed until very recently (Speakman 1971). The pro-α chains are longer at the NH_2-terminal than the α-chains by some 300 residues, the equivalent of one-third the mass of the α-chain (Fessler et al. 1975). This additional peptide lacks the high glycine and imino acid content of the α-chain and possesses instead more aspartate, glutamate, serine, tyrosine and histidine (Bornstein et al. 1972, von der Mark and Bornstein 1973). This difference in amino acid composition makes the peptide more susceptible to the action of pepsin and chymotrypsin. There is a much higher interspecies variation in these registration peptides than in the helical portions of the molecule (Bornstein 1974). The organism must have a compelling reason to devote as much as 10% of total body protein synthetic effort to the elaboration of NH_2-terminal peptides (Fessler et al. 1975).

The Speakman model proposes that the NH_2-terminal extension serves a "registration" function, i.e., that this portion of the pro-α chain

has the ability to select and line up with complementary chains during the formation of the procollagen molecule. Subsequent work on chick embryo bone procollagen has borne this out. After assembly on the ribosomes (Prichard et al. 1974), the pro-α chains are transported into the lumen of the rER (Olsen and Prockop 1974). The additional peptides initiate mutual selection of 2 α_1 and 1α_2 chains (Bellamy and Bornstein 1971). The formation of S-S bonds assures best fit and aids in vitro folding to the native structure (Fessler et al. 1975). The properly aligned chains are concurrently undergoing hydroxylation and random coil formation. As their melting temperature approaches 37°C, triple helix formation is initiated and the procollagen molecule is stabilized. Any mismatched structure is "melted out" because of thermal instability at body temperature (Fessler and Fessler 1974, Fessler et al. 1975). The presence of the "registration peptides" appears to inhibit aggregation of triple helices into fibrils within the cell, assuring secretion into the extracellular space where aggregation occurs (Bellamy and Bornstein 1971, Lapière and Nusgens 1974). Carboxy terminal extensions of pro-α chains linked by S-S bonds have been visualized by EM of segment long spacing (SLS) crystallites and molecular sieve chromatographic methods (Tanzer et al. 1974, 1975, Byers et al. 1975, Davidson et al. 1975, Murphy et al. 1975). These extensions may aid in preventing slippage of the three strands of the helix.

Triple helix formation The formation of the helix is dependent upon the degree of hydroxylation of the individual pro-α chains (Olsen and Prockop 1974, Uitto and Prockop 1974). As the

transition temperature of each chain rises, folding, followed by triple helix formation, occurs. Since interchain bonds form simultaneously, they probably serve to "lock" the helix (Schofield et al. 1974) prior to secretion. Underhydroxylation results in failure of triple helix formation because of thermal instability, and secretion is impaired because only triple helices are secreted at the optimal rate.

Disulfide bond formation   Procollagen chains are linked by S-S bonds in the "registration peptide" region during the mutual selection process. Disulfide bond formation occurs between 70 to 80% of the pro-α chains in chick embryo leg tendon by 9 to 12 minutes. Since triple helix and S-S bond formation occur almost simultaneously, the latter process may be essential for rapid helix formation in vivo (Schofield et al. 1974). However, there are indications that S-S bond formation is not necessary to effect procollagen secretion (Kerwar 1974).

Secretion of procollagen   The procollagen molecules are secreted into the extracellular space (Bellamy and Bornstein 1971, Dehm and Prockop 1971, Layman et al. 1971). The Golgi complex provides part of the transcellular secretory pathway (Olsen and Prockop 1974, Weinstock and Leblond 1974). The impairment of the secretory process by colchicine and vinblastine, and the requirement of ATP for secretion, imply the involvement of microtubules in the transport of procollagen (Dehm and Prockop 1972, Diegelmann and Peterkofsky 1972, Ehrlich et al. 1974, Bornstein et al. 1975). The final stage in secretion consists of

fusion of the Golgi vesicles carrying the procollagen with the plasma membrane in the exocytotic process.

Limited proteolysis Pulse-chase experiments with newborn rat calvaria yielded evidence of pepsin-mediated cleavage of pro-α chains (Bellamy and Bornstein 1971). Much evidence exists that the extracellular enzyme procollagen peptidase performs the cleavage in vivo (Tanzer et al. 1974, Byers et al. 1975, Murphy et al. 1975). Several intermediates in the scission process have been isolated (Fessler et al. 1975), demonstrating the presence of at least two cleavage sites in each pro-α chain (Davidson et al. 1975). It is suggested that scission of the NH_2-terminal peptides occurs in one step (Kohn et al. 1974). Subsequently there is a stepwise cleavage of the COOH-terminal peptides in which one chain at a time is snipped, by an enzyme that is probably distinct from procollagen peptidase (Davidson et al. 1975). The final form of the tropocollagen molecule is the result (Goldberg et al. 1972, Goldberg and Sherr 1973, Clark and Veis 1972, Veis et al. 1972, 1973).

Fiber formation and cross linking Cross-links are of two general types: intramolecular, binding chains of the same molecule, and intermolecular, between adjacent molecules.

The intramolecular cross link is formed when two lysine aldehydes (allysines, α-aminoadipic semialdehydes), derived from oxidative deamination of two NH_2 groups, condense to form a dehydrated aldol. This cross-link may serve as precursor to a more complex intermolecular crosslink; other than that, its significance is obscure (Bailey and Robins 1973).

One class of intermolecular cross-link may be formed by the condensation of allysine and the NH_2 group of hydroxylysine to form the aldimine dehydro-hydroxylysinonorleucine, prominent in skin collagen (Bailey and Peach 1968). Another cross-link, lysinonorleucine, is formed from the condensation of allysine and lysine. A third, dehydro-dihydroxylysinonorleucine (syndesine), is the product of the condensation of hydroxyallysine and hydroxylysine. The last two are minor components of skin collagen, but syndesine is a major constituent of tendon, cartilage and bone by virtue of its high stability (Robins and Bailey 1973).

The exact location of these cross-links on the fibers has not been unequivocally determined. If one accepts the quarter-stagger hypothesis of molecular packing and that allysine and hydroxyallysine occur only in NH_2- and COOH-terminal peptides (Rauterberg et al. 1972a, 1972b), one can conclude that cross-links occur between telopeptides of one molecule and helical regions of adjacent molecules (Zimmermann et al. 1973). Some evidence exists that allysine residues may occur in the helical portion of the molecule (Deshmukh and Nimni 1971), but cross-linkage there appears redundant since bonds involving both ends of each molecule should suffice to prevent slippage (Bailey and Robins 1973).

Molecular packing The organization of the collagen molecules into fibers was first deduced from results obtained with electron microscopy. Alternating dark and light bands indicating the native periodicity of the fibrils were seen in both native and acid-recon-

stituted fibers. Dark bands are regions where charged groups bind the heavy metal stain and light bands are regions containing primarily uncharged residues (Bruns and Gross 1974). The axial period D was established variously as 64 nm (Bear 1942) or approximately 70 nm (Schmitt et al. 1942); the variation is due to degree of fibril stretch and hydration. The currently accepted value for D seems to be 66.8 nm (Hodge and Schmitt 1960). The axial period has been accounted for by a "quarter-stagger" model (Hodge and Schmitt 1960), later modified (Hodge and Petruska 1963, Bruns and Gross 1974). The parallel adjacent tropocollagen molecules are rotated 72° and their ends displaced by the stagger distance D, 66.8 nm, the length occupied by 234 residues, due to the maximization of the electrostatic and hydrophobic interactions between the molecules in two-dimensional array (Hulmes et al. 1973). The tropocollagen molecular length of around 300 nm is approximately 4.5 D.

Collagen fibers are composed of fibrils 5 to 200 nm in diameter (Hodge 1967) forming in the extracellular matrix immediately surrounding the fibroblast (Trelstad 1975). Fibers grow by a crystallization process consisting of nucleation and growth (Wood and Keech 1960, Piez 1975).

Effect of nutritional factors and hormones on collagen synthesis

Vitamins The role of ascorbate in the synthesis of collagen was discussed previously. In addition, since it regulates the function of lysine hydroxylase, it can influence the cross-linking pattern (Robert and Robert 1969). Vitamin A is believed to cause fusion of

Golgi vesicles (primary lysosomes) with the plasma membrane (Dingle 1968). Hence vitamin A may play a role in the regulation of the equilibrium between connective tissue synthesis and degradation (Dingle 1969). Vitamin D apparently causes the ratio of dehydro-dihydroxylysinonorleucine to dehydro-lysinonorleucine to decrease with age, during the normal matrix maturation necessary for proper mineralization (Mechanic 1975).

Food intake In kwashiorkor, protein calorie malnutrition, the excretion of hydroxyproline in the urine is decreased, reflecting depression of collagen turnover resulting from a 25% loss in skin collagen. Aldehyde content of fibers is raised because of diminished cross-link formation. Similar results are seen with low calorie intake in general. High protein intake also reduces incorporation of glycine and proline into skin proteins and depresses muscle collagen biosynthesis as much as 20% (McClain et al. 1975). Certain amino acids, e.g., alanine, serine, and cysteine, may inhibit uptake of ^{14}C-proline by chelating metal ions (Blumenkrantz and Asboe-Hansen 1973).

Hormones Testosterone and the estrogens increase the proportion of soluble collagen in skin (Deyl et al. 1971). Somatotrophin promotes collagen synthesis and formation of labile intermolecular cross-links (Prockop and Kivirikko 1968, Bailey and Robins 1973, Deyl et al. 1971) and rate of degradation as indicated by urinary excretion (Prockop and Kivirikko 1968). Cortisone depresses collagen synthesis (Kivirikko 1963, Ebert and Prockop 1967) as reflected in

reduction of hydroxyproline excretion in young rats (Kivirikko and Laitinen 1965, Smith and Allison 1965).

Metabolism, turnover, and excretion of collagen

The degradation of collagen in the intercellular matrix appears to be mediated by collagenase, which cleaves the molecule at a point three-quarters of its length from the NH_2-terminal end (Harris 1974). This cleavage site is highly specific and is characterized by adjacent bulky side chains and few stable triplets (Gross 1974). Both mesenchymal and epithelial cells are capable of collagenolytic activity (Pérez-Tamayo 1970).

The degradation of collagen results in excretion of hydroxyproline in the urine (Ziff et al. 1956). From injection of ^{14}C-proline into young rats, at least three distinct hydroxyproline pools were found, with half-lives of 1, 5, and 50 to 200 days respectively (Lindstedt and Prockop 1961). Apparently the degradation of the soluble fraction furnishes a large proportion of the urinary hydroxyproline-containing peptides in young rats (Prockop 1964, Kivirikko et al. 1965, Avioli and Prockop 1967), whereas in older rats the major portion of excreted hydroxyproline is derived from insoluble collagen. Apparently only 5 to 10% of the hydroxyproline released by the degradation of insoluble collagen under normal conditions is recoverable as urinary hydroxyproline (Prockop 1964).

In urine 97% of the hydroxyproline exists as peptides (pro-OHpro and gly-pro-OHpro)(Schrohenloher et al. 1958), and only 3% as the free imino acid (Ziff et al. 1956). Actually only around 25% of the

peptides derived from collagen degradation end up in the urine. The rest are broken down to their constituent amino acids and excreted as urea and CO_2 (Weiss and Klein 1969). Pro-OHpro is excreted almost quantitatively because of inefficient peptidase activity, and any alterations in collagen breakdown are reflected in the urinary excretion of pro-OHpro-linked peptides (Weiss and Klein 1969).

Research Objectives

The primary objective of the present study was to determine the effects of lead on collagen metabolism in the rat. Inhibition of collagen biosynthesis or enhancement of collagen degradation in vivo would provide indirect evidence for the biochemical basis for a breakdown in the blood-brain barrier in lead encephalopathy since this barrier is composed, in part, of collagen. Both short-term and total synthesis of collagen was assessed by measuring the incorporation of ^{14}C-proline and unlabelled proline into the soluble and insoluble collagen in the skin of young rats exposed to lead since birth. Colorimetric and radioactivity assays of hydroxyproline and proline from both fractions were performed. In addition, the incorporation of ^{14}C-proline into the total soluble skin protein was measured to determine the effect of lead on noncollagenous protein synthesis. Since lead may inhibit collagen synthesis by reducing the extent of proline hydroxylation, resulting in formation of an underhydroxylated collagen that is more rapidly degraded, the 24-hour urinary excretion of hydroxyproline and proline was measured.

The secondary objective was to verify that lead impairs renal function in mature rats treated with lead since birth. Both glomerular filtration

rate and effective renal plasma flow were determined by measuring the plasma clearance of inulin and para-aminohippuric acid, respectively. In addition, light microscopic studies of the kidneys of control and lead-exposed rats were conducted to determine the site and extent of damage.

Finally, the iron-requiring enzymes in the heme biosynthetic pathway are inhibited by lead, and the one most sensitive to the action of lead is δ-ALA dehydrase. Since the accumulation of δ-ALA is routinely measured as an index of lead intoxication, the 24-hour urinary excretion rate of δ-ALA was determined in mature rats chronically exposed to lead.

MATERIALS AND METHODS

Experimental Animals

Timed-pregnant Wistar-Lewis albino rats (Charles River Laboratories, Wilmington, Massachusetts) were housed in standard cages in an air-conditioned colony room with alternating 12-hour light-dark cycle. Within 24 hours after parturition, drinking water containing 1.0, 1.5, or 2.0% lead acetate was substituted for tap water. Control dams received glass-distilled water. The young were weaned between three and four weeks of age and placed on the same dose level of lead as their mothers. The colony was maintained on a standard commercial diet (Teklad Mouse and Rat Diet, ARS Sprague-Dawley, Madison, Wisconsin) ad lib.

Procedures

Blood lead

Rats in each treatment group were anesthetized with pentobarbital, 25 mg/kg intraperitoneally at two, three, and four weeks of age. Blood was collected by cardiac or posterior vena-caval puncture from enough littermates to yield a total volume of 0.3 to 1 ml. Lead was assayed by the macromethod of Berman (1969). Proteins were precipitated with 10% trichloracetic acid (TCA) and the precipitate was washed with 5% TCA. The TCA-soluble fraction was obtained by pooling the supernatant and the 5% TCA washings. The pH was adjusted to 7 with 2.5N NaOH, using 0.1% bromthymol blue as indicator. The lead was chelated with 1 ml of 1% sodium diethyl dithiocarbamate, and the

chelation complex extracted with 2.5 ml methylisobutyl ketone (MIBK). The MIBK phase was aspirated into the flame of a Varian Techtron model AA-5 atomic absorption spectrophotometer. Lead concentrations of samples were determined by comparing the absorbance peaks at 217 nm with a standard curve obtained from tubes containing 0, 0.2, 0.5, 1.0, 2.0, 4.0, and 8.0 ppm lead. All glassware was soaked overnight in nitric acid and repeatedly rinsed with glass-distilled water, and all water used in making up solutions or dilutions was glass-distilled water.

Skin collagen

In order to determine the effects of lead on collagen biosynthesis in the neonate, the conversion rate of proline to hydroxyproline and the total proline and hydroxyproline content of soluble and insoluble collagen in skin were measured. Control rats and rats exposed to 1, 1.5, or 2% lead acetate were autopsied at two, three, four, and six weeks of age. Between 10 and 21 rats were examined at each age in each treatment group.

Each rat received 5 μCi (U)^{14}C-proline (Amersham-Searle, Arlington Heights, Illinois) in 0.1 ml saline subcutaneously 12 hours prior to autopsy. The rats were anesthetized with pentobarbital. The dorso-lumbar area was shaved, depilated, washed with detergent (Lux) and warm water, blotted dry, and defatted with ether. An area of skin approximately 3 cm^2 was excised, the fasciae rapidly dissected off, and the skin was divided into duplicate or triplicate samples (20 to 125 mg each), placed on glassine paper and frozen at -20°C.

After one to three hours, the samples were weighed, minced, and transferred to a glass homogenizing tube. To the tube half-immersed in crushed ice was added 1.5 ml of ice-cold 0.45M NaCl, and the sample was homogenized with a motor-driven glass pestle (Tri-R Instruments, Rockville Centre, New York). Care was taken to prevent sample warming in order to minimize heat denaturation of the collagen. The homogenates were transferred quantitatively to polycarbonate ultracentrifuge tubes with two 1-ml washings of cold 0.45M NaCl. The tubes were shaken in a cold room at 4°C for 24 hours to ensure maximal dissolution of the soluble fraction. The tubes were centrifuged at 60,000 x g for one hour at 4°C. Each supernatant was decanted into a 20-ml screw-capped culture tube, and four volumes of ice-cold ethanol was added to precipitate the soluble collagen. The tubes were stored at 4°C for 24 hours with occasional mixing to ensure maximal precipitation. The pellets containing insoluble collagen were transferred to 10-ml screw-capped culture tubes with 4 ml ice-cold 0.45M NaCl. All tubes were centrifuged at 2000 rpm for five minutes and the supernatants discarded. Four ml water was added and the pellets gelatinized at 124°C for four hours. An equal volume of 12N HCl was added and the resulting 6N HCl solutions autoclaved at 158°C 12 hours to hydrolyze the peptide chains. The acid hydrolyzates were stored at 4°C until subjected to analysis.

In the initial phase of the work, hydroxyproline and proline were separated by the solvent extraction method of Kivirikko et al. (1965). Because of the improved imino acid separation afforded by ion-exchange chromatography, the method of Klein (1970) was adopted.

Dowex-50 cation exchange resin, 200-400 mesh, 8% cross-linked (Sigma Chemical Co., St. Louis, Missouri) was washed alternately with water and 0.25N NaOH and packed as an aqueous slurry into glass columns. The columns were washed with 0.25N NaOH, clamped, and covered with 5-ml plastic disposable beakers to keep out dust. Before each use, the columns were prepared by passing approximately one column volume of water and two column volumes of 0.1M citrate buffer, pH 2.80±0.05 through them. Eluant pH was checked with pHydrion paper (Micro Essential Laboratory, New York) and the columns adjudged ready for use when pH was 2.8.

The initial calibration of each of four randomly-chosen columns was performed using a standard containing 0.1 ml (U)^{14}C-hydroxyproline, ca. 35,000 dpm, 0.1 ml(U)^{14}C-proline, ca. 50,000 dpm, and 0.1 ml each of 1 mg/ml stock solutions of unlabelled hydroxyproline and proline. Each standard was made up to 1 ml with 0.1M citrate buffer (pH 2.8) and layered on the column with a Pasteur pipette. The tube and pipette were rinsed twice with 1 ml of buffer and each ml layered after the preceding one had sunk into the resin; total volume layered was 3 ml. The eluates were collected in 10-ml aliquots with a fraction collector and assayed for proline and hydroxyproline as described below. Hydroxyproline was eluted in the fractions collected between 45 and 100 ml and proline was eluted in the 120 to 190 ml fractions. The fraction peaks were essentially identical for the four columns. The elution pattern of the imino acids is illustrated in Figure 1.

For the separation of proline and hydroxyproline in skin samples, the hydrolyzates were transferred to porcelain evaporating dishes and the HCl evaporated for 30 to 40 minutes over a boiling water bath in a hood. Care was taken to prevent overheating of the dry residues. Approximately 1 ml water was added immediately after dryness and evaporated to ensure the removal of all traces of acid, which would alter the binding of the imino acids to the resin. The residues were taken up in 1 ml 0.1M citrate buffer (pH 2.8) and filtered directly onto the resin surface. The evaporating dish and filter paper were rinsed twice with 1 ml buffer onto the resin to bring the volume layered to 3 ml. A standard similar to the calibrating standard previously described was run with each set of samples to provide recovery estimates. The samples and standard were eluted with 0.1M citrate buffer (pH 2.8). Fractions were collected as follows: 1) 0 to 45 ml - discarded; 2) 45 to 100 ml - analyzed for hydroxyproline; 3) 100 to 120 ml - discarded; 4) 120 to 190 ml - analyzed for proline. Because of low radioactivity, the hydroxyproline fraction was concentrated by gentle evaporation to a volume of 11 to 12 ml to increase isotope concentration.

The unlabelled hydroxyproline was assayed by the Klein (1970) method. To 2 ml of sample or standard in a screw-capped culture tube was added 1 ml of a freshly-mixed 1:1 solution of 0.015M $CuSO_4$-15% NaOH. After mixing, 0.5 ml of 8.4% H_2O_2 was added, and the tubes thoroughly mixed for five minutes to ensure complete oxidation of hydroxyproline. They were placed in a water bath at 70±0.5°C for

ten minutes with constant agitation to destroy residual H_2O_2. Ehrlich's reagent (35 g p-dimethylaminobenzaldehyde, 855 ml isopropanol and 145 ml 24N H_2SO_4), 2.5 ml, was added and the tubes returned to the bath for 25 minutes with constant agitation to form the chromophore. The tubes were cooled and absorbance at 540 nm read within one hour on a Beckman DB-GT spectrophotometer. Standards containing 2, 4, 8, and 12 μg were assayed concurrently.

The unlabelled proline was assayed by the method of Troll and Lindsley (1955). Approximately 10 ml eluate was shaken with 1 g Zeolite to remove interfering basic amino acids. Tubes were centrifuged at 2000 rpm for 10 minutes, and 4 ml of each supernatant pipetted into a screw-capped culture tube. To each tube was added 4 ml glacial acetic acid and 4 ml ninhydrin reagent (5 g ninhydrin, 120 ml glacial acetic acid, 80 ml 6M phosphoric acid). The tubes were heated in a boiling water bath one hour, cooled, and shaken vigorously with 4 ml benzene five minutes to extract the chromophore. After centrifugation, the benzene phase was aspirated into a cuvette and the absorbance read at 515 nm. Sample concentrations were determined from a standard curve obtained by concurrent assay of tubes containing 1, 2, 3, and 5 μg/ml.

The radioactive isotope in the fractions collected was measured by the method of Peterkofsky and Prockop (1962). To 15 ml scintillation fluid (6 g PPO, 75 mg POPOP, 500 ml Triton X-100, 1000 ml toluene) was added 2 ml of hydroxyproline fraction concentrate or proline fraction, and the radioactivity was counted in a Packard

Tri-Carb scintillation counter with an external standard. All counts were corrected for background, quenching, counting efficiency and column recovery.

Skin total protein

The effects of lead on total protein synthesis in skin were determined by measuring the incorporation of ^{14}C-proline into the proteins of the 0.45M NaCl soluble fraction of skin. Rats were injected with ^{14}C-proline and skin samples were taken 12 hours later as described in the skin collagen procedure. The skin homogenates were extracted with 0.45M NaCl, the extracts dialyzed for 48 hours against three volumes of 0.45M NaCl at 4°C, and aliquots were counted for radioactivity to determine the incorporation of ^{14}C-proline into total skin protein.

Aliquots were also taken for the determination of unlabeled protein by the method of Lowry et al. (1951). A 0.1 ml volume of homogenate was dissolved in 0.9 ml 1N NaOH and allowed to stand at 4°C for 48 hours. Then 0.1 ml of the resulting suspension was diluted with 0.9 ml water. Meanwhile reagent C was made up, using 50 ml reagent A (2% Na_2CO_3 in 0.1 N NaOH) and 1 ml reagent B (freshly mixed 1:1 solution of 1% $CuSO_4 \cdot 5H_2O$ and 2% sodium tartrate). A 2.5-ml volume of reagent C was added to each tube and the tubes were allowed to stand 10 minutes. Then 0.25 ml cold reagent D (1N Folin) was added, the tubes were allowed to stand 45 minutes, and read at 660 nm. A stock solution containing 8% total protein (5% human albumin and 3% human γ-globulin) was used to make up standards containing 80, 40, 20 and 8 μg, which were run with each sample batch.

The results, in dpm/mg protein, were normalized for body weight

because all rats received 5 uCi ^{14}C-proline. Since lead-treated rats weighed less than controls, the treated rats possessed a relatively smaller tissue pool for isotope distribution. Therefore, the dpm/mg protein of each treated rat was multiplied by the ratio of its body weight to mean control body weight.

Urinary excretion of hydroxyproline, proline, and delta-amino levulinic acid (δ-ALA)

The effects of chronic lead exposure on collagen biosynthesis and breakdown were assessed in older rats by measuring the 24-hour urinary excretion rates of hydroxyproline and proline. In addition, δ-ALA urinary excretion rates were determined to provide an index of inhibition of heme biosynthesis. Twenty-eight rats were examined between the ages of 4 and 13 months; 10 controls, 4 at 1, 11 at 1.5 and 3 at 2% lead acetate in the drinking water.

Rats were placed in cylindrical plexiglass holders, and their hind feet taped to a crossbar (Cotlove 1961). Urine samples were collected over a 24-hour period in 50-ml glass cylinders containing 1 ml 6N HCl as preservative. The urines were diluted to 50 ml with water, and 4-ml aliquots were hydrolyzed in 6N HCl for 4 hours at 138°C. The hydrolyzates were evaporated and subjected to ion exchange chromatography and assay of eluates for hydroxyproline and proline as previously described.

The δ-ALA in the urines was assayed by the Rijks (1974) modification of the Berkó and Durkó (1972) method. Nine ml diluting solution [0.25 g medicinal charcoal (Norit A, Fisher Scientific Co., Fairlawn, New Jersey), 100 ml 0.5M acetate buffer, pH 3.5] was

added to 1 ml urine to remove interfering substances and establish optimum pH for the reactions. After filtration through Whatman No. 1 paper, two 2-ml aliquots were pipetted into test tubes. Acetylacetone (2,4-pentanedione), 0.1 ml, was added to one tube; the other tube served as a blank. The tubes were heated 20 minutes in a boiling water bath to effect condensation of the δ-ALA with the acetylacetone to form pyrrole. After cooling, 2 ml Ehrlich's reagent (1.0 g p-dimethylaminobenzaldehyde, 35 ml glacial acetic acid, 8.0 ml 70% perchloric acid, and glacial acetic acid to 50 ml) was added to each tube to form the chromophore. At 15 minutes, absorbance was read at 553 nm. Concentrations in samples were estimated from a standard curve obtained from tubes containing 2.5, 5, 10, and 20 μg which were run concurrently.

Effects of lead on renal function

Among the sequelae of protracted lead exposure is a nephropathy in which proximal tubular function is severely impaired. For this reason it was of interest to measure both glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) in older rats which had been maintained since weaning on 1, 1.5, or 2% lead acetate in their drinking water. These rats and controls were examined between the ages of 4 and 13 months. The GFR was obtained by measuring the clearance of inulin from the plasma, and the clearance of paraaminohippuric acid (PAH) was used to estimate ERPF.

Rats were anesthetized with ether, a 19-gauge hypodermic needle was inserted into one of the lateral tail veins, and a 75-cm length

of polyethylene tubing (PE 10 Intramedic[R], Clay-Adams, Parsipanny, New Jersey) filled with heparinized saline, was threaded through the needle into the vein for a distance of at least 6 cm (Cotlove 1961). The tubing was sutured and taped in place, the rat restrained in a plexiglass holder, and allowed to recover from anesthesia. Two thousand units of heparin/kg body weight was administered intravenously to facilitate collection of blood. After a priming dose of 200 mg/kg inulin and 16 mg/kg PAH, they were infused at a rate of 12 mg/kg/min and 1.7 mg/kg/min respectively. The infusion solution contained 10% mannitol to insure adequate urine flow. The sustaining solution was infused at a rate of 0.05 ml/min with a constant infusion pump (Model 975, Harvard Apparatus, Mills, Massachusetts). Quantitative urine collection began immediately after the first spontaneous voiding approximately 45 minutes after the start of infusion. Urination in the rat results in virtually complete emptying of the bladder (Cotlove, 1961). Timed urine collections were made over approximately 30-minute periods. Blood samples, 0.2 to 0.5 ml, were collected near the mid-point of each urine collection period by incising a dorsal foot vein and collecting drops into a heparinized Wintrobe tube. Blood samples were centrifuged immediately after collection to prevent movement of PAH into erythrocytes, and promptly refrigerated. Urine samples were diluted to 50 ml with water, mixed, and refrigerated.

Plasma was aspirated into a clean dry 1-ml tuberculin syringe and weighed by difference to the nearest 0.1 mg. The plasma weight was multiplied by 1.0262, the specific gravity of rat plasma (Siegel

et al., 1947) to obtain the volume. Water, 0.5 ml/0.1 ml plasma, 0.3 ml $ZnSO_4$ reagent (100 g $ZnSO_4 \cdot 7H_2O$, 40 ml 6.25N H_2SO_4, and water to 1000 ml), and 0.3 ml 0.75N NaOH, were added to the plasma (Somogyi, 1930). After standing for 10 minutes with occasional mixing, the tubes were centrifuged at 2000 rpm for six minutes. The supernatants were diluted 1:10 with water and assayed for inulin and PAH as described below. Each urine sample was diluted either 1:50 or 1:100 and assayed along with the plasma filtrates.

Inulin was assayed by the method of Walser et al. (1955). To 1 ml of plasma filtrate or diluted urine was added 0.5 ml 4N NaOH. Each tube was covered with a marble and heated in a boiling water bath 10 minutes. The tubes were cooled and 3.1 ml diphenylamine reagent (14 g diphenylamine, 600 ml glacial acetic acid, 360 ml 12N HCl) was added, the marbles replaced, and the tubes returned to the bath 30 minutes for formation of the blue inulin-diphenylamine complex. After cooling, the absorbance was read at 620 nm. Standards containing 5, 10, and 20 μg were run with each sample batch.

The PAH was assayed by the method of Bratton and Marshall (1939). To 2 ml plasma filtrate or diluted urine was added 0.4 ml 1.2N HCl. After mixing, 0.2 ml freshly prepared 1 mg/ml $NaNO_2$ was added to form the diazo bond. After 5 minutes, 0.2 ml 5 mg/ml ammonium sulfamate was added to destroy excess NO_2^- (Marshall and Litchfield. 1938). After another 5 minutes, 0.2 ml 1 mg/ml N-(1-naphthyl)ethylenediamine hydrochloride was added for the coupling reaction with the diazotized PAH to produce the red chromophore. After 10 to 30 minutes, absor-

bance was read at 540 nm. Standards which were run concurrently contained 0.5, 1.0, 2.0, and 5.0 μg PAH.

Histopathology of the Kidney

In order to correlate renal functional and morphologic changes occurring during chronic lead exposure, kidneys were examined from rats exposed to 0, 1.0, 1.5, or 2.0% lead acetate in the drinking water from birth until autopsy between the ages of 6 and 18 months. Four rats in each treatment group were anesthetized with pentobarbital and the left kidney was removed. Kidneys were fixed in formalin, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin.

RESULTS

The Effects of Lead on Immature Rats

Growth and development

There was an exponential increase in the body weights of the control rats from two to six weeks. These weights averaged 26, 36, 55, and 126 g for the two-, three-, four-, and six-week old pups, respectively (Figure 2). The oral exposure of young rats to lead via the maternal milk, and to 1, 1.5 or 2% lead acetate in the drinking water at the end of the suckling phase, resulted in highly significant decreases (P<.0005) in body weight at two, three, four, and six weeks of age (Figure 2). The growth retardation was dose dependent. At the highest dose level, the body weight was approximately one-half that of controls throughout the period of study. Some retardation of development between one and three weeks was evidenced by a delay in the appearance of hair. Posterior paralysis was not observed, but urinary incontinence was indicated by the staining of the perineal area after three weeks.

Blood lead levels

Blood lead concentrations increased linearly from two to four weeks of age (Figure 3). The rate of increase was greatest at the 2% dose level, in which the blood lead values averaged 0.44, 5.34, and 9.70 ppm at two, three, and four weeks respectively. Much lower levels were obtained in the pups exposed to 1% lead acetate, although there was a linear increase with age (Figure 3).

Hydroxyproline content of soluble skin collagen

There was a linear increase in the hydroxyproline content of soluble skin collagen in the control rats. The values were 491, 1065, 1498, and 1757 μg/g skin at two, three, four, and six weeks, respectively (Figure 4). The two-week old sucklings whose mothers were drinking water containing 1.5 or 2% lead acetate, showed significant decreases of 40 to 43% (P<.01) (Figure 4). At three weeks, all lead-exposed animals had decreases of 41 to 50% (P<.05). By the fourth week, there was an increase of 17% (n.s.) of hydroxyproline in the soluble collagen in the weanlings on the low dose, while marked decreases, amounting to 23 and 51% respectively (P<.025) remained in the animals on the medium and high doses. At the end of the sixth week, the increase of 6% and decrease of 10 and 20% in the low, medium, and high dose groups, respectively, were not significant.

The trend appears to be an increasing inhibition of soluble collagen synthesis from two to three weeks in the low dose group, followed by a recovery at six weeks. The inhibition is most pronounced in the medium dose group in the third week, and in the high dose group in the fourth week. Both groups show a leveling off in the inhibition at six weeks. Some degree of dose dependence is apparent in the marked difference in the degree of inhibition between the low dose group on the one hand and the two higher groups on the other.

Hydroxyproline content of insoluble skin collagen

In the untreated rats, insoluble collagen hydroxyproline content increased exponentially from the second to the fourth week and leveled off at six weeks. The values were 1580, 2801, 8784 and 8292 μg/g skin at

two, three, four, and six weeks (Figure 5). Nonsignificant decreases amounting to less than 1% were observed in all lead-exposed sucklings at two weeks. At three weeks, decreases of 5 and 7% (n.s.) and 28% (P<.01) were measured in the 1, 1.5, and 2% groups, respectively. The decrease of 9% (n.s.) in insoluble collagen hydroxyproline that appeared in the low dose group at four weeks was in contrast to the maximal decreases of 41 and 47% (P<.005) in the medium and high dose groups, respectively. At six weeks, the weanlings had decreases of 8 and 20% (n.s.) in the 1 and 1.5% groups, respectively, and an increase of 8% (n.s.) in the 2% group.

Lead appears to exert no significant inhibition on the synthesis of insoluble collagen at the low dose level throughout the period of study. Some dose dependence is manifested in significant decreases occurring only at the two higher doses at four weeks. Partial recovery is evident in the two lower dose groups and complete recovery in the high dose group at six weeks.

Radioactivity in soluble skin collagen hydroxyproline

There was an exponential increase in the radioactivity of the soluble collagen hydroxyproline in the untreated rats from two to four weeks, followed by a decline at six weeks. The values obtained were 17, 20, 26, and 20% at two, three, four, and six weeks, respectively (Figure 6). At two weeks, the lead-induced decreases in radioactivity amounted to 2, 5, and 7% (n.s.) in the low, medium, and high dose groups (Figure 6). Nonsignificant decreases of 26 and 23% and a significant (P<.025) decrease of 41% were found in the animals exposed to 1, 1.5, and 2% lead acetate,

respectively, at three weeks. At four weeks, decreases in the radioactivity of the hydroxyproline of the soluble fraction of 37% ($P<.005$), 25% ($P<.025$) and 28% ($P<.01$) were measured in the low, medium, and high dose groups, respectively. Nonsignificant increases of 20 to 26% are apparent at six weeks in all lead-treated animals.

The inhibition of short-term soluble collagen synthesis by lead is most pronounced in the high dose group at three weeks, and in the two lower dose groups at four weeks. Reversal of this inhibition is evidenced at six weeks by the nonsignificant increases in the labeled hydroxyproline of the soluble fraction.

Radioactivity in insoluble skin collagen hydroxyproline

In the control rats, there was a slight increase in labeled hydroxyproline of the insoluble skin fraction from two to three weeks, a very pronounced increase from three to four weeks, and a decrease at six weeks. The values were 4, 6, 25, and 8% at two, three, four, and six weeks, respectively (Figure 7). The two-week old sucklings had a decrease of 46% ($P<.025$), and increases of 65% ($P<.01$) and 1% (n.s.) in the low, medium, and high groups, respectively (Figure 7). At three weeks the lead-exposed pups showed 6 and 23% (n.s.) and 36% ($P<.005$) reductions at the 1, 1.5, and 2% dose levels. A nonsignificant decrease in labeled insoluble collagen hydroxyproline of 8% and highly significant decreases of 42 and 57% occurred in the four-week old weanlings on the low, medium, and high dose levels, respectively. The six-week old rats had increased the insoluble skin collagen hydroxyproline levels over the controls by 32% (n.s.), 162% ($P<.0005$), and 80% ($P<.025$) at the

given lead dosages.

The effects of lead on new insoluble collagen synthesis are not consistent in the two-week old pups. Dose dependence appears at three weeks and becomes very pronounced at four weeks, during the period of maximal growth rate. As in the newly synthesized soluble collagen fraction, increases in the insoluble fraction are found in all lead-exposed pups at six weeks. These increases are very marked in the animals exposed to the 1.5% lead acetate dose.

Proline content of soluble skin collagen

In the control rats there was a slight decrease in the proline content of the soluble skin fraction from two to three weeks, followed by a marked increase at four weeks. Values averaged 1055, 952, and 2577 μg/g skin at two, three, and four weeks, respectively (Figure 8). In the two-week old sucklings at 1, 1.5 and 2% lead-treated groups respectively, there were nonsignificant reductions of 20 and 14% and a highly significant reduction of 79% (P<.0005)(Figure 8). Significant increases of 48 and 40% (P<.05) and 64% (P<.025) were seen at three weeks. By four weeks, the increase of proline in the soluble fraction had dwindled to 5% (n.s.) in the low-dose groups, and reversed to significant decreases of 36% (P<.025) and 35% (P<.005) at higher doses.

On the whole, there is no apparent trend in the effects of lead on the proline content of the soluble skin fraction. There are, however, marked reductions in the two-week old animals on the high dose, and in the four-week old animals at the medium and high dose levels. At three weeks the effects of lead on the proline in the soluble fraction are the opposite of those in the hydroxyproline of the same fraction. Some

parallelism is evident in the increase of both imino acids at the low dose level and the decrease of both imino acids at the medium and high dose levels at four weeks.

Proline content of insoluble skin collagen

In control rats, the proline content of the insoluble skin fraction increased exponentially from two to four weeks. The values averaged 3194, 5246, and 10468 μg/g skin at two, three, and four weeks, respectively (Figure 9). The effects of lead were very irregular. Decreases of 20% ($P<.005$) and 78% ($P<.0005$), and an increase of 99% ($P<.005$) were calculated for the 1, 2, and 1.5% lead acetate dose groups, respectively, at two weeks (Figure 9). At three weeks, there were no significant differences between control and lead-exposed rats. By the fourth week, the low dose group showed a 15% increase (n.s.), and the medium and high dose groups had decreases of 32% ($P<.005$) and 29% ($P<.025$), respectively.

As in the case of the soluble collagen proline, a consistent trend is not indicated by the above data. Some similarity in metabolic pattern of the proline in the two fractions is evidenced by the marked decreases at two weeks at the high dose level and at four weeks in the medium and high dose levels.

Radioactivity in total skin protein

The incorporation of ^{14}C-proline into the proteins of the 0.45M NaCl-soluble fraction in 12 hours was measured as an index of short-term protein synthesis. The highest rate of synthesis occurred at two weeks in both control and lead-treated rats. Control values were 827, 553, 762 and 166 dpm/mg skin protein at two, three, four and six weeks respec-

tively (Figure 10). The ^{14}C-proline incorporation into the total skin protein of lead-treated rats, normalized for body weight, was not significantly different from controls at any age examined, although some depression was observed at four weeks at the higher lead dosages. The rate of skin protein synthesis was relatively low by six weeks in all animals, concomitant with the completion of the period of rapid growth.

The Effects of Lead on Mature Rats Exposed since Birth

Body weight

The rats studied were 4 to 13 months of age. The mean body weight of control rats was 408 g for males and 277 g for females (Table 1). The body weights were significantly lower in rats exposed to lead since birth. The mean weight of rats exposed to 1.5% lead acetate was 248 g for males and 204 g for females (Table 2). These weights were 39 and 26% less than respective control weights. The weights of three male rats in the 2% group averaged 396 g, 26% less than control males (Table 2). The lower body weights of mature lead-exposed rats represent a continuation of the body weight reductions of 43% and 53% observed in the 1.5 and 2% lead acetate groups, respectively, at six weeks.

Renal function

The inulin clearances of control rats averaged 10.95 ml/min/kg (Table 1). In rats drinking 1.5 or 2% lead acetate since weaning, inulin clearances were 8.01 ml/min/kg and 6.44 ml/min/kg, respectively (Table 2). The decreases of 27% ($P < .01$) were proportional to the lead dose.

The clearance of PAH in the controls averaged 41 ml/min/kg (Table 1). The clearances in the treated rats were 28 ml/min/kg and 14 ml/min/kg in

the 1.5 and 2% lead acetate groups, respectively (Table 2).

Renal function studies were performed on three older rats (14, 16, and 22 months of age) receiving 1% lead acetate in their drinking water since weaning. In the youngest rat, the GFR of 5.34 ml/min/kg was approximately 50% that of controls, and ERPF of 40 ml/min/kg was similar to control values. In the two older rats, however, inulin and PAH clearances were so severely impaired that constant plasma levels could not be maintained by infusion. This prevented an accurate measure of renal plasma flow, since the plasma PAH levels reached tubular maximum concentrations.

Histopathology of the kidney

The morphology of the kidney of one-year old control rats was normal upon gross and light microscopic examination (Figure 11). In rats exposed to 1.5% lead acetate in the drinking water from birth to one year, the kidneys were enlarged and microscopically revealed dilation of tubules in the cortex and tubular degeneration (Figure 12). More severe renal changes were observed in rats exposed to 2% lead acetate for 10 months. Tubular degeneration and interstitial fibrosis were evident in the cortex and suggested a severe loss in functional capacity of the kidney (Figures 13 and 14). The glomeruli were not affected.

Urinary excretion of hydroxyproline and proline

The ten control rats excreted an average of 349 μg hydroxyproline and 1105 μg proline in their urine in 24 hours (Figure 15). Rats in the three lead-treated groups excreted more imino acid. For the hydroxyproline, the increases in 24-hour urinary excretion amounted to 127, 92, and 253% (all significant at the .005 level), while for proline the increases were

66% ($P<.01$), 21% (n.s.) and 87% ($P<.01$) in the low, medium, and high dose groups, respectively (Figure 15).

Urinary excretion of δ-ALA

On the average, the control rats excreted 94 μg δ-ALA in their urine in 24 hours (Figure 15). The excretion of this intermediate in heme synthesis into the urine of all lead-treated rats was substantially elevated. The increases amounted to 465% ($P<.005$), 677% ($P<.005$), and 1069% ($P<.0005$) at the given lead levels, illustrating a high degree of dose dependence.

Figure 1. The elution pattern of hydroxyproline and proline. A standard containing 0.1 ml (U)^{14}C-hydroxyproline, ca. 35,000 dpm, 0.1 ml (U)^{14}C-proline, ca. 50,000 dpm, and 100 μg each of stock solutions containing, respectively, 1 mg/ml unlabelled hydroxyproline and proline, was layered on Dowex 50-W columns and eluted with 0.1M citrate buffer, pH 2.8±0.05

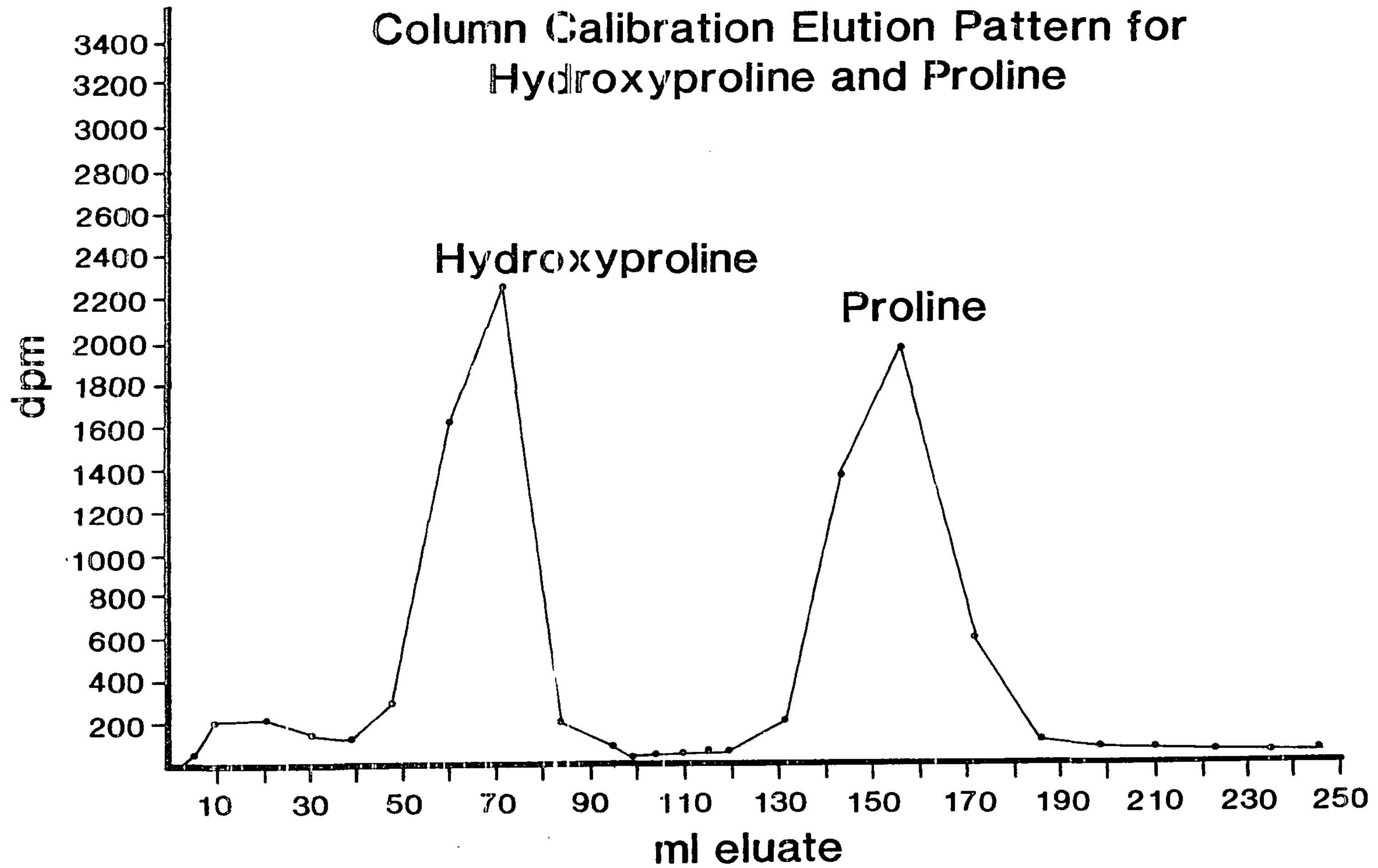

Column Calibration Elution Pattern for
Hydroxyproline and Proline
Hydroxyproline
Proline
dpm
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
10
30
50
70
90
110
130
150
170
190
210
230
250
ml eluate

Figure 2. Effect of lead acetate in the maternal drinking water on body weight in young rats. At birth, tap water was replaced with distilled water containing the indicated concentrations of lead acetate. The rats were weaned between three and four weeks of age and maintained on the same concentration of lead acetate in the drinking water

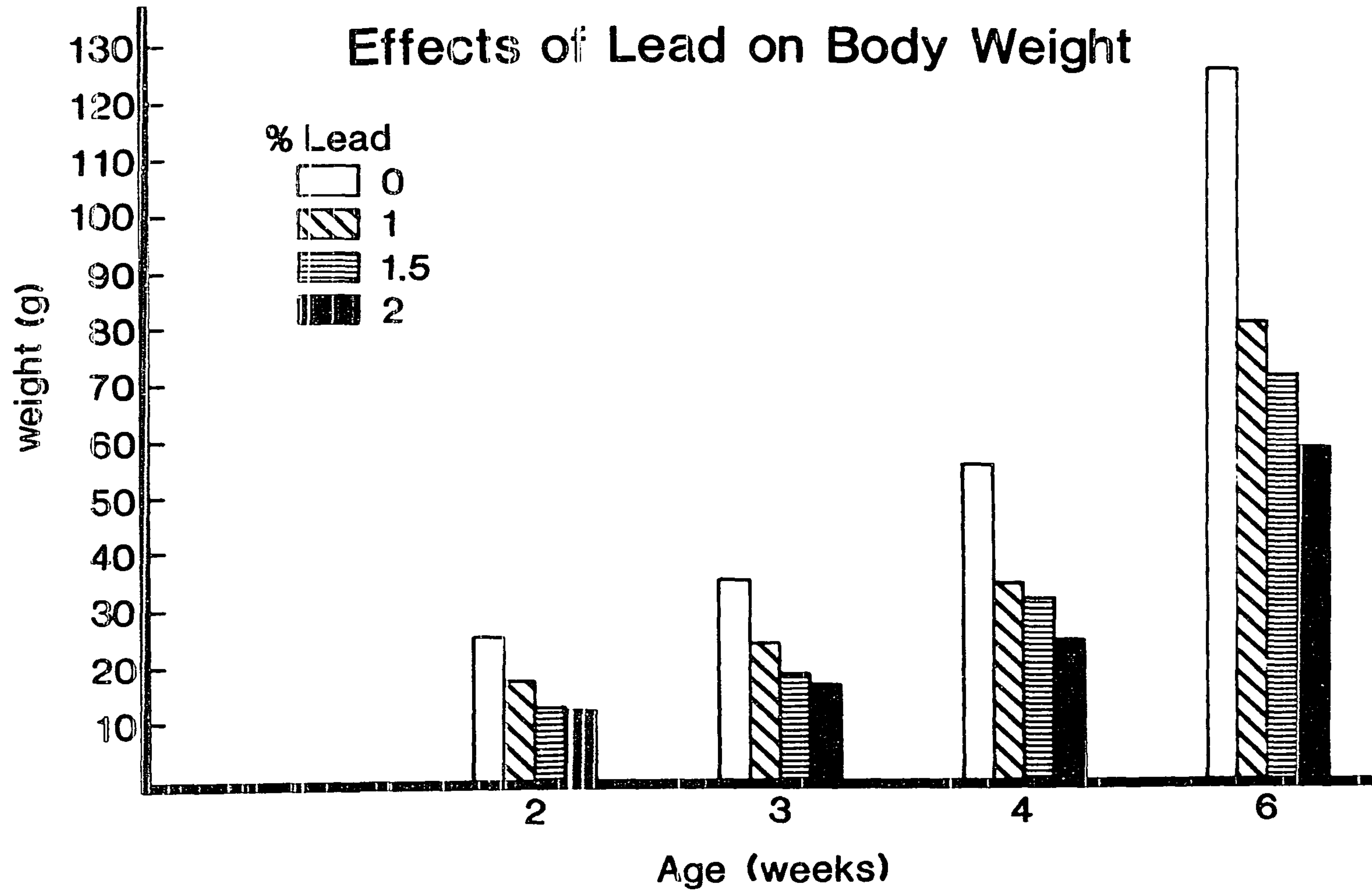

Effects of Lead on Body Weight
% Lead
0
1
1.5
2
weight (g)
130
120
110
100
90
80
70
60
50
40
30
20
10
2
3
4
6
Age (weeks)

Figure 3. Blood lead levels of suckling rats during the development of lead encephalopathy as determined by atomic absorption spectrophotometry. The maternal drinking water contained 1.0 or 2.0% lead acetate

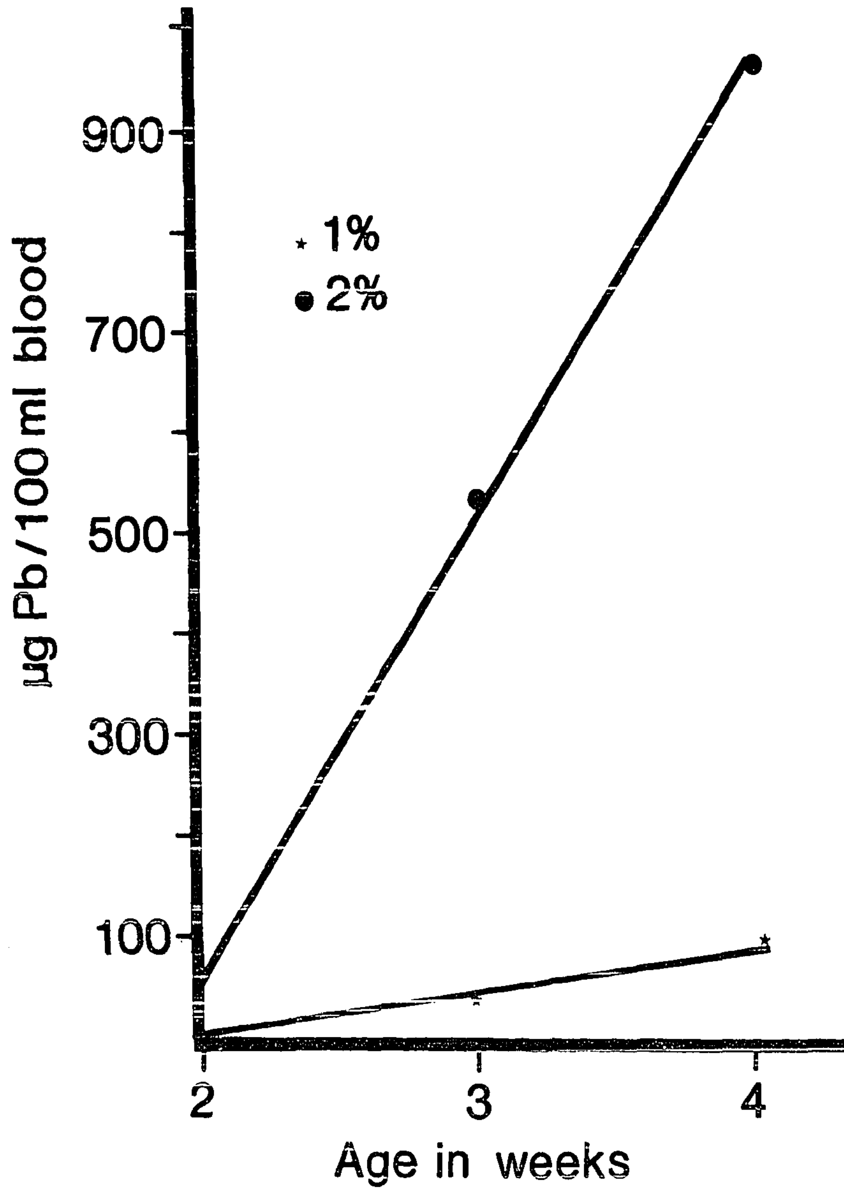
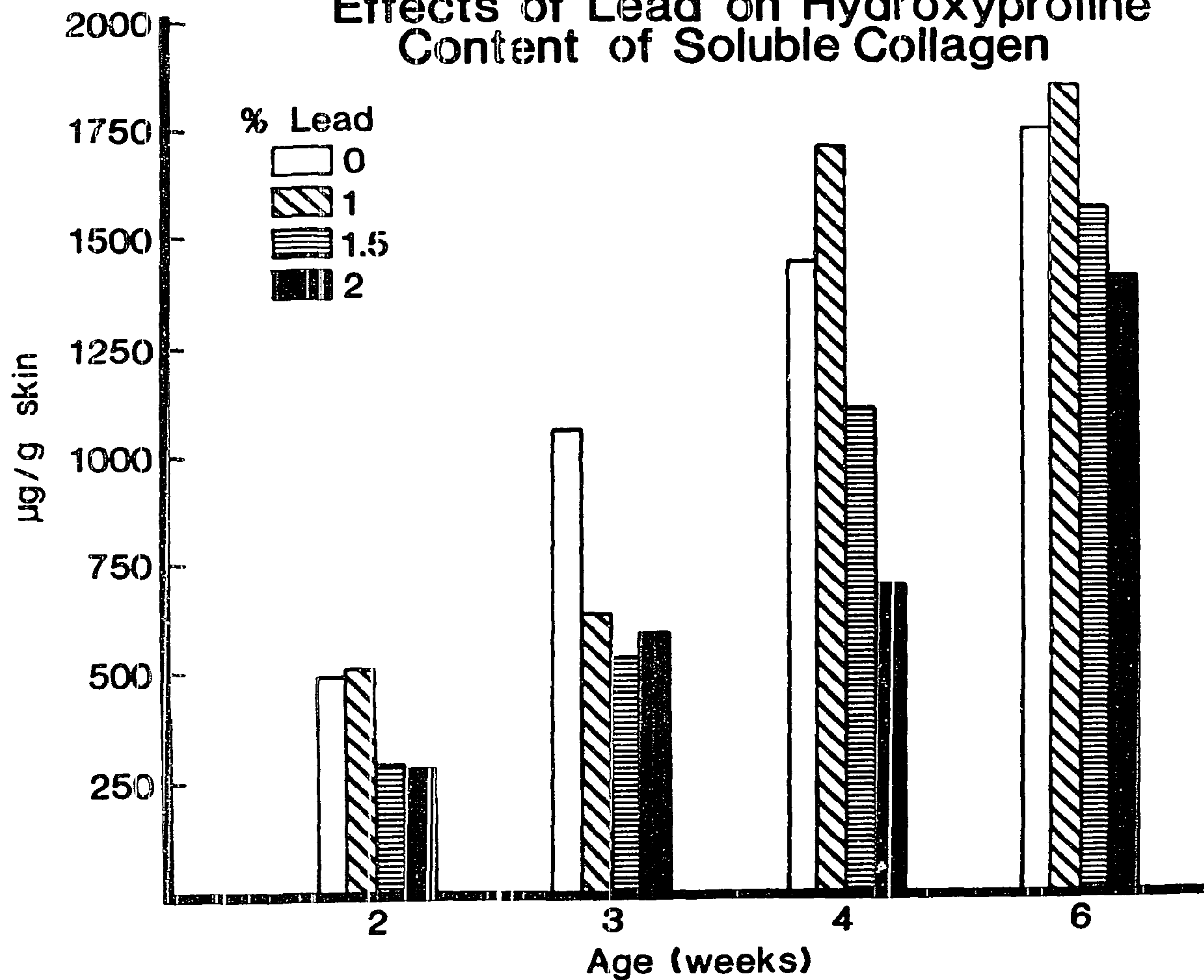

900
700
500
300
100
μg Pb / 100 ml blood
1%
2%
2
3
4
Age in weeks

Figure 4. The effects of lead acetate in the maternal drinking water on the hydroxyproline content of 0.45M NaCl-soluble skin collagen in young rats. The rats were weaned between three and four weeks of age and maintained on the same concentration of lead acetate in the drinking water



Effects of Lead on Hydroxyproline Content of Soluble Collagen
% Lead
0
1
1.5
2
2000
1750
1500
1250
1000
750
500
250
µg/g skin
2
3
4
6
Age (weeks)

Figure 5. The effects of lead acetate in the maternal drinking water on the hydroxyproline content of 0.45M NaCl--insoluble skin collagen in young rats. The rats were weaned between three and four weeks of age and maintained on the same concentration of lead acetate in the drinking water

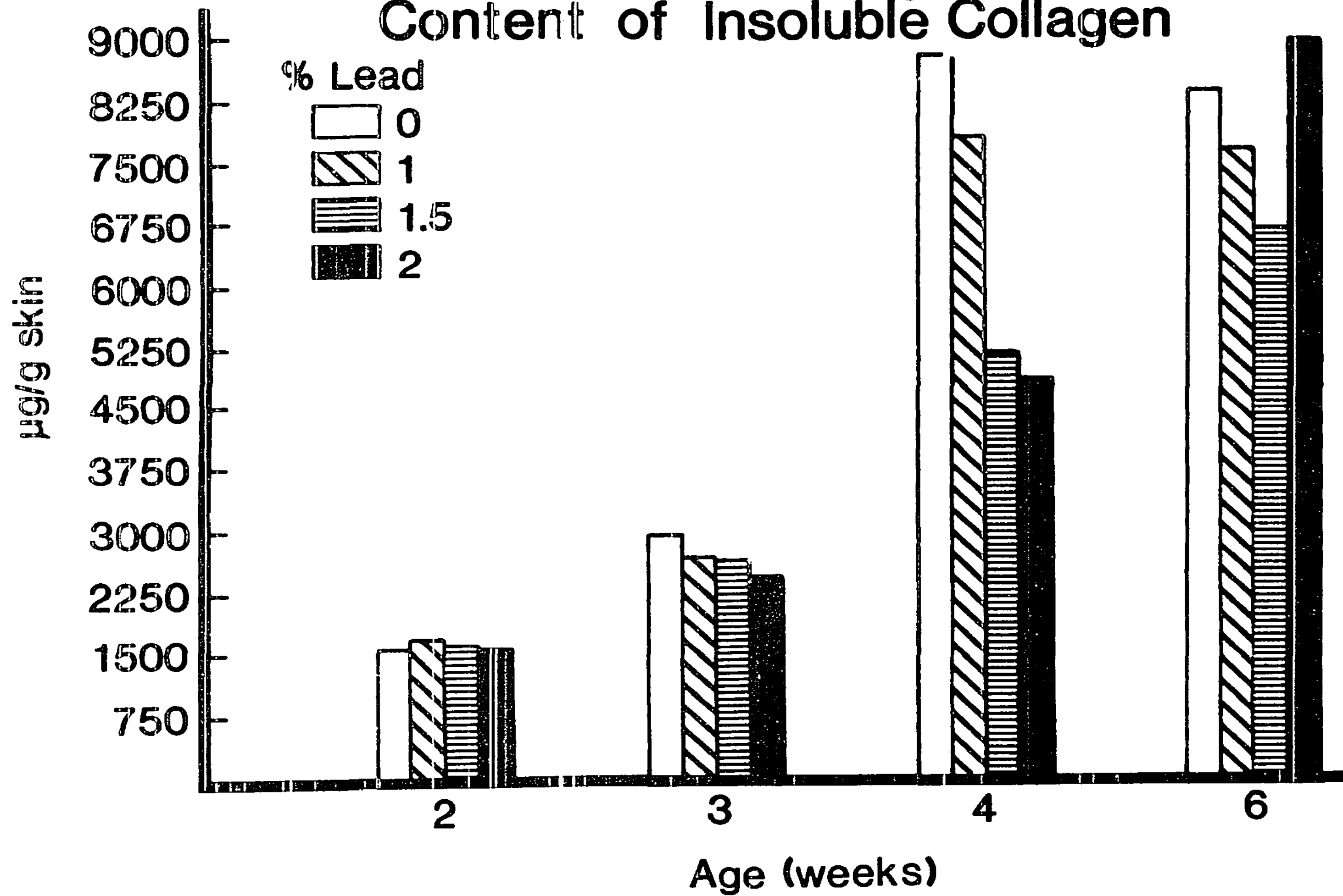

Effects of Lead on Hydroxyproline
Content of Insoluble Collagen
9000
8250
7500
6750
6000
5250
4500
3750
3000
2250
1500
750
µg/g skin
% Lead
0
1
1.5
2
2
3
4
6
Age (weeks)

Figure 6. The effects of lead acetate in the maternal drinking water on the proportion of radioactivity in the hydroxyproline of 0.45M NaCl-soluble skin collagen in young rats. The rats were weaned between three and four weeks of age and maintained on the same concentration of lead acetate in the drinking water

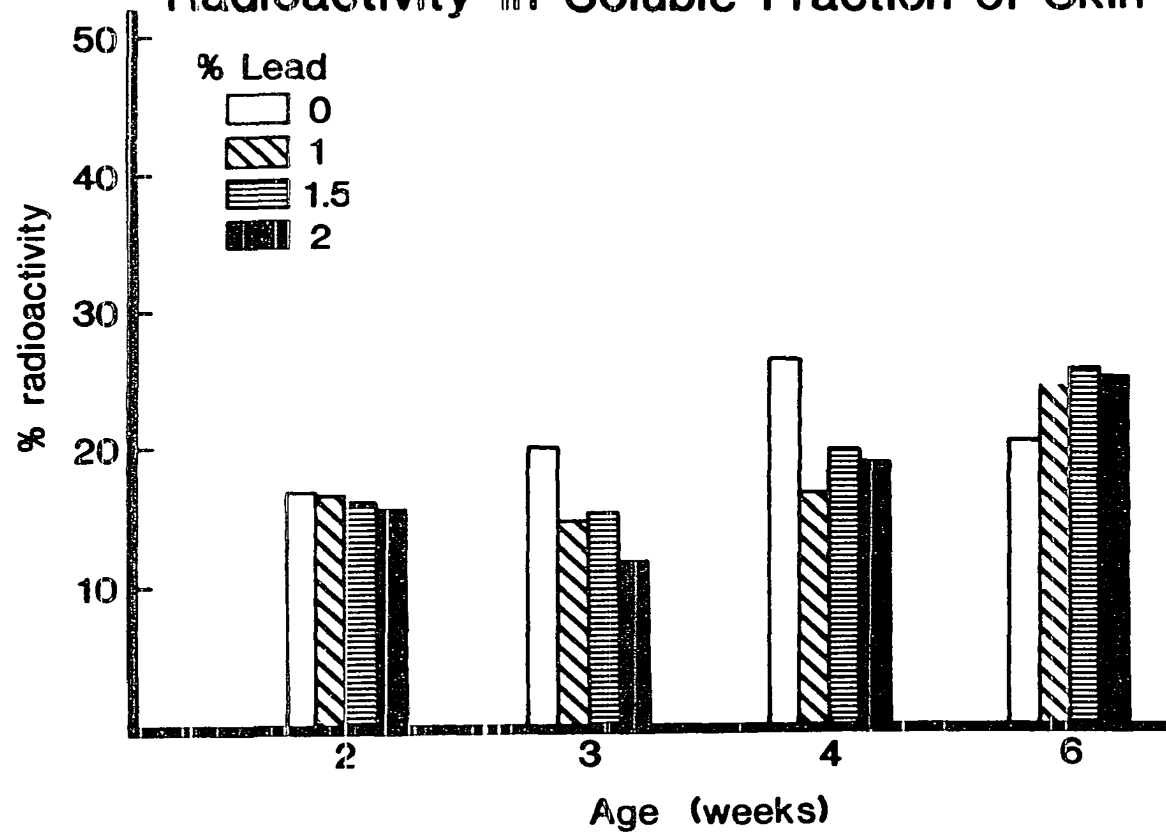
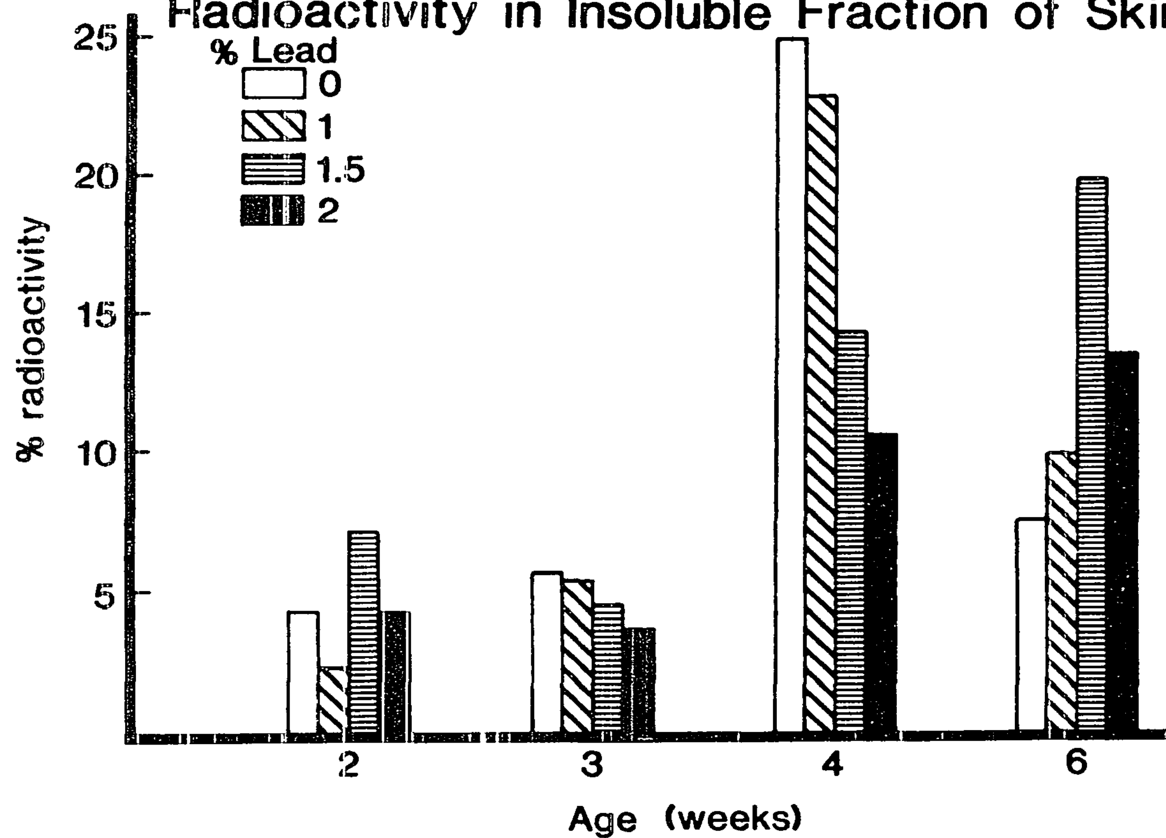

Effects of Lead on Percent of Hydroxyproline Radioactivity in Soluble Fraction of Skin
% Lead
0
1
1.5
2
% radioactivity
50
40
30
20
10
2
3
4
6
Age (weeks)

Figure 7. The effects of lead acetate in the maternal drinking water on the proportion of radioactivity in the hydroxyproline of 0.45M NaCl in insoluble skin collagen in young rats. The rats were weaned between three and four weeks of age and maintained on the same concentration of lead acetate in the drinking water



Effects of Lead on Percent of Hydroxyproline Radioactivity in Insoluble Fraction of Skin
% Lead
0
1
1.5
2
% radioactivity
25
20
15
10
5
2
3
4
6
Age (weeks)

Figure 8. The effects of lead acetate in the maternal drinking water on the proline content of the 0.45M NaCl-soluble skin collagen in young rats. The rats were weaned between three and four weeks of age and maintained on the same concentration of lead acetate in the drinking water

Effects of Lead on Proline Content of Soluble Collagen

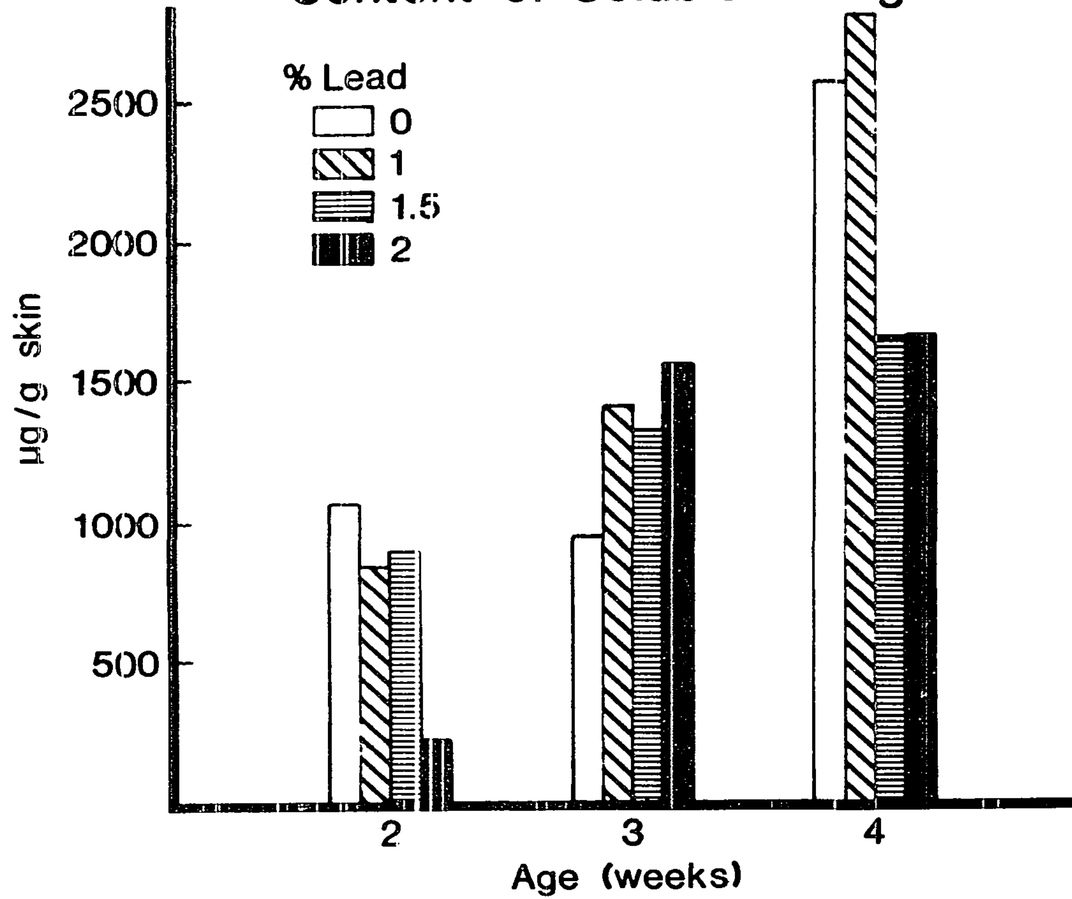
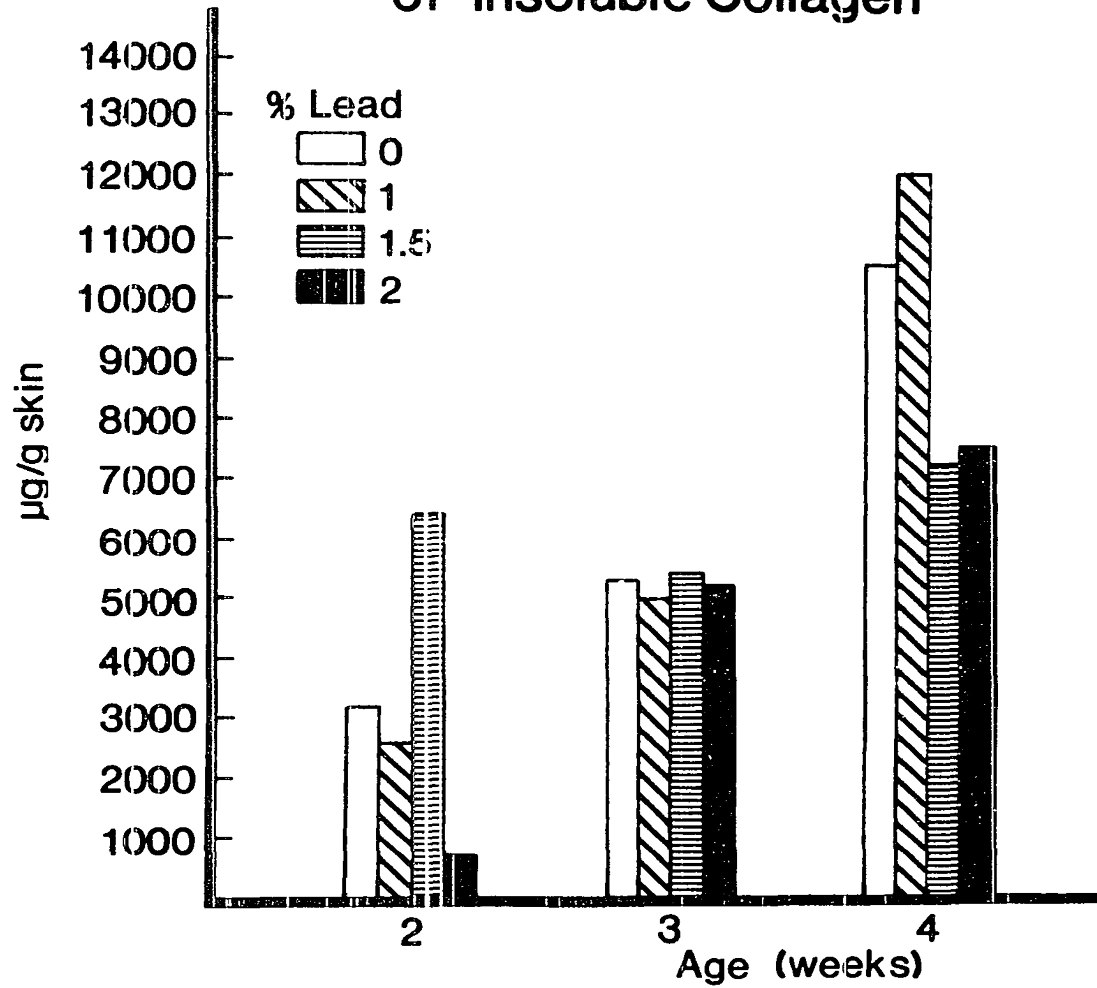


Figure 9. The effects of lead acetate in the maternal drinking water on the proline content of the 0.45M NaCl-insoluble skin collagen in young rats. The rats were weaned between three and four weeks of age and maintained on the same concentration of lead acetate in the drinking water



Effects of Lead on Proline Content
of Insoluble Collagen
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
µg/g skin
% Lead
0
1
1.5
2
2
3
4
Age (weeks)

Figure 10. The effects of lead acetate in the maternal drinking water on the incorporation of (U) ^{14}C-proline into the total protein of the 0.45M NaCl-soluble fraction of skin in young rats normalized for body weight. The rats were weaned between three and four weeks of age and maintained on the same concentration of lead acetate in the drinking water

Radioactivity in Total Protein of 0.45 M NaCl Soluble Fraction of Skin

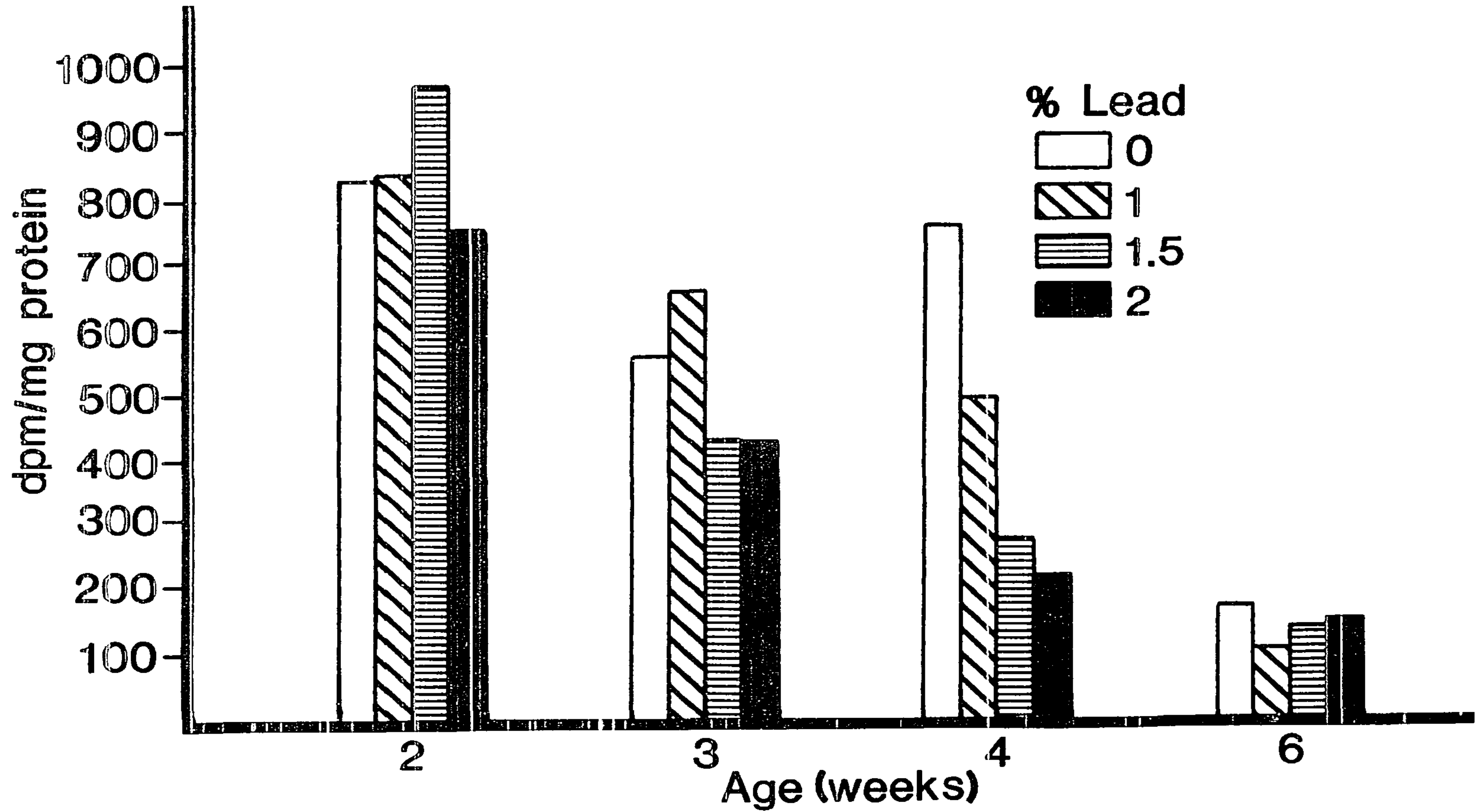
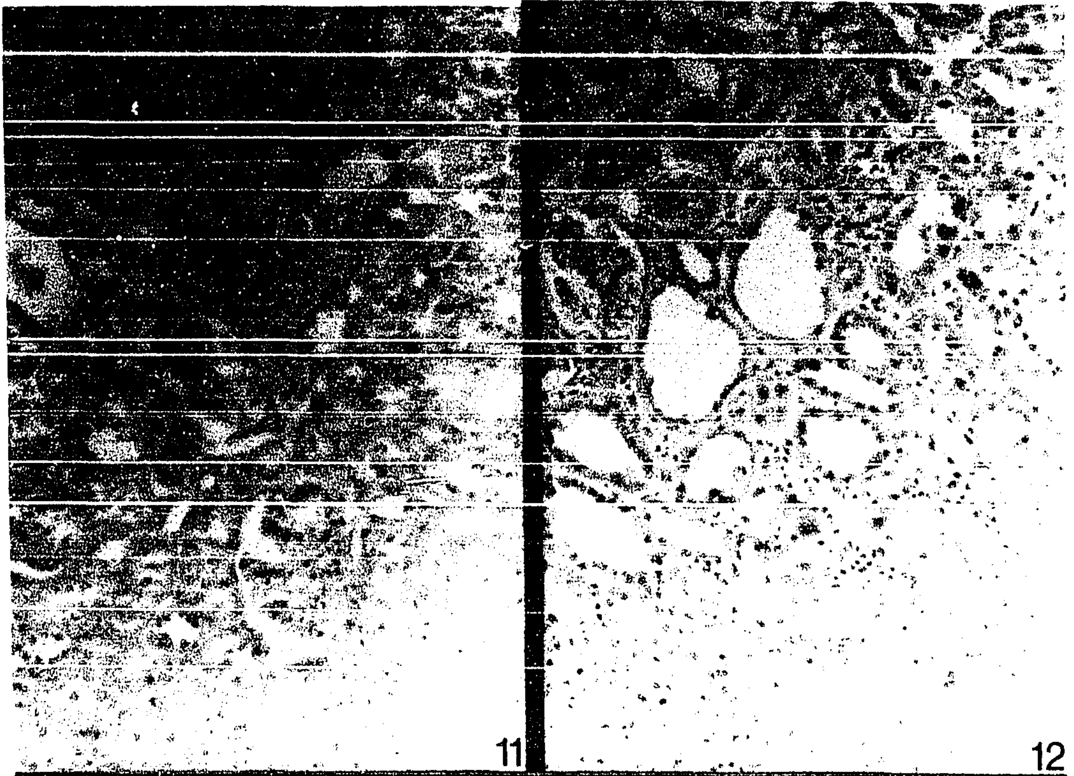


Figure 11. Renal cortex of a one-year old control rat. Normal tubular structure surrounds the glomeruli in the center and lower center. Hematoxylin and eosin, 375X

Figure 12. Renal cortex of a one-year old rat which received 1.5% lead acetate in the drinking water since birth. There are many dilated renal tubules with degeneration of the epithelium lining the tubules. Hematoxylin and eosin, 375X

Figure 13. Renal cortex of a 10-month old rat which received 2% lead acetate in the drinking water since birth. Tubular degeneration and interstitial fibrosis is present. Hematoxylin and eosin, 375X

Figure 14. Renal cortex of a 10-month old rat which received 2% lead acetate in the drinking water since birth. There is necrosis of tubular epithelium and interstitial fibrosis. Hematoxylin and eosin, 1500X


11
12

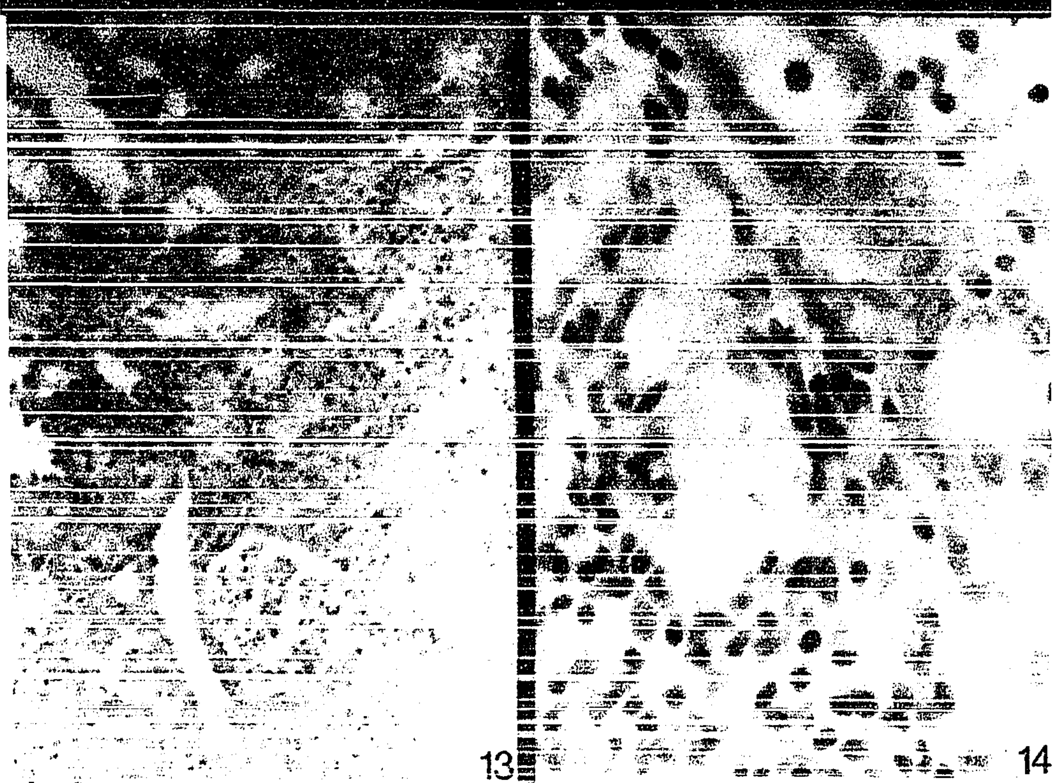

13
14

Figure 15. The effects of lead acetate in the drinking water on the 24-hour urinary excretion of hydroxyproline, proline and δ-ALA in mature rats

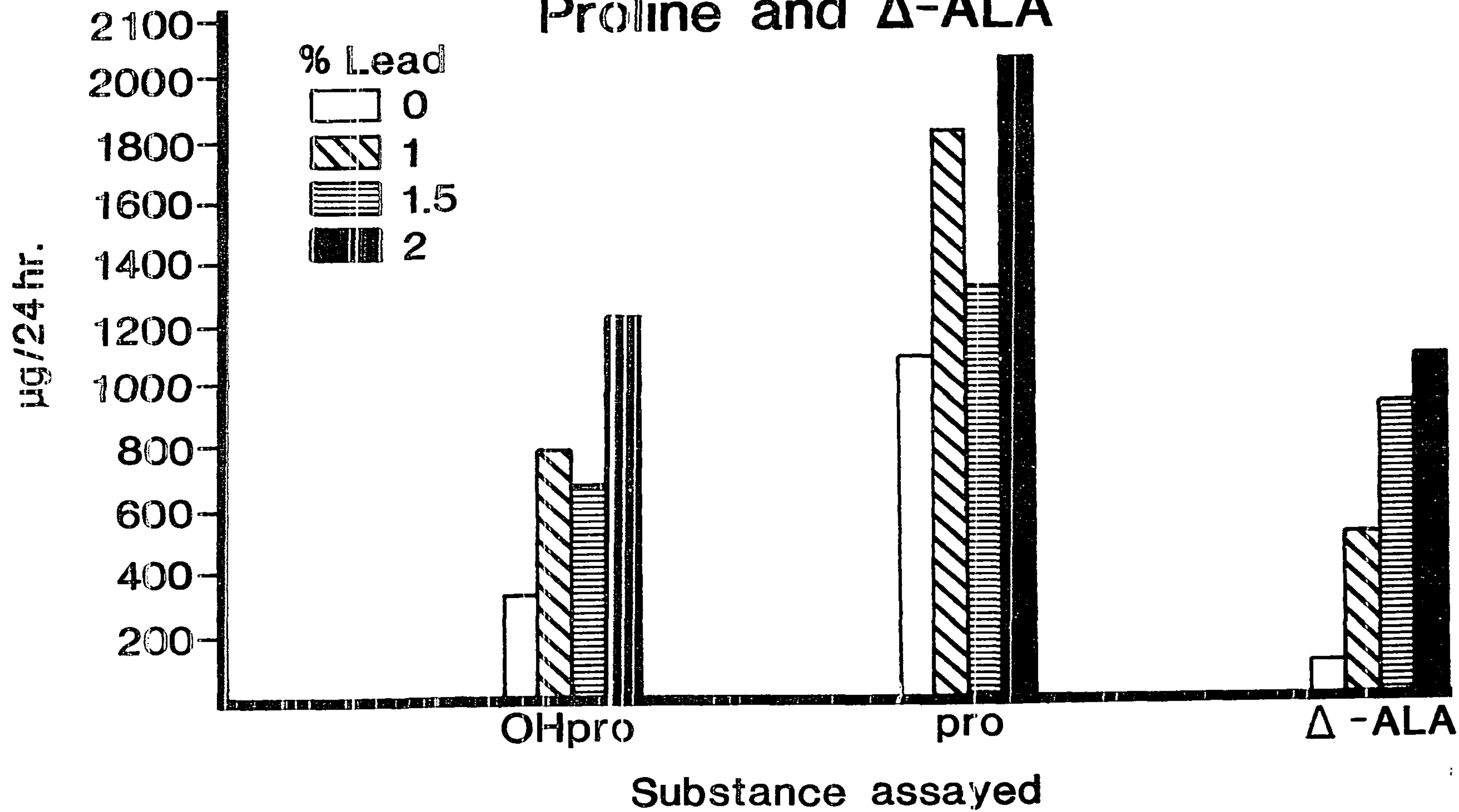

Urinary Excretion of Hydroxyproline,
Proline and Δ-ALA
% Lead
0
1
1.5
2
2100
2000
1800
1600
1400
1200
1000
800
600
400
200
µg/24 hr.
OHpro
pro
Δ -ALA
Substance assayed

Table 1

Parameters of Renal Function in Control Rats
(Glomerular Filtration Rate and Effective Renal Plasma Flow)

Rat No.	Age (mo.)	Body Weight (g)	Sex	Glomerular Filtration Rate (ml/min/kg)	Effective Renal Plasma Flow (ml/min/kg)
1	4.0	201	F	11.49	53.70
2	4.5	329	M	14.78	52.78
3	8.5	458	M	11.39	49 72
4	9.5	239	F	10.59	35.72
5	9.5	271	F	12.78	40.11
6	10.5	307	F	9.03	29.87
7	11.0	472	M	8.68	30.12
8	12.0	336	F	11.73	48.71
9	12.5	372	M	8.44	59.81
10	13.0	318	F	9.93	30.12
11	13.0	266	F	11.12	32.52
$\bar{X} \pm$ S.D.	9.8	324		10.95 ± 1.90	42.11 ± 11.12

Table 2

Parameters of Renal Function in Rats Exposed to 1.5 and 2% Lead Acetate
(Glomerular Filtration Rate and Effective Renal Plasma Flow)

	1.5% Lead Acetate					2% Lead Acetate				
	Age (mo.)	Rat B. wt. (g)	Sex	GFR (ml/min/kg)	ERPF (ml/min/kg)	Age (mo.)	Rat B. wt. (g)	Sex	GFR (ml/min/kg)	ERPF (ml/min/kg)
	8.0	303	M	4.38	38.47	9.0	322	M	7.38	16.92
	8.5	257	M	9.54	27.10	9.0	307	M	3.52	11.07
	8.5	194	F	7.90	41.10	9.0	279	M	8.41	39.28
	8.5	171	F	6.25	20.23					
	9.0	221	M	10.56	30.41					
	9.0	210	M	7.04	20.56					
	9.0	161	F	9.20	29.59					
	9.5	246	F	8.30	21.26					
	9.5	246	F	8.92	27.24					
$\bar{X}$	8.8	223		8.01**	28.44*	9.0	396		6.44*	22.42*
S. D.				1.88	7.50				2.58	14.89

* Significant at .025 level. ** Significant at .005 level.

DISCUSSION

The method of exposing suckling rodents to lead via the milk of their dams, first utilized by Pentschew and Garro (1966), has become a frequently used model of lead encephalopathy, with a few modifications (Thomas et al. 1971, Michaelson and Sauerhoff 1974a, 1974b for the rat; Rosenblum and Johnson 1968 for the mouse).

Growth retardation occurs in suckling pups exposed to lead. When lactating rats were fed a diet containing 4.5% lead carbonate and 1% lead acetate was added to their drinking water, the sucklings weighed only 34% of controls (Thomas et al. 1971). The offspring of dams consuming a 5% lead acetate diet since parturition suffered a 45 to 60% retardation in growth (Michaelson and Sauerhoff 1974a, 1974b). Since lactating rats consume an average of approximately 10 g feed and 35 ml drinking water daily (present study), the daily lead dose from a 5% lead acetate diet approximates that from drinking water containing 2% lead acetate. The average weight deficit of 53% in our six-week old weanlings in the 2% lead acetate group is therefore in agreement with the findings reported above. In addition, the dose dependency of the growth-retarding effects of lead are evident (Figure 2).

In vitro investigations may suggest a mechanism for the profound effects of lead on growth. When Swiss albino mouse fibroblast 3T6 cells were exposed to 22.9 or 33.8 μM Pb^{++}, the reduction in cell numbers per plate at 43 hours was associated with significant increases in mean generation time. The increases in cell number during the logarithmic growth phase were 153% for control cultures and 67.8 and 57.9% for cells

exposed to 22.9 and 33.8 μM Pb, respectively (Vistica 1975).

In a similar study, L-A mouse fibroblasts, a subline of L929, were exposed to lead chloride concentrations increasing in geometric progression from 2 to 128 x 10^{-5}M (20 to 1280 μM) for 2 or 7 days. Control cell numbers reached a maximum of over 8 x 10^5 cells/ml on day 7, while cells exposed to 40 or 80 μM Pb increased to only approximately 5.9 and 2.6 x 10^5 cells/ml, respectively, on day 8. Cell groups at both lead dosages exhibited fewer mitoses than controls, especially on days 2 and 4. Morphological changes included increase in cell size, attributed to a block in the G_1 phase of the cell cycle, with consequent failure of DNA synthesis and mitosis. In spite of reduced cell proliferation, lactate production was relatively and absolutely increased after an initial lag. Lactate production is directly related to lead dose, suggesting that the cell switches to glycolysis for its energy needs as the mitochondrial oxidative functions are increasingly impaired by lead (Fischer 1975).

Failure of DNA synthesis, but not of RNA or protein synthesis, was indicated by low DNA levels in the cerebella of suckling rats whose dams were consuming 5% lead acetate (Michaelson and Sauerhoff 1974b). The wet tissue concentration of lead in these cerebella was 12.53 ppm, or 6 μM, one-fourth to one-sixth of the minimal growth-inhibiting dose for Swiss albino mouse fibroblasts in culture (Fischer 1975). In the present study, the concentration of lead in the blood of four-week old pups in the 2% lead acetate group was 970 μg/100 ml, or 4.5 μM. Inhibition of DNA synthesis may be only one of the means by which lead inhibits growth.

The retardation of development evidenced by delayed hair formation and neurological impairment in the form of urinary incontinence in lead-exposed suckling rats (present study) is in agreement with other reports (Michaelson and Sauerhoff 1974b). The absence of the posterior limb paralysis that was also reported may be due to strain differences.

The acute encephalopathy produced in suckling animals by exposure to lead since birth is associated with edema of the brain tissue. Ultrastructural studies revealed extensive vascular damage primarily in the cerebellum, where evidence of extravasation of a protein-rich fluid and hemorrhages was apparent (Popoff et al. 1963, Clasen et al. 1974). Breakdown of the blood-brain barrier was indicated by the entry of the normally excluded tracer protein horseradish peroxidase (HRP) into cerebellar tissue after intravascular infusion (Vistica 1975). Failure of the HRP to penetrate capillary endothelial cell plasma membranes implicates the endothelial cell junctions as the route of entry into the cerebellum.

Ultrastructural defects were found in the endothelial cell junctions and capillary basement membranes of cerebellar capillaries of lead-treated suckling rats from two to three weeks (Vistica 1975). No defects of comparable magnitude were produced in the cerebral capillaries. The special susceptibility of the cerebellar capillaries to lead has been imputed to the delay of 7 to 10 days in the maturation of the basement membranes of the cerebellar capillaries as compared with the cerebral capillaries (Vistica 1975). The partial digestion of basement membranes and endothelial cell junction material when brain slices were incubated with

collagenase at 37°C for 36 hours (Vistica 1975) confirms earlier findings that these structures are composed principally of collagen (Kefalides 1971). Two questions arise: is it the collagen in the basement membrane which is being affected by lead, and, if so, does the lead inhibit collagen synthesis or promote collagen degradation?

The present study reveals that soluble collagen as measured by hydroxyproline content of skin is decreased in suckling rats at two to four weeks by 1.5 or 2% lead acetate in the drinking water. At the lowest level of lead (1%), decreased skin collagen content was observed only at three weeks. It is not certain whether the lower levels of soluble collagen in lead-treated rats represent impaired biosynthesis or enhanced degradation or both. The rate of hydroxylation of ^{14}C-proline of soluble collagen may be decreased as evidenced by the lower percent of hydroxyproline radioactivity in the soluble collagen at three and four weeks in all lead-treated rats. The results are consistent with the production on underhydroxylated protocollagen similar to that observed in vitro. Swiss albino mouse 3T6 fibroblasts cultured in media containing 22.9 or 33.8 μM Pb^{++} retained an underhydroxylated procollagen as indicated by increased proline-hydroxyproline ratios. The amount of this procollagen retained by the cell was linear with lead dose (Vistica 1975).

The effects of lead on the proline content of soluble collagen were less consistent, and the decreases observed did not parallel those of hydroxyproline. It should be noted that at three weeks, the lead-induced decrease in hydroxyproline was accompanied by increased proline content in soluble collagen, suggesting the synthesis of underhydroxylated collagen.

The inhibitory action of lead on the synthesis of the insoluble collagen content of skin was apparent at three weeks and maximal at four weeks. No effect was detected at two weeks. Since this fraction represents older collagen, the lag in inhibition is not surprising if it occurs secondary to the decrease in soluble collagen pools. Unlabelled proline and hydroxyproline tended to decrease in parallel in insoluble collagen in the presence of lead at three and four weeks. This indicates that the proline-hydroxyproline ratio was normal in this fraction and that underhydroxylated soluble collagen is labile and not readily converted to mature collagen, or, if converted, it is more rapidly degraded. The results of the effects of lead on the percent of hydroxyproline radioactivity in the insoluble collagen fraction are more consistent with the latter hypothesis. There was a substantial decrease in the hydroxyproline fraction of total ^{14}C-imino acid incorporated into newly formed mature collagen.

The consistent finding of maximum depression of growth and collagen synthesis in the two- to four-week period in lead-exposed rats illustrates the principle that the most active anabolic processes possess the highest susceptibility to chemical insult during development. The lack of inhibitory effects of lead on collagen synthesis at six weeks may result from the deceleration of the collagen synthetic rates that acccompanies the close of the period of active growth, and decreased permeability of the maturing cell membranes to certain metal ions. A study on gastrointestinal absorption of ^{212}Pb in young rats disclosed that over 80% of an orally ingested dose is absorbed at 16 days, 74% at 22 days, the

weaning time, and only 15% at 32 days (Forbes and Reina 1972). One contributory factor to this decline in lead absorption in the gut may be the cessation of pinocytotic activity in the columnar epithelial cells by 18 to 20 days, as revealed by the loss of ability to absorb antibodies from the maternal milk (Clark 1959).

Lack of significant differences in total protein synthesis between control and lead-treated rats at all ages examined provides support for the contention that inhibition of collagen synthesis is not due to any inhibition of general protein synthesis. The occurrence of the maximum rate of short-term protein synthesis as indicated by the 12-hour incorporation of ^{14}C-proline in the two-week old rats bears further testimony to the rapid biosynthetic activity that accompanies rapid growth. The substantial decline in the rate of short-term skin protein synthesis at six weeks provides an additional marker of the termination of the period of active growth.

In summary, the observation that lead inhibits collagen biosynthesis in vitro (Vistica 1975) has been extended to in vivo inhibition by the results of the present study. The mechanism of inhibition has been investigated in tissue culture in which lead-induced inhibition of hydroxylation of proline residues in the procollagen α-chains was observed (Vistica 1975). Enzyme assays were performed on cell sonicates of 3T6 mouse fibroblasts cultured in the presence of 1 to 15 μg/ml Pb^{++}. Assay for proline hydroxylase activity in the presence of chick embryo substrate and the cofactors α-ketoglutarate and ascorbate disclosed that the collagen retention within the cells decreased, the proline-hydroxyproline ratios

decreased, and the percentage of proline hydroxylation increased to control values as the molar ratio of Fe^{++} to Pb^{++} in the culture medium approached 5:1 (Vistica 1975). The reversal of the lead-induced inhibition of proline hydroxylation by supplementary iron by apparently competitive kinetics implies a competition between lead and iron for the same site on the proline hydroxylase molecule (Vistica 1975), with lead acting as inhibitor and iron as activator of the enzyme.

Since lead inhibits collagen biosynthesis, it is of interest to consider other agents which are known to have a similar action in tissue culture as they may provide insight into mechanisms of lead action. Considerable data exists on the effects of various chelating agents on collagen synthesis. The two Fe^{++} chelators α, α'-dipyridyl and 1,10-phenanthroline inhibit proline hydroxylase in cell cultures but simultaneous administration of these two agents increases proline hydroxylase activity in skin, gut and uterus by an unknown mechanism (Chvapil et al. 1974). The former agent inhibits collagen synthesis in quartz-dust induced granulomas in mice (Chvapil and Hurych 1969). The action of α, α'-dipyridyl was divided into two components: a short-term action depressing the proline hydroxylase activity, and a long-term one depressing collagen synthesis via a negative feedback mechanism produced by the intracellular accumulation of protocollagen (Chvapil and Hurych 1969). A number of agents were classified into three groups on the basis of their action (Blumenkrantz and Asboe-Hansen 1973). Group 1, including α, α'-dipyridyl, inhibits collagen synthesis via a primary action to depress hydroxylation and a secondary action to inhibit glycosylation of hydroxylysine. Group 2, including 1,10-phenanthroline

and tetracycline and group 3, including CaEDTA and the penicillamines, inhibit proline and lysine incorporation and hydroxylation, the latter group in a dose-responsive manner (Blumenkrantz and Asboe-Hansen 1973). Swiss albino mouse fibroblast 3T6 cells exposed to α, α'-dipyridyl secrete small molecular weight peptides due to the enhancement of collagen breakdown by the absence of the triple-helical conformation (Vistica 1975). Some agents may act by mechanisms in addition to metal sequestration; e.g., α, α'-dipyridyl decreases inorganic phosphate uptake by hepatic and renal mitochondria in the rat (Blumenkrantz and Asboe-Hansen 1973). Penicillamine tends to concentrate in tissues with high collagen turnover, such as skin, lungs, kidneys and liver, where it blocks the conversion of aldehydes to cross-links and cleaves Schiff bases (Grasedyck and Lindner 1975).

The absence of Fe^{++} mimicks the effect of the Fe^{++}-chelators, resulting in lack of hydroxylation of proline and lysine because the hydroxylases are not activated. Ascorbate deficiency enhances secretion of pro-α chains by 3T6 cells, probably because of increased dissociation of underhydroxylated pro-α chains. The lack of degradation of these chains to small peptides constitutes the major difference between the absence of ascorbate and the presence of α, α'-dipyridyl. This difference cannot be explained (Vistica 1975).

Any deficiency in α-ketoglutarate or tissue O_2 tension would impair proline hydroxylase activity and thus collagen synthesis because α-ketoglutarate produces part of the strong reducing environment required to keep iron in the divalent state, and O_2 is divided between the hydroxyl

group on hydroxyproline and α-ketoglutarate to produce succinate.

The investigation of lead effects on the rate of collagen degradation in older rats provides additional information on the impairment of this structural protein by lead. The soluble collagen resulting from new synthesis or solubilization of mature collagen in the intercellular matrix is acted upon by the enzyme collagenase, which cleaves the molecule three-quarters of its length from the NH_2-terminal end (Harris 1974). The cleavage is highly specific and facilitated by the helical configuration of the molecule (Harris 1974). The resulting two fragments are denatured at physiological temperatures (Kivirikko 1970). The ensuing loss of helical configuration renders these fragments susceptible to the action of nonspecific proteases, resulting in the production of small peptides and free amino acids.

Hydroxyproline in the free or peptide-bound form is not utilizable for new collagen synthesis because of the nonexistence of a codon for this imino acid. Therefore, only two metabolic alternatives exist; either excretion as such or degradation to simpler products. The free imino acid is rapidly oxidized in liver and kidney by a specific oxidase, resulting in the formation of Δ' -pyrroline-3-hydroxy-5-carboxylic acid, which is further broken down to urea and CO_2 via γ-hydroxy-L-glutamate (Adams 1973). The processes of tissue degradation and of hydroxyproline renal tubular reabsorption are so efficient that only 3% of total urinary hydroxyproline is found as the free imino acid (Ziff et al. 1956).

The balance of the urinary hydroxyproline, or about 97% is in the form of peptides, of which 75 to 80% are small peptides of molecular weight

700 to 900 (Kivirikkó 1970). The dipeptide pro-OHpro is excreted almost quantitatively because of inefficient peptidase activity (Weiss and Klein 1969). Both this dipeptide and the tripeptide gly-pro-OHpro are common sequences in collagen, implying that their appearance in the urine is caused by collagen catabolism (Schrohenloher et al. 1958). All peptides are rapidly cleared by the kidneys (Prockop et al. 1962) and only partially reabsorbed in the tubules (Benoit and Watten 1968).

The present study disclosed highly significant increases in the 24-hour urinary excretion of both hydroxyproline and proline in mature rats (4 to 13 months old) exposed to lead in the maternal milk since birth and 1, 1.5 or 2% lead acetate in the drinking water since weaning (Figure 15). The larger part of the urinary hydroxyproline excretion in normal mature animals is derived from insoluble collagen (Lindstedt and Prockop 1961). Yet a decrease in the rate of conversion of soluble to insoluble collagen will cause increased excretion of hydroxyproline (Kivirikko 1970). Lead-induced inhibition of collagen synthesis via underhydroxylation of the peptide chains and subsequent cleavage by intracellular proteases may result in decreased rate of insoluble collagen synthesis and enhanced degradation of the poorly hydroxylated soluble fraction.

An alternate explanation for the increased imino acid urinary excretion in the lead-treated mature rats in the present study may be the impaired function of the renal tubules indicated by the results of the renal function tests and histological studies reported below. The primary site of lead-induced renal damage appears to be the proximal

convoluted tubule epithelial cell, whose function is the reabsorption of many substances from the glomerular filtrate. Damage to these cells may produce impaired reabsorption of both the free and the peptide-bound imino acids resulting from a normal rate of collagen catabolism. However, the rate of hydroxyproline excretion in lead-treated rats was increased to a much greater extent than proline in the present study. Since hydroxyproline is derived from collagen only and proline is derived from collagenous and noncollagenous protein, the results indicate that increased degradation is, at least in part, responsible for the elevated urinary excretion rates in the presence of lead.

Chronic exposure to lead may result in nephropathy in both children and adults, with the primary lesion located in the renal tubular epithelium (Goyer and Rhyne 1973). Dysfunction of the renal tubules is manifested by the Fanconi syndrome, including glucosuria, aminoaciduria and phosphaturia in the presence of hypophosphatemia (Chisolm 1962). Aminoaciduria has been detected in rats (Sun et al. 1966). In the present study, aminoaciduria in the form of marked elevations in urinary excretion of both hydroxyproline and proline occurs in mature rats exposed to lead since birth, and may be due in part to impairment of tubular reabsorption for these imino acids.

A marked decrease in the functional capacity of the kidney was observed in the present study, as evidenced by highly significant decreases in the ability of mature lead-exposed rats to clear their plasma of inulin and para-aminohippuric acid. These decreases are dose dependent. The rate at which an inulin load is cleared from the plasma

provides an estimate of the glomerular filtration rate (GFR), inasmuch as the tubules are not involved in inulin excretion. The rate at which the kidney clears the plasma of a PAH load provides an index of the effective renal plasma flow (ERPF), since PAH is filtered at the glomerulus and secreted by the tubules. If indeed lead exposure causes injury to proximal tubular epithelium, a decrease in ERPF may result.

In an early study, 250-g albino rats treated with lead acetate, 35 mg/100 g by gavage three times a week for nine weeks, then with a quadrupled dose similarly administered for 10 additional weeks exhibited a 21% increase in tubular maximum for PAH ($^{T}m_{PAH}$) injected subcutaneously as a 6% solution. The increased $^{T}m_{PAH}$ was interpreted as due to an increase in tubular excretory mass (Pardoe 1952), possibly due to compensation by the kidney for any lead-caused damage. More recently, mature rats fed 1 to 4% lead acetate immediately after weaning for 10 to 40 weeks and tested during the course of the treatment displayed increased capability to excrete PAH when injected with 5 mg/kg intramuscularly. In the same study, renal cortical slices from 30-day old weanlings whose dams received 2 or 4% lead acetate diet since parturition likewise exhibited marked increase in uptake of PAH. Sequestration of lead in intranuclear inclusions, the low absorption of lead in the gut and the ability of the kidney to compensate for any damage caused by lead by increasing tubular excretory mass, are cited as explanations for the resistance of the rat kidney to the toxic effects of lead (Hirsch 1973). In addition, the excretion of lead by tubular secretion as well as glomerular filtration (Vostal and Heller 1968) tends to reduce blood

lead levels and total body burden of lead.

The conflict between these reports and our results may be due in part to differences in methodology. The subcutaneous injection of PAH and urine collection over a 30-minute period starting 15 minutes after injection (Pardoe 1952), or intramuscular injection of PAH and subsequent 2-hour urine collection (Hirsch 1973), utilizing an oral water load for diuresis 15 minutes before PAH injection and collection of one blood sample at the end of the urine collection period (Pardoe 1952, Hirsch 1973) might not yield steady state conditions. In the present study, blood and urine samples were collected from animals which had been infused via the tail vein at a constant rate for at least an hour so as to allow for equilibration, with an infusion solution that also contained mannitol for diuresis. This provides a more accurate gauge of renal function than a single injection or isolated tissue preparations.

The renal tubular dysfunction is correlated with impaired mitochondrial function (Goyer et al. 1970a). Ultrastructural defects include intranuclear inclusion bodies, mitochondrial swelling and vesiculation of cristae (Mao and Molnar 1967). Mitochondrial membrane distortion produced by the swelling may explain the impairment of the activities of the membrane-bound enzymes cytochrome oxidase and NADH-cytochrome c reductase observed in isolated rabbit kidney mitochondria bathed in 0.5 mM lead acetate in sucrose-Tris solution (Iannaccone et al. 1974). Uncoupling of oxidative phosphorylation, with consequent reduction in the rate of ATP synthesis and deficiency in the amount of energy available for use in active transport systems in the renal tubules may result.

Histological studies of the kidneys of mature animals chronically exposed to lead revealed intranuclear inclusions, cell vacuolation, karyomegaly, hyperchromatic cell nuclei, some cell necrosis and desquamation of strips of epithelium in the distal segment of the proximal convoluted tubule in man (Blackman 1936, Popoff et al. 1963, Clasen et al. 1974), dog (Zook 1972), rabbit (Hass et al. 1964) and rat (Pardoe 1952, Hirsch 1973). The necrosis was accompanied by numerous mitoses (Pardoe 1952) which this author interprets as evidence of the high regenerative capacity of the tubular epithelium triggered by nonspecific damage, rather than a specific stimulation of DNA synthesis by lead (Choie and Richter 1973). Present findings include dilation of tubules and epithelial cell flattening in mature rats exposed to lead since birth and drinking 1.5% lead acetate since weaning. The greater severity of the damage, consisting of tubular necrosis and some interstitial fibrosis in the 2% lead acetate group, correlates with the dose dependence of the impairment of PAH clearance. The lack of evidence of intranuclear inclusions universally reported in the literature on lead-induced renal damage may be due to the nonspecificity of the stain used. The apparent absence of pathological changes in the glomeruli of the chronically lead-exposed kidney of the mature rat is in agreement with previous reports (Pardoe 1952, Zook 1972). Since the glomeruli are apparently normal, the significant decreases in GFR observed in mature rats chronically exposed to lead in the present study may be due to the development of interstitial fibrosis in the kidney. Such fibrosis may cause impairment of blood flow in the capillaries surrounding the tubule, which in turn may cause a feedback

effect to produce a decrease in the rate of blood flow through the glomeruli.

Chronic exposure ot lead causes an anemia via the inhibition of heme synthesis. One enzyme inhibited by lead, δ-ALA dehydrase, catalyzes the conversion of δ-ALA to porphobilinogen, the second step in the pathway. This enzyme is dependent on zinc, and possesses a SH group that is essential for activity (Finelli et al. 1975). Binding of the SH group by lead is the postulated basis for the extreme susceptibility of the dehydrase to lead (Schroeder and Balassa 1961). This susceptibility is illustrated by the observation that lead in a concentration of 2×10^{-7}M depressed enzyme activity in rabbit blood (Bruin and de Jong-Heisterkamp 1968), and provides the basis for the exquisite sensitivity of the urinary test for δ-ALA as an indication of undue lead exposure (Davis et al. 1968).

In the present study, substantial increases in the 24-hour urinary excretion of δ-ALA were observed in mature rats (4.5 to 13 months of age) exposed to lead in the maternal milk since birth and to 1, 1.5 or 2% lead acetate in the drinking water since weaning. The level of excretion in the low dose group was approximately five times control and yet only half the level of excretion in the high dose group, illustrating a high degree of dose dependence.

SUMMARY

Collagen synthesis was examined at two, three, four and six weeks in rats exposed to lead via the milk of dams receiving 1, 1.5 or 2% lead acetate in the drinking water since parturition. The incorporation of ^{14}C-proline and unlabelled proline into the 0.45M NaCl-soluble fraction of skin collagen was decreased at the two higher dose levels from two to four weeks. The decrease in hydroxylation rate of ^{14}C-proline in soluble collagen evidenced by the lower percent of radioactivity in soluble collagen hydroxyproline from three to four weeks suggests underhydroxylation of the procollagen synthesized. Decreases in the insoluble collagen content of skin did not appear until three weeks. The lag of one week in the inhibition of insoluble collagen synthesis may indicate the time required for a decrease in soluble collagen pools to affect mature collagen content. Parallel decreases in unlabelled hydroxyproline and proline content of insoluble collagen at three and four weeks implies that underhydroxylated collagen is not converted to insoluble collagen or, if converted, is rapidly degraded. Lead-induced inhibition of collagen biosynthesis was no longer evident by the end of the sixth week of life.

The decreased collagen synthesis was accompanied by a general growth and developmental retardation. However, neurological symptoms were not observed in lead-treated animals except for occasional urinary incontinence.

Significant increases in the 24-hour urinary excretion of both imino acids occurs in older rats exposed to lead since birth. These

increases may be due to two factors: 1) the deleterious effects of lead on collagen metabolism, whether in the form of decreased synthesis or enhanced catabolism, and 2) the impairment of the reabsorptive capacity of the renal tubules for amino acids, suggested by decreases in effective renal plasma flow (ERPF). The observation that hydroxyproline excretion was greater than proline excretion implies that increased collagen degradation rather than increased noncollagenous protein degradation provides the major source for the elevation in urinary imino acid excretion in lead-treated mature rats.

The major lesion caused by chronic exposure to lead appears in the kidneys, specifically in the proximal convoluted tubules, as evidenced by highly significant decreases in ERPF as well as in glomerular filtration rate. Renal function impairment was accompanied by histological findings of tubular dilation and degeneration, and interstitial fibrosis at the two higher levels of lead exposure. The significant decreases in glomerular filtration rate in the absence of histological defects in the glomeruli are attributed to the presence of interstitial fibrosis, which may cause a decrease in blood flow through the glomeruli consequent to a decrease in flow through the peritubular capillaries.

The deleterious effect of lead on heme biosynthesis was verified by the significant, dose-dependent increases in the 24-hour urinary excretion of δ-aminolevulinic acid (δ-ALA) observed in mature rats under lifelong lead exposure.

Further studies that might add to the understanding of the mechanisms by which lead impairs collagen metabolism in the young could be conducted

utilizing cofactors, for example ascorbate or Fe^{++} in supplemented or deficient diets. Since the mechanism of action of these cofactors in collagen synthesis is known, their interaction with lead may yield information on the mechanisms of lead toxicity.

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