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Effects of Chronic Lead Exposure on Levels of Acetylcholine and Choline and on Acetylcholine Turnover Rate in Rat Brain Areas in vivo*

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Abstract. Rats were exposed to lead acetate from birth, and were killed at the age of 44–51 days for analysis of levels and turnover rates of acetylcholine (ACh). Steady-state levels of ACh were not altered in midbrain, cortex, hippocampus, or striatum of lead-exposed rats. Similarly, no changes in choline (Ch) concentrations were found in cortex, hippocampus, or striatum. In the midbrain, however, a 30% reduction in Ch levels was observed. Changes in specific activity of Ch and ACh were measured as a function of time in selected brain areas of rats infused with a radio-labeled precursor of Ch. Specific activities of ACh were not altered. Ch specific activities were, however, significantly elevated in all brain areas examined, as compared with age-matched control rats. The in vivo ACh turnover rate in cortex, hippocampus, midbrain, and striatum was diminished by 35%, 54%, 51%, and 33%, respectively. These findings provide direct evidence for an inhibitory effect of lead exposure from birth on central cholinergic function in vivo. Since a significant reduction of body weight was found in those animals treated with lead acetate, the alteration of central cholinergic function may partially be attributed to malnutrition observed in the lead-exposed animals.

Key words: Cholinergic function – Acetylcholine – Choline – Levels – Turnover rates – Gas chromatography – Lead poisoning – Malnutrition – Central nervous system

It has recently been demonstrated in some laboratories that rodents and monkeys, chronically exposed from birth to inorganic lead, will exhibit a behavioral hyperactivity (Sauerhoff and Michaelson, 1973; Silbergeld and Goldberg, 1973; Allen et al., 1974). Pharmacologically, such an animal model appears to respond to certain drugs in a manner similar to that seen in hyperkinetic children (Silbergeld and Goldberg, 1974a). In lead-exposed mice, the observed increase in motor activity was suppressed by the administration of amphetamines, methylphenidate, cholinergic agonists, and aminergic antagonists, and was exacerbated by aminergic agonists and the anticholinergic agent atropine (Silbergeld and Goldberg, 1974b, 1975).

Neurochemical effects of early lead exposure have been studied extensively. Recent reports using rats and mice indicate that both levels and turnover rate of brain norepinephrine are increased by lead exposure (Michaelson et al., 1974; Golter and Michaelson, 1975; Silbergeld and Goldberg, 1975; Hrdina et al., 1976; Jason and Kellog, 1977), whereas dopamine levels remain unchanged (Sobotka et al., 1975; Silbergeld and Goldberg, 1975; Golter and Michaelson, 1975; Grant et al., 1976; Schumann et al., 1977). In addition, two catecholamine metabolites, homovanillic acid and vanillylmandelic acid, were significantly increased in brain and urine of lead-exposed mice (Silbergeld et al., 1975; Silbergeld and Chisolm, 1976), indicating a probable increase in the metabolism of norepinephrine and dopamine, in vivo, respectively.

In studies dealing with the cholinergic system, Silbergeld and her coinvestigators have shown that in lead-exposed mice there is a marked decrease in high affinity uptake of Ch in forebrain synaptosomes (Silbergeld and Goldberg, 1975) and a decrease in postassium-stimulated release of both ACh and Ch from lead-treated cortical minces (Carroll et al., 1977), indicating a generalized reduction in cholinergic function in brains of lead-treated mice. According to these same investigators, the steady-state levels of ACh were not different between control and lead-treated animals. Other investigators (Modak et al., 1975; Sobotka et al., 1975; Hrdina et al., 1976) have, however, reported changes in the level of ACh and in the activity of the

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cholinergic enzymes, acetylcholinesterases, and choline acetyltransferase, in different brain areas of their lead-intoxicated experimental animals.

Whether or not lead treatment has some selective effect on ACh of Ch levels in the brain, changes in levels of a transmitter or in its enzyme activities would not necessarily allow any conclusions to be drawn regarding the effect of lead exposure on the dynamics of brain cholinergic function. For this, one must resort to an approach that provides a more dynamic index of brain cholinergic mechanisms. We therefore elected to conduct a study of ACh turnover rate in vivo, which is an important measure for monitoring the neurochemical function of the cholinergic synapses during adaptation to environmental changes imposed by drug administration or other external means (Hanin and Costa, 1976).

We have been intrigued by this potential animal model of hyperkinesis in children. We have, therefore, studied the effect of neonatal lead exposure on ACh turnover rate in selective brain areas of rats in vivo. In this report, which describes the results of such studies, our data provide direct evidence for a profound inhibitory effect of chronic lead exposure on the development of cholinergic function in the immature rat brain in vivo.

Materials and Methods

Procedures for Handling and Feeding Animals. A group of rats was exposed to dietary lead according to a procedure described earlier (Shih et al., 1977). Briefly, lactating Sprague-Dawley rats with 10 pups each (of both sexes) were supplied from Zivic-Miller Labs (Allison Park, Pennsylvania) on the day the pups were born. Each dam, with 10 pups as obtained, was individually housed in temperature-controlled animal quarters that were maintained on a 12-h light-dark cycle, with light on from 6 a.m. to 6 p.m. Eight nursing dams and their pups were randomly divided into two groups, and dietary treatment was initiated immediately. The control group of mothers was given regular chow (Wayne Lab Blox). The other group received powdered chow containing 4% lead acetate (certified grade, Fisher Scientific Co.). Thus lead was supplied indirectly to the experimental group of pups through the milk of the lactating dams (Pentschew and Garro, 1966). Food and water were provided ad libitum to both groups of animals.

At the age of 5 days, the litters were culled to eight sucklings per nursing mother. At 17 days of age the diet for the experimental group was changed from 4% lead acetate to that containing 40 ppm lead. Upon weaning (21 days of age), the pups were separated according to sex, isolated from the mother, group housed, and fed with powdered chow containing 40 ppm lead acetate. They were maintained on this diet for approximately four weeks until termination of the experiment. The body weight of the animals was measured at the time of sacrifice in order to monitor for potential effects of lead on animal nutritional development.

Experimental Procedure. At the age of 44–51 days a total of 60 animals of both sexes were used for the experiment. Phosphoryl (Me-^{14}C) Ch (44 mCi/mmol, New England Nuclear Corp., Boston, Massachusetts) was infused i.v. at a constant rate (40 μCi/kg/min) into the tail vein, according to the method described by Racagni et al. (1974). It has been demonstrated that the steady-state concentrations of Ch and ACh in brain areas do not change significantly when radiolabeled phosphorylcholine is infused under such conditions at a constant rate for 8 min. During the infusion the rats were confined in small plastic cylinders. At 4, 6, and 8 min after labeling, the rats were killed by focusing a beam of microwave radiation (2.0 kW at 2.45 GHz, Medical Engineering Consultants, Lexington, Massachusetts) on the head for 3 s. This procedure inactivates instantaneously any enzymatic activity that might induce postmortem changes of steady-state concentrations of Ch or ACh (Stavinoha et al., 1973; Guidotti et al., 1974). All of the animals were killed approximately at the same time of day, 10 a.m. – 12 noon, in order to avoid variation due to circadian periodicity (Hanin et al., 1970; Friedman and Walker, 1972; Saito et al., 1975).

Preparation of Brain for Analysis of Levels and Specific Activities of Ch and ACh. After the animal was killed by microwave fixation focused on the head, discrete brain areas (cortex, hippocampus, midbrain, and striatum) were dissected, weighed, and immediately homogenized in 3 ml ice-cold 0.4-N perchloric acid. The homogenates were centrifuged and the pH was adjusted to 4.0–4.2 with a predetermined volume of potassium acetate (7.5 N) in water. The extraction and chemical derivatization of Ch and ACh in brain tissues were then processed as described by Jenden and Hanin (1974), using butyrylcholine as an internal standard. A radio gas chromatographic (GC) assay procedure for simultaneous measurements of levels and specific activities of Ch and ACh was utilized as previously described (Hanin et al., 1973). A Packard model 825 fraction collector was attached to a Packard model 7401 GC system.

Using this set-up, 90% of the effluent from the GC column is diverted via a heated transfer line (95 C) to the fraction collector, which is maintained at room temperature. The carrier gas passes through the trapping medium (molecular sieve 5A, 45–60 mesh, Hewlett-Packard, Avondale, Pennsylvania), while the radiolabeled tertiary amines are trapped by the highly reactive molecular sieve. The flame ionization detector (FID) of the GC senses the remaining 10% of the effluent from the GC column. During monitoring of the FID response, the tertiary amines are selectively trapped as they are seen to emerge from the GC column. The trapped radioactivity of each sample is then counted by transferring the entire contents of each trap to a scintillation counting vial containing 15 ml of scintillation fluid (consisting of 15 g of PPO, 0.6 g of POPOP, 1 l of Triton-X 100, 2 l of toluene) and 2 ml of H_2O.

Radioactivity was measured with a Packard Model 3390 Tri-Carb Liquid Scintillation spectrometer. The concentration of Ch and ACh was calculated from the FID record, from the corresponding peak heights. Specific activities of each compound were then determined and reported as counts per min per nmol of substance.

Since there were no differences in the levels of Ch and ACh between male and female rats in either control or lead-treated groups, the data presented here were pooled from both sexes within each treatment group. The fractional rate constants of ACh were calculated using the finite differences method of Neff et al. (1971). The statistical comparisons of concentrations of Ch or ACh between control and lead-treated animals were performed according to the two-tailed Student's *t*-test.

Results

Postnatal lead ingestion resulted in significant retardation of the growth of the experimental rats. At the time the neurochemical study was performed (44–51 days of age), the mean body weight ± SE of 30 rats fed with lead acetate was 149 ± 3 g, while the 30 age-matched controls weighed 169 ± 3 g ($t = 4.60$, $df = 58$,

Table 1. Effect of lead exposure on regional distribution of acetylcholine and choline levels in neonate rat brain[a]

Brain region	nmoles/g wet weight[b]			
	acetylcholine		choline	
	control	lead-treated	control	lead-treated
Cortex	19.6 ± 3.04 (27)	23.5 ± 3.61 (30)	20.0 ± 2.79 (27)	23.3 ± 3.52 (30)
Hippocampus	12.4 ± 0.62 (20)	11.8 ± 0.42 (27)	52.7 ± 4.23 (25)	50.7 ± 4.17 (30)
Midbrain	30.9 ± 3.21 (21)	30.6 ± 3.60 (27)	61.3 ± 4.70 (20)	42.8 ± 2.80 (26)*
Striatum	50.0 ± 2.37 (18)	46.1 ± 2.07 (20)	40.0 ± 1.43 (18)	40.4 ± 0.94 (20)

[a] Rats were sacrificed by microwave irradiation of the head (3 s). See text for conditions of lead exposure
[b] Values represent mean ± SE for number of rats shown in parentheses
* $P < 0.001$ (two-tailed Student's t-test)

$P < 0.001$). These results replicate our previously reported observation of growth retardation in a group of older (55 – 70 days of age) rats after chronic exposure to the same dietary lead regimen as described here (Shih et al., 1977). During this period (up to 51 days) of lead treatment, no tremors or convulsions were observed in the experimental animals. However, the lead-exposed rats were noted to be more irritable and agitated when they were disturbed in the cage, or when the experimentor tried to pick them up.

The data reported in Table 1 show that chronic lead-exposure in neonate rats caused no reliable change in steady-state levels of ACh in any of the four brain areas studied (cortex, hippocampus, midbrain, and striatum). Moreover, Ch concentrations were not significantly altered by chronic lead ingestion in any of the brain areas investigated, except in the midbrain, where a 30 % reduction was found in those lead-exposed rats (compared with control rats; $t = 3.54$, $df = 44$, $P < 0.001$).

Figures 1 A and 1 B illustrate the specific activities of ACh and Ch, which were measured in the cortex and midbrain respectively, after 4, 6, and 8 min of i.v. infusion of phosphoryl (Me-^{14}C) Ch. Similar curves (not shown here in order to avoid repetition) were obtained with the hippocampus and with the striatum. In all four cases, specific activities, expressed in terms of cpm/nmol of ACh, were unaltered. At the same time, the specific activities of Ch in lead-treated pups were significantly elevated in all brain areas studied when compared with age-matched control animals.

The turnover rate of ACh, by definition, is equal to the product of the fractional rate constant, K_B, and the steady-state concentration of ACh. K_B was calculated from the following relationship derived by Neff et al. (1971):

$$K_B = \frac{2(SACh_{t_2} - SACh_{t_1})}{(t_2 - t_1)[(SCh_{t_1} - SACh_{t_1}) + (SCh_{t_2} - SACh_{t_2})]}$$

where SACh and SCh represent specific activities of ACh and Ch, respectively, and t_2 and t_1 are arbitrary time points (in this case one minute apart) selected on the figure representing specific activity changes as a function of time (e.g., in Figs. 1 A and 1 B).

Table 2. Effect of lead exposure on the relative turnover rate of acetylcholine (ACh) in designated rat brain areas

Brain area	Relative turnover rate of ACh[a]
Cortex	0.65 ± 0.11 (4)[b]
Hippocampus	0.46 ± 0.18 (4)
Midbrain	0.49 ± 0.03 (6)
Striatum	0.67 ± 0.04 (6)

[a] Expressed as ratio of K_Bs of lead-treated to control animals
[b] Values represent mean ± SE for number of determinations shown in parentheses

Since in our studies ACh concentrations were not changed by the lead treatment (see Table 1), the relative changes in K_B values should, therefore, give us an indication of the changes of the turnover rate of ACh. We have calculated K_B values in specific brain areas of lead-treated rats and in corresponding areas of control animals, and have subsequently expressed changes in ACh turnover rate as fractions of K_B in treated, as compared with control animals. Such relative changes are shown in Table 2. It is evident from Table 2 that lead exposure reduced by 35 %, 54 %, 51 %, and 33 % the in vivo turnover rate of ACh in cortex, hippocampus, midbrain, and striatum, respectively.

Discussion

The concept of lead-induced hyperactivity in animals as an analog or model of hyperkinesis is not as yet uniformly accepted because of difficulties in some laboratories in replicating the reported increase in motor activity (Sobotka and Cook, 1974; Modak et al., 1975; Grant et al., 1976; Krehbiel et al., 1976; Schumann et al., 1977). Nevertheless, it has prompted relatively extended studies on the neurochemical effects of postnatal lead exposure in animals in an attempt to

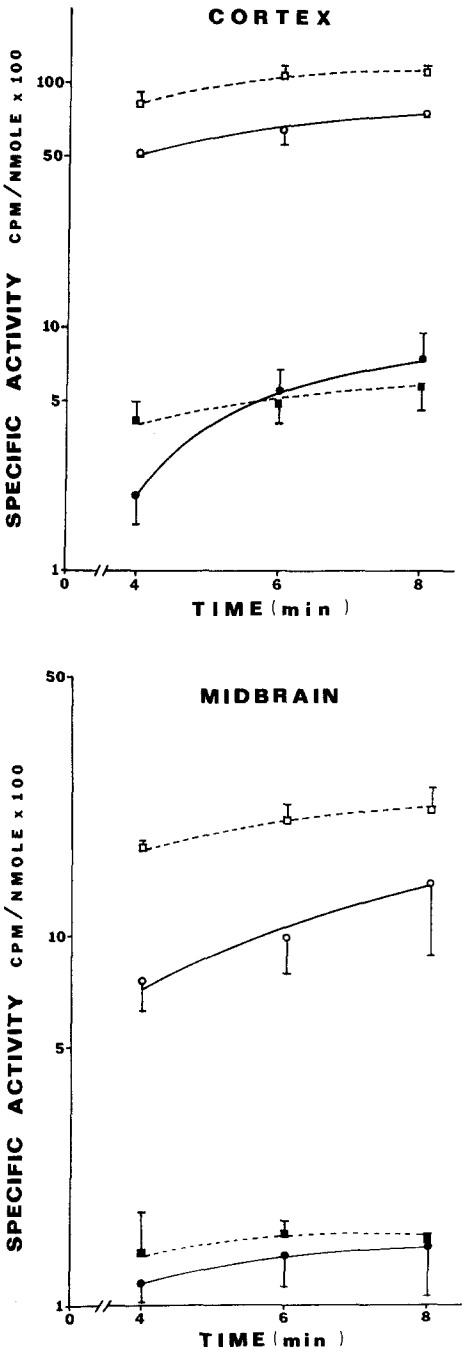

Fig. 1. Specific radioactivities of choline (○ or □) and acetylcholine (● or ■) in rat cortex (A) and midbrain (B) at various times during infusion of phosphoryl (Me-^{14}C) choline in lead-treated (*dashed line*) and control (*solid line*) animals. *Each point:* mean ± SE of 3 determinations

investigate and understand the implications of neurochemical consequences of lead poisoning in children. Such studies are extremely important, particularly in view of the fact that subclinical lead poisoning in children has in recent years been reported to be associated with long-lasting impairment of behavioral and cognitive function (David et al., 1972, 1976; De LaBurde and Choate, 1972; Perino and Ernhart, 1974; Landrigan et al., 1975) and chronic lead exposure in immature animals has demonstrated similar sequelae of impaired behavior and learning ability (Snowden, 1973; Sobotka and Cook, 1974; Brown, 1975).

Early lead exposure has been repeatedly shown to result in a significant loss in body weight of experimental animals relative to age-matched controls (our own observations; Michaelson, 1973; Clasen et al., 1974; Krigman et al., 1974; Golter and Michaelson, 1975; and others). Since our lead-exposed animals exhibited impaired growth patterns as compared to identically handled controls, it is conceivable that the early impairment of growth, caused by the lead ingestion, alters central cholinergic functions. Most recently, in fact, Michaelson and his coinvestigators (Loch et al., 1976; Michaelson et al., 1977) have suggested that the effect of lead is indirect; i.e., that the increased locomotor activity and paradoxical responses to amphetamine and phenobarbital treatment are the result of the undernutrition induced by lead exposure in these experimental animals. This notion is intriguing, but still should be considered tentative, since these observations contradict the earlier report of Michaelson and Sauerhoff (1974), in which they were able to demonstrate that lead-exposed animals and pair-fed controls do not, in fact, exhibit similar increases in spontaneous activity. Recently, the latter observation has also been confirmed by Wince et al. (1976), who compared lead-exposed rats with pair-fed controls and demonstrated, nevertheless, that the lead-exposed animals showed a significant increase in gross motor activity. Moreover, in a study by Modak et al. (1975) lead-exposed experimental rats showed a significant reduction in body weight, but no increased motor activity or even slight tremor was ever observed.

Studies across a number of laboratories, dealing with the cholinergic system, have indicated that the effect of chronic lead-exposure on central cholinergic parameters is relatively consistent. In mice, a 48% decrease in Ch high affinity uptake from forebrain synaptosomes and a decrease in potassium-induced release of Ch and ACh from lead-treated cortical minces have been demonstrated (Silbergeld and Goldberg, 1975; Carroll et al., 1977). In lead-exposed rats, the activity of both acetylcholinesterase and choline acetyltransferase were significantly altered in certain brain areas (Modak et al., 1975; Sobotka et al., 1975). The results of the present study, demonstrating diminished ACh turnover rate in rat brain areas in vivo after chronic lead exposure, further confirm the general observation from all these findings that, in lead treated animals, there is a deficit in central cholinergic function.

These findings of an inhibitory effect of chronic lead treatment on the cholinergic function in brain areas in vivo is consistent with the effects in vitro of lead on peripheral nervous tissue. Peripherally, after lead treatment, a decreased output or release of ACh has been found in vitro in the sympathetic ganglion (Kostial and Vouk, 1957; Kober and Cooper, 1975) and at the neuromuscular junction (Manalis and Cooper, 1973; Silbergeld et al., 1974). Since the effects in vitro do not depend on the nutritional status of the animals, it is unlikely in those cases that the malnutrition at early life is the major cause of the observed neurochemical alterations.

Our observation that postnatal lead treatment resulted in no change in steady-state levels of ACh in any of the four brain areas studied agrees with the observations of Silbergeld and coinvestigators (Silbergeld and Goldberg, 1975; Carroll et al., 1977) in mouse forebrain, and with the report of Modak et al. (1975) in rat cerebellum, cortex, hippocampus, midbrain, medulla-pons, and striatum. In all these cases, chronic lead exposure caused no changes in levels of ACh. The only area which did show a significantly higher level of ACh by the latter report (Modak et al., 1975) was the diencephalon-thalamic area. However, a recent paper by Hrdina et al. (1976) showed a 32–48% increase of concentrations of ACh in rat cortex of lead-treated animals. This discrepancy in the results might be due to differences in doses of lead, duration of lead exposure, time and method of administration to the suckling animals, the general conditions of the animals, the age of the animals when the studies were performed, and the means of killing the animals.

In addition to ACh, we have also investigated the effect of lead treatment on levels of Ch in various brain areas. In the present study, Ch concentration was not significantly altered by lead tratment in the brain areas investigated, except in the midbrain, where a 30% reduction was found in those lead-exposed rats. A recent report by Carroll et al. (1977) also found no change in Ch steady-state levels in the forebrain of their lead-treated mice.

Levels of Ch were unaltered in most brain areas tested, yet specific activities of Ch were elevated in lead-treated animals. This could be attributable to the overall reduction in central cholinergic activity induced by the treatment with lead. Thus, a reduced amount of radiolabeled precursor Ch would be incorporated into its radiolabeled products (phospholipids, ACh) within a specific period of time. The end result of this function could be an increased accumulation of the free radiolabeled Ch, and hence a higher overall specific activity of brain Ch pools in the lead-treated animals.

It is becoming increasingly evident from available literature, as well as from the present studies, that chronic ingestion of lead in animals causes increased aminergic function and, on the other hand, impairment of cholinergic activity. These neurochemical findings may be of significant clinical importance in view of the fact that increasing numbers of reports in the literature have documented an alarming increase in lead absorption in children, particularly as a result of paint chip ingestion (Oberle, 1969) or environmental lead pollution (Wessel and Dominski, 1977). Although a large proportion of these children shows biochemical evidence of impaired heme synthesis, few have exhibited overt clinical symptoms (NRC, 1972). It is not known whether the subclinical degree of increased lead absorption may be causing either transitory or permanent impairment of the immature developing nervous system in these young children. Therefore, these findings in experimental animals might have some very important implications in studying and evaluating the consequences of chronic lead ingestion by children.

Lead-induced hyperactivity in animals is still a tenuous analog or model of hyperkinesis in children. The similarity in the effect of pharmacologic treatment in lead-induced hyperactivity and in hyperkinesis in children is, nevertheless, quite intriguing. The CNS stimulants, amphetamines and methylphenidate, have enjoyed widespread use clinically in the symptomatic treatment of minimal brain dysfunction or hyperkinesis in children (Millichap and Fowler, 1967; Werry, 1968). These drugs also work to suppress the lead-induced hypermotor activity in animals (Silbergeld and Goldberg, 1976). If, indeed, lead-exposure causes an impairment and reduction in central cholinergic activity, then one would expect that these CNS stimulants activate the cholinergic system to reestablish the balance between cholinergic and aminergic function in the hyperkinetic subject. In fact, evidence for an activating effect of these stimulant agents on CNS cholinergic function is increasing in both animal experiments (Pepeu and Bartolini, 1968; Shih et al., 1974; Bryan and Ellison, 1975; Silbergeld and Goldberg, 1975; Shih et al., 1976) and human studies (Porges et al., 1975; Porges, 1976).

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