7. We have studied the decreased conductance EPSP in 22 experiments. In several other experiments, this EPSP was not initially observed, but could be revealed by varying one or more parameters of connective stimulation, such as pulse intensity, duration, or polarity. In a few cases, however, connective stimulation produced a slow depolarizing response that resembled the slow EPSP in its time course, but differed from it in being associated with a conductance increase. The source of this variability is not known. Most likely it is due to the fact that the connective is a mixed nerve and that several different inputs to the motor cells, with similar thresholds to electrical stimuli, are activated. Alternatively, variations in the resting K^+ conductance in the ink gland motor cells in different preparations might contribute to the variability of the decreased conductance EPSP.
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10. Increasing Mg^{2+} increased the input resistance by 30 to 50 percent in the ink gland motor cells, but this effect was not specific. The input resistance of nerve cells that do not receive the decreased conductance EPSP, such as the gill motor cell L7, was also increased by the same amount.
11. Animals were anesthetized by injection of isotonic $MgCl_2$ (25 to 50 percent of body weight) to prevent inking during dissection. The abdominal ganglion and the mantle (containing the ink gland) were excised and pinned in a recording chamber with a Sylgard floor. Seawater was perfused across the gland and channeled across the photocell to measure the inking response [for details, see (*6*)]. The siphon was stimulated by means of platinum wires sewn into the skin; the connectives were stimulated by means of Ag-AgCl electrodes built into the chamber.
12. Another factor that might contribute to the augmentation of the depolarizing pulse is the possibility that the decreased conductance EPSP may reduce the voltage threshold for spike initiation [for a discussion, see M. V. L. Bennett, B. Hille, S. Obara, *J. Neurophysiol.* **35**, 585 (1970)].
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15. The coupling coefficient from cell 1 to cell 2 (K_{12}) is given by $K_{12} = r_2/(r_2 + r_c)$, where r_2 is the input resistance of cell 2 and r_c is the junctional resistance. From this equation it follows that the greater r_c is in relation to r_2, the greater will be the enhancement of coupling due to a decreased conductance EPSP [see M. V. L. Bennett, *Ann. N.Y. Acad. Sci.* **137**, 509 (1966)].
16. We wish to thank J. Koester and M. V. L. Bennett for helpful comments on earlier drafts of the manuscript, and A. K. Hilten for help with the illustrations. This research was supported by a Foundations Fund for Research in Psychiatry fellowship 72-524 to T.J.C., career scientist award 5-K05-MH-18558-09 to E.R.K., and NINCDS grant 2-R01-NS12744-02.

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Lead Poisoning: Altered Urinary Catecholamine Metabolites as Indicators of Intoxication in Mice and Children

Abstract. *Whether neuropsychological impairment occurs in children with increased lead absorption who are without clinical symptoms is of current concern. This issue, which involves potentially large numbers of children, remains unresolved, in part because of the lack of sensitive biochemical indicators of the effects of lead on the nervous sytem. In experimental subclinical lead poisoning in mice, significant increases in homovanillic acid and vanillylmandelic acid have been found in brain and urine. In children with increased lead absorption, these acids were measured in urine collected quantitatively under controlled dietary conditions; preliminary results show fivefold increases in the daily output of these compounds. These data suggest that the altered catecholamine metabolism also occurs in children.*

Current screening programs have revealed many young children with increased lead absorption. While a high proportion of these children have biochemical evidence of impaired heme synthesis, few have clinical symptoms compatible with plumbism (*1*). It is unknown whether this subclinical degree of increased lead absorption causes either transitory or permanent impairment of the developing nervous system in the very young. Studies of children by standard psychometric and neurophysiological techniques have yielded conflicting results: some reports suggest that there is a statistically significant association between asymptomatic or mildly symptomatic increased lead absorption and subtle, but long-lasting, impairment in behavior and cognitive function (*2*), but other studies have not found lead-associated deficits (*3*). The role of lead in the causation of impaired nervous system function, in the absence of encephalopathy, is difficult to determine, in part because of the lack of sensitive neurochemical indicators of effects of lead on the nervous system (*4*). We report here initial data on the urinary output of two catecholamine metabolites, 4-hydroxy-3-methoxymandelic acid (vanillylmandelic acid, VMA), and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA), in asymptomatic and mildly symptomatic young children with increased lead absorption and biochemical evidence of disturbed heme synthesis. The preliminary results strongly suggest that catecholamine metabolism is altered in such patients.

The clinical investigations were undertaken after studies on an animal model of subclinical lead poisoning. Animal models of childhood lead poisoning have demonstrated sequelae of impaired learning and hyperactivity (*5–7*). Associated with these disorders are significant alterations in behavioral response to several drugs, particularly an attenuation or, in lead-treated hyperkinetic mice, a reversal of amphetamine-induced stimulation of motor activity (*7–9*). Neurochemically, long-term postnatal exposure of rodents to lead produces enhanced noradrenergic function and inhibition of cholinergic function in the central nervous system (*9–11*).

The observation that in vivo lead exposure alters the function of at least one catecholamine neurotransmitter provided the rationale for investigating the usefulness of measuring the catecholamine metabolites HVA and VMA in the urine of lead-treated hyperkinetic mice and of children with increased lead absorption. In mice, brain levels of HVA and VMA were also measured.

Lead-exposed mice (CD-1, Charles River Laboratories) aged 40 to 70 days were used in these studies; methods of postnatal exposure of mice to lead acetate in drinking water (5 mg/ml) are described in (*5*). The mice used in these studies were exposed continuously from birth; no decreases in brain or body weights resulted from long-term lead treatment. Mice were housed individually in stainless steel metabolism cages for 24 hours for urine collections. The cages were washed with 0.01*N* HCl and these washes were combined with urine for assays. The urine and washes acidified to *p*H 2.0 and whole forebrains ob-

Table 1. Concentrations of HVA and VMA in whole brain and urine of control and lead-treated mice (males, 40 to 70 days of age). Values are means ± standard errors of the mean. Each determination was done in duplicate.

Metabolite	Concentration in brain (μg/g, wet weight)		Brain, lead/control (%)	Concentration in urine ($\mu g\ ml^{-1} day^{-1}$)		Urine, lead/control (%)
	Control	Lead		Control	Lead	
HVA	0.092 ± 0.008 (N = 12)	0.122 ± 0.013 (N = 9)	133*	6.510 ± 1.130 (N = 11)	17.292 ± 4.216 (N = 11)	265*
VMA	0.060 ± 0.001 (N = 6)	0.089 ± 0.009 (N = 6)	148*	6.829 ± 1.632 (N = 9)	14.782 ± 2.291 (N = 11)	216*

*$P < .05$.

Table 2. Urinary HVA and VMA in children with increased lead absorption and in controls. Children with increased lead absorption were studied with a protocol approved by the Joint Committee on Clinical Investigation of the Johns Hopkins Medical Institutions; they were drawn from the Childhood Lead Clinic of the John F. Kennedy Institute, Baltimore, during June and July 1975. Data are for quantitative 24-hour urine collections obtained before treatment. Among the six children, two were hyperirritable, one was hyperactive, and the rest were clinically asymptomatic. Other pertinent data are: age range, 16 to 42 months; range in surface area, 0.43 to 0.67 m^2; hematocrit, 35 to 42 percent; blood lead concentrations, 59 to 68 μg per 100 ml of whole blood (*17*); and free erythrocyte protoporphyrin, 500 to 800 μg per 100 ml of erythrocytes (*18*). Control data were derived from inpatients at the Pediatric Clinical Research Unit of the Johns Hopkins Hospital without neurological disorders and from volunteers aged 3 to 10 years. All controls resided in suburban areas in new housing with little opportunity for lead exposure. None were anemic.

Metabolite	Concentration in urine (mg m^{-2} day^{-1})			
	Control (N = 6)		Lead-exposed (N = 6)	
	Mean	Range	Mean	Range
HVA	4.520	1.907–7.004	27.541	11.060–59.350
VMA	2.551	1.964–2.997	14.070	6.447–24.332

tained by cervical dislocation and homogenized in 0.4N perchloric acid were assayed by microscale adaptations of published methods for HVA (*12*) and VMA (*13*). Lead treatment resulted in a 33 percent increase in brain HVA and a 265 percent increase in urinary HVA; brain VMA was increased 48 percent and urinary VMA was increased 216 percent in lead-treated mice (Table 1). Since urinary HVA and VMA probably reflect peripheral catecholamine metabolism, these results suggest alterations in both peripheral and central catecholamine metabolism in mice exposed to lead. However, the possible contributory effects of lead-induced renal dysfunction on urinary values are not eliminated. The results are consistent with other data proposing enhancement of catecholamine function in experimental lead poisoning (*8–10*). It should be noted that it is impossible to determine from present data whether alterations in catecholamine metabolism are causative of lead-induced hyperkinesis in the animal model (*5*), or result from increased motor activity produced by unknown actions of lead.

Preliminary clinical data are consistent with the experimental observations that increased urinary output of HVA and VMA are associated with increased lead absorption (Table 2). These asymptomatic and mildly symptomatic children with increased lead absorption (blood lead concentrations, 59 to 68 μg per 100 ml) and biochemical evidence of disturbed heme synthesis ("free" erythrocyte protoporphyrin, 500 to 800 μg per 100 ml of erythrocytes) excreted in their urine an average of five times as much HVA and VMA as did controls without undue lead exposure. All children were on restricted diets, which excluded vanilla, chocolate, cola drinks, coffee, and tea. None were receiving medications. Results are shown in Table 2 as milligrams per square meter per 24 hours, as Voorhess (*14*) found that urinary excretion of these metabolites is related to body surface area in growing children. The values found in the controls are in agreement with published data for healthy children (*14, 15*), while the degree of elevation in the children with increased lead absorption is comparable with that reported in children with tumors of the sympathetic nervous system (*15*). An increase in urinary VMA has been reported in children with iron deficiency anemia (*16*); however, the increases found in these six children with increased lead absorption far exceed the values reported for children with iron deficiency anemia. Only one of the six children (Table 2) had evidence of mild iron deficiency anemia (hemoglobin, 10 gram percent) while hemoglobin was $\geq$ 11 gram percent in the other children studied.

The finding of significant and substantially increased excretion of these two catecholamine metabolites in children suggests, in conjunction with the animal data, that increased concentrations of lead in nonosseous tissues are associated with altered catecholamine metabolism. Further studies are required to determine the nature of this alteration and its significance to catecholamine function. Furthermore, a dose-effect relationship for this change remains to be determined for both experimental and clinical lead poisoning. These preliminary data identify neurochemical indicators of the effect of lead on the sympathetic nervous system, which may prove helpful in resolving some of the uncertainties about lead in the fields of environmental and child health.

Note added in proof: Samples from five additional children with increased lead absorption have shown similar increases in urinary HVA, while urinary VMA values have not been significantly different from controls in most of these children.

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ter-saturated ethyl acetate; a 500-μl portion was then mixed with 75 μl of 1*M* K_2CO_3. The organic phase was discarded and 50 μl of K_2CO_3 was used for assay. The VMA was converted to vanillin by adding 15 μl of 2 percent $NaIO_4$ and incubating at 50°C for 30 minutes. After cooling on ice, the reaction was stopped with 15 μl of 10 percent $Na_2S_2O_5$ and the sample was neutralized with 30 μl of 5*N* acetic acid followed by 60 μl of 3*N* $K_2HPO_4 \cdot 3H_2O$ (*p*H 7.5). Vanillin was extracted with 800 μl of toluene (scintillation grade) and 600 μl was mixed with 150 μl of 1*M* K_2CO_3. A 100-μl portion of this phase was analyzed with a Gilford spectrophotometer at 360 nm. Urinary VMA was determined on 1 ml of urine. Internal and external standards were routinely used, and recovery was between 80 and 90 percent; the results are corrected for percentage recovery. The adaptations of both methods demonstrated linear relationships between the amount of tissue or urine assayed and the concentration of VMA and HVA detected. Specificity was checked against a mixture containing norepinephrine, epinephrine, dopamine, tyrosine, 3,4-dihydroxyphenylacetic acid, 5-hydroxyindoleacetic acid, and l-(3,4-dihydroxyphenyl)alanine. Possible interference by high amounts of lead and δ-aminolevulinic acid in urine from lead-exposed children and mice was also investigated and found not to occur.

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Induction of Mitosis in Mature Neurons in Central Nervous System by Sustained Depolarization

Abstract. *DNA synthesis and mitosis have been induced in vitro in fully differentiated neurons from the central nervous system by depolarization with a variety of agents that produce a sustained rise in the intracellular sodium ion concentration and a decrease in the potassium ion concentration. Depolarization was followed in less than 1 hour by an increase in RNA synthesis and in 3 hours by initiation of DNA synthesis. Apparently normal nuclear mitosis ensued, but cytokinesis was not completed in most cells; this resulted in the formation of binucleate neurons. The daughter nuclei each contained the same amount of DNA as the diploid preinduction parental neurons; this implies that true mitogenic replication was induced.*

The hypothesis has been advanced (*1*, *2*) that intracellular cation levels associated with generation of electrical transmembrane potentials in somatic cells may be functionally involved in control of mitogenesis and, hence, of cell division. Results from studies with a variety of cell systems have supported this premise (*3*, *4*). As a corollary of the hypothesis, it was proposed (*2*) that mitogenesis might be activated in highly polarized, nondividing cells such as neurons in the central nervous system (CNS) and muscle by treatments that would produce and maintain a substantial increase in the level of intracellular Na^+ and a decrease in K^+. This proposition was recently investigated for the case of mature neurons from chick spinal cord depolarized with ouabain (*5*); DNA synthesis was induced in a significant percentage of the neuron population by a range of ouabain concentrations. The question remained, however, as to whether the observed synthesis was truly mitogenic, rather than the result of an anomalous thymidine exchange or repair activity. We now report the induction of full DNA synthesis and subsequent mitosis in much larger percentages of chick spinal cord neurons by use of more favorable ouabain treatments, and an equally effective induction of mitotic activity in such neurons by other agents that depolarize by quite different mechanisms. The daughter nuclei from the induced mitoses each contain an amount of DNA identical to that of the mitotically quiescent G_1 (or G_0) neurons; this implies that true mitogenic replication has been induced by the imposed ionic changes.

The neurons were obtained by trypsin dissociation of spinal cords from chick embryos 7 to 10 days old and cultured as described (*5*). Mature 16- to 20-day cover slip cultures of well dispersed, fully differentiated neurons were used in the experiments. The cultures contained numerous small neurons and similar-sized neuroglial cells in addition to the large, distinctive motoneurons, but primarily the latter were utilized in the present investigation to preclude any possible confusion with the glial cells; fibroblasts were also quite numerous. The motoneurons were readily identified by their large size, angular cytons with long axon and dendrites, extensively granular cytoplasm, distinctive vesicular nucleus with prominent nucleolus, and differential staining properties (*6*). The glial population in the cultures increased continuously by mitotic division up to 14 days after plating but declined thereafter, although many glial mitoses were still observable; the ratio of glia (including the numerous microglia) to neurons at the time of culture use in experiments was approximately 10 to 1. In general, the glial cells tended to aggregate in clumps while the motoneurons were well dispersed. The cultured neurons had resting potentials of −35 to −60 mv, and were entirely devoid of mitotic activity after plating.

The ability of ouabain, veratridine, and the ionophore gramicidin to induce neuronal DNA synthesis and mitosis was determined over a range of concentrations for each agent. For DNA synthesis determinations, cultures were incubated for 6 hours with medium containing the test agent and [^{3}H]thymidine (0.1 μc/ml). The cover slips were rinsed, fixed, coated with Kodak NTB-3 nuclear track emulsion, and exposed for 14 days as described (*5*). The time of onset of DNA synthesis following exposure to ouabain was determined by pulse labeling for 1 hour prior to serial fixations at 1 to 6 hours. Similar pulse labeling with [^{3}H]uridine (2.5 μc/ml) was used to study changes in RNA synthesis rate following ouabain treatment; the same autoradiographic procedures were used as with DNA, but with a 7-day exposure period. For mitotic activity determinations, cultures were incubated for 24 hours with medium containing the test agent, then fixed, stained, and examined for binucleate neurons (*7*). Control cultures were assayed as were the test cultures, but were incubated in normal culture medium without addition of the depolarizing or other agents. Several supplementary time-lapse film studies were made of treated neurons to elucidate features of the mitotic process and the behavior of daughter cells. Relative amounts of DNA in the nuclei of control phase (G_1) neurons and in the daughter nuclei resulting from induced mitoses were determined by Feulgen microspectrophotometry of individual nuclei (*8*). The nuclear DNA content of G_1 fibroblasts in the cultures was similarly determined by using an excess-thymidine blocking procedure for accumulating G_1 cells, to establish the basic diploid nuclear DNA level. The neuron cultures were Feulgen-stained by the procedure of DeCosse and Aiello (*9*), and the relative absorbency of each nucleus was determined at 550-nm wavelength by using a Zeiss fluores-