United States
Environmental Protection
Agency

Health Effects Research
Laboratory
Research Triangle Park NC 27711

Research and Development

EPA-600/S1-82-008 Nov. 1982

EPA

Project Summary

Effects of Heavy Metals on the Differentiation of Metabolic Pathways in the CNS

John J. O'Neill, George van Rossum, and Leon Salganicoff

The effects of lead and organotin on brain and spinal cord, liver or kidney in the developing or adult Sprague-Dawley and Wistar rat strains were investigated. The effects were primarily ascertained *in vitro* following lead exposure. Two concentrations of lead were present in drinking water: 200 ppm or 600 ppm as lead in lead acetate. Blood and tissue lead levels were either measured by anodic stripping voltammetry or by atomic absorption spectrophotometry (Perkin Elmer Model 303) following digestion in nitric acid.

Tissue slices prepared from the kidney cortex of animals exposed to 200 or 600 ppm lead showed that respiration rates were 15% less and ATP contents 30% less than those for control animals. Lead treatment *in vivo* had no significant effect on the ion and water contents of kidney cortex, in contrast with *in vitro* results. The ability of kidney cortex slices to restore ion and water contents after incubation at 1°C was also unimpaired after *in vivo* treatment. Longer *in vivo* exposure might be necessary for effects to become evident.

Lead sensitivity of carbonic anhydrase depended upon the organ from which it was derived. At a lead concentration of 10 ppm, there was a 50% fall (5.11± 1.46 units to 2.54 ± 0.75 units) in myelin carbonic anhydrase and a 44% fall in kidney carbonic anhydrase. The enzyme present in nerve endings was particularly sensitive and showed a drop from 20 units ± 4 to 2.3 ± 1.7 units (to 12% of normal). The nerve ending enzyme was sensitive even at 1 ppm. *In vivo* administration of lead (600 ppm) was carried out over a 3-month period. Brain myelin, synaptosomal and kidney microsomal carbonic anhydrases were measured. Consistent with *in vitro* measurements, myelin and synaptosomal activity were decreased 67% and 57%, respectively, whereas kidney microsomal carbonic anhydrase showed a relatively small change (19%) from 9.46 units to 7.6 units following 12 weeks exposure.

This Project Summary was developed by EPA's Health Effects Research Laboratory, Research Triangle Park, NC, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

The neurotoxic effects of lead, mercury, and organotin are well established at high dose levels. The controversy and source of concern is not with high concentrations but rather with levels that do not cause overt symptoms This appears to be especially true in the case of lead intoxication where the symptons are not well established. The effects on adults are qualitatively different from those on young children and the developing nervous system.

This report describes research conducted on central and peripheral effects of controlled exposure of pregnant and nonpregnant albino rats to lead and tributyl-tin at levels of 200 or 600 ppm.

The research was divided into three group projects. Project 1 focused on the influence of lead on the developing and adult central nervous system. Considerable emphasis was placed upon monitoring the development in the spinal cord morphologically, by electron microscopy, and neurochemically. Although the fetal brain, especially the cerebellum, appears to be most susceptible to lead exposure, the spinal cord, which undergoes development earlier, might be the first fetal organ to manifest an effect as a result of exposure *in utereo*.

Current literature suggests that lead and organotin compounds might have toxic effects on the energy production of cells and on the regulation of cellular ionic composition and tissue volume. Since nephrotoxicity is a prominent manifestation of lead poisoning, the use of kidney as well as hepatic tissue in these studies is especially relevant in project 2

Project 3 dealt with the effects of lead on the zinc metallo enzyme, carbonic anhydrase, which appears to assist in maintaining H^+ ion and electrolyte balance in kidney tissue. Although carbonic anhydrase is present in the intralamina of myelin in all nerve tissue and thought to be involved in controlling cerebral and spinal tissue edema, there is no general agreement about this. Because lead and some organotin compounds, especially triethyl-tin, cause cerebral edema, some attention was devoted to the forms of the enzyme which occur in brain and kidney.

Tissue lead has routinely been measured by flame absorption spectrophotometry. This may require the use of the major portion of a sample by standard instrumentation or miniaturization of the system The lack of portability of such equipment further limits its applicability to field conditions. In search of alternative methods we elected to study blood, brain, kidney, and liver tissue lead levels by Anodic Stripping Voltammetry. The basic unit is relatively inexpensive, portable, and requires a relatively small (100 microliter) sample size. The method is highly versatile and can be employed in measuring such metal ions as lead, tin, cadmium, iron, or its chelates.

Conclusions

The results of this study show that lead exhibits energy metabolism in kidney cortex and specifically alters K^+-ion transport and CA^{+2}-ion distribution in the cells, suggesting that effects on cellular energetics and/or ion transport may play a role in the nephrotoxic manifestations of lead poisoning

In contrast to findings of others regarding delayed synaptic development in the cerebral cortex of rat pups exposed to lead *in utereo*, no significant effect was observed on synaptic development in the thoracolumbar region of the developing spinal cord. Inability to demonstrate a gross morphologic effect may be due to the lack of specificity of the method. Although some features of the presynaptic and postsynaptic apparatus can be discerned, the method does not permit a study of the receptors known to be present. When the potent muscarinic binding drug, Quinuclidinyl benzilate-^{3}H (QNB-3), which has high affinity for muscarinic receptors ($K_{aff} = 10^{-13}$M), was employed, lead (600 ppm) in drinking water consumed by the pregnant nursing dam caused a slight delay in the appearance of muscarinic binding sites in spinal cord during fetal and early neonatal development

Lead poisoning can produce a devastating encephalopathy in children. The symptoms are characterized by increased intracranial pressure (ICP), convulsion and coma The metabolic basis is not understood but the disruption of fluid and electrolyte metabolism undoubtedly is involved in the development of edema responsible for the ICP. In studies in which liver tissue was exposed to tributyl-tin, this compound, which has a weak inhibitory effect on energy metabolism, was discovered to possess a hitherto unknown inhibiting effect on cell volume regulation in the liver. We suggest that this latter effect may underlie the previously observed edematous changes in the central nervous system of animals poisoned by trialkyl-tin compounds and possibly by lead as well.

Experiments in which rats were administered lead (600 ppm) in drinking water for several weeks showed that exposure to lead reduced the respiratory activity by 15%. When intact cells of kidney cortex were incubated as a slice preparation, it was determined that lead had reduced the ability of the kidney to maintain ATP levels by 30% This is consistent with other findings on mitochondria isolated from kidneys, and our results show these findings to relevant to intact cells. Further, wh more lead was added *in vitro* to slices, an extra decrease occur in respiration and ATP levels. The mini levels, however, did not differ fr those obtained by directly treating slices from control rats with lead *vitro*. This finding indicates that *in* and *in vitro* effects of lead expos were not additive and were proba acting at the same site In view of rapidity of action *in vitro*, we believe t lead acted directly as an inhibitor uncoupler of mitochondrial oxida phosphorylation Similar findings brain slices from rats treated for 20 d at 200 and 600 ppm lead also occur help to explain effects on acetylcho synthesis.

The marked inhibitory effect of l on oxidative phosphorylation v observed through *in vitro* studies mitochondria isolated from kidn cortices of control animals. Sim results obtained using rat brain m chondria from untreated anim showed brain mitochondria to be hig sensitive to lead ions. These result adult animals make it improbable t lead exposure *in vivo* reduces the s thesis of cytochromes (through est lished effects on heme synthesis) su ciently to produce a marked effect cell respiration. Rather, we believe t lead had a direct effect on respirat metabolism, the precise nature of wh is being further investigated.

Lead administered *in vivo* affec neither the water and ionic (K^+, Na^+, Ca^{2+}, Mg^{2+}) composition of the kid cortex nor the ability of slices of tissue to transport any of these ion water Thus, the reduction in ene metabolism through lead treatmen *vivo* was insufficient to limit the sup of ATP required for these homeost mechanisms. Further, the levels of l attained in the tissue were insuffic to inhibit the transport mechanis directly

Currently, rats are being treated v lead for longer periods (up to six mon to determine whether any additi effects are produced.

Lead added to kidney-cortex slice *vitro* at 50-200 μM had two effects ion exchange *in vitro*, lead reduced active accumulation of K^+ by a m mum of 30% and had no signific effect on the net extrusion of Na^+, Ca^{2+} or water or on the content of N in the tissue The partial inhibition c

accumulation becomes apparent only after 30 min. of incubation.

The lead concentration (200 μM) that inhibited K^+ accumulation by 30% also inhibited respiration and reduced ATP content by 25 to 30%. ATP reduction and K^+ transport inhibition occurred in close succession. Thus, circumstantial evidence suggests that by reducing the availability of ATP, lead caused the inhibition of ion transport. However, the fact that lead had no inhibiting effect on Na^+ extrusion was rather unusual The known inhibitory effect of lead on Na^+ and K^+- dependent adenosine triphosphatase (Na-K-ATPase) of the kidney and other cells could have accounted directly for the transport inhibition To distinguish between these two possibilities, kidney cortex slices were titrated with a specific inhibitor of mitrochondrial respiration, antimycin A, and with an inhibitor of the Na-K-ATPase, ouabain This permitted us to compare the effects of these agents when K^+ transport was inhibited by 30% with the effects of Pb^{2+} given the same reduction of K^+ transport. However, the results of the comparison were equivocal. When antimycin A was the inhibitor, 30% inhibition of K^+ transport was accompanied by a similar decrease in respiration and ATP contents but, unlike the results obtained with lead, Na^+ extrusion was also inhibited by antimycin H When ouabain inhibited K^+ transport by 30%, the extrusion of Na^+ was not inhibited, whereas respiration decreased by 25%, results similar to those with Pb^{2+}. ATP was unchanged by ouabain. The biggest difficulty using this comparison to explain the results with Pb^{2+} is that a 30% reduction of ATP levels produced by lead failed to inhibit Na^+ extrusion The temporal coincidence of inhibited K^+ transport and depressed ATP levels suggests that the latter accounts for the former, but it is clear that further experiments are required to elucidate the mechanism by which Pb^{2+}inhibits K^+ accumulation

The second effect of lead *in vitro* on ion exchange was a reduction in entry of Ca^{2+} into the slices while metabolic activity was greatly reduced, that is, to 1°C. Examination of the Ca^{2+} content of mitochondria isolated from the slices revealed that lead prevented the entry of Ca^{2+} into these organelles, apparently by inhibiting a residual, calcium-accumulating activity of the mitochondria which persisted at 1°C However, this did not account for the entire reduction in the entry of Ca^{2+} into the whole slice at this temperature. Studies of ^{45}Ca influx and efflux in the slices were performed in order to obtain more information on possible fractions of tissue Ca^{2+} that might be affected by lead We found that lead reduced the quantity of Ca^{2+} which was inexchangeable with ^{45}Ca in the medium and the quantity of Ca^{2+} in the most slowly exchanging component of the three into which the exchangeable Ca^{2+} could be divided, probably the mitochondrial Ca^{2+} This result complements our findings with the directly measured Ca^{2+} on mitochondria The inexchangeable portion may represent Ca^{2+} which is so tightly bound to nonmitochondrial tissue structures that it cannot be displaced by ^{45}Ca but which may be displaceable by Pb^{2+}. This latter fraction would then account for the nonmitochondrial portion of the Ca^{2+} uptake that was prevented by lead in the other experiments

Initially, the laboratory investigated the pathway of metabolism leading from glucose to acetylcholine syntheses. It is well established that brain and nervous tissue cannot synthesize choline and must depend on an exogenous source for supply. Equally well established is the fact that glycolysis supplies pyruvate as a precusor for Acetyl-CoA and ultimately acetylcholine formation. The problem which has confronted neurochemists for two decades is that pyruvate forms Acetyl-CoA intramitochondrially. As such it remains within the mitochondrion, because no known mechanism exists for its transport to the cytoplasm, the site of acetylcholine synthesis

We recently demonstrated that citric acid formed intramitochondrially leaves and enters the cytosol There it is cleaved by an enzyme, ATP. citrate lyase to reform Acetyl-CoA and thence to form acetylcholine We have determined that lead at either 200 ppm or 600 ppm in drinking water results in the inhibition of acetylcholine synthesis We find that at 600 ppm, the conversion of glucose to lactate and citrate decreases markedly Hence, lead interference with glycolysis and the tri-carboxylic cycle (TCA) may interfere with energy metabolism and affect neurotransmitter function.

The mechanism by which acetylcholine is derived from glucose and pyruvate has been intimately linked to brain mitochondria, since the enzyme which converts pyruvate to acetyl-CoA (Pyruvate dehydrogenase E.C. 1.2 4.1) exists exclusively intramitochondrially. Acetyl-CoA thus formed must be converted to a permeate anion in order to cross the mitochondrial membrane to the cytosol. The evidence to date strongly supports citrate as that anion; when citrate transport is interfered with, acetoacetate fulfills this role The approach used to test this hypothesis is based on the observation that glucose labeled in the carbon-6 position with either tritium (^3H) or carbon-14 (^{14}C) is converted to pyruvate without loss of either isotope. However, when acetyl-CoA is condensed with oxaloacetate (OAA), a hydrogen is lost to OAA which is exchanged and lost to water in the formation of citrate The probability that the hydrogen lost would be tritium, barring isotope discrimination, would be one-third and that would reduce the $^3H/^{14}C$ ratio to 2/3 (0.67) This ratio remains constant only in citrate carbons 4 and 5 and is drastically reduced in other molecules with each turn of the cycle within mitochondria Acetyl-CoA arising from citrate ($C-^{4-5}$) in the cytosol gives rise to acetylcholine with the same $^3H/^{14}C$ ratio (0.67), direct conversion from glucose through acetate would require the ratio to be one.

Tributyl-tin was studied for its effects *in vitro* on rat liver, since most biochemical studies of the organotin compounds have concentrated on the mitochondria isolated from this tissue Despite the reported high sensitivity of several aspects of oxidative metabolism in isolated mitochondria to tributyl-tin, we needed a concentration of 1 mM to produce a 20% decrease in respiration and ATP content in slices of liver *in vitro*. At this concentration, the net transport of K^+, Na^+, Cl^- and water declined substantially.

Much lower concentrations of tributyl-tin (1-100 μM), however, significantly reduced the extrusion of Cl^- from the liver slices, an effect accompanied by less marked inhibitions of Na^+ and water extrusion. But K^+ accumulation was completely resistant to these concentrations of the tin compound. We conclude that tributyl-tin at 1-100 μM was probably acting as a specific inhibitor of the mechanism for net extrusion of Na^+ and Cl^-, which is not coupled to K^+ uptake and which is partly responsible for the control of cell volume This effect may be related to the known effect of tributyl-tin in facilitating Cl^-/OH exchanges through biological membranes. Higher concentrations (1 mM) also affect mitochondrial energy provision and thus inhibit the ouabain-

sensitive transport of Na^+ and K^+ through the paucity of ATP If tributyl-tin is indeed a specific inhibitor of the Na^+ and Cl^- transport not coupled to K^+, it may be a most useful agent with which to study the properties of this system and hence to study volume control Its action as an inhibitor of volume control may well account for the finding of manifestations of edema in the central nervous system of animals intoxicated with trialkyl-tin compounds

Recommendations

The studies on lead have extended knowledge of the mechanism of action of this heavy metal and may be particularly relevant to the means by which it causes nephrotoxicity We have indicated that lead acts on energy provision *in vitro* by a mechanism similar to that by which it acts *in vivo* Lead causes alterations of K^+ homeostasis in the kidney cells and also affects the distribution of Ca^{2+} in them, especially with regard to the compartmentalization between mitochondria and other cell components Such effects on ion-homeostasis, especially that of Ca^{2+}, may well lead to far-reaching alterations of metabolic balance and of reabsorptive and excretory activities of the kidney cortex.

In light of the findings on brain metabolism by us and others and recent clinical evidence that there is a strong correlation between lead exposure and minimal brain damage characterized by hyperactivity that may interfere with classroom performance, further studies on this important aspect are warranted The social implications of present drug treatment of hyperactivity should be reappraised and greater emphasis be placed on monitoring of safe lead levels in drinking water to prevent such neurophysiologic damage in future young.

The action of tributyl-tin as an inhibitor of volume-controlling activity, at concentrations substantially lower than those at which it affects energy metabolism in the intact cells, provides a completely new indication of its possible mode of action. Such an action, if it also occurs in nervous tissue, could readily provide an explanation of the central nervous toxicity of the trialkyl-tin compounds. This line of investigation may well prove to be more profitable in elucidating the mechanism of toxicity of the trialkyl-tin compounds than studies of energy metabolism and other functions of isolated mitochondria.

John J. O'Neill, George van Rossum, and Leon Salganicoff are with the Temple University School of Medicine, Philadelphia, PA 19140.
***Richard J. Bull** is the EPA Project Officer (see below).*
The complete report, entitled "Effects of Heavy Metals on the Differentiation of Metabolic Pathways in the CNS," (Order No. PB 82-249 145; Cost: $9.00, subject to change) will be available only from:
National Technical Information Service
5285 Port Royal Road
Springfield, VA 22161
Telephone: 703-487-4650
The EPA Project Officer can be contacted at:
Health Effects Research Laboratory
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

☆ U S GOVERNMENT PRINTING OFFICE 1982—659-017/0863

United States
Environmental Protection
Agency

Center for Environmental Research
Information
Cincinnati OH 45268

Postage and
Fees Paid
Environmental
Protection
Agency
EPA 335