anisms in releasing prolactin. It has been shown that suckling acts at the hypothalamic level by inhibiting prolactin inhibiting factor (PIF) activity (*7*). Our study supports the concept of two different mechanisms of prolactin control since nicotine blocks the suckling-induced release of prolactin, probably by stimulating PIF, but does not block the other mode of release.

Although nicotine was completely effective in inhibiting the reflex release of prolactin, oxytocin secretion was apparently not blocked since milk release was indicated by the presence of milk in the pups' stomachs after 10, 30, and 60 minutes of suckling from nicotine-treated mothers.

These data suggest that nicotine has the ability to cause severe malfunction in milk production by blocking prolactin output in lactating mothers even though the milk release mechanism is apparently unaltered by nicotine. With the knowledge that tobacco smoking can cause severe reproductive disorders in women (*1*) the present results cannot be ignored by lactating mothers who are heavy smokers. Although the smoker's consumption of nicotine would certainly be much less than our rat's intake on a per kilogram basis, species differences can make weight-basis comparisons meaningless.

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Subtle Consequences of Methylmercury Exposure: Behavioral Deviations in Offspring of Treated Mothers

Abstract. *Overt neurological impairment is the endpoint currently used to document a case of methylmercury poisoning. No consideration is given to possible subtle consequences. Offspring from mice exposed to methylmercury on day 7 or 9 of pregnancy were apparently unaffected during postnatal development. However, subtle behavioral differences between treated and control offspring were found when the overtly normal animals were tested in an open field and evaluated in a swimming apparatus at 1 month of age. Brain weight, protein, choline acetyltransferase, and cholinesterase were not significantly altered.*

Obvious neurological dysfunction is the only criterion used in officially recognizing a case a methylmercury poisoning and in setting standards for the Allowable Daily Intake (ADI) of methylmercury. No consideration is given to possible behavioral, biochemical, carcinogenic, or other subtle effects such as lowered intellectual capacity and premature senility (*1*, *2*). We think subtle alterations are important and should be considered.

Furthermore, attention should be directed to the fetus since the unborn organism is much more susceptible to the toxic effects of methylmercury than the adult. Methylmercury easily crosses the placental barrier and is concentrated in fetal tissues; it has a greater affinity for the embryonic central nervous system than for that of the adult (*1*, *3*, *4*).

Our studies with mice from mothers exposed to methylmercury show that, although offspring are overtly normal, they are significantly different from controls when tested in an open field and evaluated in a swimming apparatus. However, data from subsequent analysis of brain weight, protein, and acetylcholine enzyme activities revealed no significant difference between treated and control offspring. As a result of our research, we believe that screening for subtle behavioral deviations (from normal) in offspring from treated mothers may be one of the most sensitive indicators of the toxic effects of methylmercury.

On day 7 or 9 of pregnancy, primiparous 129/SvSl mice (approximately 4 months old) were weighed and each was given a single intraperitoneal injection of saline or 0.16 mg of methylmercury dicyandiamide (MMD) per 20 g of body weight (*5*). This dose [approximately ⅓ to ¼ the LD_{50} (*6*)] and these days of treatment were selected because, in a previous study to determine effects of MMD on prenatal development (*4*), all offspring born of similarly treated 129/SvSl females were apparently normal. Objectives of the present study were to determine if subtle behavioral, biochemical, or neurological deviations are detectable during postnatal development of these so-called normal offspring.

One day before term (day 18), pregnant mice were put in separate cages and allowed to deliver. Young remained with their biological mother until weaned at 21 days of age. Therefore, offspring were potentially exposed to methylmercury in utero and during nursing. There was no difference in average litter size (6.70) or in appearance and weight (1.2 g) of individual young. When 30 days old, each offspring was tested in the open field on 2 consecutive days (at approximately the same time each day) and then evaluated in a swimming apparatus. At 60 days the brains were removed and weighed, and protein, choline acetyltransferase, and cholinesterase determinations were made. All evaluations were done "blind."

Treated and control mothers were observed for possible differences in rearing (nursing, retrieving, grooming, defense) of young, open-field behavior, and swimming performance. No differences were found.

A daily session in the open field (*7*) consisted of first placing the experimentally naive animal in the center of the field under a light-tight chamber for 15 seconds. The chamber was then removed and eight behavioral parameters were recorded during the ensuing 2-minute interval.

Table 1 summarizes the results of open-field testing. A two-way least-squares analysis of variance (*8*) was used to examine the behavioral responses as a function of treatment and sex. There was a significant difference

between treated and control animals with respect to four parameters: latency (time in seconds to move from center squares), defecation, urination, and backing (three or more backward steps). The sex difference was significant only on average number of defecations and urinations. No parameters exhibited significant interaction. Thus the effects of MMD and sex may be considered independently.

When control animals were placed in the center of the field they proceeded forward and began exploratory activity. Offspring from treated mothers took a significantly longer time to begin exploration, and when they did a significant number took three or more backward steps initially or during the test period. This backing phenomenon is highly unusual. Only 1 out of 19 (5 percent) control animals exhibited this strange behavior, whereas backing was observed in 10 out of 20 treated animals. All subjects were capable of proceeding forward, but 3 out of 10 treated mice traveled backward for more than half the test session (> 1 minute).

Although the difference in the other four parameters measured did not achieve significance at the .05 level, in each case the mean response for the treated offspring was lower than for control offspring (center squares entered, 29 percent lower; grooming, 52 percent lower).

In spite of the fact that all test animals were grossly indistinguishable from controls, the animals' behavior during swimming (*9*) was significantly different. In general, the 19 control offspring adjusted to the water (stopped struggling to get out) within 1 to 2 minutes, assumed a characteristic posture (Fig. 1a), and began to swim back and forth. All controls swam with front legs tucked and seldom used; hind legs were used alternately to turn or propel, with tail under water for balance and propulsion. Without exception, animals in the control group soon appeared relaxed and well-coordinated; at no time did their eyes, nose, or back sink below the surface.

Table 1. Effect of maternal methylmercury exposure on open-field behavior of offspring: Mean number of responses from 2-minute trials on 2 consecutive days beginning at 30 days of age. Abbreviations: S, saline; F, female; M, male.

Parameter	Responses			
	Treatment		Sex	
	S*	MMD†	F	M
Center latency (seconds)	5.5	7.1§	6.1	6.5
Center squares entered‡	4.1	2.9	3.7	3.3
Peripheral squares entered	34.9	34.1	35.4	33.6
Defecation	2.9	1.7§	2.9	1.7‖
Urination	0.89	0.52‖	0.92	0.50‖
Rearing	3.6	3.3	3.4	3.6
Grooming	1.3	0.65	0.58	1.3
Backing	0.03	0.37§	0.18	0.22

* $N = 19$ (8 female, 11 male). † $N = 20$ (11 female, 9 male). Mothers of test animals received 0.16 mg of methylmercury dicyandiamide per 20 g of body weight. ‡ Entering is defined as placing all four legs into any square. § $P < .05$. ‖ $P < .01$, two-way least-squares analysis of variance, no interactions significant.

In contradistinction, 12 of 20 treated offspring showed one or more signs of neuromuscular impairment while swimming. Representative of the group were frequent episodes of "freezing" in the water and floating motionless with all four legs extended and askew (Fig. 1, b and d). On occasion, the entire body, except for the head, would sink and the animal would float suspended in a vertical position (Fig. 1c). Other deviant swimming behavior, typical of the treated group, was excessive churning and splaying of all four legs with the tail whipping above and below water until the animal was able to maintain its body position. In spite of these intermittent periods of incoordination and impaired swimming ability, all treated offspring exhibited "normal" locomotion in the water. We were able to significantly ($P < .001$, two-tailed chi-square analysis) discriminate treated from control offspring by means of evaluation in a swimming apparatus.

In an attempt to correlate these subtle behavioral differences with neurotransmitter enzymes, choline acetyltransferase and cholinesterase determinations were done on the brains of 24 randomly chosen mice (equally distributed between treated and controls, males and females). A two-way (MMD and sex) analysis of variance was done for each of the following parameters: diencephalic-telencephalic weight; cerebellar weight; total milligrams of protein (*10*) in whole brain; and activity of choline acetyltransferase and cholinesterase [in micromoles per gram of protein, per hour, or micromoles per brain, per hour (*11*)]. The only significant effect ($P < .05$) was a sex difference in cerebellar weight (males > females). No significant alteration was

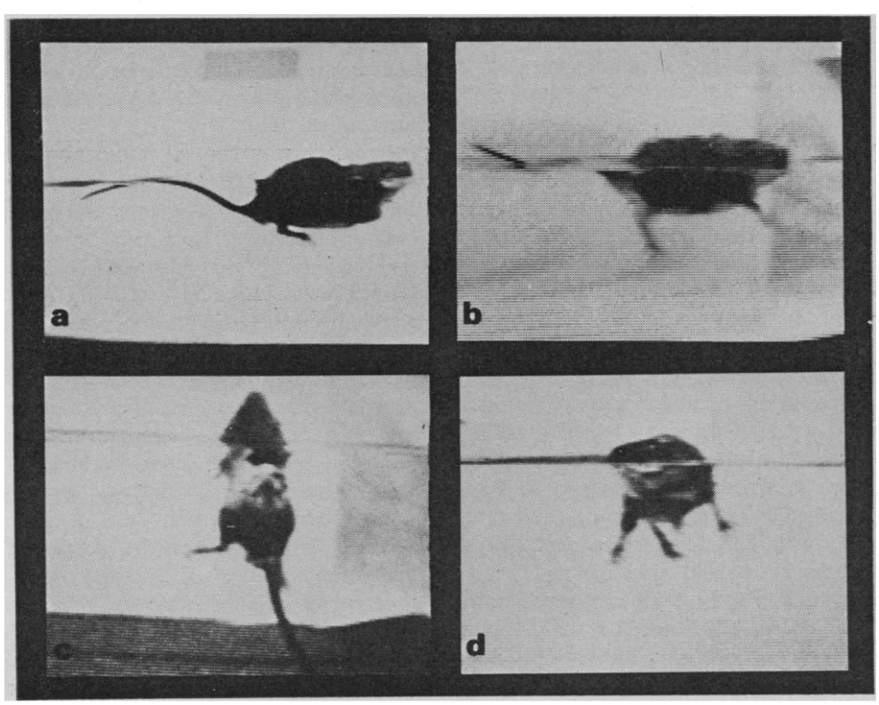

Fig. 1. Prenatal exposure to methylmercury dicyandiamide significantly affected the swimming behavior of apparently normal mice. Controls swam with (a) front legs tucked, hind legs alternately kicking to turn or propel, and tail under water for balance and propulsion (note general posture with respect to waterline). Characteristic of the treated group were frequent episodes of incoordination and impaired swimming ability such as (b) "freezing" in the water with all legs extended for periods up to 2 minutes [compare posture to that of control in (a)]; (c) floating suspended in a vertical position with only head above water; and (d) swimming with legs askew and inability to maintain normal orientation in water.

found in any parameter between treated and control offspring.

In summary, we found that in the absence of any overt sign, offspring from methylmercury-exposed mice behaved significantly different from controls when tested for subtle deviations during postnatal development. We believe there is need to examine subtle parameters when assessing the risks from methylmercury exposure. It seems likely that many people with minor symptoms or subclinical damage have gone undetected.

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Human Insulin: Facile Synthesis by Modification of Porcine Insulin

Abstract. *Human insulin differs from porcine insulin by a single amino acid—the carboxyl terminal residue of the B chain. By means of chemical and enzymatic treatment, it is possible to remove quantitatively and selectively the carboxyl terminal octapeptide from porcine insulin B chain. This fragment can be replaced by an analogous synthetic human octapeptide to give a protein which is identical to human insulin by a number of criteria. By this method, human insulin can be prepared on a large scale simply and inexpensively from porcine insulin. The method is also useful for preparing specifically labeled radioactive human insulin, as well as insulins with modified amino acid sequences, for research purposes.*

Insulin is a protein composed of two polypeptide chains linked together by two disulfide bridges. The A chain is composed of 21 amino acid residues, while the B chain contains 30, as shown in Fig. 1. It was the first protein for which chemical structure (*1*) and precise molecular weight (*2*) were determined. The complete de novo synthesis of insulin has been successfully accomplished in a number of laboratories (*3*). While these chemical syntheses represent major breakthroughs in synthetic protein chemistry, they are in no way practical for large-scale and inexpensive production of insulin. The de novo synthesis of a protein containing 51 amino acid residues is time-consuming and expensive. In addition, the yields of insulin are rather low at the stage where the A and B chains are linked together by disulfide bridges. In this regard, the method which I have devised has the special advantage that at no stage are the disulfide bridges between the A and B chains disrupted.

Up to this time, the only practical supply of insulin for treatment of diabetes mellitus has been from animal sources. The similarities in structure (*4*) and biological activity of these insulins to human insulin has made this possible. Unfortunately, immunologic intolerance to these nonhuman proteins develops in patients (*5*) and this intolerance to heterologous protein may be significant in bringing about the pathological changes that occur among diabetic patients maintained on insulin therapy for extended periods of time. It would therefore seem to be most desirable if insulin identical in structure to the human protein could be made available for such patients. To this purpose I have developed a method whereby readily available porcine insulin can be converted easily and inexpensively to human insulin.

An outline of the procedure is given below and is depicted schematically in Fig. 1:

1) Reversible blocking of all of the six carboxyl groups in porcine insulin; the four γ-carboxyl groups from glutamic acid residues and the two terminal carboxyl groups.

2) Digestion of blocked porcine insulin with trypsin. This generates a single free carboxyl group at the arginine residue in position 22 of the B chain.

3) Reversible blocking of all free amino groups with BOC (*6*) azide (*7*).

4) Coupling of the free carboxyl at position 22 to a synthetic octapeptide