Pharmacology Biochemistry & Behavior, Vol. 4, pp. 507–513.

Time Dependent Effects Produced in Chicks after Prenatal Injection of Methylmercury[1]

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(Received 2 December 1975)

HUGHES, J. A., E. ROSENTHAL AND S. B. SPARBER. *Time dependent effects produced in chicks after prenatal injection of methylmercury.* PHARMAC. BIOCHEM. BEHAV. 4(5) 507 513, 1976. Methylmercury dicyandiamide (0.05 to 10 mg/kg egg) injected into the yolk sac of fertilized chicken eggs prior to incubation produced a dose related decrease in the percentage of chicks hatched (90–57% of control). With dosage fixed at 0.5 or 5.0 mg/kg egg and injections made on Days 0, 7 or 14 of incubation, hatches were 90, 68 and 75%, respectively, for the low dose and 63, 13 and 18% for the high dose. In contrast to results obtained from chicks hatched from eggs injected on Day 0 of incubation, chicks hatched from eggs injected with 0.5 or 5.0 mg MMD/kg on Day 7 or 14 were not different from controls in a detour learning situation. Administration of 14-C methylmercury revealed maximal brain radiolabel in embryos injected on Day 0 to be 10% that seen with eggs injected on Day 7 but twice that seen with eggs injected on Day 14. We tentatively conclude that a period of maximal sensitivity to the behavior effects exists prior to Day 7 and that the mechanisms of embryolethality is different from that producing the functional deficits.

Behavioral teratology Behavioral toxicity Mercury toxicity Learning deficits Critical period

REPORTS of widespread environmental contamination and of large scale incidents of accidental poisoning in man [1, 4, 5, 7, 15, 16, 17] have prompted a number of investigations of the potential health hazards of alkyl mercurials. Studies of chronic and subacute exposures to methylmercury compounds have demonstrated that at the onset of symptoms, the concentration of mercury is greater in the liver, kidney and blood than in the brain in virtually all species examined [4, 14, 17]. Despite this, the central nervous system has been clearly shown to be the critical target organ of systemic intoxication by methylmercury [4]. The clinical picture is dominated by symptoms of neurological origin. These include ataxia, bilateral concentric constriction of the visual fields, disturbances of superficial and deep sensation, tremor, dysarthria and mental disturbances.

In general, relatively large amounts of methylmercury are needed to produce these symptoms and when they appear the course of the disease is usually irreversible, terminating in death or in the permanent disability of the afflicted individual. Such symptoms may, therefore, be considered to be somewhat coarse indicators of the underlying neuropathy of the effected organism.

A number of investigators have recently presented evidence to show that the developing organism is particularly susceptible to methylmercury intoxication and may be severely effected at dosages which leave the mothers free of apparent dysfunction [14]. In addition to causing fetal wastage, congenital malformations and mental retardation, methylmercury has been implicated in the production of more subtle behavioral deficits which appear without the obvious appearance of other functional aberrations [3, 6, 18, 19, 26, 27, 28]. Hughes, Annau and Goldberg [6], for instance, demonstrated significantly increased trials to criterion in an avoidance situation by mice born of mothers treated with 3 or more mg Hg/kg, as methylmercury hydroxyide, on Day 8 of gestation. Spyker, Sparber and Goldberg [27] administered 8 mg methylmercury dicyandiamide/kg to mice on Days 7 or 9 of pregnancy and found that offspring from the treated mothers took more steps in a backwards direction than did controls. The experimental mice also showed impaired locomotor ability and intermittent periods of incoordination during a swimming test. Zenick [28] gave 2.5 mg methylmercury chloride/kg to pregnant mice by way of their drinking water and found maze learning deficits in their progeny which persisted on retesting 3 weeks later. These deficits occurred in the absence of detectable levels of mercury in the mouse brains. In none of these studies did the mothers show functional disorders apart from the behavioral deficits. (Spyker [27], however, presents evidence that as treated progeny grew older a number of aberrations appeared, including immunologic, eye and back disorders.)

A difficulty in this sort of research lies in separating the direct effects of prenatal exposure to the methylmercury from indirect effects (such as changes in the uterine milieu, hormonal imbalances, nutritional disturbances) and from perinatal influences such as abnormal mother-pup interactions, exposure to mercury through the mother's milk, etc. While it is possible, to some extent, to control for these factors by use of cross fostering and the like [8, 21, 25], the resultant analysis is still complicated.

[1] Supported in part by grants from the USPHS (MH 08565 and ES 00760) and the Minnesota Medical Foundation (FSW-14-71).

Another approach is to use a species such as the chick, whose embryos grow outside the mother's body and are not dependent upon the mother for nurture after birth and are therefore essentially free of indirect effects from the mother and from perinatal influences upon behavior. The chick also offers the advantages of easy access to the embryo, easily controlled environmental conditions, and precocial young with relatively well developed and well-defined behavioral repertories exhibited within a few hours to days after birth [22]. Using such a model, Rosenthal and Sparber [18] were able to demonstrate the direct effects of injections of doses of methylmercury dicyandiamide as low as 0.5 mg/kg prior to incubation, which had no effect upon the percent of chicks which hatched but significantly impaired detour learning in young chicks. The results were similar in many respects to those obtained from an earlier series of experiments from the same laboratory in which it was demonstrated that small doses of the drug reserpine produced subtle biochemical and behavioral alterations upon hatched chickens; the effects were long lasting and both dose and time related, suggesting that critical periods during development might exist for biochemical and/or behavioral consequences of early insult in this species [10, 11, 21, 22, 23, 24, 25].

In this paper we present the results of a series of experiments designed to ascertain whether a behavioral effect of early exposure to methylmercury is also time related, and if so, which period(s) of embryogenesis is/are most important. We also wished to determine how much methylmercury reached the embryo and when maximal levels were attained in the embryo brain relative to embryolethal and behavioral actions of the alkylmurcurial.

EXPERIMENT 1

Effects of Methylmercury Injected at Different Times upon the Percentage of Chicks Successfully Hatched

Fertilized White Leghorn chicken eggs, obtained from a local hatchery, were candled to remove defective members, numbered consecutively and assigned to treatment groups on the basis of time of injection and dosage by the use of a table of random numbers.

Just prior to incubation 4 dosages of methylmercury dicyandiamide (MMD), freshly prepared in 0.9% saline, were injected into groups of eggs as follows: 0.5 mg MMD/kg (N = 302); 1.0 mg MMD/kg (N = 60); 5.0 mg MMD/kg (N = 298); and 10.0 mg MMD/kg (N = 75). A second series of eggs was injected with either 0.5 mg MMD/kg (N = 51) or 5.0 mg MMD/kg (N = 106) on Day 7 of incubation and a third was injected with 0.5 mg MMD/kg (N = 96) or 5.0 mg MMD/kg (N = 121) on Day 14 of incubation. For each time of injection 2 additional groups, each containing 60 eggs, served as controls. One group was not injected and the other was injected with 0.9% saline. In all cases injection was into the center of the yolk in volumes of 30 μl. The technique was essentially that of McLaughlin *et al.* [13] as modified by Sparber and Shideman [22].

The eggs were incubated in a forced air, rotating, incubator for 18–19 days and then transferred to a forced air hatcher. Temperature was maintained at 37.5°C and humidity at 85 86%. Once hatched, the chicks were placed in standard heated brooders with food and water freely available. Room temperature was kept at 25–26°C and lighting was on a 12 hr basis.

Sparber and Shideman [22], as well as March *et al.* [12], have shown that a drug injected into a fertilized egg can effect not only the viability of the embryo, but also the time of hatching. Chicks usually hatch from the 20th to the 22nd day of incubation. In several instances, the distribution of hatching for each group was monitored every 2 hr. The total hatch period was determined and divided in half. The percent hatchability of groups in each half was compared using confidence belts for proportions [2].

Results

Sixty-five percent of the saline treated eggs successfully hatched, compared with 75% of the noninjected controls. Taking the saline-injected eggs as 100%, injection of methylmercury prior to incubation produced a log linear, dose-dependent, decrease in hatchability (Fig. 1). A dose of 0.5 mg MMD/kg egg lowered the percent hatch only 12%, whereas 10 mg/kg dropped to 57% of the saline injected controls.

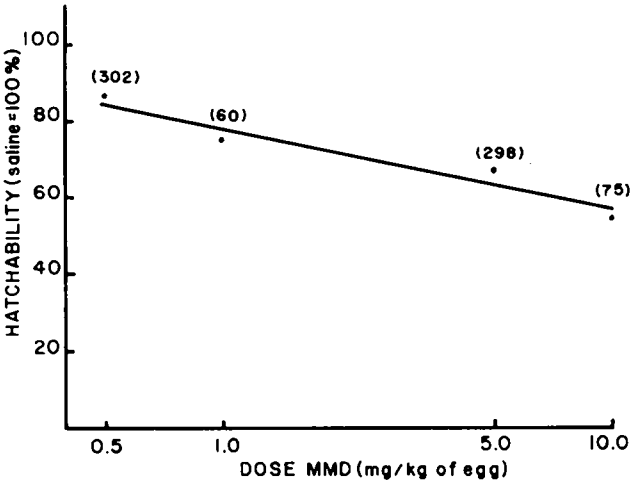

FIG. 1. Relationship between dose of methylmercury dicyandiamide (MMD) injected into fertilized White-Leghorn chicken eggs prior to incubation and the percentage of chicks which hatched (= hatchability). Numbers in parentheses represent the number of eggs injected at each dose.

Injection of MMD after 7 or 14 days of incubation produced an increase in embryolethality when compared with the effects of equivalent dosages administered prior to incubation (Fig. 2). A dose of 0.5 mg MMD/kg egg injected on Days 7 or 14 resulted in very nearly the same hatch percentage as did a dose 10 times as great, but administered on Day 0 (about 68%). Five mg/kg MMD administered on Days 7 or 14 reduced the hatch rate to 13 and 18%, respectively. This was a mortality more than 13 times greater than a dose of 10 mg/kg injected prior to incubation.

Noninjected and saline control eggs usually hatched over a period of about 32 hr, beginning after 20 days and continuing to the 22nd day of incubation. When the hatch period from first to last egg was arbitrarily divided into equal segments, about 70% of the control eggs were found to hatch during the first half (Fig. 3); 0.5 mg/kg MMD did not alter this distribution. A dose of 5 mg/kg MMD, however, significantly prolonged the hatching in the second half of the hatch period.

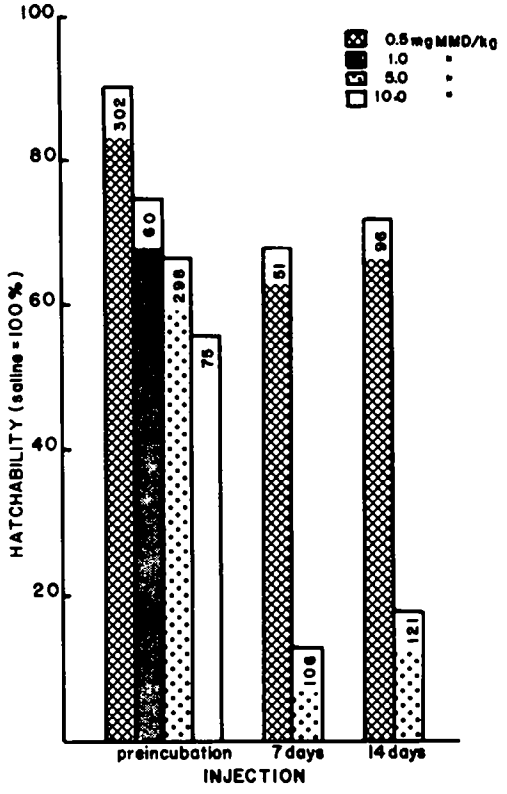

FIG. 2. The effect of methylmercury dicyandiamide (MMD) upon the percentage of chicks hatching after injection on Days 0, 7 or 14 of incubation. Numbers in the bars refer to the number of eggs in each group.

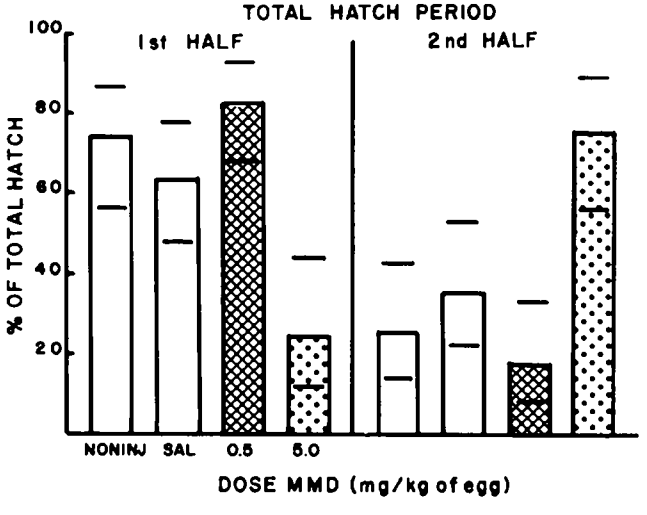

FIG. 3. Retardation of the hatching process induced by methylmercury dicyandiamide (MMD) injected prior to incubation. The total length of the hatch period was divided in half and the half in which each chick emerged from its egg was recorded. Both noninjected and saline injected controls are included for comparative purposes. Solid lines above and below the top of each bar represent 95% confidence limits.

EXPERIMENT 2

Effects of Methylmercury Injected at Different Times upon Detour Behavior

Fifteen chicks from noninjected and from saline control groups were selected at random for testing in a detour learning paradigm. Equal numbers were also selected from groups injected with 0.05, 0.5 and 5.0 mg MMD/kg prior to incubation or on Day 7 or 14 of incubation. All chicks were 7 days old at the start of the experiment and were given 1 trial daily for 5 days.

The device employed and the procedure utilized were basically that of Scholes [19] and have been described in detail elsewhere [18,23]. The apparatus consisted of a box divided by a clear Plexiglas wall into 2 compartments, designated social and isolation and connected with one another by means of a tunnel. Three or 4 chicks, deprived of food 18–20 hr, were selected at random from each treatment group and placed into the social side which contained moist chicken mash and a light bulb. As soon as all of the birds had begun to eat, one was removed and placed into the isolation compartment of the apparatus. In order to gain access to the social side the chick had to turn away from the rewarding stimuli (food, light, warmth and social contact) and detour through the tunnel. The time from the placement of the chick into the isolation chamber until its emergence into the social side was recorded in sec (response latency). If a chick did not spontaneously cross to the social compartment within 4 min, it was guided to the tunnel entrance with a wooden probe and gently forced to make the response. This was done so that all chicks travelled the same route during the experiment. A chick was allowed to remain in the social compartment 30 sec, after which it was removed and the trial terminated. If a chick did not respond within 4 min, its response latency was considered to be 240 sec. At the end of each session the chicks were allowed free access to food for 4 hr.

Results

Gross observation of the appearance and behavior of the methylmercury treated chicks failed to reveal any obvious abnormalities. The birds were indistinguishable from their saline and noninjected controls.

Results of the detour learning experiments are depicted in Figs. 4–7. As there were no significant differences in the cumulative detour response latencies between noninjected and saline control chicks, these data have been pooled for each time of injection. Chicks hatched from eggs injected prior to incubation with 0.05 mg MMD/kg produced an average cumulative detour response curve nearly identical to that of the control chicks, indicating no effect of this dosage at the time of injection. Dosages of 0.5 or 5.0 mg MMD/kg administered prior to incubation, however, produced chicks whose average cumulative detour response latencies were longer than those of the control chicks. The increased latencies became significant for both dosages by the 4th trial and continued through the 5th and final trial (Fig. 4, 2-tailed chi-square, $p<0.025$). Several factors seem to account for these longer cumulative latencies. In the first place, fewer chicks hatched from eggs injected with 0.5 or 5.0 mg MMD/kg had completed the task by the end of the last trial. Secondly, those chicks which did successfully enter

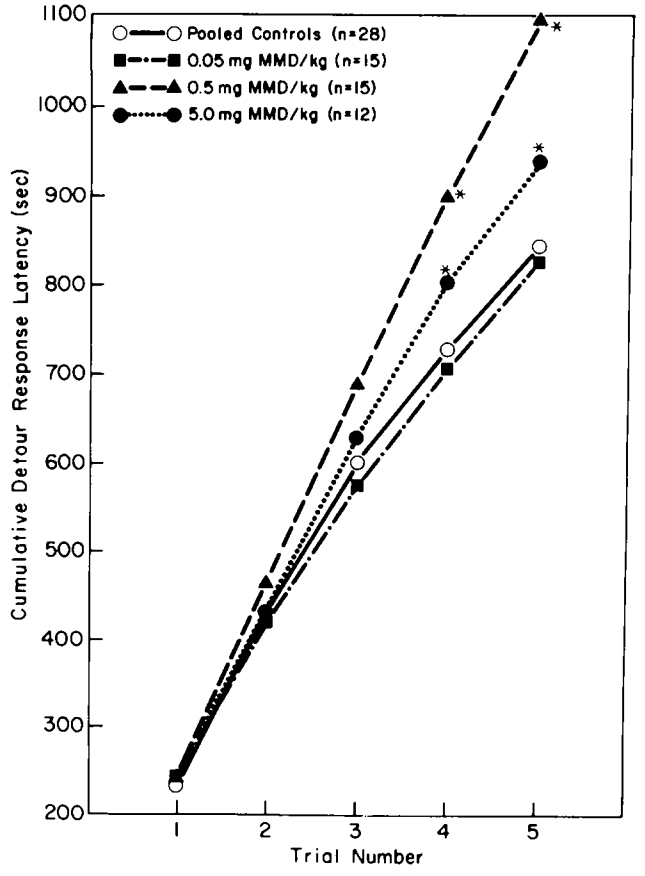

FIG. 4. Mean cumulative response latencies of chicks hatched from eggs injected with methylmercury dicyandiamide (MMD) on Day 0 of incubation and tested when 7--11 days old. Means are derived from individual performance data cumulated over successive trials. N = number of chicks in groups. All groups originally had an N of 15. Injury or mortality account for the differences in N. Data from dead or injured chicks have not been included. Presence of an asterisk denotes a value significantly different from control (chi square, $p<0.025$).

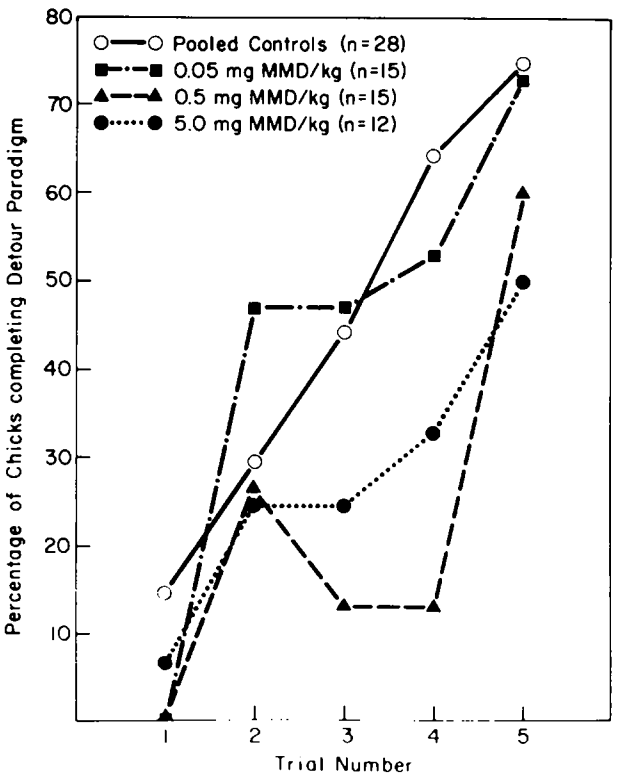

FIG. 5. Effect of methylmercury dicyandiamide (MMD) administered prior to incubation upon the percentage of chicks which responded in each trial of the detour learning paradigm. N refers to the number of chicks tested in each group. As there were no significant differences between saline and noninjected control chicks, their data have always been pooled.

the social compartment tended to do so during later trials than the control chicks or those treated with 0.05 mg MMD/kg (Fig. 5). Since there was an arbitrary 240 sec per trial ceiling for the detour latency, the measure was an underestimate of the response latency for any chick which failed to respond. Because more of the 0.5 and 5.0 mg MMD/kg treated chicks failed to respond than controls, the measure also underestimates the differences produced by administration of the methylmercury prior to incubation.

A third factor involved the likelihood that once a given bird had spontaneously crossed to the social compartment on a given trial that it would do so again on the following trial. One would predict that if learning had occurred, then the probability that a bird would respond on the following trial would be high. This seemed to be the case with the control chicks where 16 of 17 birds which had successfully completed the paradigm did so again on the following trial for a repeat percentage of 94%. This compared with 9 of 12 chicks (75%) treated with 0.05 mg MMD/kg prior to incubation and 2 of 5 (40%) and 3 of 6 (50%) chicks similarly injected with 0.5 or 5.0 mg MMD/kg, respectively. While these numbers are small, the data suggest that the differences in performance seen in the methylmercury treated birds may be due to a true learning deficit rather than simply a motor impairment. This point of view is bolstered by a lack of significant differences between the groups in the mean time taken by the birds to enter the social compartment. That is, of those birds which successfully performed, the average latency to enter the social compartment per trial was not different between control birds and those treated with methylmercury prior to incubation.

In contrast, birds hatched from eggs injected with 0.5 or 5.0 mg MMD/kg on Days 7 or 14 of incubation showed no differences from their controls in performance in the detour paradigm (Figs. 6 and 7).

Effects of Injection of ^{14}C-Labelled M thylmercury Injected at Different Times upon Levels of Mercury Obtained within the Embryonic Brain

Fertilized White Leghorn chicken eggs were injected as above with doses of 0.05, 0.1, 0.5 and 1.0 mg/kg $^{14}CH_3HgCl$ (New England Nuclear) in 0.9% saline solution on Days 0, 7 or 14 of incubation. Specific activity of the compound was 3.36 mCi/mM and radiochemical purity was greater than 97%.

Beginning with the fourth day of incubation, embryos were removed from the eggs, washed in 0.9% saline and blotted on a filter paper. The brains were then dissected

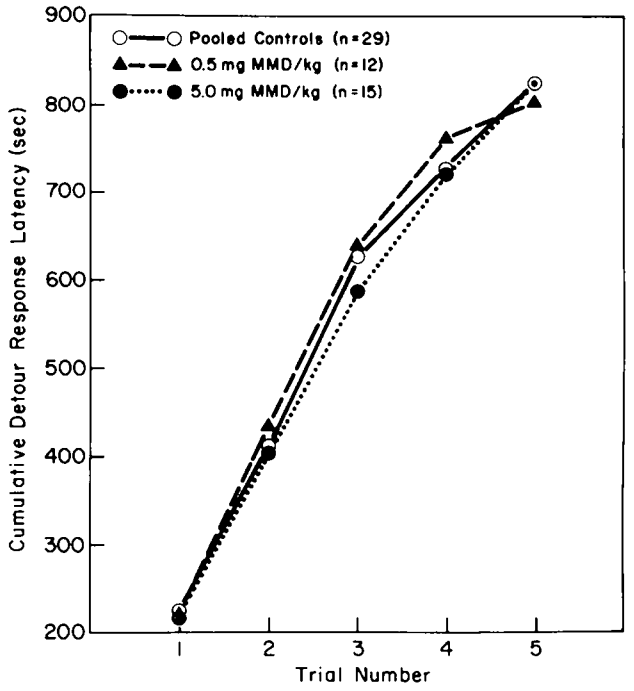

FIG. 6. Mean cumulative response latencies of 7–11 day old chicks hatched from eggs injected on Day 7 of incubation with 0.5 or 5.0 mg methylmercury dicyandiamide (MMD) per kg and their controls. Since there were no significant differences between noninjected and saline control chicks, their data have been pooled. Means have been derived from individual performance data cumulated over successive trials, N is the number of chicks in each group.

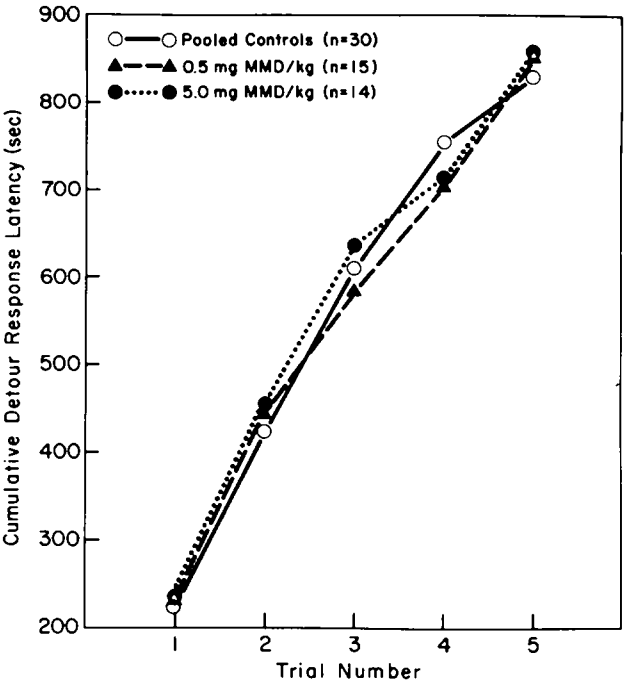

FIG. 7. Mean cumulative response latencies of 7–11 day old chicks hatched from eggs injected on Day 14 of incubation with 0.5 or 5.0 mg methylmercury dicyandiamide (MMD) per kg and their controls. Since there were no significant differences between noninjected and saline control chicks, their data have been pooled. Means have been derived from individual performance data cumulated over successive trials. N is the number of chicks in each group.

free and the brains and carcasses were washed, blotted, and then placed into separate, previously tared, glass liquid scintillation vials with polypropylene lined caps. The vials were weighed, tissue weight calculated, and 1 ml of 0.6 N NCS solubilizer (Amersham/Searle) was added per 100 mg of tissue weight. The vials were then placed in a water bath at 45° C and left to incubate over night. Next morning, after cooling to room temperature, 10 ml of 0.4% PPO in toluene was added to each vial. The vials were then dark adapted and radioactivity measured by means of a Beckman LS-230 liquid scintillation counter. In the cases of older embryos, brain and carcass tissues were separately minced and aliquots of 50–100 mg removed for solubilization and counting. Methylmercury in tissue was expressed as equivalents of $^{14}CH_3HgCl$ in mg/gm or as DPM/100 mg of tissue. One μg of $^{14}CH_3HgCl$ gave 29,719 DPM.

Results

Substantial amounts of radiolabel were already present in the embryo within 4–6 days after injection into the yolk sac prior to incubation. The amounts were dose related and showed a linear relationship when plotted on semi-log coordinates. The amount of methylmercury chloride ranged from 0.09 ± 0.02 to 1.24 ± 0.20 μg per gram of tissue wet weight (Fig. 8). At these stages of development there were no significant differences between carcass and brain levels of methylmercury when compared using a paired t-test.

By Days 7–9 of incubation peak levels of radiolabel had been attained in the embryo brains for all doses, after which there was a steady decline (Fig. 9). The maximal amounts of methylmercury, expressed on a μg/g basis, were 3.10 ± 0.56, 1.57 ± 0.16, 0.22 ± 0.05 and 0.19 ± 0.01 for the 4 doses injected prior to incubation, in descending order.

Injection of methylmercury after 7 days of incubation resulted in a rapid uptake of radiolabel into the embryonic brains, maximal brain levels having been obtained within 24 hr after administration of the compound. The quantity of mercury averaged 7 times the maximal levels found after injection of the equivalent doses on Day 0 of incubation (Fig. 9). The amounts of methylmercury in the brains were dose-dependent and followed a log-linear relationship. Brain levels steadily decreased after the first 3 or 4 days and fell off at a rate roughly parallel to that seen in the brains from eggs injected prior to incubation. There was no significant difference between the maximal amount of mercury found in the brain after injection of 0.05 or 0.1 mg/kg on Day 7 of incubation compared with the maximal amounts seen after injections of doses 10 times as large but administered prior to incubation (t-test, $p>0.05$).

Injection of 1.0 or 0.1 mg $^{14}CH_3HgCl$/kg on Day 14 of incubation also resulted in a rapid uptake of radiolabel into the brain with peak levels being obtained within 24 hr after injection (Fig. 9). These levels, however, were far lower than the maximums seen after injection of comparable doses on Days 0 or 7 of incubation. In μg/g, the greatest amounts of methylmercury in the brains were 1.01 ± 0.16 and 0.09 ± 0.02, respectively, for the higher and lower doses. This amounted to only about 1/3 of the maximal

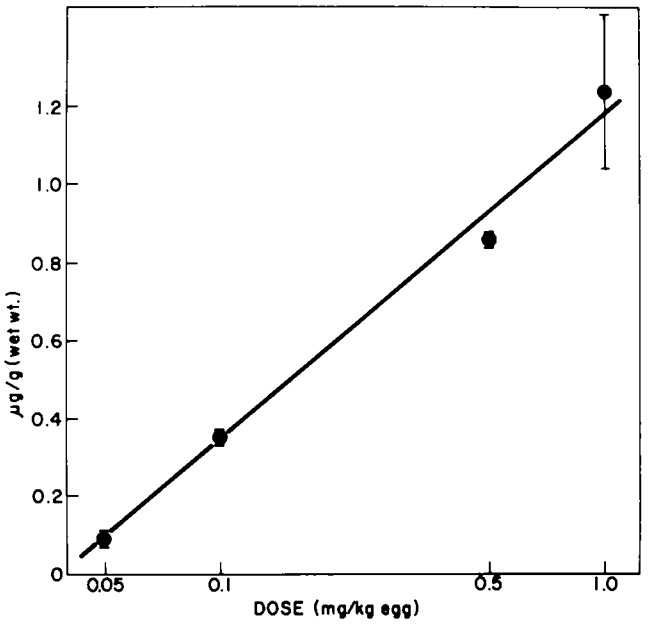

FIG. 8. Equivalents of methylmercury chloride in chicken embryos. Labelled (^{14}C)-methylmercury Cl was injected into the yolk sac prior to incubation and embryos sampled 4–6 days later. Each point represents the mean vertical lines ± SEM for 5–9 chicks. The straight line was derived by a least-squares linear regression analysis on log- transformed data.

levels attained after injection on Day 0 of incubation and 1/20 the maximal dosage seen after injection on the 7th day of incubation.

DISCUSSION

Administration of MMD into the yolk sac of fertilized chicken eggs prior to incubation produced a dose-related decrease in percent hatch and a retardation in the acquisition of detour learning by chickens hatched from eggs treated with 0.5 and 5.0 mg MMD/kg. Injection of these same 2 doses after 7 or 14 days of incubation resulted in an even greater decrease in hatch percentage but had no evident effect upon the acquisition of detour learning in those chicks which successfully hatched. We infer from these observations that the older embryo is differentially sensitive to the embryocidal effects of MMD, that the embryocidal action of MMD is different from its behavioral teratogenic action and that a period of maximal sensitivity to the detour behavioral effects exists and terminates sometime prior to the seventh day of incubation. The heightened sensitivity of the older embryo to the embryocidal effects of MMD can be seen in the markedly decreased hatches produced after injection of 0.5 or 5.0 mg MMD/kg on Days 7 or 14 of incubation compared with their effects after administration prior to incubation. Of particular relevance is the observation that a dose of 0.5 mg MMD/kg prior to incubation had only a slight effect upon the percentage of successful hatches, but the same dose given 1 or 2 weeks later gave a hatch percentage which was as low as that produced by a dose 10 times greater but delivered on Day 0 of incubation.

If there was no difference between the embryocidal and behavioral mechanisms of action of MMD, then it would be

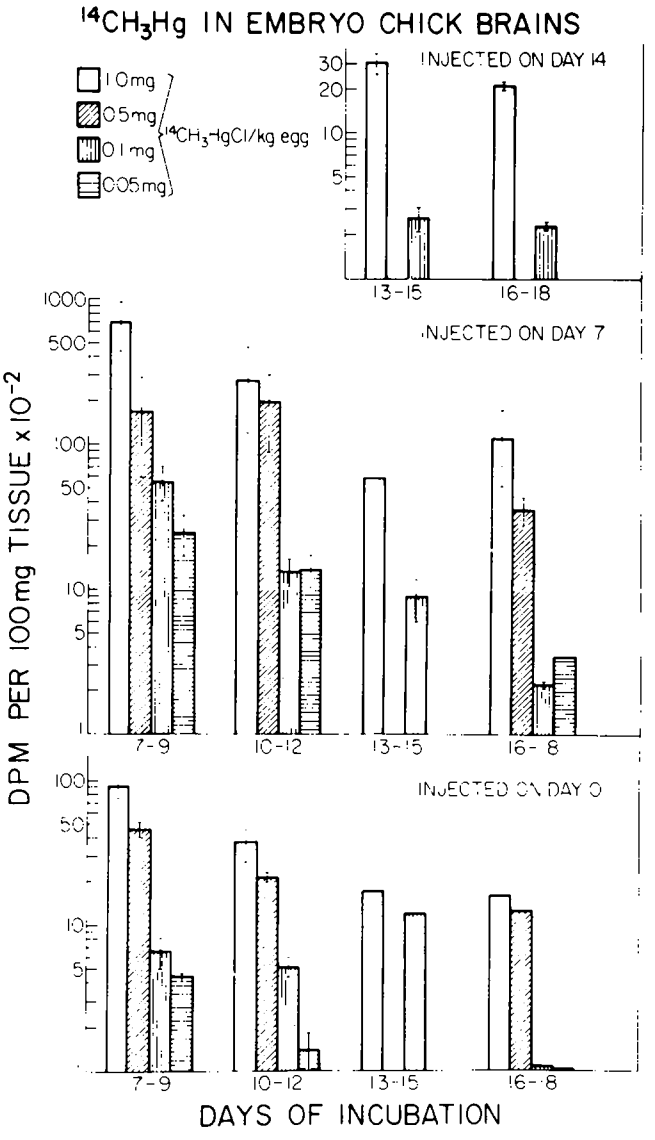

FIG. 9. Radioactivity in the brains of chicken embryos at various stages of development, after injection of carbon 14 methylmercury chloride ($^{14}CH_3HgCl$) prior to incubation (Day 0) or after 7 or 14 days of incubation. Although data are grouped into blocks of 3 days each, values for the blocks surrounding the days of injection (7 or 14) are derived from brains taken between 2 and 24 or 48 hr after injection. Vertical lines represent ± 1 SEM and histograms without estimates of variability are derived from 2 brains only. One microgram of $^{14}CH_3HgCl$ is represented by 29,719 disintegrations min^{-1} (DPM).

expected that chicks hatched from eggs injected on Days 7 or 14 of incubation would show retarded detour learning and this was not the case. It could be argued that the reason for the lack of a behavioral effect was not due to a difference in mechanism of action, but to an artifact of the increased embryolethality seen at these times. Had they survived, the affected embryos may well have exhibited retarded detour learning. Those embryos which actually did survive, therefore, might also have been unaffected with regard to the behavioral teratologic effect. Evidence against such a screening-out phenomenon comes from comparing

the effects of 5.0 mg MMD/kg injected prior to incubation with the effects of 0.5 mg/kg injected on Days 7 or 14 of incubation. In these situations virtually identical decreases in percentage were obtained (Fig. 2), but only in the case of the earliest injection was there a significant effect upon detour learning. It is of interest to note also that the maximal amount of radiolabel found in the brains of embryos after injection of 0.5 mg/kg on Day 7 of incubation was not significantly different from a dose 10 times as great, but delivered prior to incubation and that these maxima occurred at nearly the same time. These observations, coupled with the lack of behavioral effect seen after administration during the later periods of embryogenesis argue in favor of the existence of a period of maximal susceptibility to the behavioral effects which occur sometime before Day 7 of incubation. How early this period extends is difficult to ascertain from out data, but substantial quantities of radiolabel were found in the embryos as early as 4 days after injection prior to incubation and presumably the critical period can extend to at least this early time.

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