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d-Amphetamine Unmasks Postnatal Consequences of Exposure to Methylmercury *in utero*: Methods for Studying Behavioral Teratogenesis[1]

JOHN A. HUGHES[2] AND SHELDON B. SPARBER[3]

Department of Pharmacology, University of Minnesota, Minneapolis, MN 55455

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HUGHES, J. A. AND S. B. SPARBER. *d–Amphetamine unmasks postnatal consequences of exposure to methylmercury* in utero*: methods for studying behavioral teratogenesis.* PHARMAC. BIOCHEM. BEHAV. 8(4) 365–375, 1978. – Pregnant rats were given various doses of methylmercury (MM) at three different stages of gestation (Days 0, 7 or 14). Administration of 56% or 27% of the dose given on the first day (Day 0) of pregnancy to 7 or 14 day pregnant rats, respectively, resulted in equivalent concentrations of MM in 19 day old feti and 1 day and 1 week old neonates. A single, 5 mg MM/kg, oral dose on Day 0, or its equivalent on Days 7 or 14 of gestation did not produce any signs of toxicity in pregnant dams or their offspring. Weight gain of pregnant dams, litter size, litter weights at birth or at weaning and gross physical appearance were not different amongst the various treated groups and their respective controls. Operant level of bar pressing and acquisition of a discrete trial autoshape task indicated no differences with respect to operant levels, rate of acquisition or asymptotic performance resulting from exposure to MM *in utero*. The operant (autoshaped) behavior showed sex related differences to the disrupting influence of d-amphetamine (d–A); females were significantly more sensitive than males. Moreover, both males and females whose dams were treated with MM on Day 0 or 7 of pregnancy were significantly less affected by the d–A when compared with controls. Offspring born to dams given MM on Day 14 of pregnancy did not show a differential effect of d–A. It is concluded that early prenatal exposure to low doses of MM can result in behavioral consequences subtle enough to require unmasking of the effects with psychotropic drugs. Additionally, periods may exist during development when the embryo or fetus is most susceptible to behavioral or functional teratogenic effects of exposure to chemical insult. The testing procedures used in these experiments were objective, automatic and amenable for use with relatively large sample sizes compared with other operant behavior analytical methods. They also lend themselves to appropriate parametric statistical analyses, a staunch requirement for behavioral toxicological and teratological studies.

Methylmercury Behavior Behavioral teratology Prenatal Behavioral toxicology Heavy metals

ONE of the major responsibilities of individuals doing research in the newly emerging discipline of behavioral teratology [45] is the development and standardization of test procedures for assessing the potential toxicity of drugs and chemicals. We have attempted to circumvent the problems associated with having to rely upon subjectively estimated behavior changes (e.g., rating scales) and prohibitively large sample sizes because of inherent variability of the measures (e.g., open-field behavior). To do so we designed a protocol which makes use of a single apparatus (operant chamber or Skinner box) to study general exploratory activity in this novel environment and rate of learning and attainment of asymptotic performance levels of a discrete-trials autoshaped task. The rather simple nature of this task, with its automatic equipment programming requirements, results in a small loss of sensitivity but offers the greater advantage of objectivity. The experimenter does not have to shape the animals' behavior to stable baselines as is customary in behavioral pharmacological or toxicological studies, thereby introducing a potential source of bias. This is especially important in those cases where the rate or capacity to learn a task may be affected. In those instances where unconditioned and conditioned performance variables do not demonstrate

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[2] Present address: Department of Biology, William Paterson College, 300 Pompton Road, Wayne, NJ 07470.

[3] Send reprint requests to: S. B. Sparber, Department of Pharmacology, 105 Millard Hall, 435 Delaware Street, S.E., University of Minnesota, Minneapolis, MN 55455.

differences in CNS, myoneuronal, etc., function because of inherent plasticity (which is likely to be the case with toxic substances administered chronically or in low doses) challenge with psychotropic or other drugs can be used to produce behavioral disruption.

Studies of chronic and subacute exposures to alkyl mercurial 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. Despite this, the central nervous system has clearly been shown to be the critical target organ of systemic intoxication with MM [3, 7, 10, 14, 19, 32]. Exposure to MM during development has been implicated in the production of behavioral deficits without obvious concomittance of other functional aberrations in surviving organisms. In the studies demonstrating behavioral teratology, fetoxicity and/or maternal toxicity was also evident [9, 15, 16, 17, 34, 37, 38, 39, 42, 51]. We have recently presented evidence to show that, in the chick, a period of maximal sensitivity to behavioral effects of MM (as measured by performance in a detour learning situation) exists early during development and that the mechanisms by which these effects are produced differ from those which produce embryolethality [17].

A common approach in the search for periods of maximal sensitivity to functional or teratologic effects of an agent is to treat different groups of animals with equal dosages (on a mg/kg basis) but at different times during development and then to study the organisms, using appropriate methods, at some later time. Such a procedure can be misleading because administration of equal doses at different times during development may result in non-equivalent concentrations in target organs. For instance, injection of ^{14}C-labelled MM into the yolk sacs of fertilized chicken eggs on Day 0 of incubation resulted in maximal levels of radiolabel in fetal brains which were 10% of those obtained from eggs injected with the same dose on Day 7 but twice those seen with eggs injected on Day 14 of incubation [17]. Such non-equivalencies of concentration must be considered in assessing whether a given period during development is maximally susceptible to the effects of an agent or only apparently so because more of the agent is at the target organ at that time.

A number of investigations have shown transfer of MM to the fetus [2, 11, 13, 18, 22, 23, 27, 29, 43, 44, 48, 49] but none seem to have systematically examined the relationship between the amounts of MM found in the fetus and the time it was administered to the dam. Preliminary research in our laboratory and information available from the literature indicate that placental and uterine tissues attain concentrations of MM greater than all other maternal organs, with the exceptions of the kidneys and liver [2, 22, 29, 43]. This results in a concentration gradient between placenta and fetus which exists throughout pregnancy and causes the fetus to accumulate MM throughout its development. Maximal concentrations of MM thus occur at the end of gestation. In the following experiments we present evidence that these maxima differ depending upon the dose administered and the time when it is administered. An appropriate dosing schedule is then used in which equivalent concentrations of MM are produced in neonatal brains after administration of the mercurial to the mother at differnt times during gestation. When adult, the resultant progeny were tested in a single autoshaping operant task to determine if time related functional effects could be detected. Mammalian organisms frequently retain apparently normal function after extensive lesions of the CNS with functional reorganization and subsequent recovery of function occurring after destruction of as much as 98% of some brain regions [1]. A strategy useful in overriding such plasticity within the CNS has been to utilize psychotropic agents to help unmask effects of otherwise occult CNS lesions [4]. We have employed such a rationale by challenging rats performing the autoshape task with d-amphetamine (d–A) to see if deviations would occur in the behavior of MM treated animals compared with controls.

EXPERIMENT 1: RELATIONSHIP BETWEEN DOSE OF METHYLMERCURY GIVEN TO PREGNANT RATS AT DIFFERENT PERIODS OF GESTATION AND BRAIN CONCENTRATION IN FETUSES AND NEONATES

METHOD

Breeding

Eight-week old, individually ear marked, nulliparous Sprague-Dawley rats were assigned to males of proven fertility in groups of four, by the use of a table of random numbers. Females were placed with the males at 6:00 p.m. each evening and removed at 9:00 a.m. the following day, at which time vaginal material was collected by lavage with 0.9% NaCl. When sperm was detected in a lavage, that rat was presumed to be pregnant and conception was considered to have taken place at 9:00 a.m. of the same day. Pregnant rats were randomly assigned to treatment groups and housed in colony cages in groups of four. Lighting was on a 12 hr light (7:00 a.m.–7:00 p.m.), 12 hr dark basis and room temperature was maintained at 25 ± 1°C. Rats without sperm in their lavage were returned to the same male at 6:00 p.m. each evening and the procedure was repeated for a maximum of 10 consecutive days.

Intubation

Pregnant rats were treated once by gavage with ^{14}C-labelled MM as methylmercury chloride (MMC) in corn oil on Days 0, 7 or 14 of gestation (as dated from the appearance of sperm in the vaginal lavage). These times were selected because they approximately coincide with the beginnings of those times during gestation in which the rat brain is reputedly refractory to teratogenic treatment, susceptible to such treatment and resistant to it, respectively [46].

For each injection date, separate groups of rats were given 0, 0.05, 0.5 or 5 mg ^{14}C–MM/kg in corn oil. Solutions were prepared daily in concentrations such that 0.2 ml could be administered per 100 g of body weight. Concentrations were verified by radioassay prior to each injection.

An additional group of 5 rats was intubated with 5.82 mg MMC/kg on Day 0, another with 3.23 mg MMC/kg on Day 7 and a third with 1.55 mg MMC/kg on Day 14 of gestation. These doses were selected (on basis of a procedure to be described below) to give equal concentrations of MM in fetal rats on Day 19 of gestation.

Radiolabel Determination

On Day 19 of gestation 3 dams from each of the experimental and control groups were sacrificed with an

overdose of pentobarbital. The brains of 4 feti randomly selected from each rat were removed to separate, previously tared liquid scintillation vials, weighed and then solubilized overnight with NCS (1 ml per 100 mg, wet weight) at 45°C. After cooling to room temperature, 10 ml of 0.4% PPO in toluene was added per vial and radioactivity was measured using a liquid scintiliation spectrometer with 50% efficiency at 1% error using a ^{14}C-above ^{3}H discriminator.

Nineteen-day old fetal carcasses were separately homogenized without added liquid and radioactivity was determined in 0.1 ml aliquots as above.

Brains of remaining feti were removed, weighed to the nearest 0.1 mg, homogenized with 0.1 N NaOH (5:1) and utilized for protein determinations [21].

Additional groups of at least 3 rats from each of the doses administered on Day 0 or 7 of gestation were sacrificed 5 days subsequent to the intubations, i.e., on Days 5 and 12, respectively. This was done to enable us to determine if significant quantities of MM were present in the uterus prior to the second trimester in the first case and if embryos were exposed to significant quantities of MM prior to the third trimester in the second case. Uterine measures were necessary on Day 5 of gestation because embryonic tissue was too small to give meaningful counting data. Uterine levels of radioactivity at this time would presumably represent the maximal amount of radiolabel (and therefore MM) which might be found in the embryo since implantation does not occur until about Day 7. Whole embryos were utilized on Day 12 of gestation because brains were too small for meaningful counting data.

To determine whether dpm ^{14}C gave reliable estimations of MM content, brains were obtained from 9 newborn pups from 3 dams, administered to 10 mg MM/kg on Day 7 of gestation. Each brain was bisected and 1/2 was used as described above for total radiolabel determination. The other half was analyzed specifically for MM using a gas chromatographic technique.

The samples were homogenized in a mixture of 10 ml H_2O and 1 ml $CuSO_4$ (1 Molar). To each sample 5 ml H_2SO_4 – NaBr (130 g H_2SO_4 + 310 g NaBr/1) solution was added the homogenates were shaken. After sitting undisturbed for a minimum of 15 min each sample was extracted for 15 min into 10 ml of toluene. Two ml of freshly prepared cysteine solution (1% cysteine hydrochloride + 0.775 g NaAc · $3H_2O$ + 12% anhydrous Na_2SO_4) was added to the organic phase, and the mixture extracted. One ml of the H_2SO_4 – NaBr solution was added to the aqueous layer, the sample was shaken, and left undisturbed as above. The sample was then extracted into benzene and MM was determined using a Model 1440 Varian aerograph chromatogram equipped with a 63-Ni detector (column width was 32 mm, length was 2 m, column temperature 180°C and detector temperature 210°C). Column packing was 10% butanediol succinate on chromasorb W (acid washed, 60–80 mesh) and the carrier was purified N_2 at a flow rate of 40 ml/min.

The gas chromatographic procedure gave MM concentrations which were within 10% of the estimations based upon liquid scintillation counting. This indicates that essentially all of the label in our samples was present as MM and thus ^{14}C provided reliable estimations of MM content.

Equivalent Brain Concentrations

A diagram was constructed plotting the logs of the mean radioactivities obtained from 4 randomly selected fetal brains taken on Day 19 of gestation from each of 3 litters for each dose and time of injection against the logs of the dosages. Straight lines were then fitted to the points by the method of least squares as seen in Fig. 1. Analysis of the plot led to the prediction that doses equal to 56% and 27% of the amount of MM administered on Day 0 of gestation would result in the same fetal concentrations on Day 19, if administered on Days 7 and 14, respectively. We arrived at these values by drawing lines parallel to the abscissa from the brain concentration point on the least squares line obtained from the three dosages administered on Day 0, (0.05, 0.5 and 5.0 mg MM/kg) and extending them so that they intersected the lines derived from the Day 7 and Day 14 data. At each point of interaction a perpendicular was dropped such that it intersected the abscissa and the dosage so obtained was recorded. Thus a dosage of 5.0 mg MM/kg on Day 0 should give the same amount of MM in a 19 day old fetal brain as would a dose of 3.2 mg MM/kg injected on Day 7 of gestation. Ratios were then obtained by dividing each predicted dose by the value of the Day 0 injection doses and then averaging. In this manner we arrived at ratios of 1:0.56:0.27.

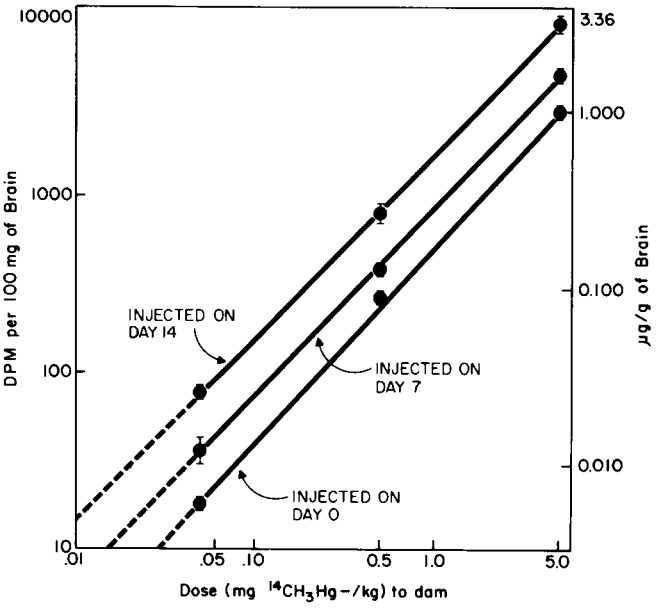

FIG. 1. Amount of methylmercury in 19 day-old fetal rat brains taken from dams injected with 5.0, 0.50 or 0.05 mg $^{14}CH_3$ Hg/kg on Days 0, 7 or 14 of gestation. Each point represents average from 3 litters and 4 feti per litter. Vertical bars are ± SEM. Lines drawn by least squares fit of log transformed data.

Alternatively, the data of Fig. 1 may be replotted as log radioactivity in fetal brains at time of sacrifice versus time (in days) since the dam was dosed. Biological half-times are then calculated for each dose and these are used to obtain k in the relationship $Bt = B_0e^{-kt}$. This considers elimination of the original body burden of the organic mercurial (i.e., the dose administered to the dam) to be a first-order process dependent upon k(=0.69/t½) and t (time since dosing, in days). Bt, the fetal brain concentration at time t is empirically measured at the time of sacrifice of those

dams administered the MM on Day 0 of gestation. This value is substituted into the equation, which is rearranged and solved for B_o, the amount of MM necessary to administer to the dam to arrive at the fetal brain concentration, Bt, after 5 days. We found $t\frac{1}{2}$ = 8 days and obtained ratios of 1:0.56:0.30, nearly identical to those obtained by the graphic method.

To test these predictions, we injected 5.82 mg ^{14}C–MMC (5.0 mg MM/kg) on Day 0, 3.23 mg/kg on Day 7 and 1.55 mg on Day 14 after the appearance of sperm in the vaginal lavages of randomly selected, separate groups of rats. Since uptake into the fetus occurs throughout gestation, we reasoned that if brain concentrations were equivalent on Day 19 of development, they should also be equivalent at birth. We therefore allowed the dams to deliver their litters. At birth, the number of pups in each litter was determined, the litters were weighed and then reduced in size to 3 males and 3 females each, so that each pup would have equal nutritional opportunities. The excess pups were sacrificed and determinations were made of the amounts of radiolabel present within the neonatal brains. One week later a male and a female from each litter were sacrificed and determinations of brain radiolabel were again made.

RESULTS

We detected no signs of MM intoxication in any of the treated dams, their feti or their pups as measured by gross physical appearance, body weight, number of viable feti on Days 12 or 19 of gestation, or number of pups born.

As may be seen in Fig. 1, fetal tissue collected on Day 19 showed a log-log linear relationship with dosage for each time of injection. Radiocarbon activities in the brains of feti whose mothers were injected on the day that sperm cells were found in the lavage (Day 0) were less for each dosage than the corresponding values obtained from feti whose dams were injected on Day 7 and these, in turn, were less than those from feti whose mothers were injected on Day 14 of gestation.

Analysis of the protein content of the treated and control feti revealed no difference when examined by dosage or by time of administration. Average protein content amounted to 9.11 ± 0.22% (means SEM) of brain wet weight.

Homogenates of the fetal carcasses contained an average of 1.16 times more radioactivity (expressed as dpm/100 mg of tissue) than the corresponding brains. When we compared fetal to placental MM concentrations by dividing the average amount of MM (as dpm/100 mg) in 3 feti by the concentration of radioactivity in the placentae with which they were associated, we found that placental concentrations were about 1-1/2 times greater than fetal concentrations in the 5.0 and 0.5 mg MM/kg cases (Table 1). Ratios obtained from placentae and feti from dams treated with 0.05 mg MM/kg, however, indicated that placental concentrations were greater than 3 times that of the fetus.

Table 2 shows the average concentrations of MM (as dpm ^{14}C/100 mg) in whole embryos and uteri or placentae from rats administered 0.05, 0.5 or 5.0 mg ^{14}C–MM/kg by gavage on Days 0, 7 or 14 of gestation and sacrificed 5 days later. While average uterine concentrations obtained from dams 5 days after intubation on Day 0 were nearly identical with placental concentrations from animals treated on Day 7 of gestation, the amounts found 5 days after intubation on Day 14 were nearly twice as great. An even greater difference was seen in fetal concentrations from dams intubated on Day 14 and sacrificed on Day 19 compared with embryos from rats sacrificed 5 days after being treated on Day 7 of gestation (embryonic tissue on Days 5 of gestation proved to be too small to give meaningful counting data).

TABLE 1

RATIO OF RADIOLABEL IN 19 DAY-OLD FETI TO THEIR RESPECTIVE PLACENTAE FROM DAMS ADMINISTERED ^{14}C-LABELLED METHYLMERCURY CHLORIDE ORALLY ON DAYS 0, 7 OR 14 OF GESTATION

Day of Gestation	Dose of Methylmercury (mg/kg) 0.05	0.50	5.0
0	0.178 ± 0.015	0.520 ± 0.031	0.570 ± 0.074
7	0.221 ± 0.022	0.631 ± 0.072	0.694 ± 0.050
14	0.287 ± 0.060	0.688 ± 0.077	0.545 ± 0.052
grand mean, by dose	0.229 ± 0.032	0.631 ± 0.049	0.630 ± 0.046

*3 Feti and placentae were removed from each dam. Ratios were obtained be dividing (dpm ^{14}C/100 mg placenta). Each value is the mean ± SEM of 3 litters.

No significant differences were detected in the amounts of radioactivity in the brains of newborn pups whose dams had been injected on Days 0, 7 or 14 with doses of MM calculated to result in equivalent brain concentrations (ANOVA, $p>0.05$). A week later, brain concentrations were still equivalent but had declined to about one-third of the newborn values (Fig. 2a). Absolute (total brain radioactivity, however, remained approximately the same when measured at birth compared with one week later, Fig. 2b). This indicates little dynamic change in distribution from the growth at these early ages and to the synthesis of protein within the neonatal brain acting to incorporate any diffusible MM before it could be redistributed or excreted.

EXPERIMENT 2: EFFECTS OF MM ADMINISTERED AT DIFFERENT TIMES DURING GESTATION UPON BEHAVIOR

METHOD

Administration of MM and Treatment of Dams

Pregnant rats were administered MM as the dicyandiamide (A. B. Casco, Stockholm, Sweden) or saline by gavage on Days 0, 7 or 14 of gestation (as dated from the appearance of sperm in the vaginal lavage). Solutions were freshly prepared with 0.9% NaCl in concentrations such that 0.2 ml could be administered per 100 g of body weight. The dicyandiamide of MM was used because of its low volatility and high water solubility.

Rats treated on Day 0 of gestation received 5.00 mg MM/kg, those intubated on Day 7 were given 56% of this dose (i.e., 2.78 mg MM/kg) and those intubated on Day 14 received 27% (1.33 mg MM/kg) of the Day 0 dose. Results from Experiment 1 had indicated that these times of administration and dosages of MM would result in the birth of progeny each containing approximately 1 μg MM/gm of brain (Fig. 2).

TABLE 2

RADIOLABEL IN WHOLE EMBRYOS AND UTERI OF SPRAGUE-DAWLEY RATS GIVEN ^{14}C-METHYLMERCURY ON DAYS 0, 7 OR 14 OF GESTATION AND SAMPLED 5 DAYS LATER

DPM/100 mg tissue (above)* and least squares estimate (below)† for each time of injection	0.05 mg Methylmercury/kg		0.5 Methylmercury/kg		5.0 Methylmercury/kg	
	Placenta	Embryo	Placenta	Embryo	Placenta	Embryo
Injected on Day 0	104 ± 9‡ 94	—	596 ± 45‡ 713	—	5942 ± 306‡ 5431	—
Injected on Day 7	110 ± 3 105	21 ± 4 20	962 ± 262 1041	232 ± 5 273	10693 ± 57 10278	4152 ± 807 3823
Injected on Day 14	316 ± 145 271	91 ± 8 91	1444 ± 73 1460	1032 ± 153 1005	16517 ± 720 14176	10809 ± 1009 10954

*Mean ± SEM derived from 3 feti from each of 3 litters for each group.
†Estimated from least squares regression analysis of log transformed data from each time period.
‡Uterine concentrations.

Once dosed, the animals were housed in colony cages in groups of 4. Cage assignment and distribution within the animal room was by way of a randomization procedure in an effort to minimize unexplained variation due to environmental conditions. The rats were weighed individually on Days 7, 14 and 19 of gestation but were otherwise unmolested apart from food and water changes. On Day 19 they were removed to individual plastic nesting cages where they remained until they had delivered their pups.

Treatment of Progeny

At birth the number of pups in a litter, their weights and sexes were recorded and then the litter was culled to a standard size of 3 females and 3 males.

In order to differentiate between the direct effects of MM upon the fetus from possible indirect effects due to changes in the mother or to postnatal exposure by the mother's milk, litters from dams treated on Day 0 of gestation were: (1) left with their biological mothers; (2) fostered to mothers which had received the same treatment as the biological mothers and which had themselves delivered litters within the same 24 hr period; or (3) cross fostered to mothers which had been treated reciprocally to the biological mother (i.e., litters born of mothers treated with MM on Day 0 of gestation were cross fostered to mothers which had received saline on Day 0 and vice versa).

Pups born of rats treated with MM or saline on Days 7 or 14 of gestation were reared by their biological mothers. If there were indirect consequences upon the young due to effects of MM upon the mother, then such effects would most likely be detected in animals treated with MM on Day 0 of gestation. These rats would have received the greatest amount of MM on a mg/kg basis and would have been exposed to the compound for the greatest length of time. Fostering and cross fostering of litters born to rats dosed in the latter periods of gestation would have increased size and complexity of the experiment without offering significant additional information.

Apart from weekly cleaning of the nest boxes and weighing of the pups, dams and their litters were undisturbed during the next 21 days, at the end of which time the pups were weaned, individually ear marked and distributed, by sex, to colony cages in groups of 4.

Behavioral Apparatus and Methods

The autoshaping procedure consists of random presentation to a naive animal of an exteroceptive stimulus associated spatially with a response manipulandum and which is immediately and predictably followed by a second stimulus, usually food. With repeated presentations the animal will eventually emit contact responses to the manipulandum, having become autoshaped to the exteroceptive stimulus. The procedure has been utilized to study acquisition of an operant response in a variety of species including pigeons, chicks, quails, dogs, monkeys, fish and rats [5, 12, 30, 35, 40]. It is easily automated, eliminates the necessity for hand shaping, allows for relatively quick screening of animals, and produces discrete data which may be interpreted unambiguously.

Our method was modified from that of Stiers and Silberberg [41] in that only one retractable lever was utilized and presentation of the lever itself served as the exteroceptive stimulus. The apparatus was comprised of standard BRS/LVE (Beltsville, MD) rodent boxes and isolation chambers, each containing a retractable lever on the right front panel which was connected to a sensitive contact circuit. Environmental control and data acquisition were accomplished with a NOVA II computer (Data General, Southboro, MA), BRS/LVE "Interact" peripherals and their "ACT I" software package (BRS/LVE, Beltsville, MD).

At 6 months of age a male and female were selected at random from each litter and housed individually under conditions of food deprivation. When within 80% of their free feeding weights operant baseline behavior was measured for each animal during 2 consecutive sessions. One minute after placement of a rat into an initially darkened experimental chamber, a light was lit above the retractable lever and the lever was extended on a random inerval (RI) schedule designed so that the average interval between presentations was 90 sec, with a minimum interval of 45 and a maximum of 135 sec. A 15 sec extension of the level

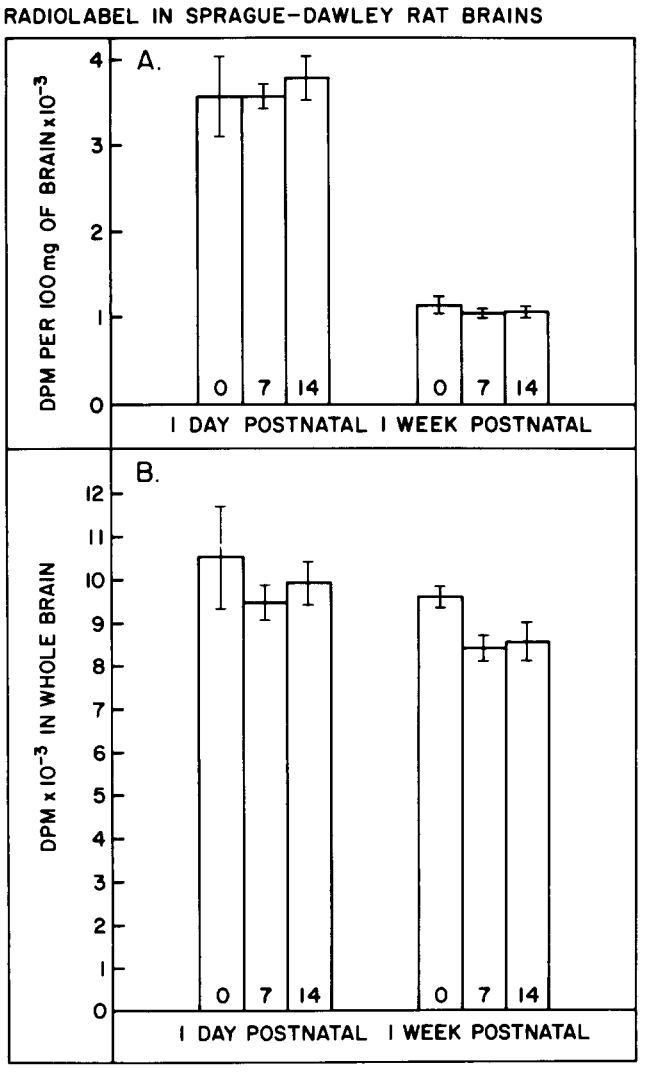

FIG. 2. Amounts of radioactivity in the brains of rat pups sacrificed 1 day or 1 week after being born to dams injected with 5.82, 3.23 and 1.55 mg $^{14}CH_3HgCl$/kg, p.o., on Days 0, 7 and 14 of gestation, respectively. Each bar is the mean of at least 3 litters and 2 pups per litter. Vertical lines represent SEM. One μg of CH_3Hg is represented by 36,852 disintegrations per minute (dpm). A. Concentration of methylmercury per 100 mg of brain weight. B. Concentration of methylmercury.

constituted a trial and 32 trials comprised a session. Contacts with the lever during the 2 baseline sessions were recorded, but had no programmed consequences with respect to the rat.

On 2 days following the baseline sessions, each rat was trained to approach and eat from a food chute in the front panel of the experimental chamber by presenting food (45 Noyes Precision Food Pellets, P. J. Noyes, Lancaster, NH) on the RI 90 sec schedule, but with the lever kept retracted.

On the 5th and subsequent days the lever was presented as in the baseline sessions, in addition, if the rat made contact with the lever it was immediately withdrawn, a food pellet was presented and a new trial was initiated. Should the rat have failed to respond within 15 sec, the lever was retracted, a pellet was presented and a new trial was initiated. Each daily autoshaping session consisted of 32 trials and the number of responses emitted and the time taken to make contact with the lever (latency to respond) was recorded.

Simultaneous with extension of the lever, the stepping pen of a cumulative recorder (Ralph Gerbrands, Arlington, MA) was activated in 0.05 sec increments. Retraction of the lever reset the stepper, thus providing a graphic representation of latency to respond for each trial. Examples of such records can be seen in Figs. 3a and 3b.

Amphetamine Challenge

When a given rat had attained an arbitrary criterion of at least 30 responses ($\geq$90%) in a session for 5 sessions, amphetamine challenges were begun. Animals whose dams had been treated on Day 0 of gestation were injected, IP, with doses of 0, 0.5, 1.25, 2.5 or 3.75 mg d-amphetamine SO_4 (d–A/kg in 0.9% NaCl, every 3 days just prior to the start of an experimental session. Administration of the d–A was in an ascending order, a descending order or one of several randomized orders for each of the rats comprising a given treatment group (i.e., males or females exposed in utero to MM or saline and reared by biological, fostered, or cross fostered dams).

Males whose dams had been exposed on Days 7 or 14 of gestation were treated, in randomized order, with 0, 2.5 or 3.75 mg d–A/kg and their female counterparts with 0, 1.25 or 2.5 mg d–A/kg. These doses were selected, on basis of our Day 0 results, as being capable of differentiating between animals exposed in utero to MM and those which had been exposed to saline.

Statistics

Data analyses were carried out using factorial ANOVAs and Newman-Keul's studentized range comparisons [47].

RESULTS

Effects of Treatment of Dams on Day 0 of Gestation

Non-behavioral measures. None of the 12 dams treated with MM gave indication of intoxication on gross observation, nor were significant differences detected between these rats and controls with respect to the amount of weight gained during gestation, litter sizes or litter weights at birth or at weaning.

Behavioral measures. Analyses of the baseline operant and acquisition data revealed no differences when the rats were compared by nature of treatment in utero or by nature of the treatment given to the mother which reared the pups. Significant differences were detected, however, when the animals were compared by sex. Female progeny were more active than males in both operant sessions, as measured by the number of trials in each session in which at least one lever contact was made or the number of sessions taken to attain the criterion of 30 or more responses (Table 3).

Neither males or females showed significant diminution of responding after IP injection with 0, 0.5 or 1.25 mg d–A/kg just prior to the start of the behavioral sessions. Doses of 2.5 or 3.75 mg/kg, however, resulted in a significant diminution in the number of responses per rat

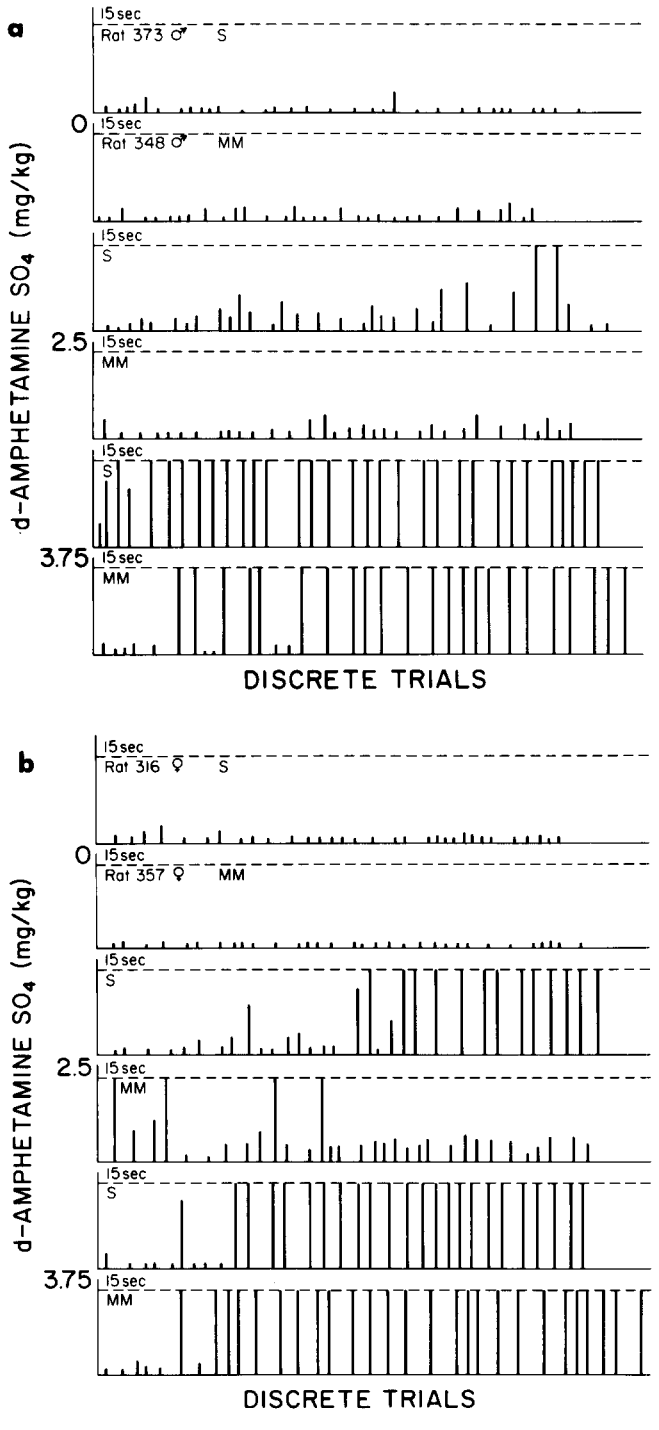

FIG. 3. Individual examples of the effects of d-amphetamine upon autoshaped responding by rats whose dams were gavaged with 5 mg MM/kg or with saline on Day 0 of gestation. Each vertical deflection (obtained by stepping a cumulative recorder pen at 0.05 sec intervals) represents the time taken to contact the lever in a given trial. Maximal excursion (horizontal dotted lines) was 15 sec, indicating failure to respond. There were 32 trials in each session. (a) Data from an experimental (MM) and control (S) male. (b) Data from an experimental (MM) and control (S) female.

when compared with the session in which they received saline. In addition, females exposed in utero to saline and treated with 2.5 mg d–A/kg and males similarly exposed in utero and treated with 3.75 mg d–A/kg were found to have responded significantly fewer times than their MM exposed counterparts (Table 4). There was no evidence for an effect due to fostering, $F(2,18) = 0.358$, $p = 0.7$ for males; $F(2,18) = 1.576$, $p = 0.234$ for females.

Examination of the individual records from each rat (representative examples of which are presented in Figs. 3a and b) suggested that the effect of d–A challenge was manifested by normal responding soon after injection followed by a sudden cessation which persisted through the end of the session. We measured the number of trials before each rat ceased to respond entirely and found that females exposed prenatally to MM and challenged with 2.5 mg d–A/kg ceased responding after an average of 28.8 ± 1.8 M ± SE) trials compared with 20.2 ± 2.8 trials for their saline controls and that males exposed in utero to MM ceased to respond after 13.9 ± 2.1 trials when challenged with 3.75 mg d–A/kg while their controls ceased in 5.8 ± 1.1 trials. By taking the differences between the means of the mercury and saline treated animals and multiplying by the average time between trials (90 sec) we found that MM treated males and females ceased to respond between 12 and 13 min (729 and 780 sec, respectively) later than their saline counterparts.

When the data were examined in terms of the mean latencies (in seconds) to respond to the lever when presented, administration of d–A just prior to beginning an autoshape session resulted in a linear increase with dosage (Fig. 4). Factorial analysis indicated significant overall effects due to dosage of amphetamine, nature of exposure in utero and sex, with no evidence for effects due to fostering or to the order of injections. These results compare well with those obtained from the analysis using discrete trials in that the animals could be differentiated on basis of the treatment given to their dam and on basis of sex. Furthermore, post hoc Newman-Keul's studentized range tests revealed significant differences between the 0 mg d–A/kg dose in the mean latencies to respond by females exposed in utero to saline or to MM when challenged with 1.25, 2.5 or 3.75 mg d–A/kg. Males derived from saline treated dams showed similar effects, but only the 2.5 and 3.75 mg d–A/kg latencies differed from control in the case of males exposed in utero to MM (Fig. 4). The mean latency data were thus not completely redundant with the discrete trials analyses in that it could differentiate effects of 1.25 mg d–A/kg, a dose which produced no significant differences when number of trials per session was the criterion.

Effects of Treatment of Dams on Day 7 of Gestation

Non-behavioral measures. None of the 9 dams treated with MM gave indication of intoxication on gross observation, nor were significant differences detected between these rats and controls with respect to the amount of weight gained during gestation, litter sizes, or litter weights at birth or at weaning (Table 1).

Behavioral measures. Analyses of the baseline operant data revealed no differences when the animals were compared by nature of treatment in utero or by sex. Females exposed prenatally to MM attained the criterion of 300 or more responses significantly sooner than controls,

TABLE 3

OPERANT LEVELS OF ACTIVITY AND ACQUISITION OF AN AUTOSHAPE RESPONSE BY SPRAGUE-DAWLEY RATS BORN TO DAMS TREATED WITH METHYLMERCURY OR SALINE ON DAYS 0, 7 OR 14 OF GESTATION (MEANS ± SEM)

	Females		Males	
	Methylmercury	Saline	Methylmercury	Saline
Operant Session 1: Trials with Contact				
Day 0	3.1 ± 0.9*	2.3 ± 0.5*	1.3 ± 0.3	1.0 ± 0.3
Day 7	1.9 ± 0.6	1.6 ± 0.5	3.1 ± 1.5	1.8 ± 0.6
Day 14	2.3 ± 0.6	1.4 ± 0.8	2.0 ± 0.7	2.0 ± 0.4
Operant Session 2: Trials with Contact				
Day 0	2.3 ± 0.3*	2.1 ± 0.3*	0.6 ± 0.2*	0.8 ± 0.3
Day 7	2.1 ± 0.4	2.0 ± 0.4	2.3 ± 0.9	0.8 ± 0.6
Day 14	1.4 ± 0.5	1.3 ± 0.5	2.5 ± 1.2	1.4 ± 0.5
Sessions to Attain Criterion				
Day 0	1.7 ± 0.3*	1.7 ± 0.2*	2.7 ± 0.5*	3.0 ± 0.8*
Day 7	1.5 ± 0.3	2.6 ± 0.3	1.7 ± 0.3	3.0 ± 1.2
Day 14	2.5 ± 0.4	1.9 ± 0.2	1.6 ± 0.3	3.0 ± 0.9

*Differ significantly from other sex, ANOVA, $p<0.05$

$F(1,14) = 7.1$, $0.05>p>0.01$. Male rats exposed to MM also had lower mean numbers of sessions to criterion, but the difference was not significant (Table 3). As shown in Table 4, male rats exposed in utero to MM responded significantly more often than controls when challenged with 3.75 mg d–A/kg, $F(1,16) = 5.49$, $0.05>p>0.01$, but not with 2.50 or 0 mg/kg. Females exposed prenatally to MM responded significantly more often than controls when injected with 2.5 mg d–A/kg, $F(1,14) = 5.05$, $0.05>p>0.01$, but not when challenged with 1.25 or 0 mg/kg. Male rats were less affected by the amphetamine than females, and signified differences were detected when the sexes were compared during the autoshape session in which they had been injected with 2.5 mg d–A/kg, $F(1,32) = 7.41$, $0.01>p>0.001$.

MM exposed females ceased responding after an average of 14.6 trials and those exposed to saline after 6.8 trials in the session in which they had been challenged with 2.5 mg d–A/kg. The mercury treated animals thus ceased responding about 11.7 min later than did controls. Similarly, male rats treated with 3.75 mg d–A/kg and exposed in utero to MM from Day 7 of gestation ceased responding after an average of 14 trials while their saline exposed counterparts did so after 8.2 trials. The MM treated rats,

TABLE 4

EFFECTS OF D-AMPHETAMINE SO_4 UPON NUMBER OF RESPONSES (MAXIMUM OF 32) MADE IN AN AUTOSHAPE SESSION BY SPRAGUE-DAWLEY RATS BORN TO DAMS TREATED WITH METHYLMERCURY OR SALINE ON DAYS 0, 7 OR 14 OF GESTATION

	Dose of d-Amphetamine SO_4 (mg/kg, IP)	Females		Males	
		Methylmercury	0.9% NaCl	Methylmercury	0.9% NaCl
Dam	0	31.9 ± 0.1	31.9 ± 0.1	32.0 ± 0	31.9 ± 0.1
intubated	0.5	31.7 ± 0.9	31.9 ± 0.1	32.0 ± 0	31.9 ± 0.1
on day 0	1.25	26.6 ± 2.8	27.5 ± 2.51	31.9 ± 0.1	29.2 ± 2.0
of gestation	2.5	17.4 ± 2.81*	12.2 ± 2.45	22.6 ± 2.3	16.0 ± 3.2
n=12, each group	3.75	6.6 ± 1.8	6.6 ± 2.1	10.1 ± 2.1*	4.2 ± 0.9
Dam intubated	0	31.9 ± 0.1	32.0 ± 0	32.0 ± 0	31.8 ± 0.2
on day 7	1.25	25.4 ± 3.3	22.4 ± 2.9	—	—
of gestation	2.5	10.4 ± 1.9*	5.8 ± 2.3	17.6 ± 3.8	14.4 ± 3.5
n=9, each group	3.75	—	—	10.4 ± 2.7*	4.0 ± 0.3
Dam intubated	0	32.0 ± 0	31.9 ± 0.1	32.0 ± 0	31.9 ± 0.1
on day 14	1.25	20.4 ± 4.4	25.0 ± 2.4	—	—
of gestation	2.5	10.8 ± 3.6	9.4 ± 1.4	23.9 ± 4.2	20.1 ± 4.0
n=8, each group	3.75	—	—	10.1 ± 2.6	6.0 ± 2.4

*Differs significantly from control, ANOVA, $p<0.05$.

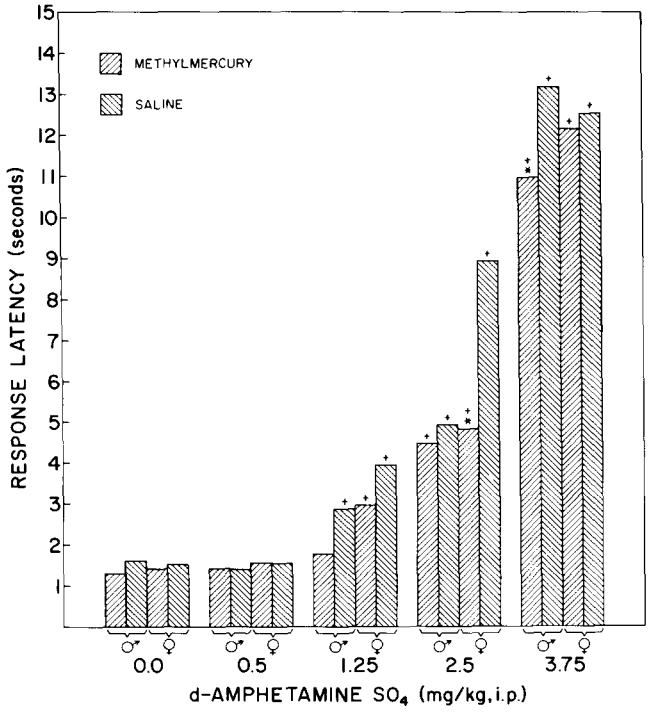

FIG. 4. Effects of d-amphetamine upon mean latency to respond upon presentation of the lever in the autoshape task. Results from 12 males and 12 females derived from dams treated with 5 mg MM/kg on Day 0 of gestation and a like number of animals from dams treated with saline. An asterisk indicates a significant difference ($p<0.05$) between MM and saline exposed rats of like sex at the indicated dose of d-amphetamine. A cross denotes a significant difference ($p<0.05$) within each group; (i.e., males exposed in utero to MM and their controls and the female counterparts to these) between latencies obtained with the indicated ar:oung of d-amphetamine and those obtained when the animals were dosed with 0.0 mg d–A/kg.

therefore, did not show the effects of the amphetamine until about 8.7 min after saline males had ceased to respond.

Repeated measure ANOVAs followed by Newman-Keul's studentized range comparisons, showed that the increases in latencies were significant for each dose of d–A. Significant differences were found between mean latencies to re pond by male rats treated with 3.75 mg d–A/kg and exposed in utero to MM and their controls, $F(1,16) = 5.159$, $p = 0.019$. Females could be differentiated with respect to the nature of their exposure in utero at a dose of 2.5 mg d–A/kg, $F(1,14) = 7.586$, $p = 0.016$. A significant sex effect was found when latencies to respond in the session with 2.5 mg d–A/kg were compared, $F(1,32) = 5.188$, $p = 0.030$.

Effects of Treatments of Dams on Day 14 of Gestation

Non-behavioral measures. None of the 8 MM treated dams showed signs of intoxication on gross observation. Nor were there significant differences between these rats and controls with respect to weight gain during pregnancy, litter sizes or litter weights.

Behavioral measures. Analyses of the baseline operant data revealed no significant differences when the animals were compared by nature of treatment in utero or by sex, nor were there significant differences in the number of sessions needed to attain the criterion of 30 or more responses (Table 3).

When challenged with d–A, no differences attributable to the nature of exposure in utero were found with regard either to the numbers of responses in sessions (Table 4) or the mean latencies to respond. Examination of mean number of responses emitted in the session with 2.5 mg d–A/kg revealed a significant overall effect due to sex, $F(1,28) = 11.999$, $p = 0.002$.

DISCUSSION

MM administered orally in doses of 5.0, 2.78 and 1.33 mg/kg to pregnant rats on Days 0, 7 and 14 of gestation, respectively, produced equivalent concentrations of the alkylmercurial in the brains of 19 day feti and in 1 and 7 day-old neonates. These dosages produced no signs of toxicity within the maternal organisms or their progeny as measured by gross phsyical appearance, weight gain during pregnancy, sizes and weights of litters at birth or culled litter weights through weaning. Operant baseline measures and acquisition of an autoshape task did not differ between animals exposed in utero to MM and those similarly exposed to saline. Challenge with d–A, however, revealed significant functional differences between contemporaneous saline exposed controls and otherwise normal appearing rats whose dams had been treated with MM on Days 0 or 7, but not those exposed to MM on Day 14 of gestation. The lack of an effect in animals whose dams were treated on Day 14 and the failure to demonstrate an effect attributable to cross fostering of pups born to dams treated on Day 0 of gestation indicated that the functional difference occurred in utero and that the stage of development of the fetus was a determinant. The data do not allow us, however, to distinguish between a direct effect of the MM upon the fetus and an indirect effort due to alteration of the maternal-placental-fetal milieu. The possibility of a direct action upon the fetus is suggested from the demonstration that administration of MM to fetilized eggs prior to hatching will produce behavioral changes in resultant chicks and that these effects are also upon the time of administration of the compound [17].

Our finding (Table 1) that placental to fetal ratios were greater in the case of the lower dosage (0.05 mg MM/kg) than with the 2 higher doses is in keeping with Löfroth's [20] hypothesis of an increasingly selective concentration of mercury into the fetus at moderate or high exposure levels. This would explain why ratios for the lower dose were higher after administration later in pregnancy since there would be more MM present in placental and uterine tissue and therefore, more would be transferred to the fetus. Childs [6] reported a similar relationship with mice in an analysis relating amount of mercury in fetal tissue to maternal concentration after exposure to MM by incorporation of the compound into their diet.

Radioactivities measured in placentae 5 days after injection on Day 14 of gestation were greater than those seen in placentae 5 days after injection on Day 7 or uteri 5 days after injection in Day 0. The same was true of feti measured on Day 19 after injection on day 14 compared with those measured on Day 12 after injection on Day 7 (Table 2), suggesting that incorporation of radiolabel (and

therefore MM) into the fetus is increased during the latter stages of pregnancy. This finding is in agreement with those of Yang *et al.* [49], who found a 3-fold increase in fetal uptake from Days 17–20 after forced feeding of MM to Sprague-Dawley rats on Day 16 of gestation, Mansour *et al.* [22] who detected increased fetal uptake in Holtzman rats 24 hr after MM on Day 20 compared with an equal time after injection on Day 15 and Olson and Massano [28] who found increased amounts of MM in fetal CFW mice whose dams were administered the compound on successively later days during gestation. Yamaguchi and Nunotani [48], however, have reported a decrease of concentration of MM in fetal Wistar rats during Week 3 of pregnancy compared with Week 2, despite a placental concentration almost 5 times greater than that of the fetus.

In using radioactivity (dpm ^{14}C) as a means of monitoring MM in tissue we have assumed that there is negligible biotransformation of the radiolabelled compound. We feel this assumption is justified because the values of MM obtained by our method were essentially the same as those obtained from specific extraction and gas chromatographic analyses. Additionally, the levels of activity we have found in fetal, uterine and pup tissue are well within the range of those reported in the literature using flameless atomic absorption spectroscopy and gamma scintillation counting of ^{203}Hg labelled MM [27, 40, 50].

We have developed a means by which we were able to obtain equivalent concentrations of MM within the brains of rat pups from Day 19 of gestation through at least one week postnatally, even though injection took place at one of three different periods of gestation. Progeny from dams intubated with MM on Days 0 or 7 of gestation differed from those whose dams were intubated with saline in that the MM treated animals were resistant to the effects of challenge with d–A. This functional deficit seemed to be time related, since pups whose dams were intubated on Day 14 of gestation did not differ from their controls. We cannot establish, at this time, whether the effect is primarily one upon the central nervous system or upon the periphery. That peripheral mechanisms may play a role in the delayed amphetamine effects comes from the finding [33] that hepatic cytochrome P–450–dependent-mono-oxygenase systems were depressed in adult male rats exposed prenatally to MM on Days 0 or 7, but not in those whose dams were treated on Day 14 of gestation. Depressed metabolism, however, would be expected to produce higher levels of d–A in the brains of MM exposed animals. These animals should, therefore, have shown greater behavioral effects due to amphetamine than controls. Our finding that equal doses of d–A (on a mg/kg basis) produced less behavioral disruption in MM derived rats may thus represent a conservative estimate of postnatal behavioral differences.

Our data for differential responsiveness of MM exposed animals should not be construed as a demonstration that this agent is toxic only to the young embryo or young fetus. With higher doses MM undoubtedly affects mature organisms of every species exposed to it. However, the data do support the notion that the particular behavior variable used is most sensitive to insult during early development. Perhaps the anatomical or biochemical substrate of one or more components of the task requirement is being transcribed or transduced during this early stage of development.

In these experiments MM was used because of the more than ample evidence of its capacity to produce congenital neurological sequelae in humans. Hopefully, the demonstration of similar effects in infrahuman species, at apparently harmless doses, will add the credence necessary for acceptance of data from similar studies in which lower life forms are exposed to agents as yet unproven vis a vis their ability to produce disasters similar to those caused by MM and thalidomide.

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