Journal of Toxicology and Environmental Health, Part A Current Issues

ISSN: 0098-4108 (Print) (Online) Journal homepage: www.tandfonline.com/journals/uteh19

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To cite this article: Joan M. Spyker & David L. Avery (1977) Neurobehavioral effects of prenatal exposure to the organophosphate Diazinon in mice, Journal of Toxicology and Environmental Health, Part A Current Issues, 3:5-6, 989-1002, DOI: 10.1080/15287397709529633

To link to this article: https://doi.org/10.1080/15287397709529633

Published online: 20 Oct 2009.

NEUROBEHAVIORAL EFFECTS OF PRENATAL EXPOSURE TO THE ORGANOPHOSPHATE DIAZINON IN MICE

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Pregnant mice were given a daily dose of 0, 0.18, or 9.0 mg Diazinon per kilogram body weight throughout gestation. Mothers of all dose groups gave birth to viable, overtly normal offspring. However, pups born to mothers receiving the higher dose of the organophosphate grew significantly slower than controls and remained significantly smaller at 1 month of age. Offspring of mothers receiving the lower dose apparently were unaffected, but systematic behavioral testing revealed subtle deviations from normal developmental ontogeny as shown by significant delays in the appearance of the contact placing reflex and of sexual maturity (descent of testes or vaginal opening). Mature offspring of mothers exposed to either dose of the pesticide displayed impaired endurance and coordination on rod cling and inclined plane tests of neuromuscular function. Offspring from the 9.0 mg/kg group, in addition, had slower running speeds in a Lashley III maze and less endurance in a swimming test. Brains obtained after sacrifice at 101 days of age revealed neuropathology in the forebrains of offspring born of mothers exposed to the higher dose. Despite functional impairments in offspring from the lower dose group, no corresponding brain pathology was observed by examination under the light microscope.

INTRODUCTION

Organophosphate insecticides are potent enzyme inhibitors that exert a generalized cholinergic action by inactivating central and peripheral cholinesterases. Subacute intoxication with an anticholinesterase agent disturbs and reduces a wide variety of behavioral outputs (Bignami et al., 1975). Tolerance develops rapidly, however, and such behavioral effects are usually transient. Glow and co-workers (1966) reported that the rat can perform complex learned behaviors even after reduction of brain cholinesterase (ChE) by diisopropyl fluorophosphate to 20% of normal. If overt cholinergic symptoms (e.g., salivation, tremor) are avoided by appropriate titration and timing of the dose, chronic subclinical

The authors wish to thank Dr. Robert MacPhail and Dr. Bernard Weiss for their critical reading of the manuscript and Dr. Louis Chang for his help in interpreting the neurohistological data.

This work was supported by Environmental Protection Agency contract 68-01-1925.

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Journal of Toxicology and Environmental Health, 3:989–1002, 1977

organophosphate intoxication usually cannot be detected by conventional behavioral testing in the laboratory (Bignami and Gatti, 1969). In a study by Spear et al. (1975), no subjective symptoms were noted in agricultural workers repeatedly exposed to anticholinesterase pesticides despite moderately depressed blood ChE activity.

The relative insensitivity of behavioral indexes to low-level chronic organophosphate exposure may have encouraged the conclusion that such exposures are without permanent neurological sequelae (Rodnitzky et al., 1975). Such an interpretation ignores the sensitivity of the developing organism to neural insult and the possibility of delayed adverse effects (Spyker, 1975). The symptoms of acute organophosphate intoxication are much more severe in young animals than in adults. LD_{50} values in immature animals have been reported to be only 1/100 of those obtained in the adult for some organophosphate compounds (Brodeur and DuBois, 1963). The developing organism may also be more sensitive to the induction of functional neural deficits.

To test this hypothesis, a prototypic organophosphate, Diazinon, was selected for prenatal treatment in the mouse. Diazinon is metabolically activated *in vivo* (Schrader, 1963) to yield the very potent enzyme-phosphorylating agents diazoxon and tetraethylmonothiopyrophosphate. The oral LD_{50} in adult mice for technical grade Diazinon is approximately 82 mg/kg (Bruce et al., 1955). Death ensues from respiratory arrest resulting from irreversible ChE inhibition following the formation of stable covalent bonds between the esterase and the phosphorus moieties.

Chronic exposure of adult rats to 0.1 mg/kg-day of Diazinon does not induce neurohistological damage observable with the light microscope (Bruce et al., 1955). Oral administration of 0.125 or 2.25 mg/kg Diazinon to pregnant hamsters on days 6–8 of gestation does not result in dose-related embryotoxic or teratogenic effects (Robens, 1969). Administration of Diazinon to gravid rabbits from day 5 to day 15 of gestation in doses of 7 or 30 mg/kg-day does not result in embryotoxicity or teratogenicity (Robens, 1969).

Daily oral administration of 0.1 mg/kg Diazinon does not depress blood ChE in the adult rat (Bruce et al., 1955). Diazinon and its metabolites are rapidly excreted, mainly in urine; at least 95% of an oral dose in rats is excreted within 168 hr (Mucke et al., 1970). Examination of tissue taken from rats chronically exposed to Diazinon by incorporation in the diet does not reveal pesticide accumulation within any tissue compartment tested (Mucke et al., 1970).

Based on the toxicological data cited above, Diazinon has been classified as a moderately toxic, short-lived organophosphate suitable for widespread agricultural use on food crops. Field workers, many of whom are women of childbearing age, receive frequent occupational exposure to the compound. The following study was conducted in mice to determine whether prenatal exposure to low levels of Diazinon might impair reflex

ontogeny or produce subtle neurobehavioral dysfunctions evident only as the offspring of exposed mothers mature.

MATERIALS AND METHODS

Groups of six adult female F_2 hybrid mice[1] were caged with individual F_2 males at 6:00 p.m. Vaginal plugs were checked at the onset of the 12 hr light interval (7 a.m.) and mated animals were randomly assigned to one of three groups. At the onset of the light cycle on each succeeding day of gestation animals were exposed to a daily dose of 0, 0.18, or 9.0 mg/kg body weight of technical grade Diazinon[2]. Mice were treated by using a nontraumatic oral dosing technique developed in this laboratory. Diazinon was incorporated in peanut butter at a uniform concentration calculated to provide the daily dose in approximately 1 ml of food. Pregnant individuals were weighed daily, an appropriate volume of peanut butter was dispensed to provide the calculated dose, and the animal was isolated in a small cage with the spiked diet. Consumption of the peanut butter was usually immediate despite the free availability of food (Purina lab chow) and tap water in the home cage. After consumption of the ration, mice were returned to the group cages and left undisturbed until the next weighing.

On day 19 after mating, animals were isolated in maternity cages. Dosing was continued daily until parturition. At birth, pups were examined for viability and gross birth defects. Within 6 hr after birth, offspring were randomized within treatment groups to give each treated or control mother four male and four female pups from a like-treated dam. Cross-fostering procedures (Spyker and Spyker, 1977) were not employed because no deviant maternal-offspring interactions were observed and the rapid excretion of Diazinon by the mother (Mucke et al., 1970) minimized contamination of the milk.

Offspring were weighed daily and checked for morbidity and mortality. Offspring were tattooed on the foot (Avery and Spyker, 1977) to permit identification of individual pups. Reflex ontogeny was evaluated by daily scoring for the presence or absence of each of seven physical or behavioral landmarks of development. Pups were tested for ability to roll from the supine to the prone position (simple righting) within 30 sec of being placed on a smooth surface. Acceleration righting was tested by scoring the ability to land in an upright posture on a padded surface when

[1] The F_2 dihybrid is produced by mating the four most common inbred mouse strains (female C57BL/6 X male A/JAX, female C3H/He X male BALB/c) and then crossing the F_1 hybrid BA (F_1) and HC (F_1) to yield the BA·HC (F_2) dihybrid. Mating the F_2 animals produces an F_3 outcross that is vigorous and well buffered against spontaneous congenital deformities but possesses a known genetic history and replicable gene pool. The dihybrid outcross was developed at and obtained from the National Center for Toxicological Research, Jefferson, Arkansas.

[2] Research Lot MG8-FL741305, obtained by courtesy of Ciba-Geigy.

dropped 25 cm in a supine position. Contact placing was scored by judging the ability of the pup to climb over a 2 cm barrier placed under its chin. Physical maturity was evaluated by recording the date of opening of the eyes and ears and the date of the first appearance of hair on the dorsal surface. Sexual maturity was judged by recording the date of vaginal opening or testes descent.

Offspring were weaned on postnatal day (PN) 28 and housed in groups of six similarly treated mice of the same sex. Animals were uniquely and permanently identified by tail tattoo at weaning. Henceforth, all data were collected in a blind manner. On PN 38 function of the visual system, including depth perception and acuity, was tested using a visual cliff apparatus (Lafayette Instrument Co.) equipped with a 2-cm-wide test platform. The side on which each mouse voluntarily stepped down was recorded (shallow or deep) during a 3 min trial. Audition was evaluated next by visual scoring of a startle response to the presentation of a 95 dB SPL burst of white noise for 200 msec. Finally on PN 38 olfactory sensitivity was scored by recording the presence or absence of a withdrawal response when an ammonia-soaked swab was presented in front of the animal's nose.

Neuromuscular coordination was evaluated on PN 50 by scoring swimming ability during a 10 min session in a 25 X 10 X 25 cm glass tank filled with water at room temperature. Sessions were videotaped to allow subsequent scoring by two independent observers. Swimming activity was scored by recording the amount of time (seconds) spent swimming actively during the 10 min observation period. A qualitative score for swimming style or ability was assigned on a ten-point scale by two experienced independent observers (Spyker, 1975).

Locomotor strength and endurance were tested on PN 60 by using a rod cling apparatus. Pups were placed near the end of a 4-mm-diameter horizontal dowel suspended 40 cm above a padded surface. A stopwatch was used to record the interval between placement and falling (cling time). Mice enduring a 1 min trial were further stressed by swinging the 25-cm-long rod to a vertical position over the pivot point at the end opposite the mouse. Again, cling time was recorded. On PN 65, a second test of endurance was conducted, using a rotarod (Stoelting treadmill 51300). Three massed trials were run with the rotarod adjusted to yield a treadmill speed of 1.5 m/min. Endurance on each trial was automatically recorded by the timer and treadle on the treadmill.

On PN 70 offspring were tested with an inclined plane apparatus. Individual mice were placed on a horizontal surface made of cotton fabric stretched on a wooden frame. The platform was then slowly rotated to the vertical position (0-90° from horizontal). The axis of rotation was perpendicular to the long axis of the mouse's body. The angle of the surface at which the mouse lost its grip on the cloth and began to slide backward was recorded.

Exploratory activity was observed on PN 75 using an open-field apparatus. The open field consisted of a 50 X 50 X 10 cm illuminated enclosure. The floor was made of black formica divided into nine equal squares. Locomotor activity was recorded on a counter activated by the breaking of infrared light beams separating the squares. The number of beam interruptions and the number of defecations and urinations during a 5 min trial were recorded. On the next day (PN 76), the open-field test was repeated with the addition of a 30 sec auditory stimulus presented during the third minute of the trial. The stimulus (a solenoid-activated alarm bell) has previously been shown to elicit audiogenic seizures in susceptible animals (Finger and Schlosberg, 1941).

Beginning on PN 87, mice were deprived of food and reduced to 80% of their free-feeding weight. Individual mice were trained to run in a 130-cm-long straight alley for food reinforcement. Ten trials were given on each of two consecutive days, with one 45 mg food pellet (Noyes) dispensed on each trial. Sufficient food was dispensed in the home cage to maintain the criterion weight. After completion of this pretraining procedure, all mice were tested in a Lashley III maze modified for semiautomatic operation (Fig. 1). A fourth alley was added to the standard design and photocells were placed to count errors automatically and to record running latencies. Magazine-type start and goal boxes as well as automatic pellet feeders speeded the operation of the maze by allowing sequential testing of up to five mice in a group. All data were recorded by automated equipment. Mice were run on five consecutive days, receiving 5 trials on day 1, 7 trials on day 2, and 11 trials on days 3, 4, and 5 for a total of 45 trials.

All animals were sacrificed at 101 days of age. Mice were rapidly decapitated and the brains removed for histological study. The tissue was fixed in Bouin's solution, washed in lithium carbonate, then dehydrated and embedded in paraffin with the aid of an automatic tissue processor. Serial sagittal 7 μm sections were cut and stained by the hematoxylin and eosin (H & E) method; brain sections were examined and photographed under the light microscope.

RESULTS

Pregnant mice exposed to Diazinon gained significantly less weight ($p < 0.05$) during gestation than animals assigned to the control group (Table 1). Generally, mothers receiving 0.18 mg/kg-day Diazinon had a smaller ($p < 0.05$) litter size than mothers receiving either 0 or 9.0 mg/kg-day. The birth weight of pups born of mothers exposed to the pesticide was not significantly decreased, but the rate of weight gain by pups born to mothers receiving 9.0 mg/kg-day was significantly ($p < 0.05$; analysis of variance) reduced (Fig. 2).

Daily testing for physiological and behavioral development revealed

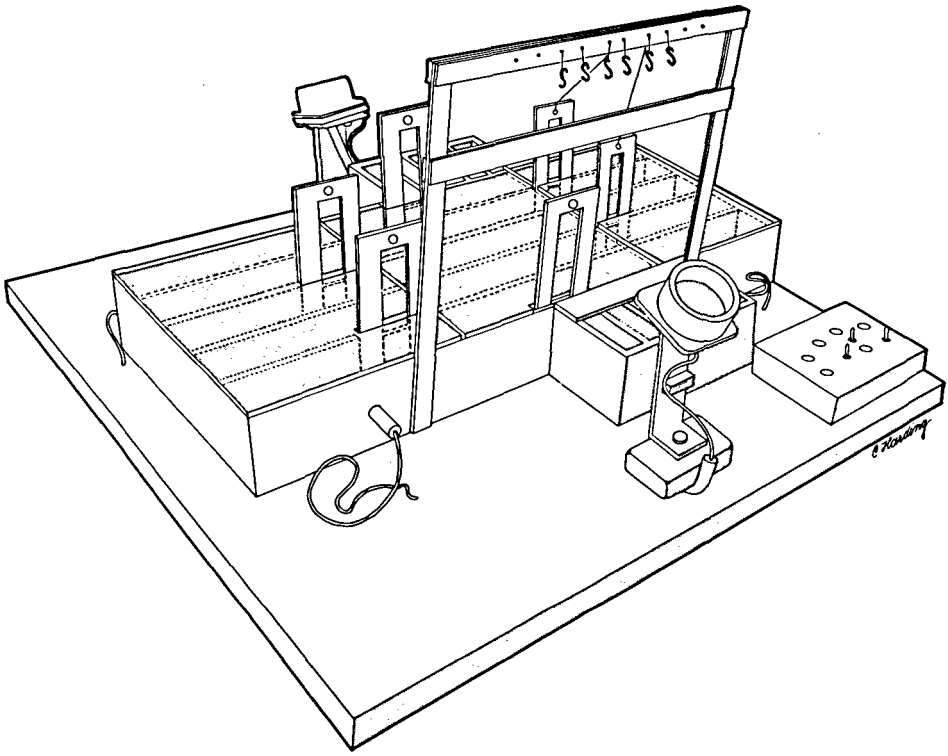

FIGURE 1. Lashley III maze modified for semiautomatic operation. Sliding start-goal boxes and automatic feeders allow up to five mice to be tested simultaneously. Errors and running speeds are recorded by using infrared photocells.

TABLE 1. Gestation and Birth Statistics

Measure	Dose of Diazinon (mg/kg body weight)[a]		
	0	0.18	9.0
Maternal weight gain during pregnancy, g	17.9 ± 1.0[b]	15.4 ± 0.9[c]	15.4 ± 0.6[c]
Number in litter	8.3 ± 0.3	6.6 ± 0.7[c]	8.3 ± 0.2
Length of gestation, days	19.3 ± 0.2	19.7 ± 0.1	19.6 ± 0.1
Number of mothers	22	21	19
Number of offspring	159	133	132

[a] Administered to mothers of test animals.
[b] Mean ± SE.
[c] $p < 0.05$ compared with the control group by t-test.

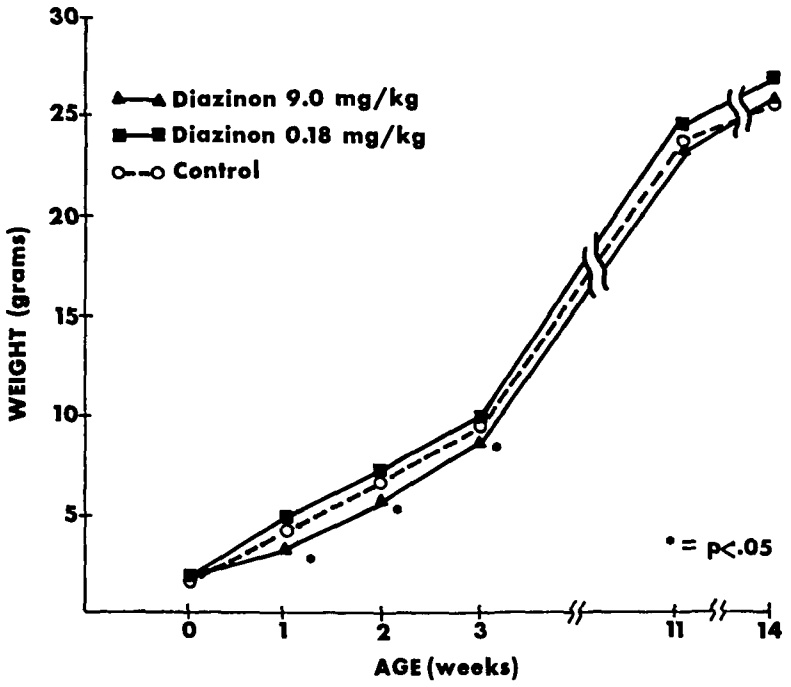

FIGURE 2. Body weight of offspring of mice exposed to 0, 0.18, or 9.0 mg/kg-day Diazinon. Rate of weight gain of pups born of mothers receiving the higher dose is significantly ($p < 0.05$) slower than that of offspring receiving either the vehicle or the lower dose. Even at 14 wk of age, offspring in the higher dose group did not reach the weight of the control group.

some evidence of retarded development among the offspring of treated animals (Table 2). The contact placing ability and sexual maturity of both male and female animals born of mothers exposed to 0.18 mg/kg-day Diazinon were significantly ($p < 0.05$) retarded compared with the development of control offspring. In contrast, the offspring of mothers treated with 9.0 mg/kg-day did not show any abnormalities in the ontogeny of development.

Sensory function assessments in the adolescent mouse revealed no treatment-related effects of pesticide exposure on either the auditory startle response or the olfactory aversion test. On the visual cliff test, male and female offspring of mothers exposed to 0.18 mg/kg-day Diazinon generally made more errors than the offspring of control mothers, although the difference was not significant ($p < 0.1$; $\chi^2 = 4.76$, df = 2). Female offspring were significantly affected ($p < 0.05$; $\chi^2 = 6.2$, df = 2), with only 11 of 20 treated animals making the correct choice while 17 of 20 control animals selected correctly. Offspring of mothers exposed to 9.0 mg/kg-day of the pesticide showed no significant deficit in visual cliff performance.

TABLE 2. Ontogeny of Development

Measure	Dose of Diazinon (mg/kg body weight)[a]		
	0	0.18	9.0
Simple righting	3.2 ± 0.4[b]	3.0 ± 0.1	2.7 ± 0.4
Hair coat	4.9 ± 0.2	4.9 ± 0.1	4.2 ± 0.1
Contact placing	11.0 ± 0.4	13.3 ± 0.4[c]	9.1 ± 0.3
Acceleration righting	10.9 ± 0.3	11.2 ± 0.9	11.1 ± 0.4
Eye opening	13.6 ± 0.4	13.3 ± 0.1	14.3 ± 0.3
Ear opening	13.9 ± 0.3	13.3 ± 0.2	14.7 ± 0.3
Sexual maturity	28.4 ± 0.4	30.0 ± 0.4[c]	26.8 ± 0.6
Number of offspring	67	48	44

[a] Administered to mothers of test animals.
[b] Postnatal day of appearance (mean ± SE).
[c] $p < 0.05$ compared with the control group by Mann-Whitney *U*-test.

No treatment-related effects on swimming style were recorded. Blind scoring of videotape records of the sessions revealed no systematic differences in posture or pattern of swimming. A dose-related treatment effect was observed for swimming activity, with no apparent effect observed for offspring of mothers exposed to 0.18 mg/kg-day but a significant ($p < 0.05$) activity increase for offspring of the 9.0 mg/kg-day group. This effect was more evident for treated male offspring. These data are presented in Table 3.

Prenatal exposure to Diazinon resulted in increased ($p < 0.05$) rod cling endurance. This effect was apparent for both male and female offspring from the 0.18 and 9.0 mg/kg-day treatment groups. Some effect of Diazinon exposure during development also seems evident in the

TABLE 3. Measures of Endurance and Coordination

Measure	Dose of Diazinon (mg/kg body weight)[a]		
	0	0.18	9.0
Swimming activity, sec	200 ± 23[b]	190 ± 19	243 ± 34[c]
Rod cling endurance, sec	65 ± 4	78 ± 6[c]	78 ± 8[c]
Rotarod endurance, sec	1,006 ± 621	407 ± 161	103 ± 56
Inclined plane, degrees	120 ± 7	104 ± 8[d]	109 ± 8[d]
Number of offspring tested	40	20	20

[a] Administered to mothers of test animals.
[b] Mean ± SE.
[c] $p < 0.05$ compared with the control group by one-way analysis of variance and post hoc analysis.
[d] $p < 0.01$ compared with the control group by one-way analysis of variance and post hoc analysis.

rotarod test of running endurance. Mean running times for the offspring of treated mothers were markedly lower than control values, but the high variability of the control subjects precluded statistical confirmation of this difference. The inclined plane apparatus provided a more consistent test of treatment-related neuromuscular impairment. A statistically significant ($p < 0.01$) reduction in the maximum angle obtained before falling was observed for both treatment groups.

No Diazinon-related effects on behavior were observed in the open-field apparatus. No differences were observed in the number of squares entered, or in the number of defecations or urinations. No audiogenic seizures were induced in the treated groups, although two occurred in the control group.

Both male and female offspring of mothers exposed to 9.0 mg/kg-day Diazinon displayed impaired running performance in the Lashley III maze. Although running speeds of treated animals were indistinguishable from those of control subjects on the first day of testing, a clear impairment was observed as learning progressed. Running speeds on the last ten trials for most of the animals of the 9.0 mg/kg-day group were significantly slower ($p < 0.05$) than those for the control grup. This decrease in running speed was not paralleled by an increase in the frequency of errors. Neither the running speed nor the error rate of mice exposed to 0.18 mg/kg-day Diazinon differed from those of the offspring of mothers of the control group. These data are presented graphically in Fig. 3.

Examination of brain tissue following sacrifice revealed morphological abnormalities among the offspring of mothers exposed to 9.0 mg/kg-day Diazinon. Focal defects could be observed in the forebrain in the area extending from the anterior commissure to the anterior olfactory nucleus (Fig. 4A). In five of the eight brains examined, dense aggregations of chromatin-containing cells (Fig. 4B) were observed that bore no obvious neuroanatomical relation to normal brain structures. Such abnormalities were not observed among the brains of offspring born to mothers receiving either 0 or 0.18 mg/kg-day Diazinon.

DISCUSSION

Diazinon cannot be described as a classical teratogen. No gross morphological anomalies were seen in the offspring, although several mothers exposed to Diazinon delivered abnormally small numbers of pups. The rate of maternal weight gain was reduced for all pregnant animals receiving the pesticide. These findings agree with previous reports of the effects of prenatal exposure to other organophosphate compounds, such as methyl parathion (Fish, 1966) and parathion (Talens and Woolley, 1973).

The behavioral deficits observed in offspring of mothers exposed to Diazinon indicate that prenatal exposure to organophosphates may produce subtle dysfunctions not readily detectable until later in life.

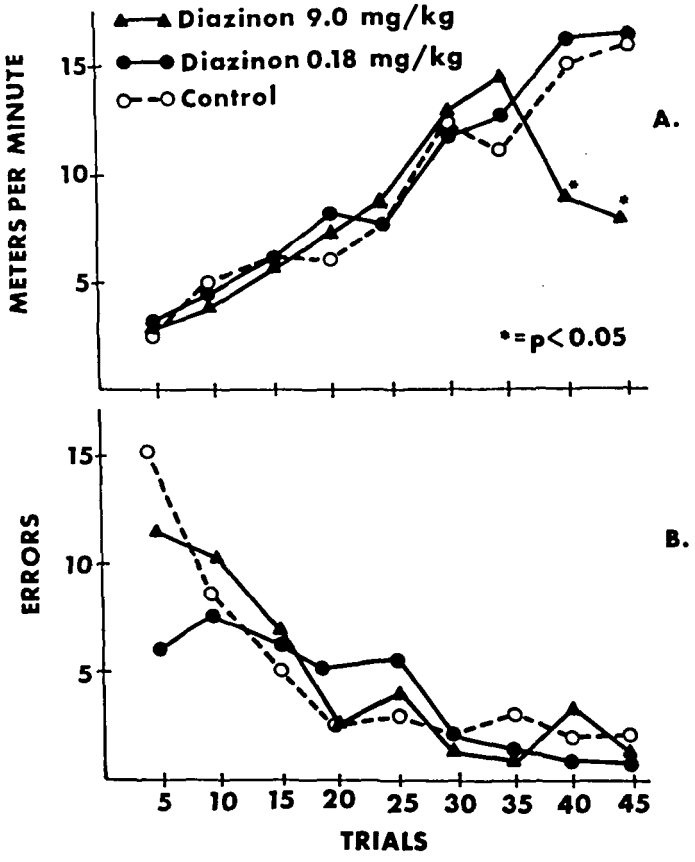

FIGURE 3. Running speed and error frequency in the Lashley III maze for offspring of mice treated with 0, 0.18, or 9.0 mg/kg-day Diazinon. Mice exposed to 9.0 mg/kg-day ran significantly ($p < 0.05$) slower on the final 15 trials than did mice in the other two groups.

Within the range of doses tested, the level of exposure affected the *pattern* of behavioral deficits observed rather than the *magnitude* of the particular deficit. Although the rate of weight gain shown by pups born to mothers of the 0.18 mg/kg-day group appeared normal, their development was retarded. Statistically significant differences were found in two measures; however, one might expect a spurious significance given the large number (14) of comparisons made (Table 2). Offspring of the 9.0 mg/kg-day group were retarded in physical growth but the test protocol failed to detect a functional deficit.

In the later tests of neuromuscular function, offspring exposed to either the 0.18 or the 9.0 mg/kg-day dose of Diazinon were significantly impaired in their performances, although the deficit was more apparent for mice exposed to the higher dose. The constellation of defects suggests an

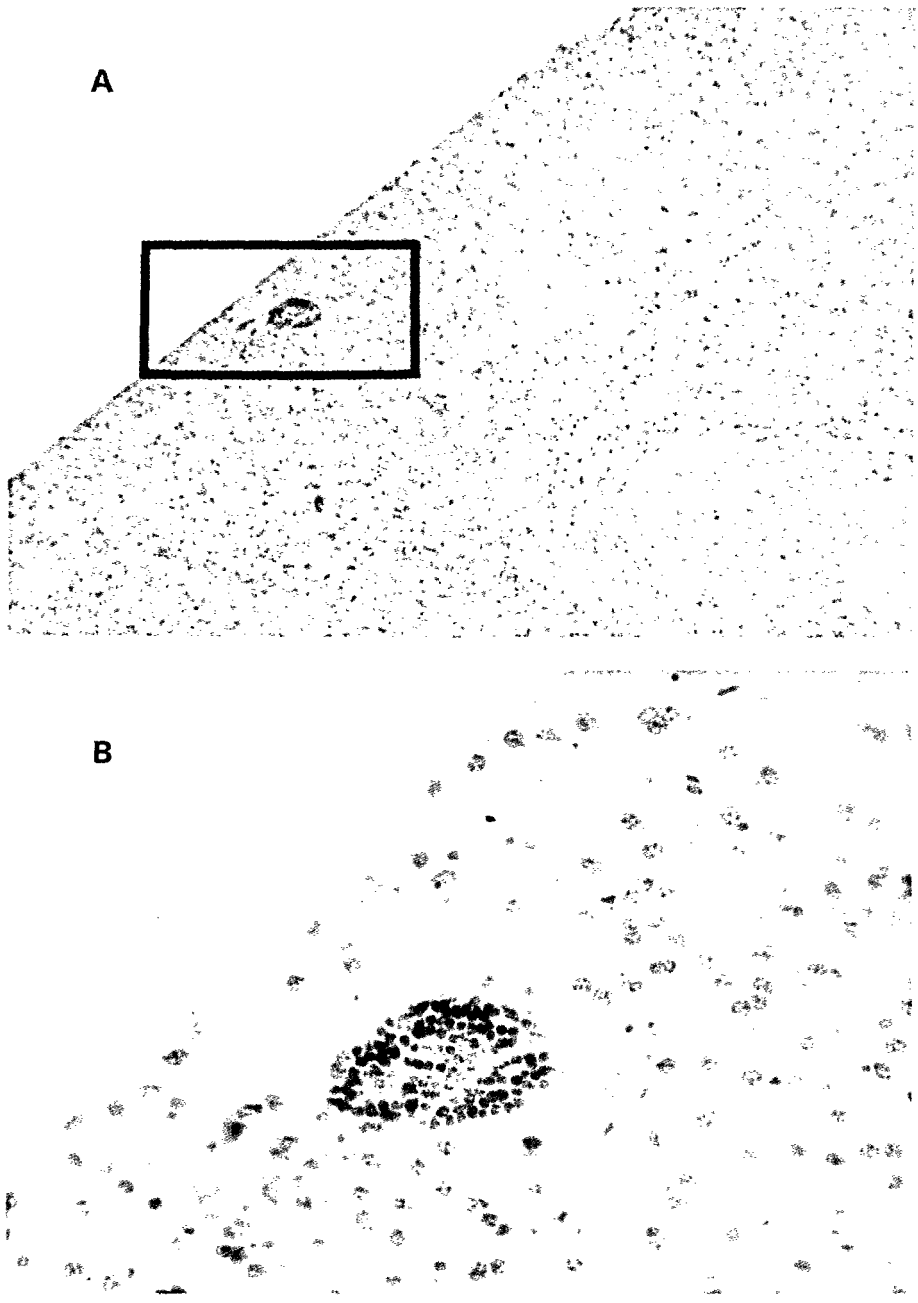

FIGURE 4. Micrograph of forebrain of 101-day-old mouse from mother treated with 9.0 mg/kg-day Diazinon. (A) Cluster of aberrantly located cells near the olfactory bulb, H & E, X 40. (B) Detail from (A) showing abnormally shaped, chromatin-containing cells that bear no obvious neuroanatomical relation to normal brain structures, H & E, X 100.

impairment of neuromuscular strength and/or coordination. A standard open-field test, generally accepted as giving a valid measure of activity and emotionality in rodents (Hall, 1934), failed to detect any effects of prenatal exposure to Diazinon at either dose. This finding supports a previous report (Al-Hachim and Fink, 1968a) that even high prenatal doses of parathion fail to influence later open-field behavior. In the present experiments, no treatment-related effects on audiogenic seizure incidence were observed when the test was administered to mature animals born of treated mothers. In a previous report (Al-Hachim and Fink, 1967), seizure frequency was found to be increased in the offspring of parathion-treated mothers, but the effect was transient and disappeared by the time offspring reached 20 days of age.

Maze running speed of offspring of mice exposed to 9.0 mg/kg-day Diazinon was significantly reduced late in training, but without a parallel increase in frequency of errors. These results might suggest that the treatment primarily impaired motor performance in the maze rather than actual learning of the task. This interpretation is consistent with an earlier finding that prenatal parathion exposure does not impair learning of a two-way shuttle avoidance task (Al-Hachim and Fink, 1968b). The impairments of neuromuscular function observed in the concurrent tests also suggest sensory-motor or neuromuscular rather than cognitive deficits. This explanation, however, fails to account for the later diminution of running speeds. Offspring of animals exposed to 9.0 mg/kg-day Diazinon initially matched the speed of the control offspring; only late in the week of testing did their performance deteriorate.

Animals exposed to 9.0 mg/kg-day Diazinon had forebrain damage observable under the light microscope. Effects of damage in this area on behavior cannot be predicted reliably because functions of the prefrontal cortex are only poorly understood (Grossman, 1973). Thus, the relationship of the behavioral changes that were found to the neuropathology in the forebrain is unknown.

Low-level prenatal exposure to methylmercury has been reported to result in pathological changes evident by use of the electron microscope but not apparent at the light microscope level (Chang et al., 1977). Studies to examine cellular ultrastructure are in progress to determine whether morphological changes not observable under the light microscope are present in these animals prenatally exposed to Diazinon.

The preceding data indicate that exposure of the fetus to low levels of the organophosphate Diazinon may result in subtle functional deficits in apparently normal animals, which are detectable only by systematic behavioral evaluation. Exposure to a higher level of the compound results in brain histopathology as well as behavioral impairment; however, mortality was still not increased.

The mechanism responsible for the effects reported above is not apparent. The treatment may have influenced maternal-offspring inter-

action, which has been shown to lead to permanent behavioral defects in the young (Denenberg et al., 1962). However, this interpretation would not explain the observed neuropathology. The central nervous system abnormalities observed among the offspring of mothers exposed to 9.0 mg/kg-day strongly suggest a direct prenatal effect of the compound on neuronal differentiation. Alternatively, anticholinesterase agents have been shown to possess a nonspecific regulatory effect on growth in the rodent (Fant and Harbison, 1977), perhaps by an influence on placental transport of nutrients. Thus, the reported dysfunctions and neuropathologies may have resulted from *indirect* interference of Diazinon with neural development by impairment of placental transport and maternal regulation of fetal growth, or from *direct* action of the organophosphate on cholinergic development of the fetus.

Although more than a single mechanism may be responsible for the observed deficits, it is apparent that, in mice, Diazinon can cause subtle and long-lasting neurobehavioral impairments in the offspring of exposed mothers.

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Received September 12, 1977
Accepted September 27, 1977