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Behavioral Effects of Perinatal Exposure of Chlordimeform in Rats

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Chlordimeform, a novel acaricide-insecticide belongs in the pesticide group known as the formamidines. One of the properties of chlordimeform which makes it a unique choice in this behavioral project is its noted ability to disrupt behavior in insects. As a result, chlordimeform is not only lethal to insects by direct killing action but from disruption of certain aspects of insect behavior. Adult rice stem borers when exposed to chlordimeform reacted with marked excitation. As a result, the insects were incapable of normal egg-laying behavior (HIRANO et al., 1972). In contrast, young larvae exposed to chlordimeform displayed different types of behavior which were collectively described as general sedation (MATSUMURA and BEEMAN, 1976). The inactivity of the larvae rendered them incapable of finding proper sites for boring. Animals exposed to the pesticide also show a diversity of reactions. Cattle treated with chlordimeform for tick control reacted in a sedated manner whereas dogs exposed to the compound became quite excited (MATSUMURA and BEEMAN, 1976). Rats exposed to high doses of the compound were hyperexcited for 5 to 10 minutes, then gradually fell into a state of sedation. While still in the sedated phase, poisoned rats were placed in an open area. The animals failed to exhibit the normal escape reaction of running to the nearest corner or wall, but remained motionless in a characteristic low posture unless externally stimulated (BEEMAN and MATSUMURA, 1973).

To date, no research has been undertaken which investigates chlordimeform's capacity as a behavioral toxicant. The present study was designed to examine behavior of rats exposed prenatally and postnatally to chlordimeform. Comprehensive judgement criteria were employed in order to get as complete a picture as possible. Early evaluations included maturational tests (righting reflex and swimming behavior) in addition to records of the general physical condition of all animals (i.e. body weight, age of eye opening, general appearance). Later tests involved a valid test of learning ability, (including learning, reasoning, and problem retention) in addition to a test of motivation. Each testing was followed by histological examinations of all major organ systems. The use of a variety of maturational and behavioral measurements at various periods in the rat's lifespan should significantly increase the sensitivity of this behavioral study.

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MATERIALS AND METHODS

Subjects and Rearing Conditions

Pregnant rats were purchased from Holtzman Rat Co. (Madison, Wisconsin) on Day 1 of gestation. Day one sperm positive analysis was done by the supplier. A total of 29 offspring (15 controls, 7 males and 8 females; and 14 treated rats, half male and half female) from six of these mothers participated in the behavioral research. Ample mothers were purchased so that each experimental group contained three mothers with eight pups each equally divided among the sexes. If a particular treatment group did not have the necessary eight pups or the correct ratio of males to females, cross fostering within each treatment group was used to correct the problem. Mothers were randomly divided into the treatment conditions. When the animals arrived, they were housed individually in standard litter cages and all were given the control diet until the fifth day of gestation. On the fifth day, the pesticide-treated diets were introduced. The five day delay helped insure implantation of the eggs in the female before the introduction of the chemical, which, in some cases, could cause spontaneous abortions. Offspring were maintained in the same treatment condition as their mothers and of the eight pups per mother, five were randomly selected to continue in the later behavioral tests. The final statistical analyses included only animals who had participated in all phases of the study.

Prior to the actual behavioral testing a preliminary investigation was conducted in the same manner as outlined above. In addition a twenty-four hour surveillance of the mothers was maintained during the delivery period. The mothers and offspring were kept for three weeks observation.

Upon arrival, all pregnant rats were fed Purina® Rat Chow in mash form until Day 5 of gestation. At this time, the animals were randomly divided into either treatment or control group. The treatment group was given chlordimeform via their diet. Daily dietary intake of chlordimeform was 100 µg/kg per rat or ten times the safe level set by the World Health Organization (ANONYMOUS, 1972). Because of the relative instability of chlordimeform, the diet was prepared weekly and stored in a -20°C freezer.

Behavioral Testing

Behavioral testing began when the offspring reached seven days old. Two different behavioral testing times were employed in the study; early development testing with the rat pups tested on postnatal Days 7 through 17 and motivational, learning, and retention tests on Days 70 through 90. The early developmental tests consisted of two tasks: a swimming test (SHAPIRO et al., 1970) and a righting reflex task (EAYRS and LISHMAN, 1955). During the early testing, a record of the pup's general appearance and day of eye opening was made.

On Day 7, the rats were color coded so that no two rats had the same sequence of colors. Each rat retained this code for the duration of the study. Further description of the behavior tasks were as follows:

Swimming Test. The swimming task was conducted in an aquarium half-filled with lukewarm water. Daily, beginning on Day 7, the rat pups were separated from their mothers and placed in a drying cage. Each pup was dropped into the aquarium from about 25 cm. The rat was then given a score on his performance. DORCEY's (1972) scoring method was used in preference to that employed by SHAPIRO (1970). An arbitrary score of "0" through "4" was assigned on each trial, "0" representing lack of swimming ability and "4" mature swimming ability.

Righting Reflex. Prior to the swimming task, each rat received three righting-reflex trials on Days 7 through 17. On each trial, the rat was held upside down by the neck and lower back and dropped approximately 30 cm. onto a cotton pad. The righting reflex was considered present if the rat landed on all four legs, receiving a score of "1". Otherwise it was scored as "0".

Symmetrical Maze Testing. The symmetrical maze, a replica of the one described by DAVENPORT et al. (1970) was employed for the motivational testing, maze problem learning, and problem retention. The standard testing procedure outlined and described by Davenport was utilized. The only departures from the standard procedure were the use of 1- rather than 2-pellet rewards (Noyes 45 mg. food pellets) and the use of test problem T-6, T-9, T-10 and T-11. In addition, the animal's performance was viewed via closed circuit television in an adjacent room so as not to disturb the performance of the animal.

Maze testing began when the animals reached 70 days old. At this time, the animals were reduced to between 80% and 85% of their Day 66 body weight and maintained at this reduced weight for the duration of the study. Water was continuously available at all times in the experiment.

Maze testing began with the motivational test, which investigated motivational differences between the control and treatment groups. Such differences would have a direct bearing on the results of the learning tests. The motivational test was equivalenet to P-1 of the four practice problems. The average of the first six running times of P-1 gave a reliable assessment of the animal's motivational level. There were three additional practice problems (P-2, P-3 and P-4) given before the four test problems (T-6, T-9, T-10 and T-11) were administered. The practice and test problems were administered at the rate of no more than one problem per rat per day. The four practice problems were given in set lengths of twelve trials each. In the four test problems, the rats were run until they reached criterion on that particular test problem (criterion consisted of four out of five trials with no errors)

or received a maximum of fifty trials without reaching criterion. Several measures of behavior and learning ability were obtained from the maze-running data. The number of errors made by a rat are a significant measure of learning ability. A record was also kept of the number of retraces or re-entry into the "start" endbox. This information can be used as a measure of abnormal behavior. Other analyzable measures of learning ability were the number of trials necessary and the total time elapsed before the problem was mastered.

Two days after an animal had completed a particular test problem, it was given five more trials on that same problem. The two day delay gave a measure of the animal's problem retention. No new test problems were introduced in the two day interval. Again, the number of errors, the number of retraces and the total time were recorded and analyzed. All of these test measurements provided fairly comprehensive and extensive information as to the learning ability and behavioral capacities of each treatment group.

Histological and Stastical Analysis: When all of the behavioral tests were completed, the animals were maintained at the 80% to 85% of normal body weight range until sacrificed. All of major organ systems were removed, visually examined, and replaced in 10% buffered formalin. Unfixed wet tissue weights were made of the following organs: liver, kidney, lung, heart and brain. Sections of the following tissues - brain, thyroid, heart, lung, liver, kidney, adrenal, stomach, small and large intestines and bladder were fixed and embedded in paraplast. The processing was done with an Autotechnicon. The embedded tissue was sectioned at 6 microns.

The histological analysis was used mainly as a screening procedure for abnormal tissue which could account for any behavioral changes. It was not intended to be used as an intensive study of pathology from insecticide exposure, since the levels used in the project were far below levels observed to cause gross pathological changes in the organs. Thus, one slide from each of the organs was stained with hematoxylin and counter stained with eosin. The duplicate slides were stained with appropriate stains for the particular tissue. All of the slides were examined by a pathologist, who also prescribed the appropriate stains in the special cases where unusual tissue changes were observed.

An exact analysis of variance was performed on all of the behavioral parameters. Pairwise analyses were performed when the obtained F ratio was significant at the $p > .05$ level (pairwise T-Test).

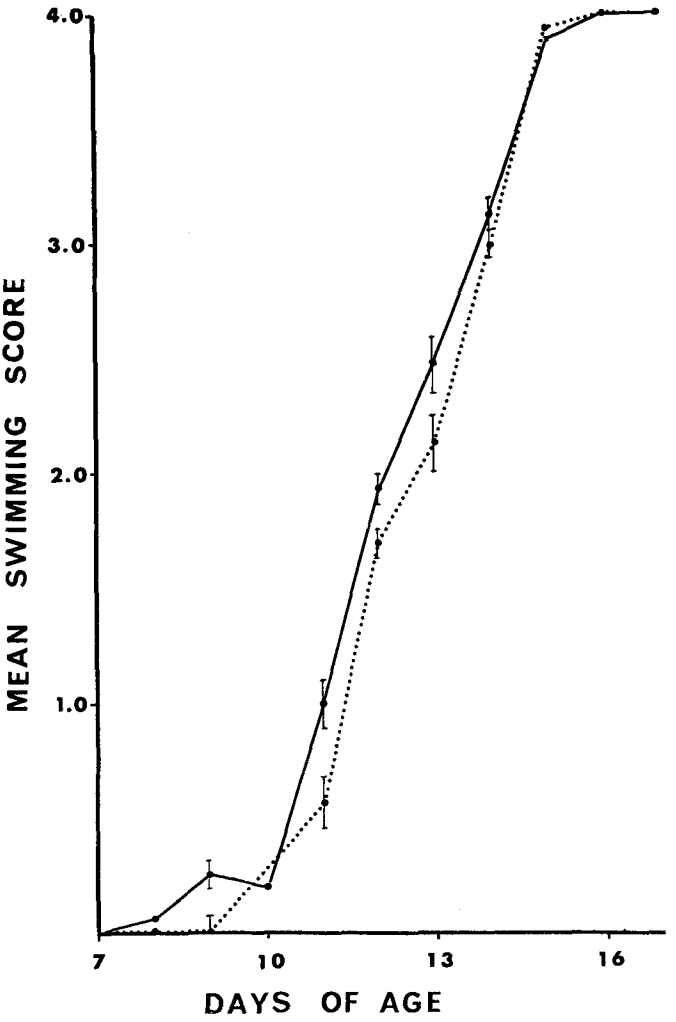

Figure 1. Mean swimming scores for the animals on the control diet in the chlordimeform experiment (represented by a solid line) and the animals fed the chlordimeform-treated diet (represented by a dotted line) on Days 7 through 17). (Means ± Standard Deviation).

RESULTS

Physical Development

The early record of animal weight on Days 7, 10, 13 and 16 showed no significant differences in weight between control and chlordimeform-treated animals, age of eye opening for controls and treatment groups occurred around Days 14-15, which is within the normal range for the white rat. No difference in physical appearance were seen between any of the animals in the study.

Early Behavioral Tests

Retarded maturation was observed in the chlordimeform-fed group with respect to swimming behavior. Analysis of variance of the summed scores over Days 9 through 15 showed significantly slower overall development of swimming ability by chlordimeform-treated animals than the controls ($F = 9.34$; $df = 1.25$; $p < .005$), (Figure 1). Day by day analysis of the swimming scores showed significant differences between the groups on Day 9 ($p < .05$) and Day 11 ($p < .025$).

The early behavioral testing revealed significant differences between the control and chlordimeform-treated offspring. The maturation of swimming ability was significantly impaired in the chlordimeform group. The development of swimming ability is a combination of acquisition of new behaviors and inhibition of others. For example, the ability of the animal to swim in a coordinated fashion with all four legs is a behavior the animal functionally develops. However, the mature swimming behavior requires that the rat learn to inhibit movement by the forelegs (SHAPIRO et al., 1970). The chlordimeform-treated animals displayed unusual swimming behaviors in that they often reverted back to earlier stages, even though they initially swam in a normal fashion. The specific behavior in point was the unusual tendency for the rat to suddenly arch its head downward and swim with its nose underwater in an uncoordinated fashion after previously swimming adequately with its nose above the water's surface. The arching of the head out of the water may be a combination of increasing muscle strength and coordination and an inhibition of the certainly deleterious behavior of swimming with the nose under water. Thus, chlordimeform may not only be interfering with the development of normal swimming behavior, but it may be doing so because of an ability to interfere with the inhibition of earlier swimming behaviors.

The maze tests revealed no significant differences between the control and the chlordimeform groups. An explanation for the early retardation and then lack of differences seen later in life may be due to the relative volatility of chlordimeform and the ease in breakdown and excretion in the adult animal (SEN GUPTA and

TABLE 1

Measures of retention in symmetrical maze problems, T-6, T-9, T-10 and T-11

(Mean ± Standard Deviation) (*Significantly superior, $p < 0.05$)

Test problems			Retention test	
Measure	Control	Chlordimeform	Control	Chlordimeform
Mean errors				
T-6	48.7 ± 5.7	51.1 ± 5.9	3.8 ± 0.6	5.3 ± 0.6
T-9	51.7 ± 4.8	51.4 ± 5.0	4.4 ± 0.9	3.7 ± 0.9
T-10	37.3 ± 3.9	39.7 ± 4.0	8.9 ± 1.1	8.3 ± 1.1
T-11	9.0 ± 1.0	9.1 ± 1.0	1.5 ± 0.3	1.6 ± 0.3
Mean retraces				
T-6	7.7 ± 1.3	8.5 ± 1.4	0.27 ± 0.2	0.5 ± 0.2
T-9	3.5 ± 0.8	4.2 ± 0.8	0.2 ± 0.2	0.6 ± 0.2
T-10	4.1 ± 0.6	2.7 ± 0.7	0.5 ± 0.2	0.3 ± 0.2
T-11	3.1 ± 0.7	2.6 ± 0.7	0.3 ± 0.2	0.3 ± 0.2
Mean trials				
T-6	27.7 ± 2.4	31.0 ± 2.5	---	---
T-9	21.9 ± 1.6	23.0 ± 1.6	---	---
T-10	16.9 ± 1.7	19.3 ± 1.8	---	---
T-11	10.1 ± 0.9	9.8 ± 1.0	---	---
Mean running time				
T-6	532.7 ± 60.5	647.6 ± 63.1	57.8 ± 5.4	68.4 ± 5.7
T-9	427.4 ± 36.5	486.0 ± 38.1	64.0 ± 6.2	76.5 ± 6.5
T-10	358.4 ± 40.1	361.2 ± 41.8	104.6 ± 11.5	99.3 ± 12.0
T-11	224.9 ± 29.6	226.8 ± 31.0	88.3 ± 7.8	100.2 ± 8.2

and KNOWLES, 1970). The immature rat probably lacked the capacity to metabolize the compound, and the increased toxicity was manifested in retarded swimming behavior.

Motivational and Maze Tests

There were no significant differences between controls and treatment groups found in the test of motivation.

There were very few significant differences between the controls and the chlordimeform-treated animals on any of the behavioral parameters tested (Table 1) and these differences occurred between the males and females of the two test groups. No conclusions can be drawn on the basis of the sporatic significant differences.

Anatomical and Histological Data

Representative sections of heart, lung, gastrointestinal tract, liver, kidney, brain, spleen and bladder were examined from at least one-half the animals in each group. No significant histologic alterations were observed in any organ systems, aside from the expected post-mortum changes. Liver and kidney weight were calculated as percentages of body weight for all animals. Again, no significant differences were seen between any of the ratios.

DISCUSSION

The swimming task was found to be the most sensitive indicator of behavioral changes from chlordimeform exposure. Studies with dieldrin and toxaphene also confirmed the sensitivity of the swimming test (OLSON, 1977). Under a similar experimental condition OLSON and BOUSH (1975) found that rats fed a diet containing a low level of naturally occurring mercury were significantly inferior to controls in developing swimming ability in agreement with the data obtained by SPYKER et al. (1972), who used a slightly different swimming test. In these cases offspring exposed prenatally to merhylmercury were significantly different from controls when tested for subtle behavioral deviations during postnatal development though they did not exhibit any overt poisoning symptoms. Most of the treated offspring showed one or more signs of neuromuscular impairment while swimming. Swimming ability represents an adaptive response to a life threatening situation which requires coordination and the integration of a series of reflex responses. The complexity of swimming behavior may account for its sensitivity to disruption by low doses of insecticides, pesticides, and other environmental contaminants such as mercury.

The known sensitivity of the immature organism to toxicants coupled with the possibility of no later overt symptoms of poisoning in the older animal necessitates the development of not only very sensitive testing parameters but tests which cover different periods in the animal's lifespan. One of the greatest difficulties in

behavioral research is the selection of tasks which may best reveal possible subtle behavioral alterations from toxicant exposure. The swimming test has consistently supported the sensitivity of the immature animal to toxicants.

The importance of behavioral toxicology in uncovering subtle effects of very low levels of any toxicants cannot be overlooked. Changes in behavior may serve as the earliest indications of potential damage to organisms and their environment, perhaps at a time when the process could still be reversed. In addition, behavioral changes may affect an organism's capacity to function. Behavioral and both perinatal and long-term evaluations of organisms exposed to chronic levels of environmental contaminants are essential for a thorough assessment of their impact on human health and the environment.

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