Pharmacology Biochemistry & Behavior, Vol. 13, pp. 429–434. Printed in the U.S.A.

Retinotoxic Effects of Diaminodiphenoxybutane in Rats

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Received 11 April 1980

NOGUCHI, Y., S. SATO, T. ANDO AND S. CHIBA. *Retinotoxic effects of diaminodiphenoxybutane in rats.* PHARMAC. BIOCHEM. BEHAV. 13(3) 429–434, 1980.—The effects of diaminodiphenoxybutane (DAPB) on visual function in rats were studied using behavioral, electrophysiological and histological techniques. A light-dark discrimination test was conducted by an operant behavioral method. DAPB did not modify intensity detection threshold at an intravenous dose of 35 mg/kg, but elevated it at doses of 50 and 70 mg/kg. Secondly, DAPB did not produce any changes in either electroretinogram (ERG) or visually evoked potential (VEP) at the dose of 35 mg/kg, but a marked decrease in the amplitude of the ERG a- and b-waves appeared after the doses of 50 and 70 mg/kg. The latency of the VEP first wave was also prolonged dose-dependently. Finally, retinal lesions were revealed in rats receiving 70 mg/kg. These results indicate that DAPB has a toxic effect on the retina in rats.

Diaminodiphenoxybutane Retinotoxicity Operant behavior Electroretinogram Visually evoked potential Rat

THE visual toxicity of some drugs, e.g., chloroquine, quinine, and ethambutol, is well known. In order to assess the visual toxicity of these drugs, three main approaches—operant behavioral [1, 12, 16, 17, 26], electrophysiological [5, 18, 19, 32], and histological [15, 20, 21, 27]—have been used. From the screening point of view, the operant behavioral approach is one of the most promising methods which can provide precise information on input-output functions of organisms.

Diaminodiphenoxyalkanes were developed as schistosomacidal drugs, but have not been marketed because of their visual toxicity [2, 3, 4, 7, 11, 14, 22, 23, 25, 29]. Edge *et al.* [11] reported that there is a marked species difference in susceptibility to this visual effect. Monkeys treated with oral doses of diaminodiphenoxypentane showed some impairment of vision as observed by abnormal feeding behavior and marked dilatation of the pupils. In cats given an intravenous dose, loss of the blink reflex and marked dilatation of the pupils with almost complete loss of the pupillary reflex were observed, but not in mice, rats, guinea pigs, and rabbits given an oral high dose. On the other hand, Nakajima [22] studied the toxic effect of diaminodiphenoxyalkanes on the eyes of rabbits by electrophysiological methods and was able to record a depressed electroretinogram (ERG).

On the basis of these findings, the present study was undertaken to detect the visual toxicity of diaminodiphenoxybutane (DAPB) in rats by an operant method adopting a conditioned suppression paradigm [28]. Further, the ERG and the visually evoked potential (VEP) from visual cortical areas were recorded to establish a direct relationship between behavioral and electrophysiological changes. Finally, a histological study was conducted to verify the location of the lesions and thus to aid in correlating damage with visual dysfunction.

EXPERIMENT 1

METHOD

Animals

Nine adult, male Ta:Wistar rats weighing approximately 300 g at the time of drug administration were used. For drug injection, a silicone rubber cannula was implanted into the right external jugular vein as described by Weeks [30]. The animals were assigned arbitrarily to one of three groups (3 animals/group) and housed individually in stainless steel cages. They were maintained at approximately 90% of their free-feeding body weights by water deprivation throughout the experiment.

Apparatus

Four operant test chambers (Lehigh Valley Electronics Inc., 143–23) mounted in sound- and light-attenuating cubicles were used. The front panel of the chamber was equipped with a lever that required a pressure of approximately 20 g to operate and a stimulus light (diameter=1.2 cm, maximum intensity=1.75 log mL) located 3.3 cm above the lever. The intensity of the light was adjusted by a rheostat and measured by a phototube illuminometer (Toshiba, PI-301). A small liquid dipper located behind the front panel of the chamber presented a cup of milk (approximately 25 μl) to the animal. An electric shock (range: 3.4–6.2 mA, 0.2 sec duration) was delivered through the grid floor of the chamber by a

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shock generator/scrambler (Lehigh Valley Electronics Inc., SGS-004). The lever and the liquid dipper were illuminated at approximately 9 lux by a house light (24 VDC) mounted 18 cm above the floor throughout the experiment. A small exhaust fan at the sidewall of the cubicle continuously circulated air through the chamber.

A minicomputer with the UDC system (Digital Equipment Co., PDP 8/E) controlled experimental contingencies. Lever pressings were recorded on the minicomputer and on cumulative response recorders (Ralph Gerbrands Co., G-3110). All such equipment was located in an adjoining room.

Procedure

Rats were trained to press a lever for delivery of a cup of milk under a random ratio schedule with a probability of reinforcement of 5% (RR 20 schedule). After responding under the RR schedule had stabilized, a stimulus light was presented for 1 min (conditioned stimulus period: CS period) at intervals averaging 3.5 min (2–5 min, non-CS period). In the CS period, a 3-sec extinction period was followed by a 57-sec punishment period in which an electric shock was given randomly after an average of 20 lever pressings (RR 20 punishment schedule). The animals were thus trained to discriminate between presentation and non-presentation of the stimulus light (intensity=1.6 log mL). The experimental session consisted of 20 CS periods and 20 non-CS periods.

In the visual discrimination test session, no electric shock was given to the rats and the intensity of the stimulus light was decreased in 0.2 log mL steps until a luminance at which the rats pressed the lever frequently enough to estimate threshold. Then, the intensity level was increased and finally oscillated around the threshold. To estimate the threshold, suppression ratios (SRs) were calculated as follows:

SR=(B-A)/B

A: response rate during the CS period.

B: response rate during the non-CS period.

Intensity detection threshold was defined as the luminance of the stimulus light at which the SR equaled 0.90. Thresholds were measured at 10 days, 1 month, and 3 months after drug administration and compared with those measured before drug administration using a two-way layout, two-tailed rank sum test [31].

In the rats receiving 70 mg/kg, an auditory discrimination test was also conducted. The procedure of the auditory discrimination test was the same as the visual discrimination test mentioned above, and an interrupted tone (2.8 KHz, 75 dB SPL), instead of a stimulus light, was used as the CS. The SRs were calculated to evaluate the auditory discrimination capacity.

Drug Administration

Diaminodiphenoxybutane·2HCl synthesized in our Medicinal Research Laboratories was dissolved in physiological saline and administered intravenously through the cannula using an infusion pump (Razel, A-99). The doses tested were 35, 50 and 70 mg/kg. Each animal was given a single dose.

RESULTS

After 20 to 30 sessions, lever pressings during the CS period were completely suppressed and conditioned suppression was developed.

The intensity detection thresholds for rats receiving DAPB are shown in Table 1. The mean threshold for the three rats receiving a dose of 35 mg/kg was not different from that determined before drug administration. The mean threshold for the three rats receiving 50 mg/kg, however, was elevated from 0.71 ± 0.18 log mL (mean ± SD) to higher than 1.75 log mL 10 days after the administration. The mean thresholds determined 1 and 3 months after drug administration were 0.98 ± 0.55 and 0.87 ± 0.66 log mL, respectively.

TABLE 1

EFFECTS OF DAPB ON INTENSITY DETECTION THRESHOLDS IN RATS

Dose mg/kg IV	N	Pre-drug	Post-drug		
			10 days	1 month	3 months
35	3	0.53 ± 0.15*	0.52 ± 0.19	—	—
50	3	0.71 ± 0.18	>1.75†	0.98 ± 0.55	0.87 ± 0.66
70	3	0.71 ± 0.21	>1.75†	>1.75†	1.07 ± 0.57

*Mean ± SD log mL.

Significantly different from pre-drug value: †$p<0.05$.

The mean threshold for the three animals receiving 70 mg/kg was elevated from 0.71 ± 0.21 log mL to higher than 1.75 log mL 10 days after drug administration. Three months later, the mean threshold for these animals was lowered to 1.07 ± 0.57 log mL.

The auditory discrimination test was conducted in the rats receiving 70 mg/kg 10 days after drug administration. Conditioned suppression to the auditory conditioned stimulus was not affected and the mean suppression ratio of the three rats was 0.98 ± 0.02.

All the rats receiving DAPB showed no signs of visual dysfunction such as loss of the blink or pupillary reflexes or dilatation of the pupils.

EXPERIMENT 2

METHOD

Animals

Nine adult, male Ta:Wistar rats weighing 300 to 350 g were used. Each rat was implanted with a cannula as in Experiment 1. Rats were assigned arbitrarily to three groups (2–4 animals/group). All animals were housed individually in stainless steel cages and allowed free access to both food and tap water.

Surgical Procedure

Each rat was anesthetized with ketamine (50 mg/kg, IM) and stereotaxically implanted with cortical electrodes for VEP recording under sterile conditions. Cortical electrodes consisting of stainless steel screws were threaded through the calvarium to make contact with the dura overlying the occipital lobe. All electrodes were attached to the skull with dental acrylic. The animals were allowed 2 weeks to recover from surgery prior to recording.

Apparatus

ERG and VEP recordings were conducted in a darkened and electrically shielded room. A photostimulator (Nihon Kohden, MSP-2R) was used to present a light flash stimulus of 100 μsec duration and 0.3 to 2.0 joule in intensity. A xenon lamp (diameter=20 cm) was placed in front of the animal's face and 30 cm distant as measured from the position of his

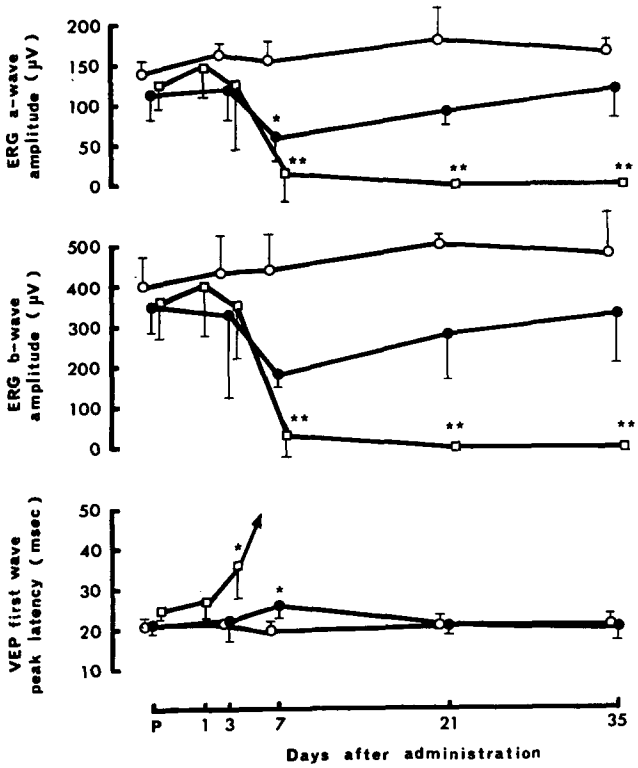

FIG. 1. Effects of DAPB on amplitudes of ERG a- and b-waves and peak latency of VEP first wave (mean ± SD) in rats. ○——○, 35 mg/kg (N=2); ●——●, 50 mg/kg (N=3); □——□, 70 mg/kg (N=4). In the four rats receiving 70 mg/kg, the VEP was depressed so markedly that peak latency could not be detected in two rats at 7 days and in all rats at 21 and 35 days after drug administration. Significantly different from pre-drug value (P): $*p<0.05$, $**p<0.01$.

left eye. The ERG was recorded through contact lens electrodes consisting of double gold rings embedded in a transparent resinous lens, and the VEP through the previously implanted cortical electrodes. Single ERGs were amplified by a signal processor (San-ei, 7T07A; time constant=0.1 sec, sweep time=20 msec/div.) and photographed with a camera (King, CRT). The stored VEPs to 10 stimuli were averaged by the signal processor and photographed.

Procedure

Prior to recording, each animal was kept in the darkened and electrically shielded room for 15 hr. Under a red light (4–5 lux), the animal was fixed to a restraining support (Shinano, SN-688) and then the pupil of the left eye was dilated by instillation of 0.5% tropicamide (Santen, Mydrin®-P) and the eye was anesthetized tropically with 0.4% oxybuprocaine (Santen, Benoxil®). Thereafter, the contact lens electrode for ERG recording was applied. After the animal had remained for 10 min in the dark, ERG and VEP recordings were begun. Light stimulus was given every 1 min in ERG recording and every 15 sec in VEP recording. ERG and VEP recordings were made at 24 hr before and 3, 7, 21, and 35 days after drug administration, unless described otherwise.

The effects of DAPB were evaluated by a two-way analysis of variance (ANOVA) for repeated measures (pre-drug, 1, 3, 7, 21, and 35 days after drug administration). If there was a significant difference, post-drug values were compared with the pre-drug value using the procedure of Dunnett for multiple comparison [9,10].

Drug Administration

The procedure for drug administration was the same as in Experiment 1.

RESULTS

Figure 1 illustrates the changes in amplitude of ERG a- and b-waves and in peak latency of VEP first waves in rats receiving DAPB. ANOVA indicated that neither ERG nor VEP was affected in the two rats receiving 35 mg/kg. In the three rats receiving 50 mg/kg, the amplitude of the ERG a-wave was depressed, $F(4,8)=5.31$, $p<0.05$, and the peak latency of the VEP first wave was slightly prolonged, $F(4,8)=4.50$, $p<0.05$. As shown in Fig. 2, the ERG was depressed 7 days after drug administration, though the VEP remained relatively unchanged. In the four rats receiving 70 mg/kg, the amplitudes of both the ERG a-wave, $F(5,15)=19.0$, $p<0.01$, and the b-wave, $F(5,15)=29.3$, $p<0.01$, were markedly depressed. The peak latency of the VEP first wave was also markedly prolonged, $F(2,6)=6.25$, $p<0.05$. Furthermore, the VEP in the rats 21 days after drug administration was so markedly depressed that the peak latency could not be detected. As shown in Fig. 3, the peak latencies of the ERG a- and b-waves and the VEP first wave were prolonged 24 hr after drug administration. The amplitudes of the ERG a- and b-waves were depressed and the VEP was also progressively reduced during 3 to 7 days after drug administration. Twenty-one days later, both the ERG and VEP were completely depressed and showed no significant improvement even 5 weeks after dosing.

EXPERIMENT 3

METHOD

Animals

The retinas of three rats receiving DAPB at a dose of 70 mg/kg were examined 4 months after drug administration and compared with those of non-treated rats.

Procedure

The animals were anesthetized with ether and their eyeballs were removed. The sclera of the eye was incised at pars plana after immersion in 4% glutaraldehyde for 1 min, and the eye was fixed with the same fixative for 15 min and then preserved in 5% Formalin as described by Okisaka [24]. The tissue was then rinsed and dehydrated by immersion in 70, 80, 90, 95, and 100% aqueous glycol methacrylate (aqueous GMA: glycolmethacrylate 100 ml, polyethylene glycol 5 ml, and benzoylperoxide 0.15 g) and embedded in prepolymerized GMA and then polymerized by ultraviolet light (Toshiba, FL-20 DL, average wavelength=3,000 Å) as described by Cole *et al.* [6]. The polymerized blocks were trimmed and sectioned at 2 to 3 μ in thickness by a pyramitom (LKB, pyramitom-11800), and sections were stained with hematoxylin and eosin.

RESULTS

The retina of the non-treated rats showed a stratiform arrangement consisting of several layers, such as rod and

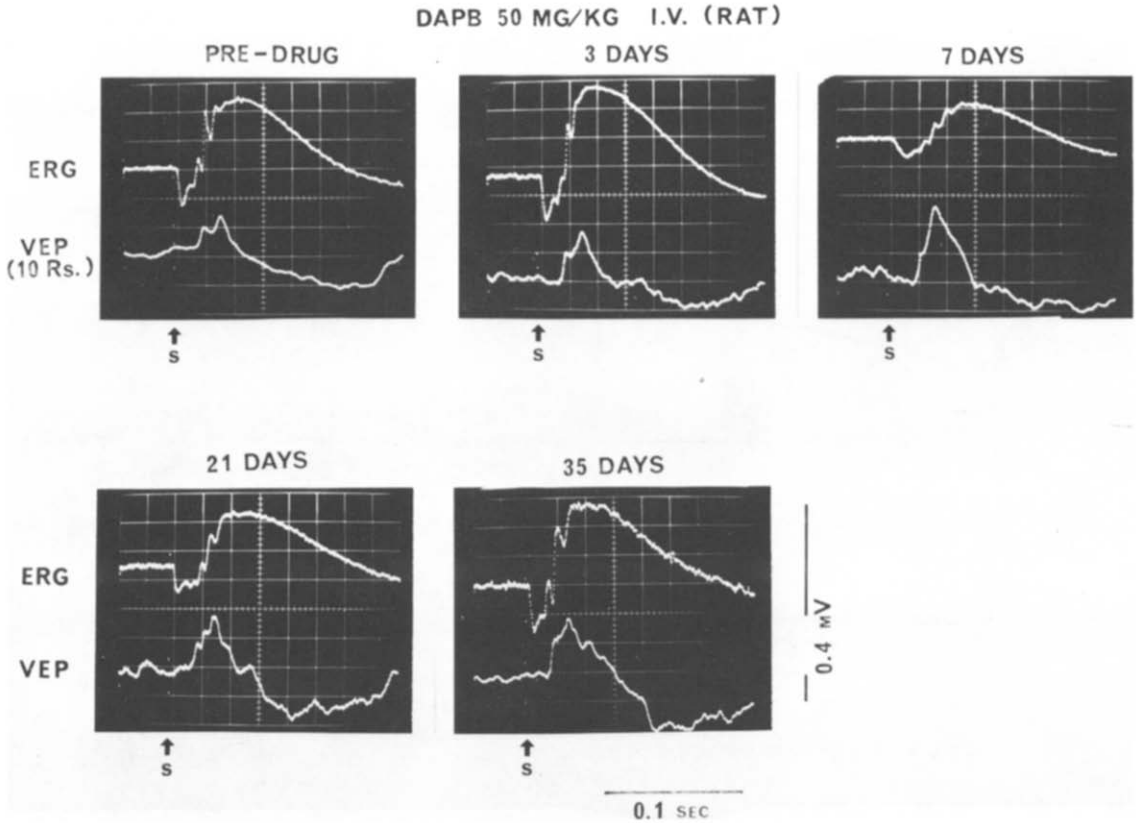

FIG. 2. Typical waveforms of the ERG (upper tracing, evoked by a single light stimulus) and the VEP (lower tracing, evoked by 10 light stimuli) in a dark-adapted rat receiving DAPB at 50 mg/kg. A slight depression of the ERG a- and b-waves was noticed at 7 days after drug administration, although the VEP remained relatively unchanged. Arrow marks (S) indicate light stimuli.

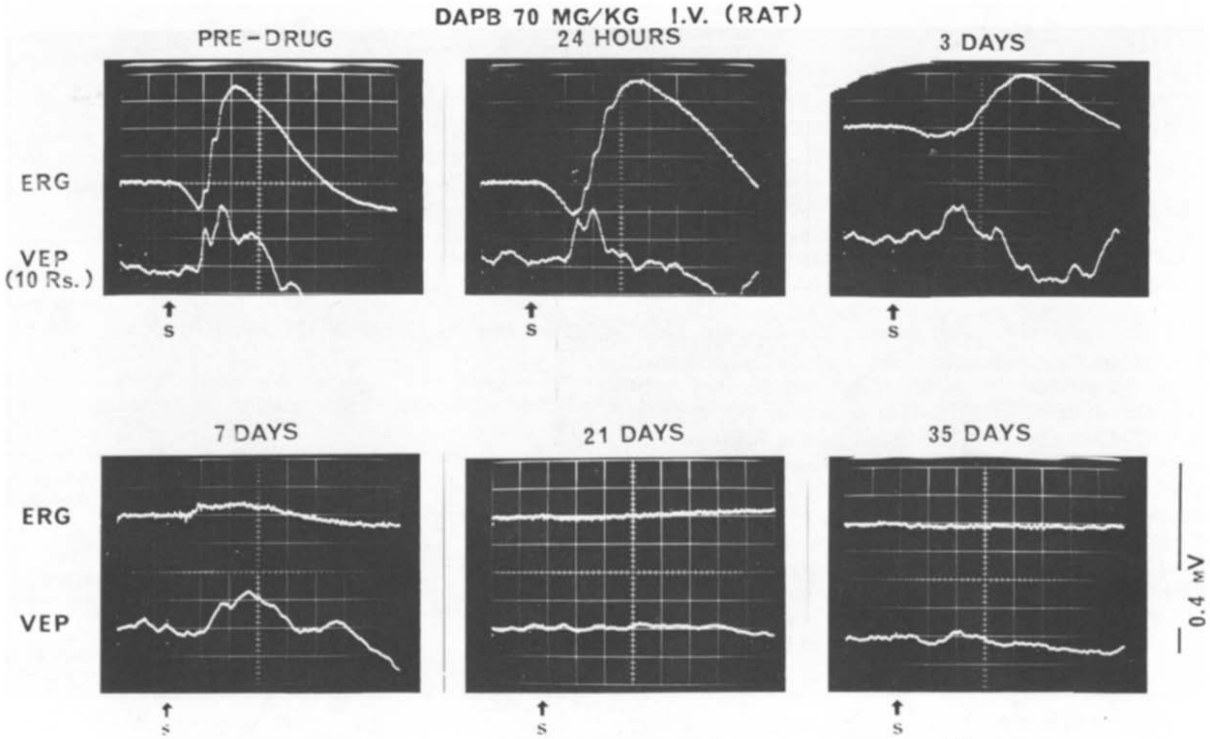

FIG. 3. Typical waveforms of the ERG and VEP in a rat receiving DAPB at 70 mg/kg. Both the ERG and VEP were progressively depressed and did not show any significant improvement even 5 weeks after drug administration. Other details as in Fig. 2.

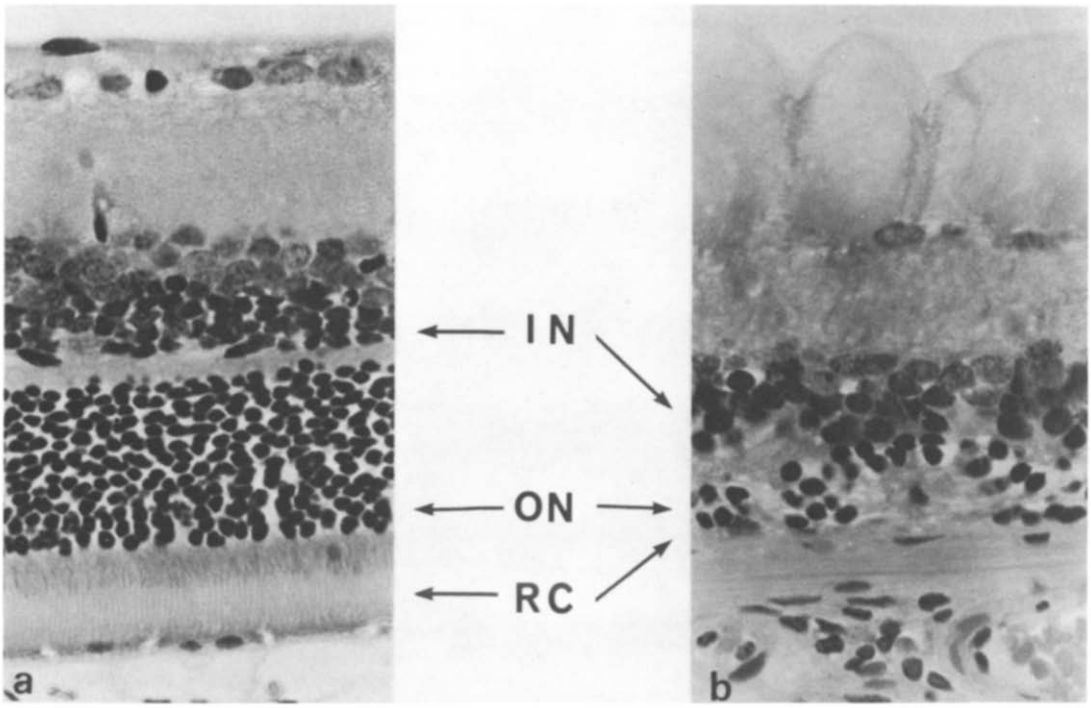

FIG. 4. (a) Light micrograph of the retina from a non-treated rat (×780). (b) Light micrograph of the retina from a rat receiving DAPB at 70 mg/kg (×780). Note the marked decrease in the number of cells in the outer nuclear layer and loss or thinning of the rod and cone layer. Abbreviation: IN, inner nuclear layer; ON, outer nuclear layer; RC, rod and cone layer.

cone layer, outer nuclear and plexiform layers, inner nuclear and plexiform layers, pigment epithelial cell layer, etc., and no abnormality was noticed.

As shown in Fig. 4, the retina of the rat receiving 70 mg/kg showed loss or decrease in the number of cells in the outer nuclear layer and loss or thinning of the rod and cone layer. In addition, slight swelling of pigment epithelial cells was sporadically noted. Although the inner layers remained relatively unchanged, retinal lesions were severe around the nerve-papilla, but not in the peripheral area. Such lesions were revealed in all the rats examined histologically.

DISCUSSION

The results of the present study demonstrated that single intravenous doses of DAPB decreased visual function in rats without affecting gross behavior. The fact that the rats treated with DAPB did not show any abnormal behavior such as loss of blink or pupillary reflexes was in agreement with the results of a previous study in rats [11]. The previous study demonstrated that diaminodiphenoxyalkanes produced a loss of the pupillary reflex and marked dilatation of the pupils in monkeys, dogs, and cats, but not in rabbits, guinea pigs, rats, or mice. As mentioned above, no significant gross behavioral changes were found in rats receiving DAPB. However, the marked loss of light discrimination capacity was detected and depressed ERG was recorded in the present study. Thus, it would appear that it is almost impossible to detect the visual toxicity in rats on the basis of the gross behavioral changes.

In the visual discrimination test, the intensity detection thresholds were markedly elevated after drug administration. These elevated thresholds indicated that DAPB depressed visual function by acting on the visual organs, because DAPB lacked general attenuating effects on the conditioned suppression [8,13] as judged from the results on the auditory system in the present study. This explanation is supported by the findings that the VEP remained relatively unchanged until the ERG was markedly depressed. Similar depression of the ERG was reported in rabbits [22]. The ERG is the graphical recording of the retinal action potential, whereas the VEP furnishes very useful information concerning the function of the retina and the conduction pathways. Therefore, the depressed ERG in association with depression of the VEP suggests the retina as the primary site of action.

The histological data also demonstrated that the outer retinal layers were the site of the lesions. This finding correlated well with the operant behavioral and electrophysiological changes.

The marked depression of the ERG was recorded about 3 to 7 days after the intravenous injection of DAPB. Such a delayed onset of action implies that DAPB would be stored somewhere in the body, presumably in the fat tissue, and slowly released into the bloodstream, demethylated, and left to act on the retina as previously suggested [22].

Three months after the dosing, the elevated intensity detection thresholds of the treated rats had a tendency to lower to the pre-drug value, in spite of the fact that the severe outer retinal lesions were revealed at the end of the experiments. This tendency may be explained by the possibility that the decreased visual sensitivity is improved by an excessive development or action of another structure in the central nervous system.

Ashton [3] reported that diaminodiphenoxyalkanes damaged the outer retinal layers of the cat such as the outer

nuclear layer, the rod and cone layers, and the pigment epithelial layer but not the inner retinal layers and the optic nerve. In the present study, likewise, the outer retinal layers were degenerated but the inner retinal layers remained relatively unaffected.

It is concluded that DAPB reduces visual function in rats and the primary site of action is the retina. In addition, the operant behavioral and electrophysiological methods used in the present study were found to be sensitive and useful for detecting the visual toxicity in rats.

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