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THE EFFECT OF DDD ON BARBITURATE AND STEROID-INDUCED HYPNOSIS IN THE DOG AND RAT*

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Abstract—The administration of *p,p'*-DDD to rats produces a marked decrease in sleep times of a variety of barbiturates and steroids. These effects are associated with an increased level of liver hexobarbital-metabolizing enzyme activity and proliferation of smooth endoplasmic reticulum. The administration of *p,p'*-DDD to dogs decreases hexobarbital sleep times but prolongs those of pentobarbital. Plasma half-lives of these barbiturates suggest that sleep times are related to DDD-induced changes in their rates of metabolism. The prolonged pentobarbital sleep time can be prevented by concomitant administration of cortisone acetate.

A HIGH mortality rate was noted by Nichols *et al.*[1] in dogs anesthetized with pentobarbital after being fed technical grade (1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2-dichloroethane(*o,p'*-DDD). These investigators demonstrated a prolonged period of hypnosis to a standard dose of pentobarbital in *o,p'*-DDD-treated dogs, but no effect in *o,p'*-DDD-treated rats. On re-evaluating the effect in rats with purified *o,p'*-DDD, Straw *et al.*[2] found *o,p'*-DDD produced markedly reduced pentobarbital sleep times and an increase in liver pentobarbital-metabolizing enzyme activity. When the *o,p'*-DDD-treated rats were given ethionine concomitantly, the increased enzyme activity was prevented, suggesting the DDD-induced change was due to an increase in enzyme population. Because of the discrepancy in results in rats and because the dog is sensitive to the adrenal necrotizing effects of DDD while the rat is resistant,[3] we have studied these two species further.

MATERIAL AND METHODS

Male Carworth Farm (CFE) rats weighing 35–40 g were used in this study. The DDD isomers and metabolites‡ were dissolved in corn oil and administered once daily by intraperitoneal injection. Control animals received an equivalent volume of corn oil only. The dogs used were healthy male and female mongrels weighing 8–12 kg. DDD dissolved in corn oil was administered to dogs orally in gelatin capsules. Sleep times were determined 24 hr after the last dose of DDD. The quantity of hypnotic

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‡ The *o,p'*-DDD, bis (*p*-chlorophenyl)-acetic acid, and 1,1-bis(*p*-chlorophenyl)-2,2-dichloroethene were purchased from the Aldrich Chemical Co. Milwaukee, Wis.

agent and route of administration is given in Tables 1 and 2. The return of the righting reflex was taken as the end point for sleep times in the rat. The end point in the dog was recorded when the animal first attempted to stand up. The length of zoxazolamine paralysis in rats was the elapsed time from i.p. injection (100 mg/kg) to the return of the righting reflex.

TABLE 1. EFFECT OF DDD ON BARBITURATE HYPNOSIS IN RATS

Treatment	Dose (mg/kg)	Days*	No. of animals	Hypnotic drug (mg/kg i.p.)	Sleep time (min ± S.E.M.)
Corn oil		3	8	Hexobarbital 150	97 ± 4
p,p'-DDD	200	3	8	Hexobarbital 150	13 ± 2
Corn oil		16	7	Hexobarbital 275	95 ± 6
p,p'-DDD	200	16	8	Hexobarbital 275	40 ± 2
Corn oil		30	6	Hexobarbital 275	56 ± 4
p,p'-DDD	200	30	6	Hexobarbital 275	26 ± 3
Corn oil		3	6	Hexobarbital 150	142 ± 8
p,p'-DDD	100	3	6	Hexobarbital 150	27 ± 3
m,p'-DDD	100	3	6	Hexobarbital 150	34 ± 4
o,p'-DDD	100	3	6	Hexobarbital 150	20 ± 4
Corn oil		3	6	Hexobarbital 150	91 ± 15
1,1-bis (*p*-chlorophenyl)-2,2-dichloroethene	200	3	7	Hexobarbital 150	9 ± 8
bis(*p*-chlorophenyl)-acetic acid	100	3	6	Hexobarbital 150	45 ± 17
Corn oil		3	6	Pentobarbital 35	126 ± 2
p,p'-DDD	200	3	6	Pentobarbital 35	0

* One dose per day.

TABLE 2. EFFECT OF *p,p'*-DDD ON BARBITURATE HYPNOSIS IN DOGS

Treatment	Dose (mg/kg)	Days*	No. of animals	Hypnotic drug (mg/kg i.v.)	Sleep time (min, range)
Corn oil		3	4	Hexobarbital 40	56 (47–61)
p,p'-DDD	200	3	4	Hexobarbital 40	11 (0–20)
p,p'-DDD	200	14	3	Hexobarbital 40	35 (33–37)
p,p'-DDD	200	44	2	Hexobarbital 40	52 (48–56)
Corn oil		3	3	Pentobarbital 23·5	101 (90–120)
p,p'-DDD	200	3	4	Pentobarbital 23·5	187 (160–202)
p,p'-DDD	200	14	2	Pentobarbital 23·5	203 (190–216)
Corn oil		3	2	Secobarbital 20	193 (191–194)
p,p'-DDD	200	3	2	Secobarbital 20	324 (318–331)

* One dose per day.

The half-life of plasma hexobarbital and pentobarbital was determined in the fasting state by the method of Brodie *et al.*,[4] that of phenylbutazone by the method of Burns *et al.*[5] In a few instances, the half-life was determined with pentobarbital-2-^{14}C. After intravenous administration of the drug, nonmetabolized pentobarbital was extracted[4] with heptane, the solvent evaporated, and the residue dissolved in scintillation fluid and counted in a liquid scintillation spectrometer. Results from either method were comparable.

The rate of side-chain oxidation of hexobarbital and of sulfoxidation of chlorpromazine by rat liver was studied with 9000-*g* supernatants. The supernatant was obtained by centrifuging at 2° a homogenate of one part liver and two parts 150 mM KCl.

Glucose 6-phosphate, 1·6 mM; NADP, 0·1 mM; $MgCl_2$, 2·6 mM; nicotinamide, 3·0 mM; and potassium phosphate buffer, 100 mM, pH 7·4, plus supernatant equivalent to 333 mg liver in a total volume of 5 ml, were incubated at 37° for 30 min in an air atmosphere. The drugs were added at 1 mM. After the incubation, the unchanged hexobarbital[4] and chlorpromazine[6] were assayed.

For electron microscopy, rats were lightly anesthetized with methoxyflurane and dogs were killed by air embolism. Immediately upon reaching insensibility, the animals were opened by longitudinal abdominal incision and thin slices of liver and adrenal removed, placed in cold (0°) 2% osmium tetroxide buffered with *s*-collidine to pH 7·4 and minced into blocks of 1 mm^3 or less. After fixation in cold, buffered osmium for 2 hr, tissues were dehydrated in a graded series of alcohols and embedded in Epon 812 or in an Epon–Araldite mixture. Ultrathin sections with gray to light-yellow interference colors were sectioned on an LKB ultramicrotome with glass knives, "stained" with lead or uranium, and examined in an RCA 3G electron microscope (40-μ objective aperture and 50 kV).

RESULTS

The response to administering *p,p'*-DDD to rats can be seen in Fig. 1. At a dose level of 100 mg/kg, equivalent results were obtained with *p,p'*, *o,p'*, and *m,p'*-DDD. Rats given 200 mg *p,p'*-DDD/kg daily for 3 days had sleep times which were 13 per

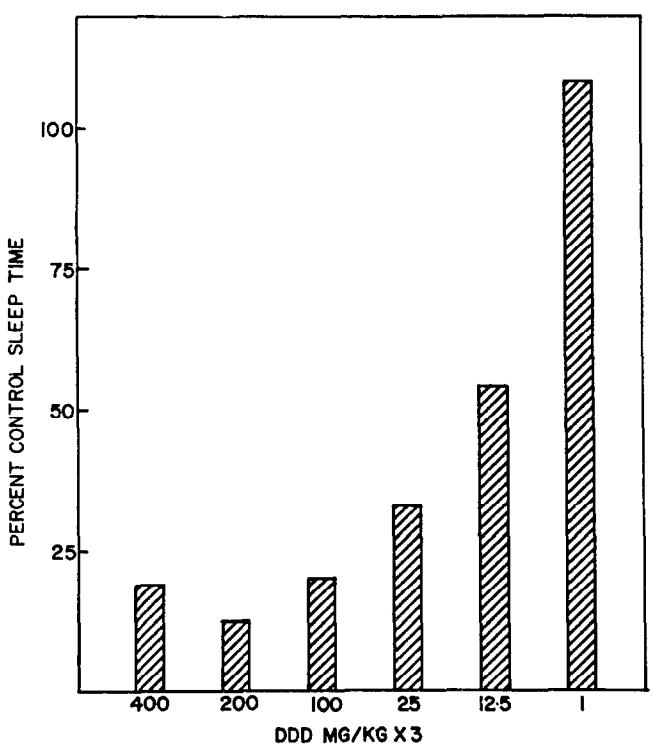

FIG. 1. Dose response to *p,p'*-DDD administration in rats. The sleep times were induced by hexobarbital (150 mg/kg).

cent of the control value (Table 1). Rats receiving only two injections slept 19 per cent, and rats receiving one injection slept 33 per cent of control values, indicating an appreciable effect within 24 hr of the first injection. When the injections were continued daily for 16 and 30 days, the sleep times were 43 and 46 per cent respectively, which were longer than after just 3 days of treatment. After only 3 days of *p,p'*-DDD administration, the decreased sleep time remained for 2 weeks; after 30 days of treatment, the decrease lasted for 2 months. In *p,p'*-DDD-treated rats, decreased sleep times were seen with a wide variety of hypnotic agents (Fig. 2). Similar effects were

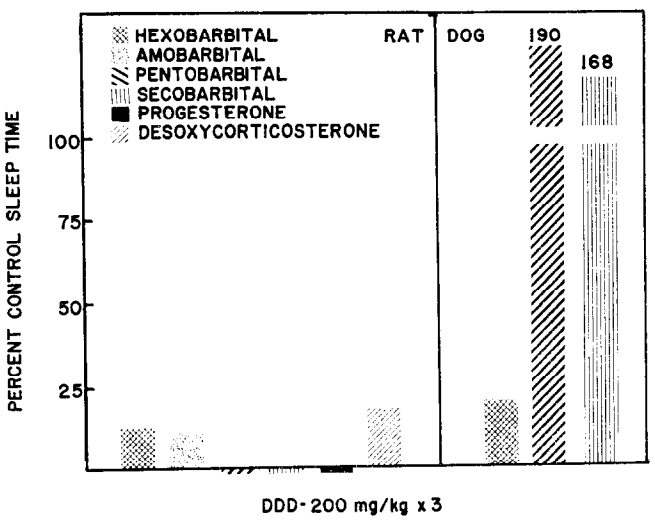

FIG. 2. Effect of *p,p'*-DDD on sleep times induced by a variety of barbiturates and steroids. In the rat the hypnotic agents were given i.p. in the following doses: sodium hexobarbital (150 mg/kg), sodium pentobarbital (40 mg/kg), sodium amobarbital (80 mg/kg), sodium secobarbital (70 mg/kg), progesterone (160 mg/kg), and desoxycorticosterone (150 mg/kg). In the dog, the sodium pentobarbital (23 mg/kg) and sodium secobarbital (20 mg/kg) were given i.v.

A value less than zero per cent of control sleep time indicates none of the DDD-treated animals in that group went to sleep.

induced by 1,1-bis(*p*-chlorophenyl-2,2-dichloroethene and bis(*p*-chlorophenyl)-acetic acid. The administration of *p,p'*-DDD was accompanied by a marked increase in hexobarbital, but not chlorpromazine-metabolizing enzyme in the liver (Table 3).

TABLE 3. EFFECT OF DDD ON RAT LIVER DRUG-METABOLIZING ENZYMES

Treatment	Dose (mg/kg i.p.)	Days*	No. of animals	Drug metabolism enzyme activity	
				Hexobarbital (μmoles g^{-1} hr^{-1})	Chlorpromazine (μmoles g^{-1} hr^{-1})
Corn oil		3	6	2·79 ± 0·6†	4·53 ± 0·15
p,p'-DDD	200	3	6	4·10 ± 0·7	4·76 ± 0·15

* One dose per day.
† Standard error of the mean.

The length of zoxazolamine paralysis was also reduced by treating rats with *p,p'*-DDD (Table 4).

Although the *p,p'*-DDD-treated dog showed a decreased sleep time with hexobarbital, the sleep time was markedly prolonged when pentobarbital or secobarbital was the hypnotic agent (Table 2). The latter persisted for at least 2 months after only

TABLE 4. EFFECT OF DDD ON RAT ZOXAZOLAMINE PARALYSIS

Treatment	Dose (mg/kg i.p.)	Days*	No. of Animals	Zoxazolamine paralysis (min ± S.E.M.)
Corn oil		3	6	225 ± 28
p,p'-DDD	200	3	6	112 ± 10

* One dose per day.

3 days of *p,p'*-DDD treatment. Consistent with these results was the shortened half-life of hexobarbital (Fig. 3) and lengthened half-life of pentobarbital in the *p,p'*-DDD-treated dog (Fig. 4). Concomitant therapy with subcutaneously administered cortisone acetate (25 or 50 mg daily) but not oral cortisol (20 mg every 12 hr) tended to return

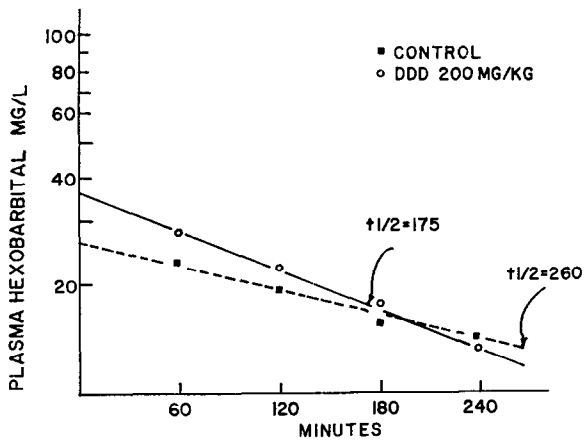

FIG. 3. Half-life of hexobarbital in *p,p'*-DDD (200 mg/kg)-treated and control dogs. The results are the mean of four dogs in each group.

the half-life of pentobarbital in the DDD-treated dog to control levels (Fig. 4). If the *p,p'*-DDD feeding of dogs was continued past 3 days, the decreased hexobarbital hypnosis returned to pretreatment levels after 30 days. The pentobarbital hypnosis, however, continued to be prolonged after 30 days of *p,p'*-DDD administration. The plasma half-life of phenylbutazone was also increased after DDD treatment in the dog (Fig. 5).

Electron microscopy

It is well known that the amount and distribution of smooth endoplasmic reticulum, like the size and shape of mitochondria, vary in different zones of the hepatic lobule

(Fig. 6). While all zones of the lobules were examined in the present studies, for comparative purposes, sections from midzonal areas were primarily used.

Rat. At 3 days of treatment with *p,p'*-DDD, the liver cells of the rat showed consistent and conspicuous increase in vesicles of smooth endoplasmic reticulum (Fig. 7), though occasional strata of rough endoplasmic reticulum still remained. Few lipid

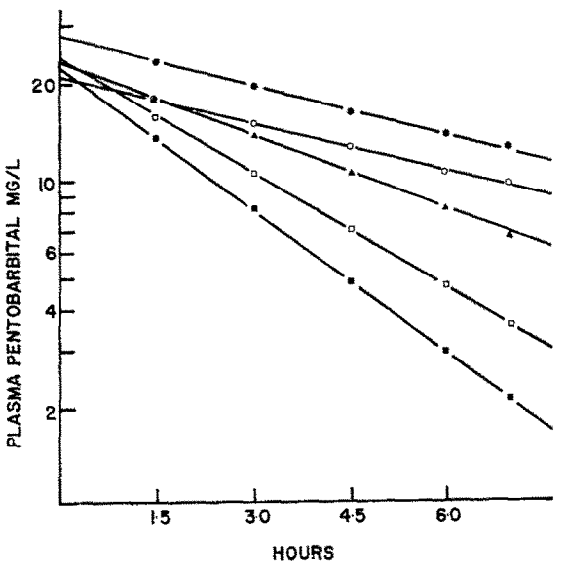

FIG. 4. Pentobarbital half-lives in dogs (each curve is mean of four dogs): ○ — — ○ *p,p'*-DDD ($t_{1/2}$ = 7·5 hr); ● — — ● *p,p'*-DDD + cortisol, 20 mg twice daily ($t_{1/2}$ = 6·5 hr); ▲ — — ▲ *p,p'*-DDD + cortisone acetate, 25 mg daily ($t_{1/2}$ = 4·15 hr); □ — — □ *p,p'*-DDD + cortisone acetate, 50 mgdaily ($t_{1/2}$ = 2·75 hr); ■ — — ■ control ($t_{1/2}$ = 2·25 hr).

droplets were present in the cytoplasm. There were no apparent alterations in mitochondria, microbodies, lysosomes, Golgi areas, or nuclei. By 16 days of treatment and thereafter, there was not only an additional increase in smooth endoplasmic reticulum over that present at 3 days but the increase was more uniform from cell to cell throughout all blocks of tissue examined. At the later intervals, other cell organelles remained unaltered.

The rat adrenal remained normal during the entire experiment (Fig. 8).

Dog. At three days of *p,p'*-DDD-treatment, liver cells of the dog showed a moderate increase in smooth endoplasmic reticulum but less abundant than in the rat (Figs. 9 and 10). At this interval, all other organelles were unremarkable. By 15 days, there was further proliferation of smooth endoplasmic reticulum with no other apparent ultrastructural abnormality. By 44 days, approximately 70–80 per cent of the mitochondria contained diffuse striations in their matrices (Fig. 11), but no abnormality in amount or distribution of smooth endoplasmic reticulum could be detected (Fig. 12).

In contrast to the rat, the dog adrenal glands showed advanced destruction of cells of the zona fasciculata (Fig. 13) at all intervals studied.

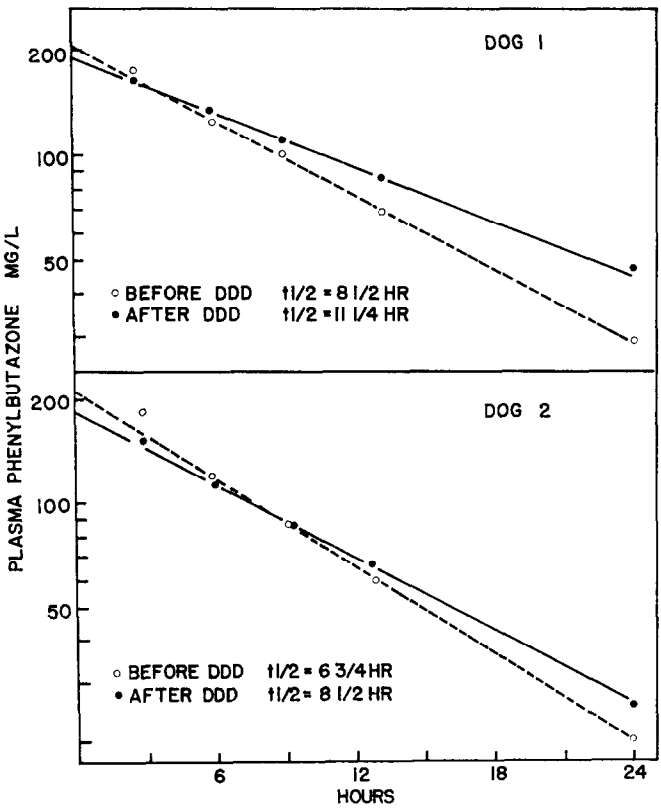

FIG. 5. The plasma phenylbutazone half-life was determined in the same dogs initially and several weeks later after receiving *p,p'*-DDD (200 mg/kg) for the 3 preceding days. The dose of phenylbutazone was 100 mg/kg i.v.

DISCUSSION

The rat liver responds to the administration of *p,p'*-DDD with a marked increase in smooth endoplasmic reticulum. Ortega[7] has previously demonstrated a similar change in the liver of rats treated with 1,1-bis(*p*-chlorophenyl)-2,2,2-trichlorethane (*p,p'*-DDT). The change is associated with an increase in the rate of metabolism of a variety of hypnotic agents but not chlorpromazine. The increase in enzyme activity presumably associated with the proliferation of the smooth endoplasmic reticulum appears to be nonspecific and can be induced by several metabolic products of DDD. Even bis(*p*-chlorphenyl)-acetic acid, which is reported to be the final product of *p,p'*-DDD metabolism[8] in the rat, retains the ability to induce a reduction in the length of hexobarbital hypnosis in this species. However, this metabolite does not appear to be as effective as *p,p'*-DDD in reducing hexobarbital sleep times. These results substantiate the reported decrease in hexobarbital[9] and pentobarbital[2] sleep times induced by *o,p'*-DDD. Our data also demonstrate that *p,p'*-DDD is capable of decreasing the sleep times of a variety of other hypnotic agents.

After 3 days of *p,p'*-DDD ingestion, the dog also has a marked increase in the smooth endoplasmic reticulum of the hepatocytes. This increase is accompanied by a decrease

in hexobarbital sleep time and plasma half-lite, suggesting an increased rate of hexobarbital metabolism, although other explanations are possible. However, with continued administration for 44 days, there is a return to pretreatment levels of the smooth endoplasmic reticulum. Concomitant with the change in endoplasmic reticulum is a return of the hexobarbital-induced hypnosis to control levels. The lack of proliferation of smooth endoplasmic reticulum and prolongation of hexobarbital sleep times suggests that, in the dog, DDD may stimulate its own metabolism to a level at which it is no longer effective. In the rat, the proliferation of smooth endoplasmic reticulum persists for at least 30 days despite continued daily administration. Changes in mitochondria in the dog occur with prolonged DDD treatment and may signify a decrease in the energy-regulating ability of the hepatocyte. Similar mitochondrial changes are prominent in livers of some humans with fatty metamorphosis of the liver, diabetes, and other diseases.[10]

Although the induced increase in smooth endoplasmic reticulum is associated with a decrease in hexobarbital sleep times, there is a prolongation of the pentobarbital sleep time. The plasma pentobarbital half-life is increased, suggesting a decreased rate of metabolism of this drug. Further evidence that a difference exists in the handling of these two barbiturates is seen in the following experiment. A pregnant dog was fed 200 mg *p,p'*-DDD/kg for the last 3 days of her pregnancy. When the puppies were 10 days old, sleep times were determined and compared to puppies of a similar size and age from an untreated mother. When hexobarbital was the sleep-inducing drug, the *p,p'*-DDD-treated pups slept 10 per cent of the control time. When pentobarbital was the agent, the treated pups slept 245 per cent of the control time.

The dichotomy in the plasma half-lives and presumably in the rates of metabolism of the two barbiturates suggests that they are metabolized by different enzymes, although it does not rule out other possibilities such as differences in the rates of tissue uptake or storage. In the dog, over 70 per cent of pentobarbital is hydroxylated in the methylbutyl side chain.[11] Hydroxylation of the methylbutyl side chain is also the major mode of metabolism of secobarbital.[12] The metabolism of hexobarbital is primarily oxidation of the cyclohexenyl side chain.[13] The cyclic and unsaturated structure of the side chain of one as compared to the straight-chain, saturated structure of the other barbiturates could account for a difference in enzyme specificity.

The dog (Fig. 13) is quite sensitive to the adrenal-necrotizing effects of *p,p'*-DDD;[3, 14] the rat (Fig. 8) is resistant and does not demonstrate a decreased secretion of adrenal glucocorticoids[15] as does the dog. Hexobarbital sleep times in adrenalectomized rats are prolonged and can be returned to control levels by the administration of glucocorticoids.[16] These results suggest that the prolonged plasma pentobarbital half-life may be due to the marked deficiency of glucocorticoid produced by *p,p'*-DDD in the dog. The results shown in Fig. 4 substantiate the importance of glucocorticoid in maintaining the level of pentobarbital-metabolizing enzyme. Similar corrections of *o,p'*-DDD prolongation of pentobarbital half-lives by parenteral glucocorticoids have been obtained by Waters *et al.*[17] Lack of response with cortisol may reflect inadequate dosage or the short biologic half-life of this glucocorticoid when it is administered orally as the alcohol. *p,p'*-DDD also produces prolonged sleep times and a decreased rate of metabolism of pentobarbital in mice, which cannot be reversed

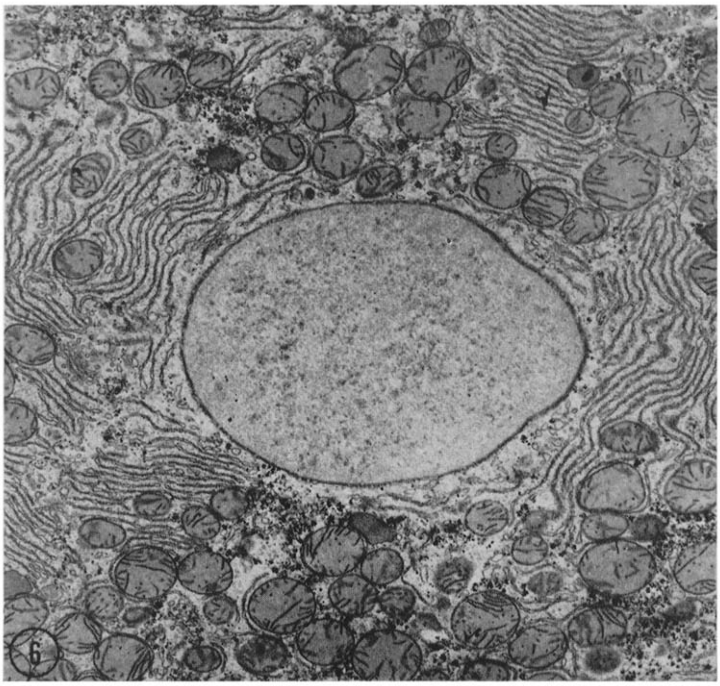

FIG. 6. Normal rat liver. Granular endoplasmic reticulum and mitochondria are abundant while smooth endoplasmic reticulum is inconspicuous. Lead hydroxide; × 8000.

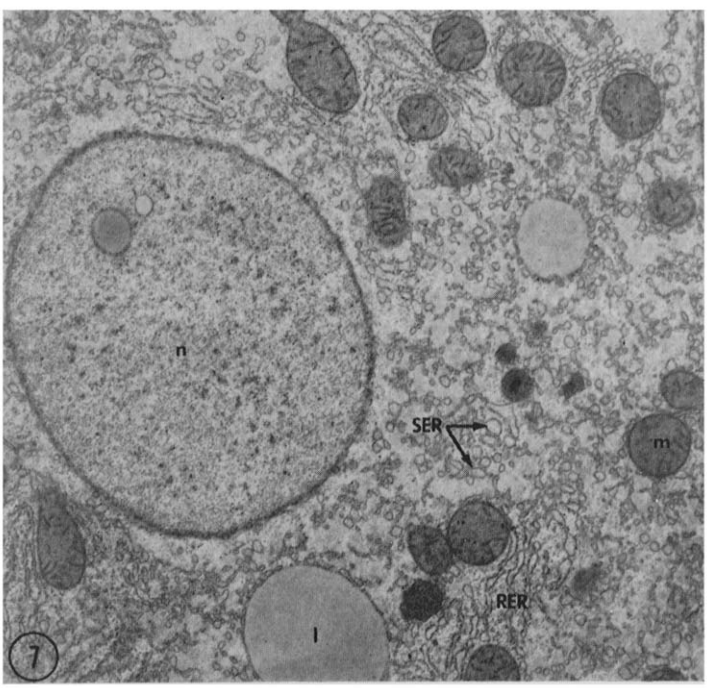

FIG. 7. Rat liver cell after 3 days' treatment with *p,p'*-DDD. Numerous vesicles of smooth endoplasmic reticulum (SER) are present throughout most of the cytoplasm. Occasional strata of rough endoplasmic reticulum (RER) are present in the more peripheral portions of the cell; l = lipid, m = mitochondria, n = nucleus. Lead hydroxide; 8600.

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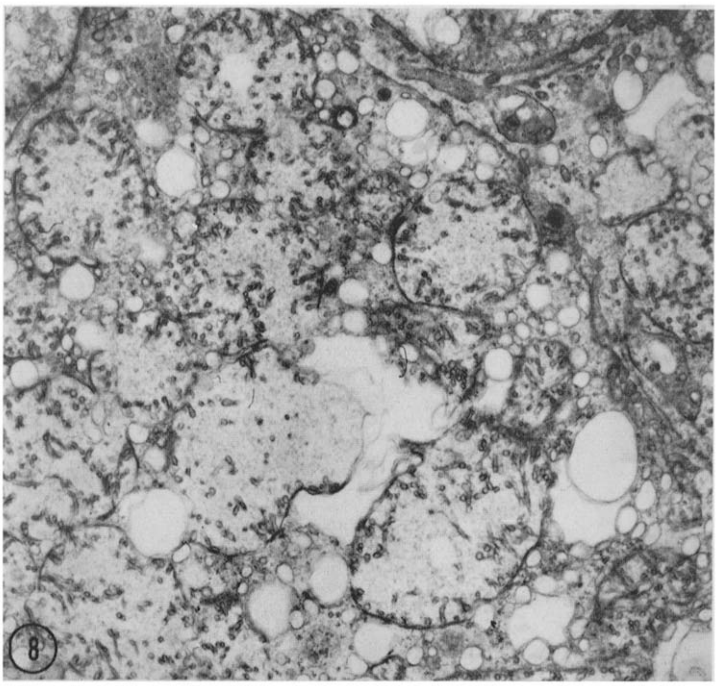

FIG. 8. Zona fasciculata of rat adrenal after 16 days' treatment with *p,p'*-DDD. The cells are comprised predominantly of irregular membranous sacs. There is no evidence of cell injury. Lead hydroxide; × 12,000.

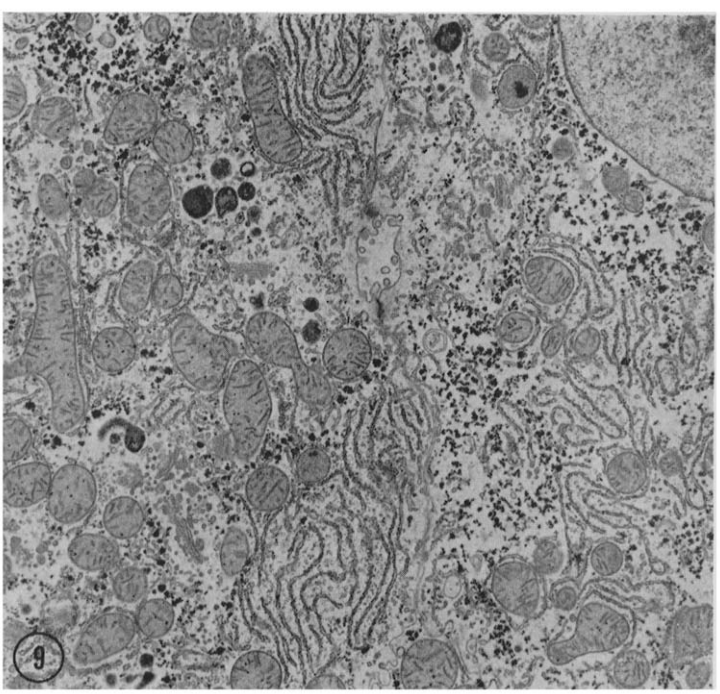

FIG. 9. Normal dog liver. The mitochondrial profiles and the interior matrix are normal. Glycogen is abundent. Virtually all the endoplasmic reticulum is of the rough-surfaced variety. Lead hydroxide; × 6700.

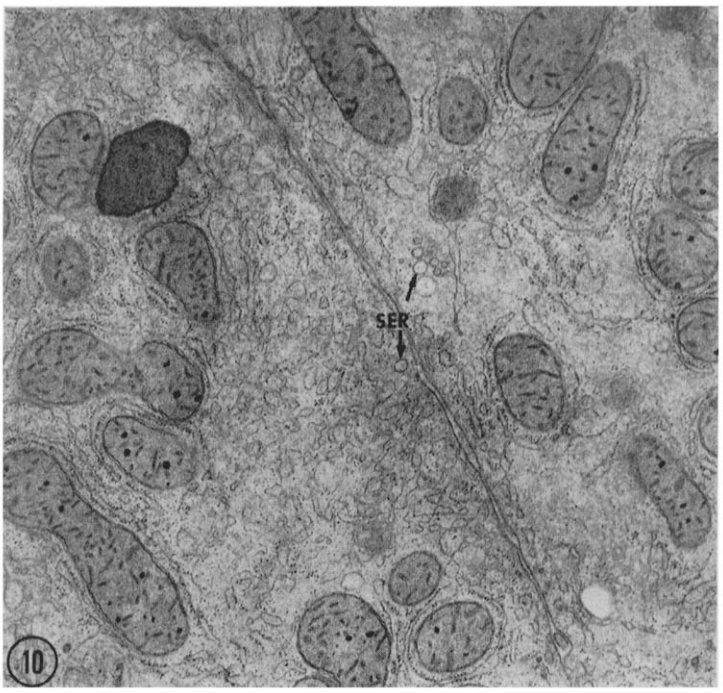

FIG. 10. Dog liver cell after 3 days' treatment with *p,p'*-DDD. A number of vesicles of smooth endoplasmic reticulum (SER) are apparent at the periphery of two adjacent cells. The increase in smooth endoplasmic reticulum is not so marked as in rat liver. Lead hydroxide; × 12,000.

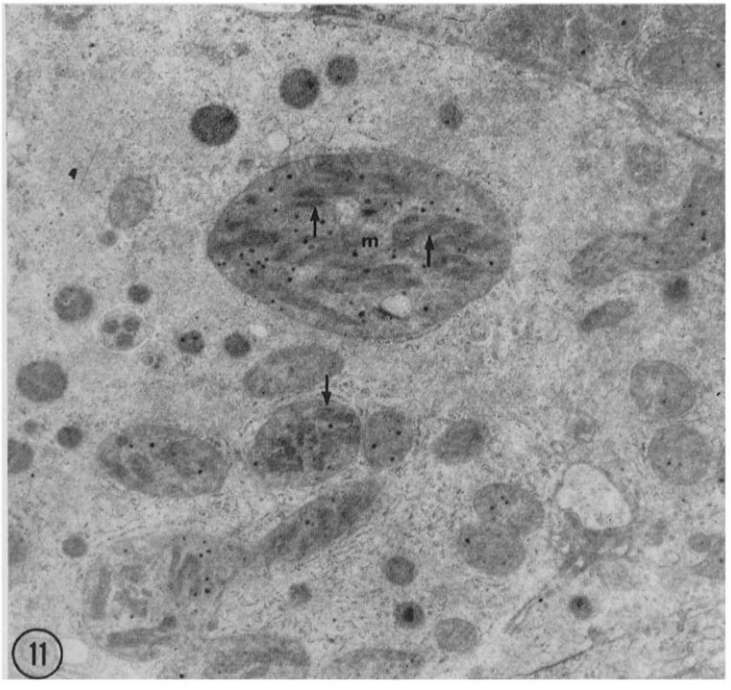

FIG. 11. Dog liver 44 days after treatment with *p,p'*-DDD. The mitochondria (m) are enlarged, of irregular shape, and possess matrix striations (arrows). Lead hydroxide; × 8600.

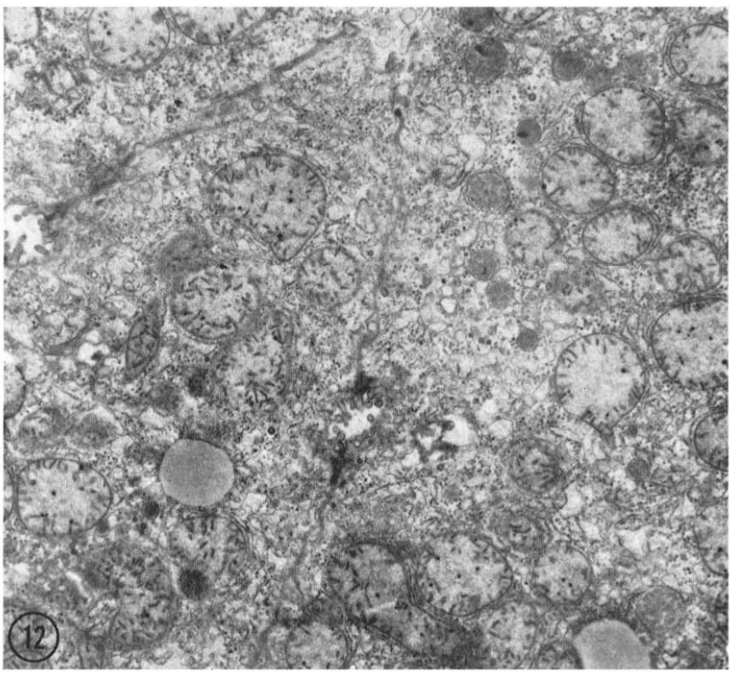

FIG. 12. Dog liver 44 days after treatment with *p,p'*-DDD. Hyperplasia of smooth endoplasmic reticulum is no longer discernible. Lead hydroxide; × 6700.

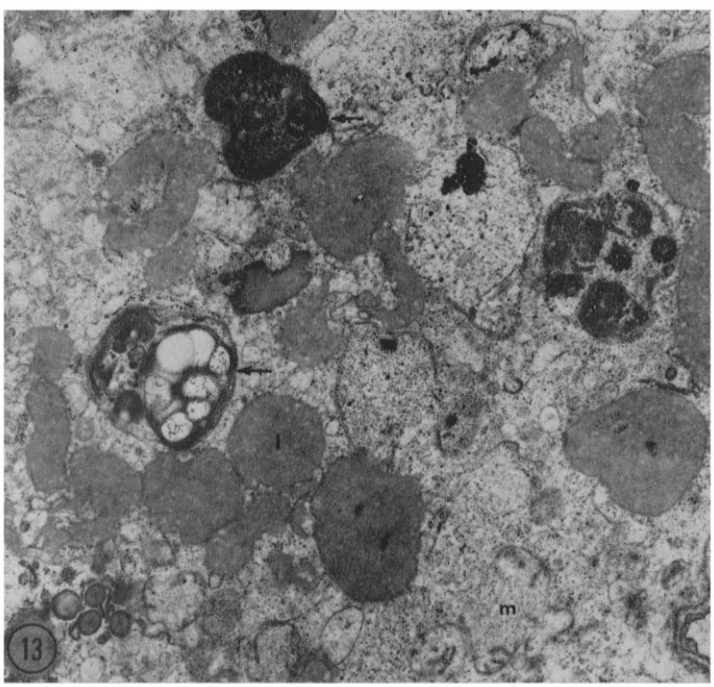

FIG. 13. Dog adrenal after 3 days of *p,p'*-DDD treatment. Cells of the zona fasciculata are necrotic and most details of cell structure are lost. Mitochondrial remnants (m) and several bodies resembling lysosomes (arrows) are apparent; l = lipid. Lead hydroxide; × 12,000.

by treatment with cortisone, although in this species, hexobarbital sleep times are also prolonged.[18] Previous studies[9] have also indicated that DDT and DDD inhibit drug metabolism in the mouse.

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