Philipp. Ent. 7(2) 129-158 ISSN 0048-3753 October 1987

EFFECTS OF ACUTE TREATMENTS OF CALAMUS OIL, β-ASARONE, AND DIMETHOXYPROPENYLBENZENES IN LABORATORY RATS[1]

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ABSTRACT

The systemic toxicity of acute exposure to β-asarone, European calamus oil, and DMPB isomers was examined in the present study. All five substances produced CNS depression but the onset and severity of symptoms appeared to be somewhat influenced by the methoxy substitution pattern. Treated animals gained less weight than controls. This was especially true for β-asarone and 2,4-DMPB. The results suggest that body weight depression was caused by change in appetite as well as diminished efficiency of food utilization.

Specific target organ response was not established with any of the test substances although thymus weights of rats in both age groups were significantly reduced with β-asarone treatment. When thymic tissues were examined, single cell degeneration was noticeable in rats given repeated dose of β-asarone. 2, 3-DMPB and 2,4-DMPB also caused thymic atrophy. Other organs whose weights were reduced significantly include the liver, spleen and heart. Liver examination showed lymphocyte aggregates and infiltration indicative of possible inflammatory reaction in 2,4-DMPB and 2,3-DMPB-treated groups. Mild pyknosis was noted in rats given β-asarone. Other tissues appeared normal.

Significant increase in organ weights was observed in rats given multiple doses of β-asarone, 2,4-DMPB, and European calamus oil. In the absence of consistent pathological changes, the increased weights of the liver, kidneys, and adrenals could be primarily due to the normal compensatory mechanisms of these organs to increased workload. Both kidneys and adrenals participate in the alarm reaction of the organism to acute stress. Hepatic microsomal protein and cytochrome P-450 levels were not elevated enough to explain the boserved hepatomegaly. None of the test substances were hepatotoxic as indicated by histological data and normal SGOT and SGPT levels. One major reason is the susceptibility of these compounds to hepatic degradation and rapid excretion.

The present study, thereby demonstrates that even with acute intraperitoneal administration, the various compounds did not produce significant toxic effects.

Key words: asarones, calamus oil, systemic toxicity, thymic degeneration

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[1] Received for publication 15 April 1986. Portion of the dissertation submitted by the senior author to the University of Wisconsin-Madison, Madison WI, U.S.A. for the degree of Ph.D. (Entomology) under the supervision of the junior author.

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INTRODUCTION

The insect control potential of calamus oil obtained from the rhizomes of the sweetfly, *Acorus calamus* L. and its active component β-asarone, has been well-documented (Ramos 1985, Ramos-Ocampo and Hsia 1986a, 1986b). Inspite of this, calamus oil or any botanical compound proven effective in laboratory and field trials may still not be recommended for large-scale production and usage if their potential hazards to man, his domestic animals and his environment have not been assessed adequately.

In the absence of epidemiological data, toxicological studies are generally conducted in laboratory animals and results are extrapolated to determine safety to humans. With calamus oil and asarone isomers, studies in various animal species dealt mainly with their effects on the central nervous system (CNS) (Dandiya et al. 1959, Sharma et al. 1961, Dhalla and Bhattacharya 1968). Chronic feeding studies conducted by the United States Food and Drug Administration revealed higher incidence of rate leiomyosarcomas in rates exposed to both calamus oil and β-asarone (Taylor et al. 1967, Habermann 1971). On the other hand, Koul et al. (1977) mentioned that calamus oil is a nontoxic chemical but presented no evidence to support their claim. It is possible that these workers based their statement on its long history of usage in Ayurvedic medicine of India.

Although various effects of calamus oil and β-asarone have already been reported, no information on the systematic toxicity as a result of acute exposure can be found in the literature. The damaging potential of both substances to the liver also requires further studies. In addition, toxicological data on the synthetic dimethoxypropenylbenzene (DMPB) analogues are non-existent. The intent of the present study is to further examine the potential toxic effects of calamus oil, β-asarone, and DMPB analogues in a mammalian system. Specifically, experiments were designed to determine the influence of these substances on the activity of the hepatic cytochrome P-450 enzymes and serum transaminases, and the histopathological changes in various organs. Other relevant information was also gathered, i.e., the influence of the substances on the animal's food consumption, body and organ weights, and behavior.

MATERIALS AND METHODS

Test substances

Five methoxypropenylbenzene analogues and European calamus oil were used in the succeeding experiments. β-Asarone, z-2,3-dimethoxypropenylbenzene (DMPB), z-2,4-DMPB and z-2,5-DMPB were synthesized in this laboratory as previously reported (Sunarjo 1981). β-Asarone was purchased from Aldrich Chemical Co. (Milwaukee, WI) while European calamus oil (Lot T434) was a gift from Fritzsche, Dodge, and Olcott, Inc. (New York, NY). Reagent grade solvents were used in the preparation of the various solutions.

Animals and their treatments

Test animals were one- or two-month old, male Sprague-Dawley rats purchased from Harlan-Sprague-Dawley Laboratories (Madison, WI). While in the laboratory, they were housed in pairs in standard stainless steel mesh cages and exposed to a 12-h light-dark cycle. Food (Purina® Rat Chow) and water were provided ad libitum (5 days duration) except on the last day when food was withdrawn.

Two sets of protocols were used to identify the toxic properties of various chemicals in rats. In the initial study, animals were treated with a single dose of either β-asarone or one of the DMPB analogues (z-2,3-DMPB, z-2,4-DMPB, or z-2,5-DMPB). Two dosage levels with four animals in each group were used except the 250 mg/kg β-asarone group which had only 2 animals. This unequal number of observations resulted from the original intention of using a 500 mg/kg level. Unfortunately, the 2 rats given this dose died 7 hours after receiving the chamical. Consequently, dose levels of 100 and 250 mg/kg were selected for subsequent experiments. Individuals rats (121 ± 1 g) received chemical treatments of either 100 or 250 mg/kg b.w. Animals in the control groups received 2 ml/kg b.w. of corn oil (vehicle). In the second study, groups of four animals each (271±2 g) were given chemicals daily for 5 consecutive days. Two treatment groups received either β-asarone or 2, 4-DMPB; a dose level of 100 mg/kg b.w. was used. The other two remaining groups were treated with either 100 or 200 mg/kg b.w. of calamus oil. As before, the control group received 2 ml/kg b.w. of corn oil. All chemicals tested were disssolved in corn oil and were administered intraperitoneally to the animals. Throughout the experiments, body weights were recorded daily. Food consumption was also monitored among rats in the first study and the behavior of rats was noted.

At the termination of each experiment, all animals were anesthetized with pentobarbital (50 mg/kg b.w.) and killed via cardiac puncture. Necropsies were performed and the liver, thymus, adrenals, kidneys, spleen, heart, and testes were excised and examined grossly. Body and organ weights were recorded. Sections of these organs were then fixed in 10% buffered formalin or WARF fixative, in the case of liver. All tissues samples were embedded in paraffin, sectioned at 6 um, and stained with hematoxylin-eosin for light microscopic evaluation (Luna 1968).

Hematological parameters

Blood samples were collected by cardiac puncture prior to necropsy. in order to examine the hematotoxicity of the test compounds, hematocrit and hemoglobin values were determined among rats in the first experiment. The procedure described by Mangrum (1975) was used. Blood samples collected from the animals in the second experiment were used to determine serum transaminase activity. In order to assess the possibility of liver injury induced by the test compounds, serum glutamic-oxalacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT) activities were

measured using a Sigma SGOT/SGPT assay kit (Karmen 1955, and Wroblewski and La Due 1955). In these methods, the transaminase reactions are coupled to specific dehydrogenase enzymes so that the ketoacids resulting from transamination are coverted to their corresponding hydroxyacids by means of reduced NAD. As the reaction proceeds, NADH is oxidized to NAD and the resulting decrease in absorbance at 340 nm is followed in a Varian Techtron UV-VIS spectrophotometer, Model 635.

Preparations of liver microsomes

Liver sample was taken from each animal for the determination of microsomal protein concentrations and cytochrome P-450 levels. Liver microsomes were prepared by the method described by Hsia and Kreamer (1979). Briefly, livers were chilled in ice-cold 0.9% (w/v) saline solution, patted dry, minced with sterile scissors, and homogenized with 4 volumes of 50 mM Tris buffer-0.15 M KCl (pH 7.2) in a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 10 minutes at 10,345 g (10,000 rpm in SS-34 rotor, of Sorval RC2-B), the supernatant collected and recentrifuged for 90 minutes at 40,000 g (25,000 rpm in a SW 27.1 rotor of Beckman Ultracentrifuge, Model L3-50). The resulting microsomal pellet was resuspended in 30% glycerol-Tris-KCl buffer (0.4 ml/g wet liver). Aliquots of the suspension were transferred into small vials and stored at -80 °C until needed.

Biochemical assays

Protein concentrations were determined by the method of Schacterle and Pollack (1973) using bovine serum albumin (Fraction V, Sigma) as the standard. The microsomal suspension was incubated at 55° C with Folin-Ciocalteau phenol solution and alkaline copper reagent. After 5 minutes, the absorbance was read at 650 nm and protein concentration was determined from the standard curve. Cytochrome P-450 levels were assessed by the differential spectral assay of Omura and Sato (1964). Briefly, 0.15 ml microsomal suspension was added to 2.85 ml Tris-KCl buffer and bubbled with CO gas for 1 minute. Half of the sample was reduced with sodium dithionite and used as sample blank. The CO-saturated suspension was scanned from 500-400 nm and the absorbance maxima determined. Cytochrome P-450 values were computed using an extinction coefficient of 0.9 nmoles/cm and reported as nmoles P-450/mg protein.

Statistical analysis

Data were analyzed using the Mann-Whitney U test, which is a nonparametric analogue to the t-test for 2 independent samples (Siegel 1956). The test is based on ranks of individual observations rather than actual values. The Mann-Whitney U test was chosen becaue the central limit theorem seems inexplicable with very small samples used in the present study. Significance was assessed at $p < 0.05$ level ($n_1 n_2 = 4, 4$) or at $p = 0.067$ ($n_1 n_2 = 2, 4$).

RESULTS AND DISCUSSION

Gross observations

After the rats received a single dose of any of the various substituted propenylbenzenes or European calamus oil (100-250 mg/kg), the following signs of toxicity were observed within 15 minutes to 2 hours; ptosis, dazed eyes, tachypnea, unresponsiveness to touch and noise, sweating, piloerection, and depression. The onset of symptoms, however, varied among the different compounds tested: 15 minutes for β-asarone, 30 minutes for calamus oil, and 1-2 hours for the DMPB isomers. After 24 hours, all toxic signs disappeared in rats given a single dose of the chemicals but the scrawny appearance persisted until necropsy. For rats receiving repeated doses of calamus oil, β-asarone, and 2, 4-DMPB, the various symptoms reappeared following each injection.

These observations are in agreement with those already reported for β-asarone and calamus oil (Dhalla et al. 1961, Dandiya and Menon 1964, Dhalla and Bhattacharya 1968, and Oswald et al. 1969) and further support the depressant effects of the drugs on the nervous system. Precocene II, a compound structurally related to β-asarone, produces similar symptoms (Hsia et al. 1981). Oswald et al. (1969) postulated that the asarone isomers, just like safrole, elemecin, eugenol, and myristicin, are converted to amphetamine-like metabolites responsible for the psychotropic action attributed to this group of substituted propenylbenzenes. Since the DMPB analogues produced similar effects as β-asarone and calamus oil, these chemicals are classified as CNS depressants. However, considering that the onset of symptoms for the various DMPB analogues is delayed and the magnitude of effects are milder than β-asarone (2, 4, 5-TMPB), the possible existence of SAR is indicated. A definite correlation requires further evaluation. Dandiya et al. (1962) and Seto and Keup (1969) stressed the importance of the alkyl side chain and the relative position of the methoxy groups in sedation. Moreover, the latter workers gave importance to the presence of 3 methoxy substituents for hypnotic-potentiating activity. Although the results presented here are inconclusive, preliminary observations indicate that the number of methoxy groups influences CNS depression significantly.

Body weight and food consumption

One of the most sensitive criteria of stress is body weight change and provides a useful initial estimate of toxicity (Jackson, 1962). To determine whether the various propenylbenzenes affect feeding and growth, food intake and percent weight gain of rats in the initial study were recorded at 2-day intervals. The results presented on Table 1 showed that, in general, treated rats gained less weight than control rats over the 4-day period. Both dose levels of β-asarone and DMPB isomers except 2, 5-DMPB at 100 mg/kg reduced weight gains markedly at day 2 following treatment. The 2, 4-isomer at 250 mg/kg still caused significant suppression of weight gain by day 4.

Table 1. Food consumption and weight gain of rate receiving a single i.p. dose of methoxypropenylbenzenes[1]

Chemical	Dose (mg/kg)	Rats per treatment (n)	Initial body weight (g)	Food Consumption (g)		% Weight Gain		Food Utilization Efficiency (g/g wt gain in 4 days)
				Day 2	Day 4	Day 2	Day 4	
2, 4, 5-TMPB	250	2	132.5± 3.5	15.0± 0.0***[2]	34.5± 0.0	4.2± 0.4***	15.4± 1.1***	3.41± 0.0***
(β-asarone)	100	4	119.5± 4.4	26.5± 1.4*	32.8± 1.6	7.1± 1.2*	11.6 ± 0.4	2.53± 0.05**
	0	4	124.3± 5.7	33.3± 0.1	33.8± 1.6	12.5± 0.1	10.2± 0.8	2.44± 0.11
2, 3- DMPB	250	4	123.3± 1.4	28.8± 2.8*	34.3± 2.2	8.7± 1.0*	11.9± 0.3	2.46± 0.08*
	100	4	124.0± 3.1	36.0± 0.0*	37.0± 1.5	12.1± 0.8**	12.2± 0.2	2.28± 0.04
	0	4	123.8± 0.9	37.5± 0.3	37.8± 0.5	15.0± 0.8	10.7± 0.9	2.23 ± 0.01
2, 4-DMPB	250	4	114.8± 2.9	27.0± 0.9*	27.0± 2.0*	5.6± 1.9*	5.5± 0.5*	4.24± 0.66*
	100	4	124.3± 5.4	25.5± 1.2*	29.8± 0.7*	3.6± 0.3*	9.9± 1.2	3.22± 0.33**
	0	4	110.8± 2.5	30.5± 0.3	33.5± 0.3	12.9± 1.8	12.4± 1.2	2.12± 0.12
2, 5-DMPB	250	4	124.3± 5.9	29.3± 2.5*	38.0± 2.0	7.0± 1.4*	13.3± 1.3	2.55± 0.17
	100	4	118.0± 3.7	31.5± 0.6*	38.3± 0.2	10.4± 1.8	15.8± 0.8	2.11± 0.02
	0	4	119.3± 0.9	36.8± 0.5	40.0± 0.9	13.9± 1.2	13.8± 1.4	2.18± 0.01

[1] Values represent mean ± S.E., Male Sprague-Dawley rats received a single i.p. dose of the test compounds. Control rats received 2 ml/kg corn oil (vehicle). Date were recorded at 2-day interval.

[2] Significantly different from respective controls (Mann-Whitney U test).

* $p = 0.014$ ($n_1 n_2 = 4, 4$)

** $p = 0.029$ ($n_1 n_2 = 4, 4$)

*** $p = 0.067$ ($n_1 n_2 = 2, 4$)

Table 1 further shows that rats receiving the various chemicals had reduced food consumption compared to controls. On a daily average basis, treated rats consumed 4-9 g less food than rats receiving only corn oil. The bulk of this decrease occurred significantly during the first 2 days after receiving a single dose of the chemical. With the exception of 2, 4-DMPB, treated animals had sufficiently recovered by day 4 such that differences in food consumption between treated and control groups are no longer statistically significant. It appears that this particular isomer influenced food intake and body weight gain more than the rest of the test compounds.

Parallel results obtained between food consumption and percent weight gain strongly suggest that the observed depression in weight of the various treatment groups is a direct effect of decreased food consumption. Habermann (1971) reported similar observations in rats fed with diets containing 400-2,000 ppm β-assarone for 2 years. In their study, the food consumption of treated rats was lower than that of controls to a degree consistent with body weight depression. Uehleke and Brikschulte-Freitas (1979) established a similar correlation with the essential oil of myrtle. During oral application of the oil, body weights of rats were found to be reduced by the second or third day of treatment. These workers concluded that the effect was due to central depression or narcosis, which reduced food and water intake.

As a measure of food utilization efficiency, the ratio of total food consumed to total weight gained in 4 days was determined and summarized (Table 1). β-Asarone and 2, 4-DMPB-treated rats have significantly less efficient food utilization capability than the control. In general, treated rats required 50-100% more food per gram weight gain. It seems, therefore, that decreased efficiency in food utilization as well as change in appetite are responsible for loss of weight in treated rats as compared to controls.

β-Asarone and 2, 4-DMPB were tested further in 2 groups of rats which received 100 mg/kg for 5 consecutive days. At the same time, 2 other groups of rats were given either 100 or 200 mg/kg calamus oil. The results of this experiment (Table 2) show that all test compounds caused significant reduction in weight gain of rats. This effect may be due to CNS depression as pointed out by Uehleke and Brinkschulte-Freitas (1979) with the oil of myrtle . Although food consumption data were not noted in the present experiment, the depressant effects produced by repeated application of the test compounds could severely affect food intake, which would result in reduced body weight gain as observed in this study. In the case of calamus oil, severe weight loss was noted by several reseachers whether the chemical was administered by stomach intubation once (Jenner et al. 1964) or mixed with the diet for 18 weeks (Hagan et al. 1967) or two years (Taylor et al. 1967, and Habermann 1971). Although growth depression was dose-related, Taylor and co-workers (1967) found that food intake was only slightly depressed at the highest dose levels of 2,500-5,000 ppm calamus oil and the degree of depression could not account *in toto* for the observed weight loss. On the other hand, Habermann (1971) reported that body weight depression was consistent with decreased food consumption in rats given a diet contain-

Table 2. Weight gain of rats treated with European calamus oil, 2, 4-DMPB, and 2, 4, 5-TMPB for 5 consecutive days.[1]

Chemical	Dose (mg/kg)	Initial body wt (g)	% Weight Gain Day 2	Day 4
2, 4, 5-TMPB (β-asarone)	100	273.3±0.7	−3.7±0.6*	1.0±0.7*
2,4-DMPB	100	272.0±3.9	1.1±0.9*	3.6±0.3*
Calamus oil	100	270.3±6.2	3.2±0.3*	3.6±1.5*
	200	274.0±6.8	1.2±1.3*	4.3±0.9*
Control	0	271.5±7.5	6.7±0.8	7.2±0.5

[1] Values represent mean ± S.E. of 4 observations. Male Sprague-Dawley rats received 5 daily injections of the test chemicals. Control rats were given 2 ml/kg corn oil (vehicle). Data were recorded at 2-day interval.

*Significantly different from control at p = 0.014 level (Mann-Whitney U test).

ing 400-2,000 ppm β-asarone for 2 years. It is clear, therefore, that growth retardation in any toxicity experiment can result either from physiological response to diminished food intake or from the toxic influences of the drugs. The use of elaborate pair-feeding experiments will give a better insight into the underlying mechanism(s) and will improve the value of this type of toxicological data (Scharer 1977 and Oishi et al. 1979).

Organ weights and histology

To determine whether the test chemicals cause specific target organ response in rats, major organs from various treatment groups were weighed and examined microscopically.

Table 3 shows that animal weights in the initial study were not statistically different at necropsy. Fasting apparently reduced the variability in weights that was observed among the various treatment groups (Table 1) during the course of the experiment. Among the different organs examined, thymus and liver weights were significantly lower in rats treated with β-asarone and 2, 3-DMPB as compared to controls. Table 3 further shows that spleen and thymus weights were markedly reduced in 2, 4-DMPB-treated groups. Slightly heavier testes were also noted in rats given 100 mg/kg 2,4-DMPB. The 2, 5-isomer produced no significant effects on organ weights.

These results show the thymus as the organ most consistently affected by the various propenylbenzenes. Thymic atrophy (20-30% weight reduction) was significant in 3 of the 4 chemicals tested. It appears to be an extremely sensitive indicator of the toxic influence of certain chemicals. Luster et al. (1982) claimed that thymic degeneration commonly occurs following expo-

Table 3. Organ weights of rats treated with a single i.p. dose of methoxypropenylbenzenes.[1]

Chemical	Dose (mg/kg)	Rats per treatment (n)	Terminal body wt. (g)	Organ Weight (g)						
				Liver	Spleen	Kidneys	Adrenals	Thymus	Heart	Testes
2,4,5-TMPB	250	2	124.5±0.5	4.61±0.24*[2]	0.40±0.04	1.22±0.04	0.038±0.001	0.34±0.01***	0.50±0.02	1.76±0.14
(β-asarone)	100	4	123.3±2.8	4.85±0.06	0.44±0.07	1.18±0.07	0.034±0.002	0.36±0.03**	0.51±0.02	1.84±0.05
	0	4	134.8±6.3	5.12±0.29	0.47±0.06	1.18±0.07	0.036±0.002	0.48±0.04	0.53±0.03	1.82±0.17
2,3-DMPB	250	4	127.3±3.2	5.07±0.19	0.49±0.02	1.30±0.04	0.035±0.001	0.42±0.02*	0.56±0.01	1.68±0.05
	100	4	136.3±1.1	4.83±0.07*	0.52±0.04	1.33±0.01	0.032±0.001	0.54±0.04	0.55±0.03	1.74±0.01
	0	4	135.5±0.7	5.14±0.09	0.56±0.04	1.40±0.04	0.033±0.001	0.58±0.04	0.58±0.01	1.69±0.06
2,4-DMPB	250	4	112.5±4.7	4.77±0.20	0.47±0.04**	1.04±0.03	0.035±0.003	0.29±0.02*	0.44±0.02	1.80±0.05
	100	4	121.3±5.2	4.65±0.15	0.59±0.06	1.07±0.05	0.038±0.002	0.31±0.02*	0.46±0.02	1.82±0.03
	0	4	116.8±2.0	4.63±0.10	0.62±0.05	1.06±0.03	0.035±0.001	0.40±0.02	0.45±0.02	1.71±0.04
2,5-DMPB	250	4	124.5±4.8	5.03±0.21	0.49±0.02	1.23±0.06	0.035 ±0.002	0.46±0.03	0.51±0.01	1.58±0.07
	100	4	127.8±4.0	4.93±0.24	0.50±0.02	1.22±0.04	0.033±0.002	0.52±0.02	0.48±0.03	1.50±0.07
	0	4	137.3±4.5	5.20±0.20	0.51±0.01	1.24±0.04	0.033±0.001	0.49±0.01	0.54±0.03	1.61±0.08

[1] Values represent mean ±S.E. Male Sprague-Dawley rats received a single i.p. dose of the test compounds. Control rats were given 2 ml/kg corn oil (vehicle). Necropsy was performed on fasted animals 5 days after treatment.

[2] Statistically significant from respective controls (Mann-Whitney U test).

* $p = 0.014$ ($n_1 n_2 = 4,4$)

** $p = 0.029$ ($n_1 n_2 = 4, 4$)

*** $p = 0.067$ ($n_1 n_2 = 4, 2$)

sure to a large number of xenobiotics. When the thymic tissues from various treatments were examined, no significant morphological changes between control (Fig. 1A) and treated groups, e.g., 100 mg/kg-treated rat (Fig. 1B) were noted. Inspite of reduced thymus weights in rats given β-asarone, 2, 3-DMPB or 2, 4-DMPB, histology revealed well-defined cortex and medulla and the absence of any apparent abnormalities. Microscopic changes in the livers of propenylbenzene-treated animals were not remarkable. There was an indication of increased number of pyknotic cells in the liver of β-asarone-treated groups (Fig. 2B). Pockets of lymphocytes in the hepatic periportal region of rats given 2,4-DMPB (Fig. 3B) and 2,3-DMPB (Fig. 4) were also noted. These findings, however, were not associated with any apparent morphological irregularities in liver architecture. Other organs examined microscopically were the spleen, kidneys, and adrenals but no treatment-related effects were observed.

When β-asarone and 2, 4-DMPB were administered repeatedly to rats, a different organ weight profile was observed (Table 4). In β-asarone-treated group, adrenal weights increased significantly while heart weights were reduced as compared to controls. Furthermore, thymus weights were still statistically lower than controls. Inspite of body weight depression, reduction in heart and thymus weights may be related to β-asarone treatment. Histological examination of the thymus in this particular group showed higher incidence of single cell degeneration in the cortex (Fig. 5B) as compared to control (Fig. 5A). The pockmarked appearance was not observed in rats given a single dose of β-asarone (Fig. 2B). T-lymphocyte degeneration might be caused by direct cytotoxic effects of the compound or as a consequence of cell-mediated immune reactions. Although heart histology was not included in the present study, it is also interesting to speculate about possible cardiac damage resulting from β-asarone treatment. Myocardial degeneration in rats fed diets containing β-asarone has been reported (Habermann 1971). This is characterized by varying degrees of necrosis of the muscle fibers, early fibrosis, and infiltration with mononuclear cells. Table 4 further shows that contrary to the results of the initial study, absolute weights of the thymus, spleen, and testes were not affected by repeated dose of 2, 4-DMPB. Adrenal weights, on the other hand, were significantly higher than controls. Microscopic examination of adrenals (Fig. 6B), spleen (Fig. 7B), and kidneys (Fig. 8B) of either β-asarone- or 2, 4-DMPB-treated animals revealed no apparent morphological changes as compared to their respective controls (Figs. 6A, 7A, 8A). In the liver of an animal given 2, 4-DMPB, an area of focal necrosis with lymphocytic infiltration was observed (Fig. 9B). These pockets of lymphocytes, indicative of inflammatory reactions, were also noted in rats given a single dose of 2, 4-DMPB and 2, 3-DMPB (Figs. 3B and 4).

The influence of β-asarone and 2, 4-DMPB on organ weights appeared to be age-dependent. In the first study using 4-week old weanling rats, reduced organ size was noted for the thymus, spleen, and liver. Adrenal enlargement, in addition to reduction in thymus and heart weights, was observed in 8-week old rats. Alteration in susceptibility to chemical assault between rats

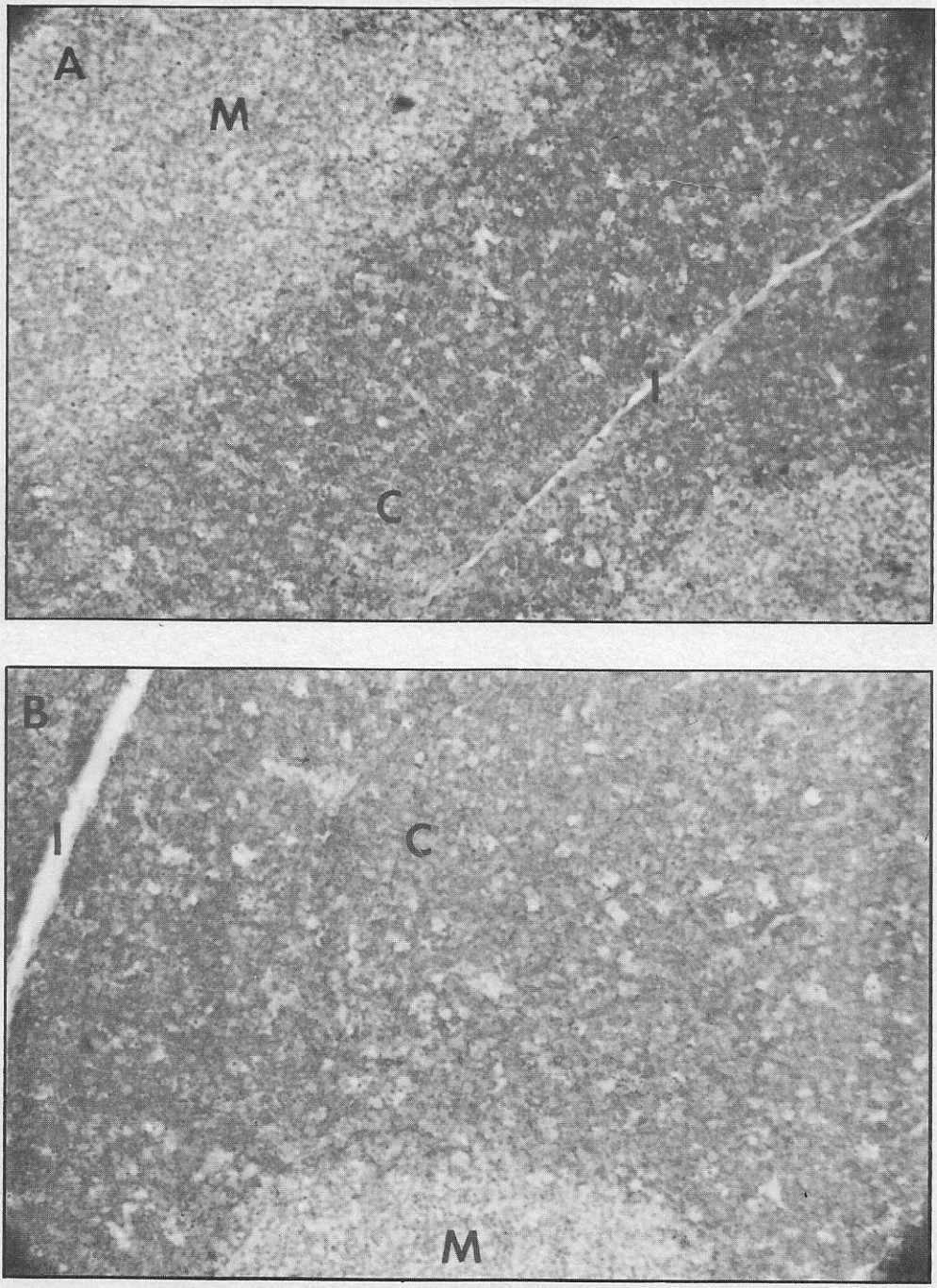

Figure 1. A Thymus from control animal exhibiting well-defined cortex (C), medulla (M), and interlobular connective tissue (I), X49. B. Thymus from animal receiving a single 100 mg/kg dose of 2,4-DMPB, X49. Note the absence of apparent morphological abnormalities as compared to normal thymus.

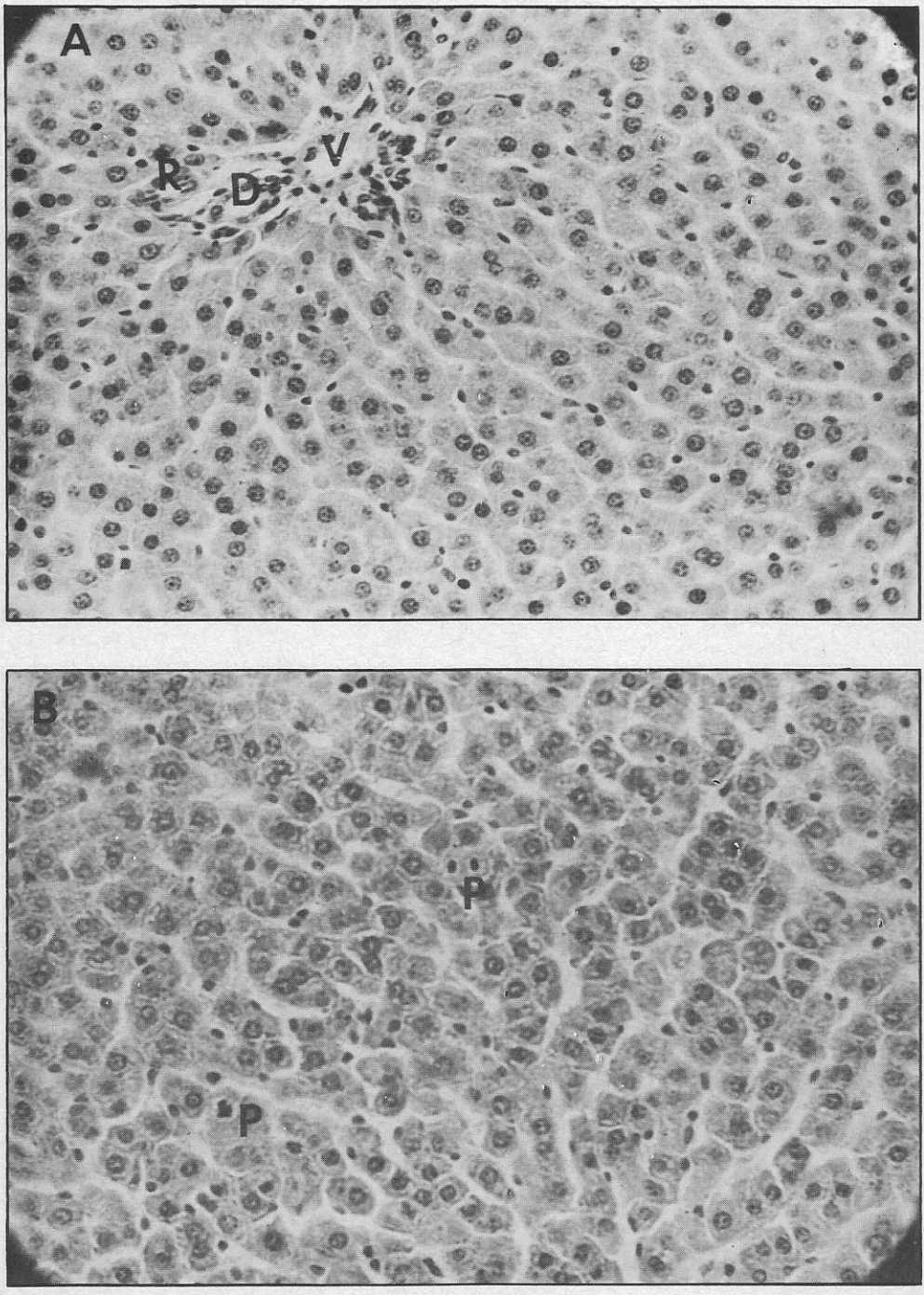

Figure 2. A. Liver from control animal showing the portal triad consisting of portal veins (V), artery (R), and bile duct (D), X126. B. Liver from animal receiving a single 250 mg/kg dose of β-asarone, X126. Note several pyknotic nuclei (P).

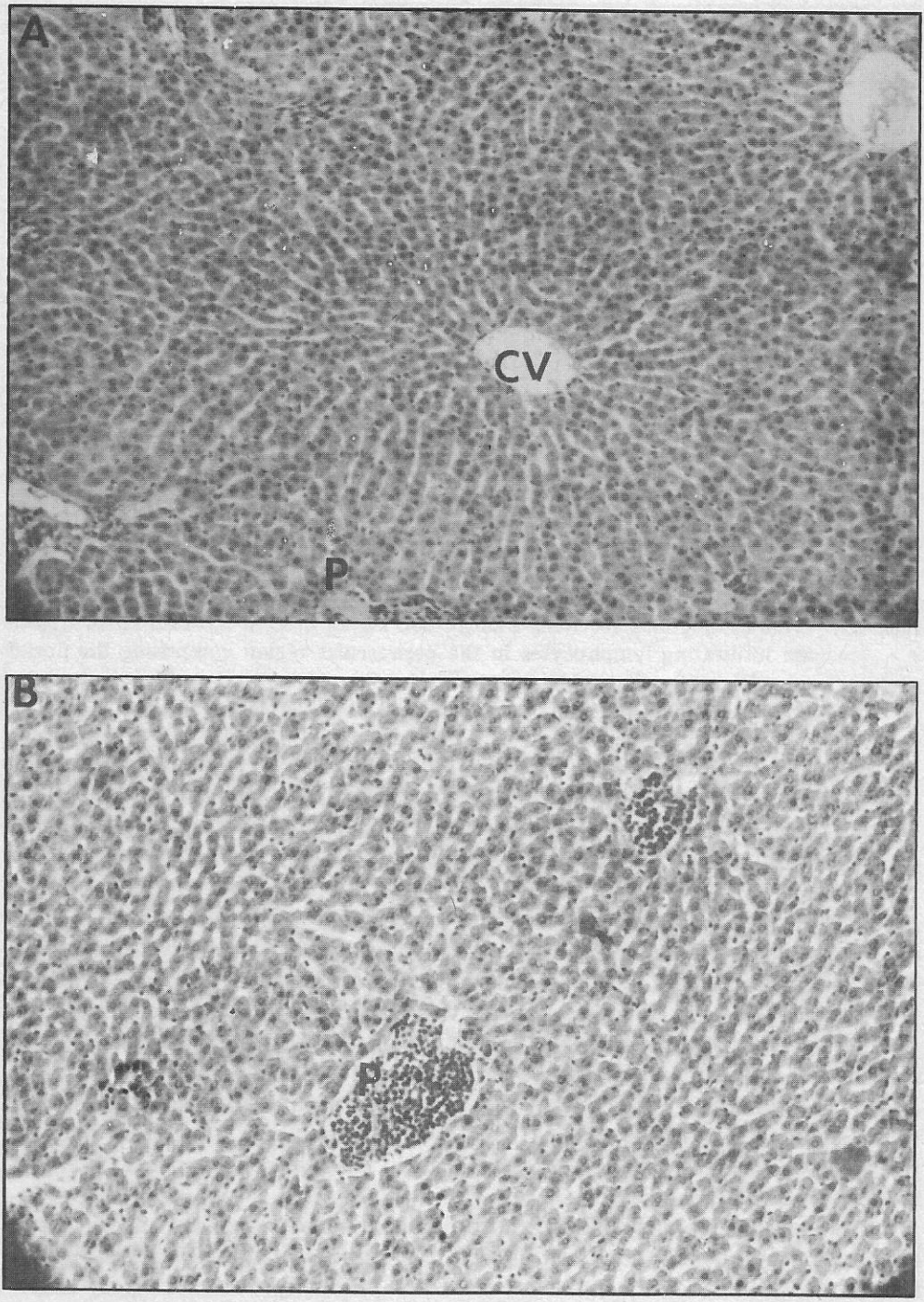

Figure 3. A. Liver from control animal showing the central vein (CV) and portal triad (P), X49. B. Liver from animal receiving a single 250 mg/kg dose of 2,4-DMPB, X49. Note the pockets of lymphocytes in the perivascular area of the liver.

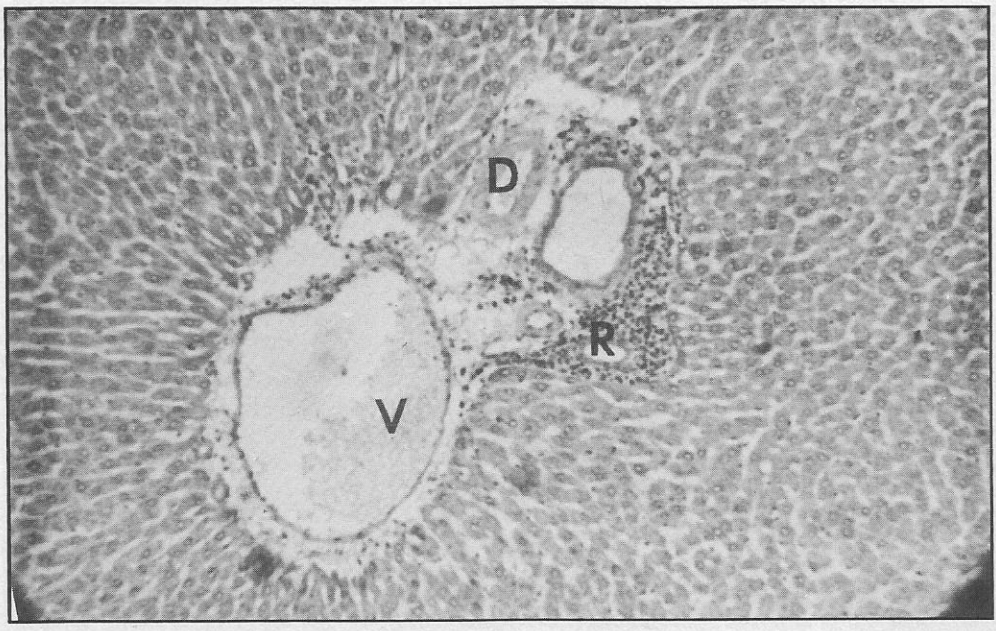

Figure 4. Liver from animal receiving a single 250 mg/kg dose of 2,3-DMPB, X49. Note the infiltrating lymphocytes in the perivascular region comprising the portal vein (V), artery (R) and bile duct (D).

of comparable ages as the present study have also been reported by Constantopoulos and Boyd (1968) and Hsia et al. (1982). It appeared that the differential response of the two age groups is due to increases in body weights and the concomitant changes in the metabolic activity of growing animals (De Castro and Boyd 1968, and Boyd 1972).

The effects of European calamus oil on the organ weights of 8-week old rats were also examined in the present study. Table 4 shows that the oil significantly increased liver and kidney weights of treated rats as compared to controls. Reduction in spleen size was also noted. Histological examination of these particular tissues revealed the absence of morphological abnormalities in rats repeatedly treated with either dose of calamus oil. The liver (Fig. 10), kidneys (Fig. 11), and spleen (Fig. 12) appeared normal. On the other hand, the cortical area of the thymus of calamus oil-treated animals (Fig. 13) showed the single cell degeneration that was also apparent in β-asarone-treated rats (Fig. 5B).

In the absence of consistent pathological changes in many of the tissues examined, it appears that the observed differences in organ weights of rats treated with the various test chemicals are primarily determined by organ workload (Frazer 1962, Goldberg 1966, and Weil 1969). Adrenal enlargement with β-asarone or 2, 4-DMPB treatment may be caused by elevated levels of glucocorticoids. A significant correlation between adrenal hyper-

Table 4. Organ weights of rats treated i.p. with European calamus oil, 2,4-DMPB, and 2,4,5-TMPB for 5 consecutive days.[1]

Chemical	Dose (mg/kg)	Terminal body wt. (g)	Organ Weight (g)						
			Liver	Spleen	Kidneys	Adrenals	Thymus	Heart	Testes
2,4,5-TMPB (β-asarone)	100	256.5±4.0*[2]	10.68±0.36	0.56±0.05	2.15±0.05	0.065±0.005**	0.36±0.04**	0.92±0.02*	3.26±0.13
2,4-DMPB	100	268.0±2.3	10.11±0.16	0.66±0.03	2.32±0.03	0.056±0.002**	0.58±0.05	0.99±0.02	3.42±0.13
Calamus Oil	100	270.3±2.6	10.23±0.14	0.68±0.03	2.46±0.10*	0.054±0.004	0.61±0.03	0.97±0.04	3.33±0.21
	200	268.8±4.6	10.69±0.04*	0.67±0.01**	2.52±0.12	0.062±0.007	0.60±0.03	1.00± 0.03	3.46±0.07
Control	0	279.3±3.5	9.78±0.27	0.78±0.04	2.27±0.03	0.48 ±0.002	0.53±0.06	1.07±0.05	3.46±0.16

[1] Values represent mean ±S.E. of 4 observations. Male Sprague-Dawley rats were given 5 daily injections of test compounds. Control rats received 2 ml/kg corn oil (vehicle). Animals were fasted and sacrificed one day after last injection.

[2] Significantly different from controls (Mann-Whitney U test).

* p = .014

**p = .029

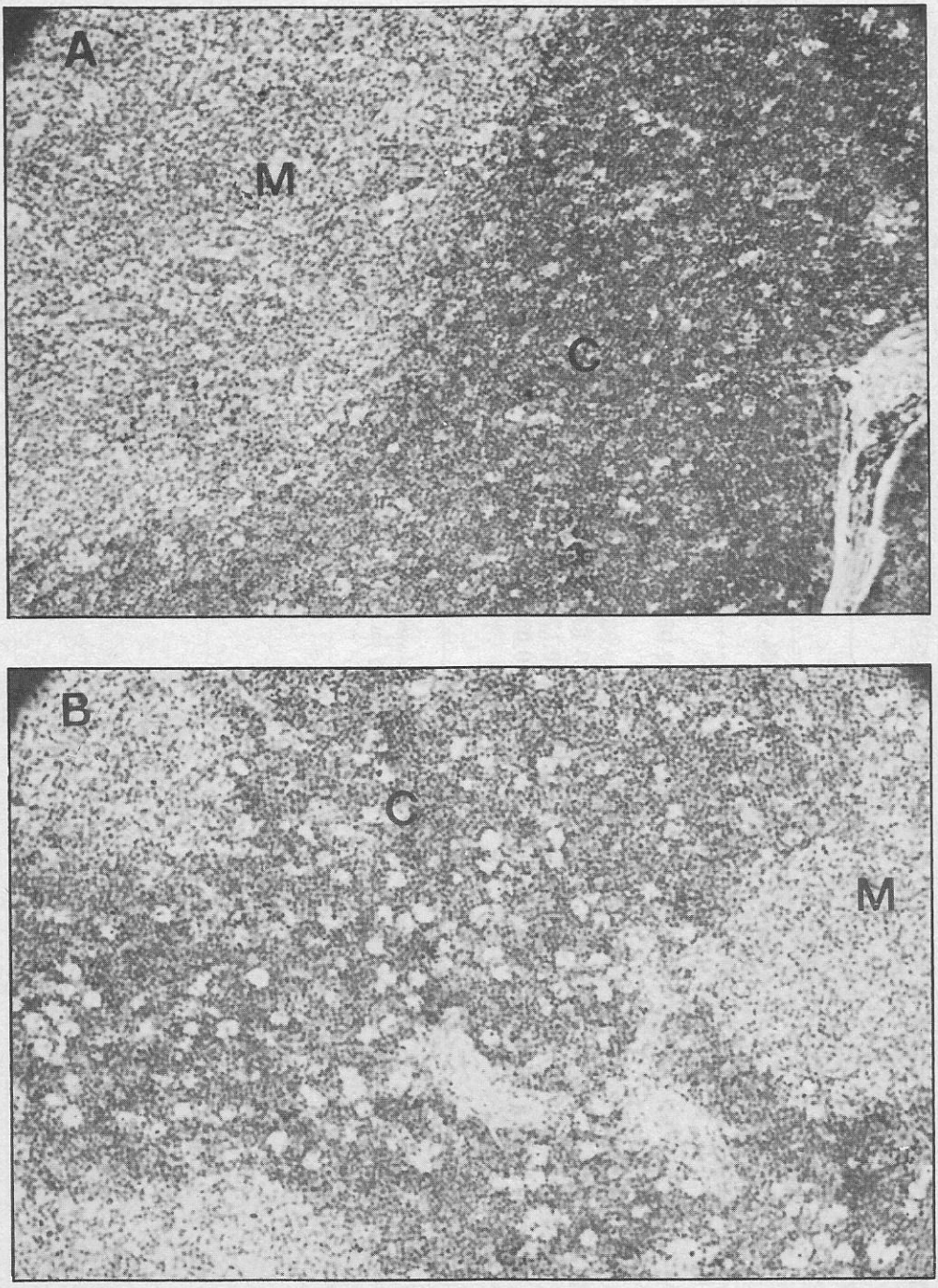

Figure 5. A. Thymus from control animal receiving multiple dose of corn oil, showing well-defined medulla (M) and cortex (C), X49. B. Thymus from animal receiving multiple dose of 100 mg/kg β-asarone, X49. Note the pockmarked appearance of the cortex.

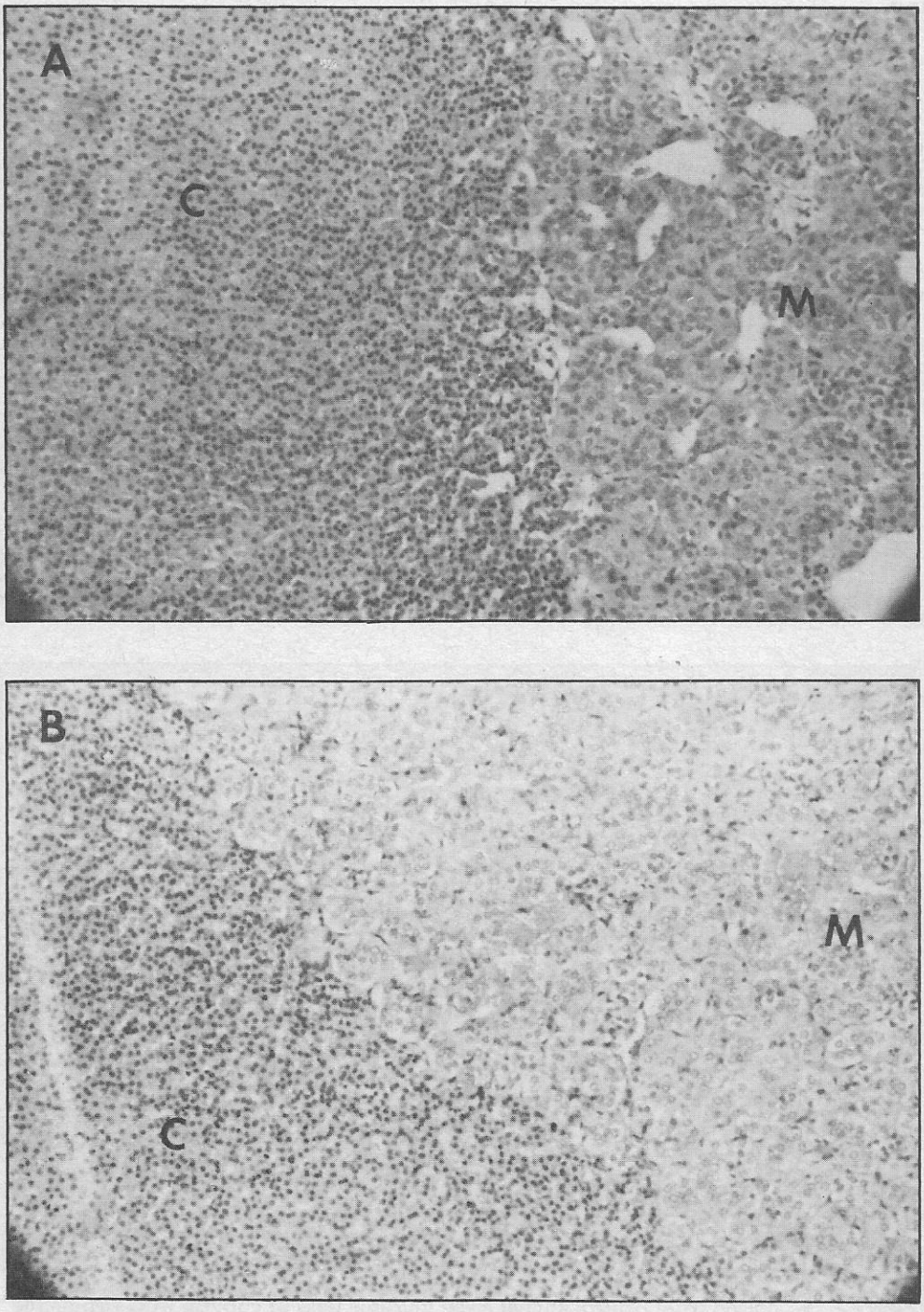

Figure 6. Adrenals from control (A) and 100 mg/kg 2,4-DMPB-treated (B) rats, multiple dose, X49. No apparent abnormalities were observed in the cortex (C) and medulla (M) of both rats.

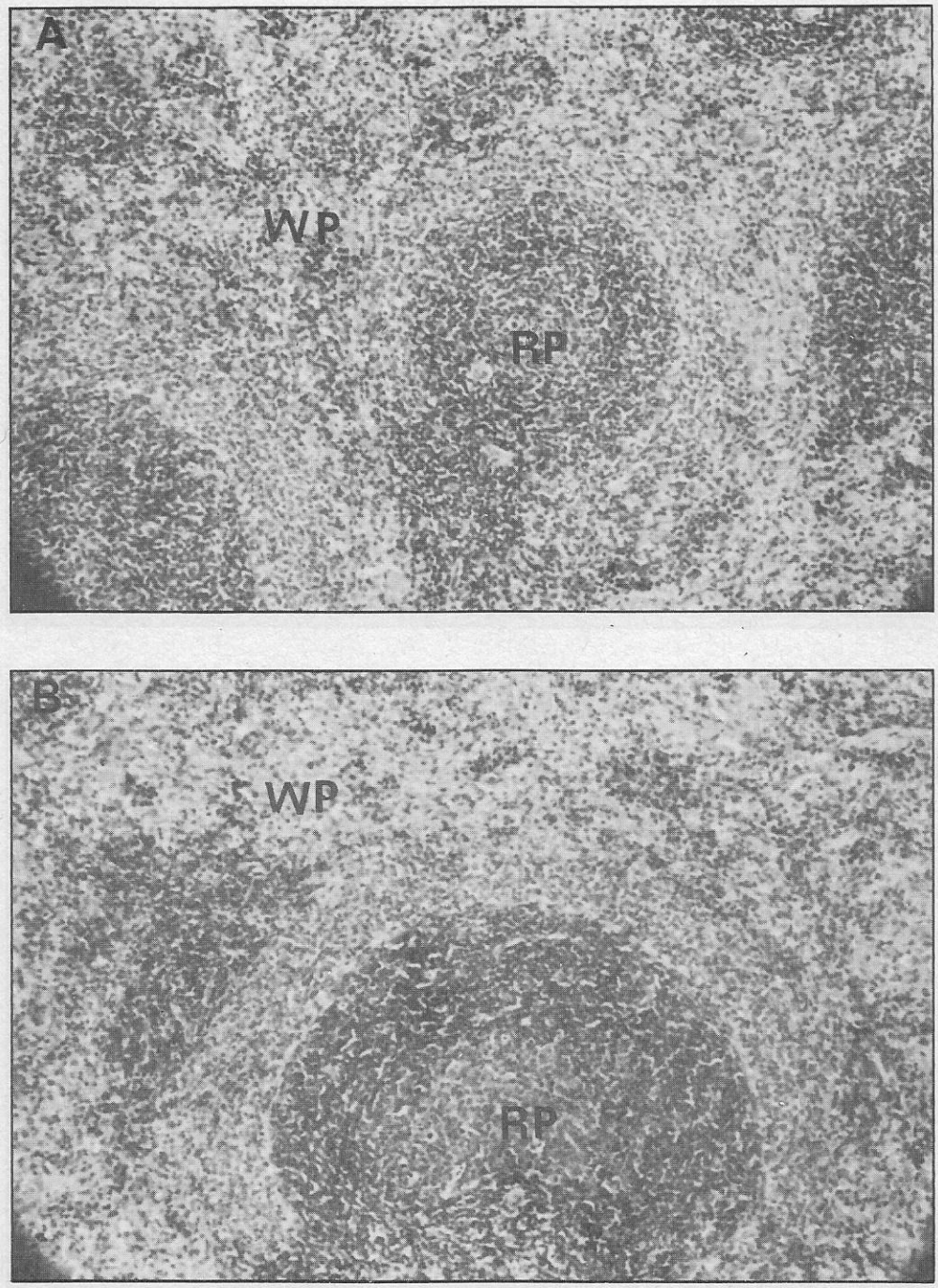

Figure 7. A. Spleen from control animal given multiple dose of corn oil with well-define areas of white pulp (WP) and red pulp (RP), X49. B. Spleen from animal given multiple dose of 100 mg/kg 2,4-DMPB, X49. Note the absence of apparent morphological abnormalities as compared to control.

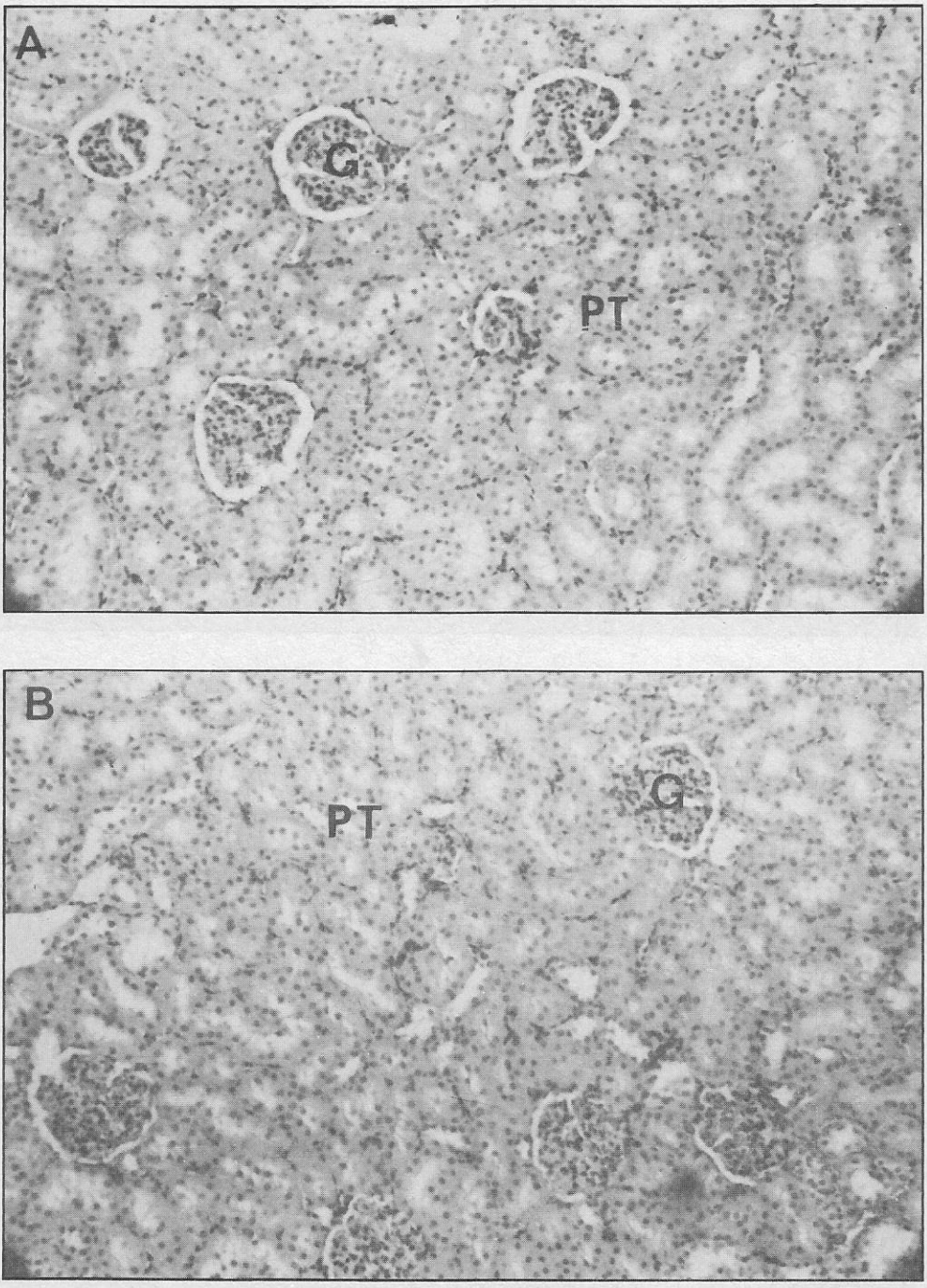

Figure 8. A. Kidney from control animal given multiple dose of corn oil, X49. B. Kidney from animal given multiple dose of 100 mg/kg β-asarone, X49. No apparent differences were noted between treated and control animals. Parts shown include glomerulus (G) and proximal tubule (PT).

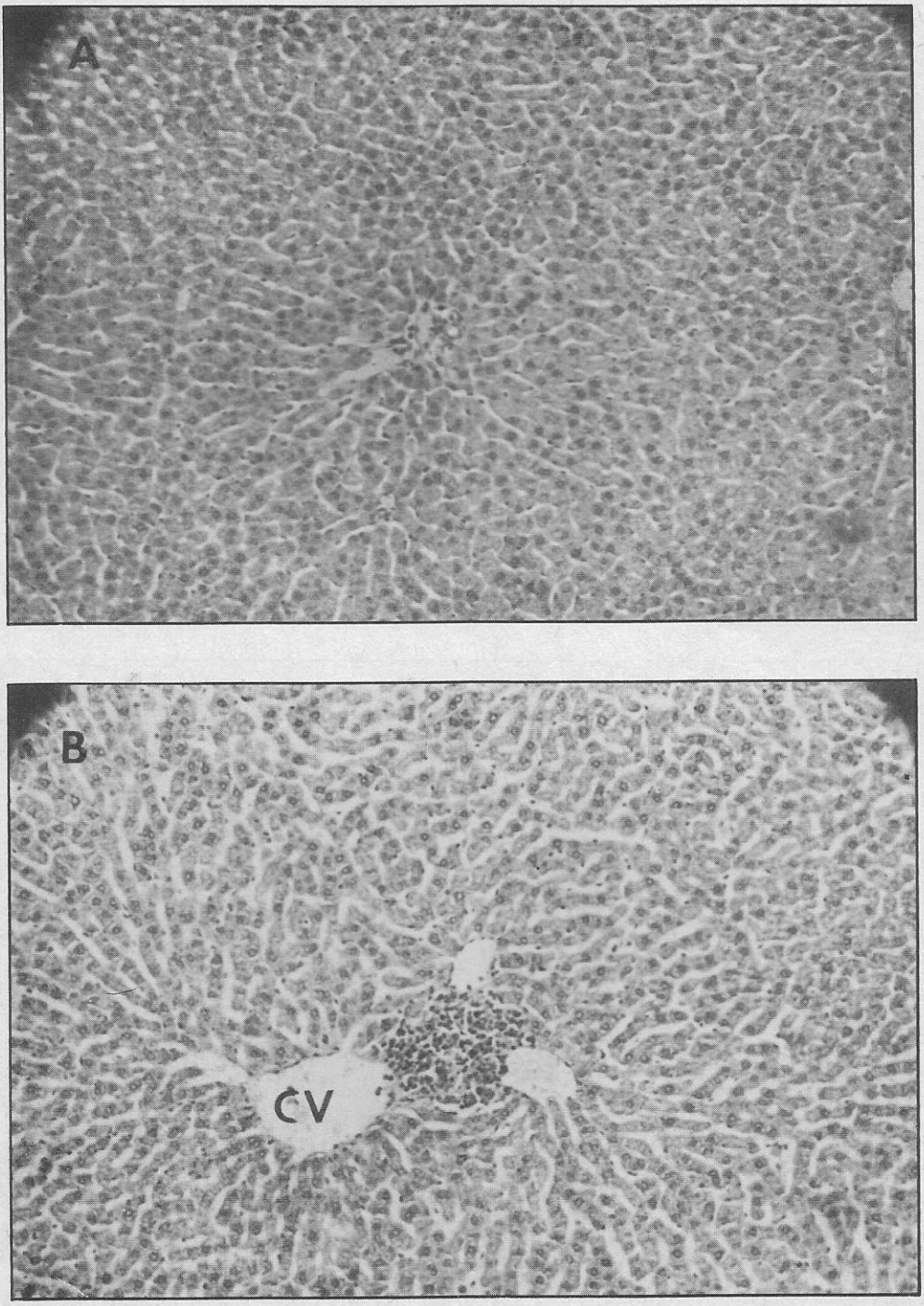

Figure 9. A. Liver from animal receiving multiple dose of corn oil, X49. B. Liver from animal receiving multiple dose of 2,4-DMPB (100 mg/kg), X49. Note the lymphocyte aggregate between central veins (CV).

trophy and increased glucocorticoid secretions has been reported in rats given dexamethasone, oxisuran, and cholera toxin (Krotkiewski and Bjorntorp 1975, Morse et al 1975, and van Dijk et al. 1975). These workers also observed thymic atrophy and destruction of lymphocytes in treated animals.

In the present study, the relation between adrenal enlargement and thymic atrophy was noted but not explored. Only β-asarone-treated rats exhibited thymic degeneration in both experiments. It is possible that thymic atrophy could be produced by undernutrition because significant weight reduction was also evident. Alternatively, since the thymus in the β-asarone treatment group revealed higher incidence of single cell degeneration (Fig. 5B), it would be interesting to determine whether the catabolic effects of prolonged administration of these two chemicals on the thymus is the result of increased glucocorticoid secretion or production. To obtain more meaningful data, the effects of growth retardation on these particular organs must first be fully identified. Moreover, experiments using adrenalectomized animals will give a better understanding of the role played by adrenal glands in thymic atrophy.

Kidney enlargement in rats exposed to calamus oil may be due to its normal compensatory response to the presence of this substance. Kidneys primarily govern the excretion of foreign chemicals and are, therefore, highly specialized in protecting themselves from the effect of xenobiotics during elimination (Stygles and Iuliucci 1981). Liver stimulation was noted in rats receiving calamus oil and β-asarone in the present study. Likewise, hepato-

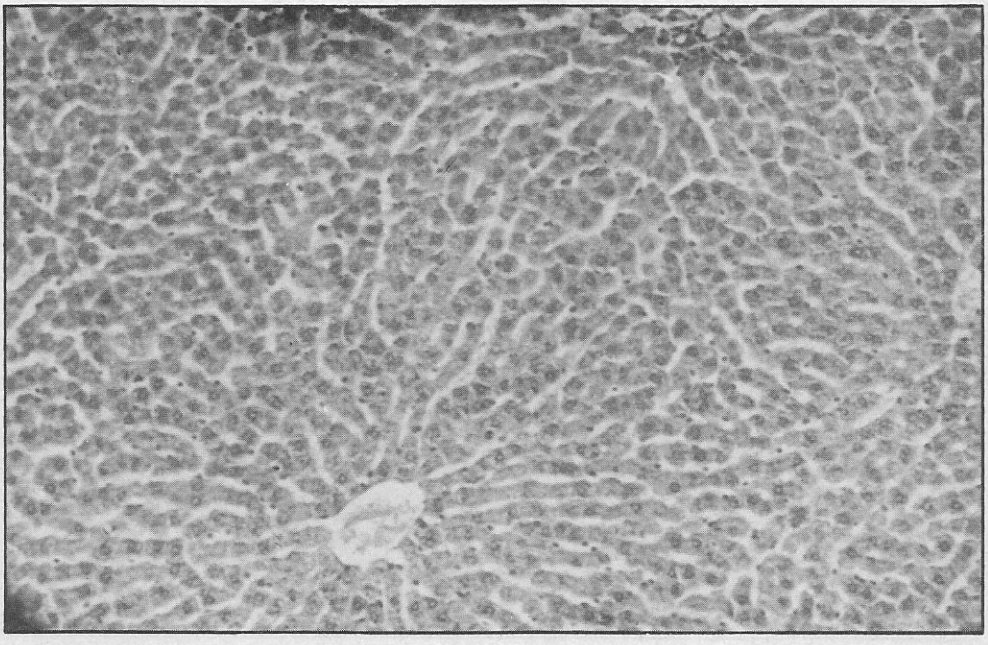

Figure 10. Liver from animal given multiple dose of 100 mg/kg European calamus oil, X49. Note the absence of any apparent morphological abnormalities in liver architecture.

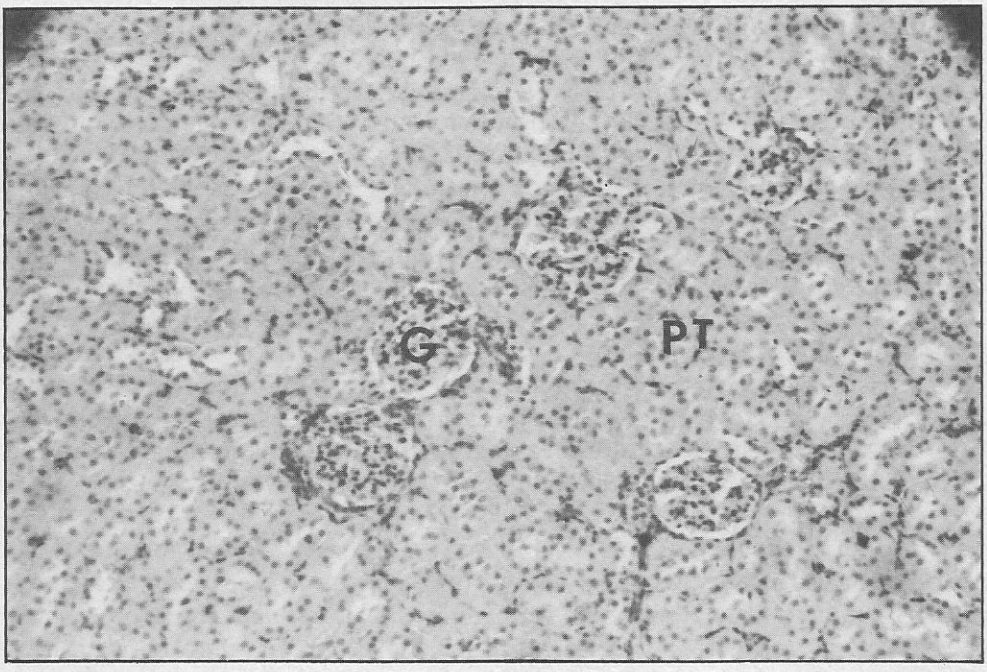

Figure 11. Kidney from animal given multiple dose of 200 mg/kg calamus oil, X49. Note the normal appearance of the glomeruli (G) and proximal tubules (PT).

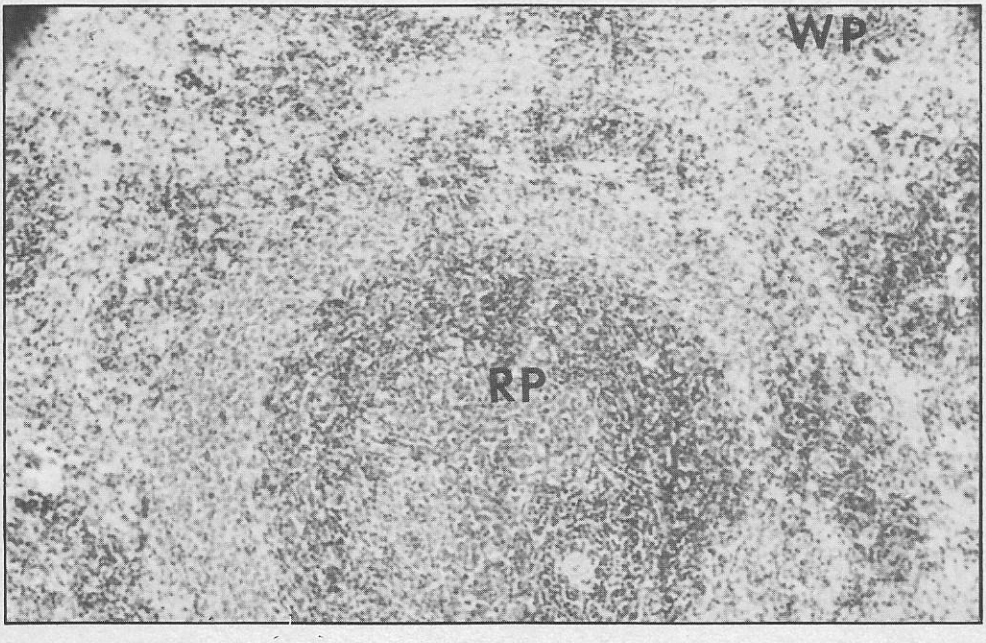

Figure 12. Spleen from animal given multiple dose of 200 mg/kg European calamus oil, X49. No overt changes are evident in both white pulp (WP) and red pulp (RP) areas of the spleen.

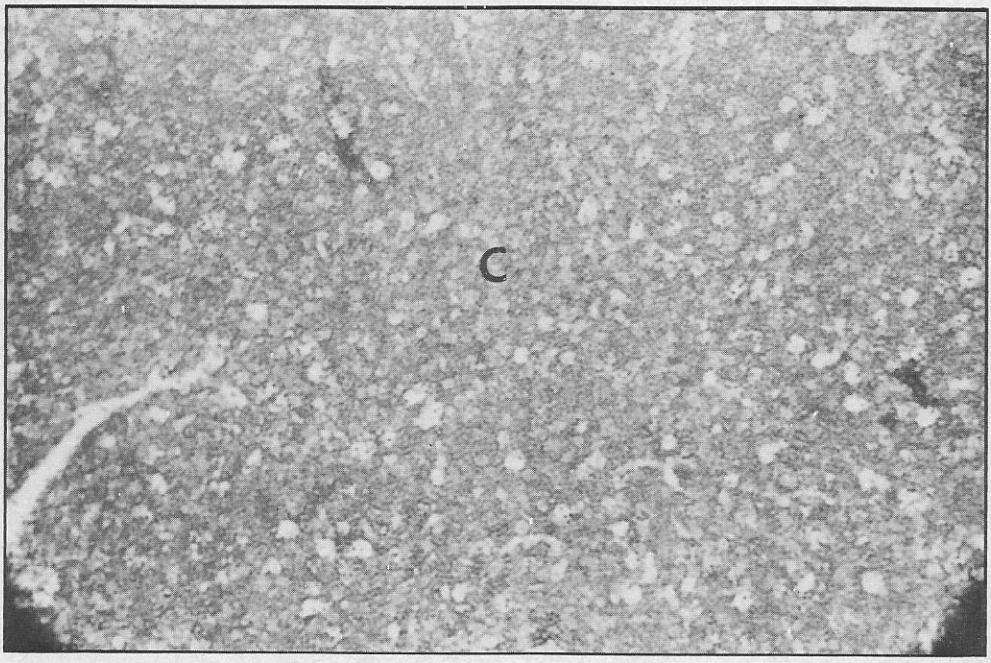

Figure 13. Thymus from animal given multiple dose of 200 mg/kg European calamus oil, X49. Note the pockmarked appearance of the cortex (C).

megaly has been demonstrated in rats given safrole (Parke and Rahman 1970), cedarwood (Wade et al 1968), terpenoids (Parke and Rahman 1969, and oil of myrtle (Uehleke and Brinkschulte-Freitas 1979). These reports tend to support the idea that in general, essential oils and fragrant materials possess liver-stimulating property. These workers also claimed that the resultant increase in liver weight was due to induction of hepatic microsomal enzymes. Because of the central role played by the liver in the metabolism of xenobiotics, the influence of the various chemicals on this organ was investigated further and will be discussed more fully in a separate section later.

Hematology

The hematocrit and hemoglobin values of the blood of rats receiving a single dose of the various propenylbenzenes are presented in Table 5. No treatment-related changes in both parameters were observed except in hemoglobin count of 2,5-DMPB-treated rats. These results are consistent with other published data which showed that neither prolonged feeding nor acute stomach intubation of calamus oil or β-asarone caused significant changes in various hematological parameters in rats (Hagan et al. 1967, Taylor et al. 1967, and Habermann 1971).

Table 5. Hematocrit and hemoglobin values of rats receiving single i.p. dose of methoxypropenylbenzenes.[1]

Dose (mg/kg)	Hematocrit (%)				Hemoglobin (g%)			
	2,4,5-TMPB	2,3-DMPB	2,4-DMPB	2,5-DMPB	2,4,5-TMPB	2,3-DMPB	2,4-DMPB	2,5-DMPB
250	45.1 ± 1.1	47.1 ± 1.5	44.0 ± 3.0	46.5 ± 1.5	11.0 ± 0.1	8.4 ± 0.6	10.2 ± 0.3	13.3 ± 0.1*[2]
100	44.2 ± 3.2	46.3 ± 1.4	46.8 ± 2.6	46.0 ± 1.6	11.2 ± 0.5	8.4 ± 0.5	10.6 ± 0.3	13.0 ± 0.1*
0	43.2 ± 1.1	45.3 ± 1.3	46.1 ± 1.8	44.7 ± 1.1	10.6 ± 0.4	7.1 ± 0.3	10.2 ± 0.4	10.8 ± 0.8

[1]Values represent the mean ± S.E. from 4 animals except the 250 mg/kg 2,4,5-TMPB (β-asarone) treatment group with 2 animals. Male Sprague-Dawley rats received single i.p. dose of the various chemicals. Animals were fasted and sacrificed 5 days after treatment. Controls received 2 ml/kg corn oil (vehicle).

[2]Significantly different from respective controls at $p = 0.014$ (Mann-Whitney U test).

Liver damage assessment

The liver is particularly vulnerable to chemical assault. Zimmerman (1978) listed several factors responsible for this: 1) its anatomical proximity to blood supply coming from the digestive tract; 2) its ability to concentrate and biotransform foreign chemicals; and 3) its role in the excretion of xenobiotics and other metabolites into the bile. As discussed in the preceding section, no dramatic histologic changes were observed in the hepatic tissues even though significant effects in liver weights were observed in rats given β-asarone, calamus oil, and 2,3-DMPB. Not suprisingly, toxicologists have long recognized the need for more sensitive indicators of chemical assault other than conventional histopathologic examination. In cases where functional and structural changes could occur independently in an organ, as suggested by Ahmad (1953), the use of function tests is indispensable. Cornish (1971) however claimed that liver function tests are not particularly sensitive due to the reserve functional capacity of this organ. In general, 20-50% impairment is necessary before abnormalities develop (Cornish 1971).

In the present study, the effects of the test chemicals on the liver were further evaluated using the activities of serum transaminases and the concentrations of microsomal protein and cytochrome P-450. The use of serum enzyme as an aid in the detection of organ damage is based on the premise that enzymes released from injured cells will get into the bloodstream where they can be measured (Cornish 1971). In particular, the assays reflect hepatic sensitivity to parenchymal injury (Zimmerman 1978).

Table 6 summarizes the results of protein and cytochrome P-450 determinations conducted with hepatic microsomal preparations obtained from the initial study. Although liver weights were significantly lower in groups given 250 mg/kg β-asarone and 100 mg/kg 2,3-DMPB, the concentrations of microsomal protein and cytochrome P-450 were not statistically different from controls. Slightly elevated levels of microsomal cytochrome P-450 were noted in rats given 100 mg/kg β-asarone. In the absence of significant hepatomegaly, enzyme induction was not strongly indicated. Moreover, this result may be influenced by the significantly low level of microsomal protein in this particular treatment group. Alternatively, this reduction in microsomal protein content suggests the possible deleterious effects produced by β-asarone on hepatic enzyme systems other than cytochrome P-450. Table IV-6 also shows significantly elevated levels of microsomal protein in rats given 2,3-DMPB at 250 mg/kg and significantly depressed cytochrome P-450 levels in rats given 2,5-DMPB at 250 mg/kg.

Data presented in Table 7 indicate that β-asarone, European calamus oil, and 2,4-DMPB do not possess liver-stimulating property and are not hepatotoxic using this particular test protocol. The activities of SGOT and SGPT were not statistically different from the control values. For some reasons, SGPT level in calamus oil-treated rats (200 mg/kg) was lower than control. Repeated administration of these 3 substances also failed to affect the levels of cytochrome P-450 and microsomal protein. Oswald et al.

Table 6. Hepatic microsomal P-450 and microsomal protein concentration in rats receiving a single dose of methoxypropenylbenzenes.[1]

Chemical	Dose (mg/kg)	Rats per treatment (n)	Liver wt (n)	Cytochrome P-450 (nmoles/mg protein)	Protein (mg/g wet liver)
2,4,5-TMPB	250	2	4.6 ± 0.24***[2]	0.81 ± 0.06	15.8 ± 1.7
(β-asarone)	100	4	4.85 ± 0.06	1.12 ± 0.08**	13.5 ± 0.8*
	0	4	5.11 ± 0.29	0.86 ± 0.05	17.1 ± 1.3
2,3-DMPB	250	4	5.07 ± 0.19	0.76 ± 0.09	14.1 ± 1.2**
	100	4	4.83 ± 0.07*	0.74 ± 0.07	12.4 ± 0.7
	0	4	5.14 ± 0.09	0.81 ± 0.02	11.0 ± 1.1
2,4-DMPB	250	4	4.77 ± 0.20	1.21 ± 0.12	15.2 ± 2.1
	100	4	4.65 ± 0.15	1.22 ± 0.05	18.3 ± 1.4
	0	4	4.63 ± 0.10	1.28 ± 0.13	16.6 ± 0.5
2,5-DMPB	250	4	5.03 ± 0.20	0.94 ± 0.06**	16.3 ± 1.6
	100	4	4.93 ± 0.24	1.28 ± 0.12	12.5 ± 1.1
	0	4	5.20 ± 0.20	1.38 ± 0.17	11.9 ± 1.4

[1] Values represent mean ± S.E. Rats were given a single i.p. dose of test compounds. Control rats received 2 ml/kg corn oil (vehicle). Animals were sacrificed 5 days after injection.

[2] Significantly different from respective controls (Mann-Whitney U test).

* $p = 0.014$ ($n_1 n_2 = 4,4$)

** $p = 0.029$ ($n_1 n_2 = 4,4$)

*** $p = 0.067$ ($n_1 n_2 = 2,4$)

Table 7. Hepatic microsomal protein, microsomal cytochrome P-450 and serum transaminase activities in rats given European calamus oil, 2,4-DMPB, and 2,4,5-TMPB for 5 consecutive days.[1]

Chemical	Dose (mg/kg)	Liver wt (g)	Protein (mg/g wet liver)	Cytochrome P-450 (nmole/mg protein)	SGOT (Karmen units/ml)	SGPT (Wroblenski-LaDue unnits/ml)
2,4,5-TMPB (β-asarone)	100	10.68 ± 0.36	18.27 ± 0.77	0.68 ± 0.03	107.69 ± 5.16	23.09 ± 0.39
2,4-DMPB	100	10.11 ± 0.14	21.03 ± 0.93	0.71 ± 0.01	110.3 ± 5.95	19.23 ± 2.38
Calamus Oil	100	10.22 ± 0.14	20.65 ± 0.88	0.74 ± 0.06	117.67 ± 8.03	21.21 ± 0.30
	200	10.69 ± 0.14*	23.86 ± 0.76	0.65 ± 0.06	104.99 ± 8.04	17.56 ± 1.77*
Control	0	9.65 ± 0.32	20.55 ± 1.16	0.84 ± 0.19	108.81 ± 8.96	23.79 ± 1.12

[1] Values represent mean ± S.E. of 4 observations. Male Sprague-Dawley rats were given 5 daily i.p. injections of the chemicals. Controls received 2 ml/kg corn oil (vehicle). Animals were fasted and sacrificed day after last injection.

*Significantly different from controls at p = .014 (Mann-Whitney U test).

(1969) have shown that the asarone isomers are rapidly metabolized in the rats. Following i.p. or oral administration of these two chemicals, maximum excretion occurred after 24-48 hours. Because of their rapid degradation, potentially hepatotoxic levels were not attained.

ACKNOWLEDGMENTS

This research was supported by the NCPC-USAID Training Grant per Crop Protection Loan No. 492-T-045. Sincere appreciation is extended to Dr. M.T. Stephen Hsia for academic guidance and additional financial assistance.

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