

ORIGINAL ARTICLE

INDUCTION OF HEPATIC DRUG-METABOLIZING  
ENZYMES BY PROPOXYPHENE

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SUMMARY

Pretreatment of mice with propoxyphene napsylate (PN, 200 mg/kg, PO, twice daily for 5 days) shortened hexobarbital sleeping time without affecting the brain barbiturate concentrations. It also produced an increase in liver weight, microsomal protein, hepatic microsomal cytochrome P-450 content and aminopyrine N-demethylase activity. These increases were prevented by simultaneous administration of actinomycin D (200 µg/kg, IP) or puromycin dihydrochloride (80 mg/kg, IP). The inductive effect was found to be dose-dependent up to 200-250 mg/kg; at higher doses the effect was less apparent. The effect reached its maximum in 3 days and then regressed to normal in about 4 days after cessation of the drug. Kinetic studies provided support for the concept that the enzymes from both normal and PN-treated livers were the same. However, this dosage schedule of PN had no effect on aniline hydroxylase activity except at a maximum inductive dose of 200 mg/kg, PO. When examined under an electron microscope, PN could cause proliferation of the smooth endoplasmic reticulum of the hepatocytes as well as a high degree of accumulation of lipid droplets. All of these findings thus suggest that the narcotic analgesic when given subacutely may not only possess several microsomal inductive properties similar to phenobarbital but also a certain degree of hepatotoxicity.

Propoxyphene is a common analgesic drug widely used throughout the world. Its use, however, is frequently associated with suicide and accidental deaths, especially when taken in conjunction with other drugs (1-3). Severe propoxyphene intoxication is associated with rapid onset

of generalized central nervous system depression, respiratory depression, cardiotoxicity and convulsions (3-5). Recently, Peterson et al (6) have found that propoxyphene when given acutely can behave as a potent inhibitor of hepatic microsomal mixed-function oxidases, acting in a manner similar to SKF 525-A. This finding thus provides evidence that deaths related to propoxyphene overdose could be a consequence of pharmacokinetic drug interactions in which propoxyphene or its metabolites, by inhibiting the metabolism of other drugs, may produce more profound toxic effects than those due to the sum of the pharmacodynamic effects of the ingested drugs. Furthermore, these investigators (6) have also reported that chronic administration of propoxyphene could increase the rate of its own metabolism as well as that of aminopyrine and aniline, thus suggesting the drug as an enzyme inducer.

In this communication, the inductive effects of propoxyphene on mouse microsomal drug-metabolizing enzymes and liver cell morphology are described. Evidence is presented to suggest that this analgesic when given subacutely possesses a number of microsomal inductive properties similar to phenobarbital as well as a certain degree of hepatotoxicity.

#### MATERIALS AND METHODS

##### Experimental Animals

Adult male New Zealand mice of about 60 days of age and weighing 30-35 g were used in the study. The animals were supplied by the Animal Center of the Faculty of Science, Mahidol University. All animals were allowed to access to food and tap water ad libitum until 24 hours before sacrifice, during which time they were allowed access to water only.

Propoxyphene napsylate (PN) was freshly prepared everyday, before use, as a 10 mg/ml solution in distilled water. Mice were given

the drug PO at the doses indicated, twice daily at 8-9 A.M. and 4-5 P.M. for 5 consecutive days except where specifically noted. Approximate volumes of the vehicles (distilled water) were administered to the control animals. The animals were killed 24 hours after the last dose of PN.

### Studies with Inhibitors of Protein Biosynthesis

#### 1. Actinomycin D

Actinomycin D, an inhibitor of m-RNA synthesis, was administered intraperitoneally to male mice at the dose of 200  $\mu$ g/kg (once daily at 4 P.M. for three days) 30 minutes prior to the administration of propoxyphene napsylate (200 mg/kg, twice daily for 5 days). Actinomycin D (20  $\mu$ g/ml) was freshly prepared in 5 % ethanol just prior to use. It was found that this 5 % ethanol employed had no effect on aminopyrine N-demethylase activity. The animals were sacrificed by abdominal aorta drainage under light ether anesthesia 24 hours after the last dose of propoxyphene napsylate.

#### 2. Puromycin dihydrochloride

Puromycin dihydrochloride (80 mg/kg, IP, once daily for 5 days) was given at 30 minutes before propoxyphene napsylate. The animals were then intubated with 200 mg/kg propoxyphene napsylate, twice daily for 5 days as before. The inhibitor of protein biosynthesis was freshly prepared in distilled water (10 mg/ml) just before use. Control animals received an equal volume of the vehicle. The animals were killed as described above.

### Sleeping Time

The sleeping time is considered as the time interval between the loss and the regain of righting reflex (7). In this study, hexobarbital sleeping time was determined by an intraperitoneal administration of 120 mg/kg hexobarbital sodium to the control and PN-treated

groups. The animals were pretreated orally with 200 mg/kg propoxyphene napsylate, twice daily for 5 days. Hexobarbital sodium solution (10 mg/ml) was prepared in distilled water just before use.

#### Determination of Brain Hexobarbital

The awakening brain hexobarbital levels were determined from animals killed by decapitation by the method of Vesell (8) which is a modification of that of Cooper and Brodie (9).

#### Enzyme Assays

All enzyme assays were done according to the method described previously (10,11); see Unchern and Thithapandha (20). Microsomal protein was determined by the method of Lowry et al (12), with bovine serum albumin as the standard. Cytochrome P-450 in the microsomal pellet was estimated by the method of Omura and Sato (13).

#### Electron Microscopic Study

Mice were fasted overnight and sacrificed by ether anesthesia in the following morning. A small strip of liver was cut off by a sharp scissor and immediately transferred into the fixative (2% glutaraldehyde in Millonig buffer, pH 7.4). The strip was divided by a razor into small pieces of about 1 cubic mm each and placed in a vial containing about 3 ml of the cold fixative for 1 hour; then they were postfixed in cold 1%  $\text{OsO}_4$  in 0.1 M phosphate buffer, pH 7.4 for another hour. Subsequently, the tissue blocks were dehydrated in graded series of ethanol and infiltrated with propylene oxide plastic mixture of 1:2 overnight. The tissues were embedded in Epon 812 poured into a polyethylene capsule and polymerized at 60°C for 48 hours. The polymerized block were trimmed and thin sections were cut by a Sorvall "Potter-Blum" MT-1 ultramicrotome fitted with a glass knife. The sections (1-2  $\mu$ ) were mounted on carbon coated copper grids and dried at room temperature. They were

stained with uranyl acetate and counter-stained with lead citrate. The ultra structures were examined and the micrographs were obtained by using a Hitachi electron microscope, type HS-8, operated at 50 KV.

#### Statistical Analysis

All statistical analyses were performed according to the multiple comparison technique as described by Newman and Keuls' test (14), with the level of significance  $P < 0.05$ . Regression analysis was used in drawing lines in Lineweaver-Burk plots and determinations of kinetic constants.

### RESULTS

#### Effects on Hepatic Drug-Metabolizing Enzymes

The experiment described in Table 1 indicates that the interaction between propoxyphene napsylate (PN) and hexobarbital is pharmacokinetic in nature. Mice pretreated with PN (200 mg/kg, PO, twice daily for 5 days) were found to display statistically shorter hexobarbital sleeping time than that of the control group. The brain barbiturate levels on awakening of these two groups were not different (Table 1). These findings thus suggested that the shortening effect of PN on hexobarbital sleeping time was possibly due to an enzyme induction.

As shown in Table 2, the oral administration of 100, 200 and 300 mg/kg PN, twice daily for 5 consecutive days, to adult male mice produced a significant increase in liver weight, microsomal protein, cytochrome P-450 content, aminopyrine N-demethylase and aniline hydroxylase activities. Indeed, the increase in enzymatic activities produced by PN was maximal at the dose of 200 mg/kg, PO, twice daily for 5 days (Table 2, Fig. 1), and this dosage schedule was chosen for studies in subsequent experiments. At the higher doses (250 and 300 mg/kg), PN produced a somewhat decreased response and its stimulatory effect on

Table 1 Hexobarbital Sleeping Time and Awakening Brain Levels  
in Control and Propoxyphene Napsylate-Pretreated Mice

Treatment	Sleeping Time (min)	Awakening Brain Level ( $\mu$ g/g)
Control	48.03 $\pm$ 2.01 (13)	38.71 $\pm$ 1.82 (5)
Propoxyphene napsylate	17.07 $\pm$ 1.22 (13) *	39.20 $\pm$ 2.41 (5)

Male mice weighing 30-35 g were treated orally with 200 mg/kg propoxyphene napsylate, twice daily for 5 days. Control animals were given an equal volume of distilled water. Twenty hours after the last dose, 120 mg/kg hexobarbital sodium was given intraperitoneally; both the sleeping time and awakening hexobarbital brain levels were then recorded. Each value represents mean  $\pm$  S.E. of animals as indicated in parentheses. \* $P < 0.05$  (from control)

aminopyrine N-demethylase disappeared at doses higher than 350 mg/kg (Fig. 1). As shown in Table 3, it was found that prior intraperitoneal administration of actinomycin D or puromycin completely abolished the PN-stimulated increase in aminopyrine N-demethylase activity, thus suggesting PN as an enzyme inducer.

#### Characterization of the Induced Aminopyrine N-Demethylase

The activity of aminopyrine N-demethylase from PN-treated mice at various time intervals during and after the treatment is shown in Fig. 2. The inductive effect of PN was apparent even at 1 day of pretreatment, and maximum induction (180 % of control) was attained with the 3-day pretreatment schedule (Fig. 2). At 2 days after the 5-day pretreatment, the significant increase in enzyme activity was still evident (120 % of control). It was found that the increased enzyme activity gradually declined to normal during the 2-4 days after cessation of the 5-day PN treatment.

**Table 2** Effects of Propoxyphene Napsylate Treatment on Various Parameters of Drug Metabolizing Enzyme Systems

Propoxyphene Napsylate (PO, twice daily for 5 days)	Liver Weight (% Body Weight)	Microsomal Protein	Aminopyrine N-Demethylase Activity <sup>a</sup>	Aniline Hydroxylase Activity <sup>b</sup>	Cytochrome P-450
mg/kg	%	mg/g liver			nmole/mg protein
Control	4.87 ± 0.03	21.50 ± 1.21	197.41 ± 13.64	41.91 ± 3.95	0.410 ± 0.045
100	5.70 ± 0.06 *	27.60 ± 1.29 *	218.66 ± 11.28	43.72 ± 1.77	0.384 ± 0.075
200	5.60 ± 0.06 *	27.70 ± 1.64 *	270.29 ± 9.54 *	51.36 ± 2.45 *	0.807 ± 0.061 *
300	6.30 ± 0.06 *	28.90 ± 2.09 *	254.93 ± 21.60 *	46.69 ± 4.24	1.009 ± 0.075 *

Values are expressed as mean +SE. from 4 determinations. a= nmole formaldehyde formed/mg protein/30 min; b= nmole p-aminophenol formed/mg protein/20 min. \* p < 0.05 (from control)

**Table 3** Effects of Inhibitors of Protein Biosynthesis on the Propoxyphene-Stimulated Increase in Aminopyrine N-Demethylase Activity

Treatment	Aminopyrine N-Demethylase (nmoles HCHO formed/mg protein/30min)
Control	209.63 ± 10.20
Propoxyphene napsylate (PN)	287.38 ± 14.55 *
Propoxyphene napsylate + Actinomycin D	218.36 ± 20.43
Propoxyphene napsylate + Puromycin dihydrochloride	171.79 ± 4.85

Male mice weighing 30-35 g were treated intraperitoneally with 200 µg/kg actinomycin D or 80 mg/kg puromycin dihydrochloride, once daily for 5 days at 30 minutes prior to the administration of PN (200 mg/kg, twice daily for 5 days). Twenty hours after the last dose of PN, the animals were sacrificed for the determination of aminopyrine N-demethylase activity. Each value represents mean + S.E. from 4 separate determinations. \* P < 0.05 (from control)

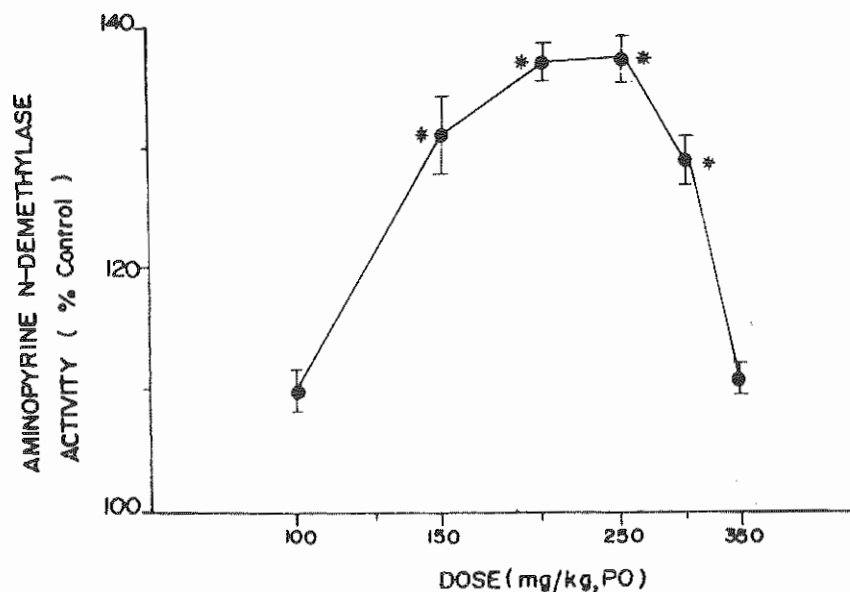


Fig.1 Dose-response increase in aminopyrine N-demethylase activity produced by PN in mice. The animals were treated with PN at the doses indicated, twice daily for 5 days. Each point represents mean  $\pm$  S.E. from 4 separate determinations. The asterisk indicates a significant difference ( $P < 0.05$ ) from control.

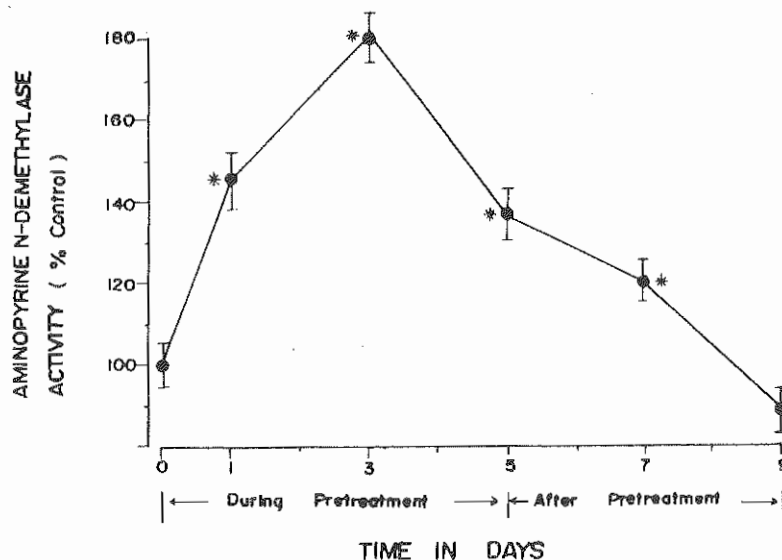


Fig.2 Time-course of induction of hepatic aminopyrine N-demethylase activity in male mice. The animals were treated with PN (200 mg/kg, PO, twice daily) for 1, 3 and 5 days. They were sacrificed 24 hours after the last dose, and at 2 and 4 days after the last dose of the 5-day pretreatment schedule. Each point is mean  $\pm$  S.E. from 4 separate determinations.



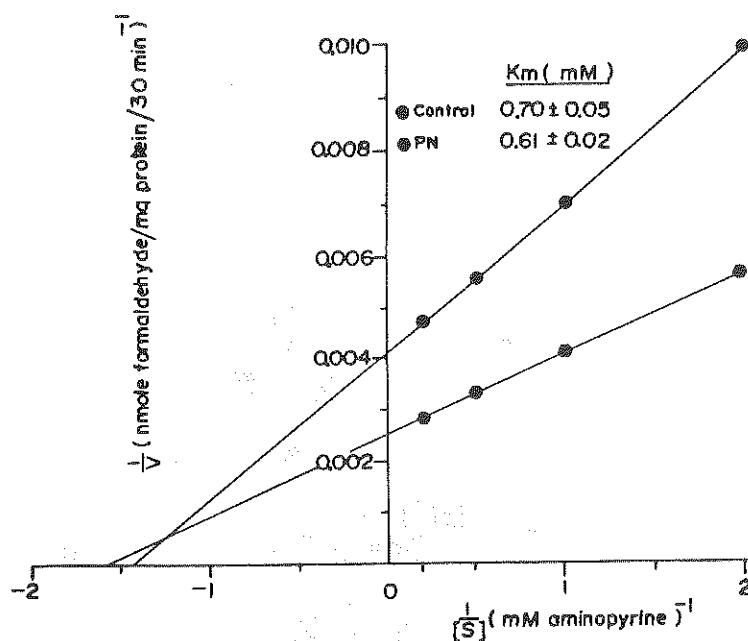


Fig. 3 Lineweaver-Burk plot of aminopyrine N-demethylase in hepatic microsomes from control and propoxyphene napsylate-treated mice.

Each point is mean  $\pm$  S.E. from 3 separate determinations in a single microsomal pool obtained from 4 control and 4 PN-treated animals (200 mg/kg, PO, twice daily for 5 days)

The Lineweaver-Burk plots for the activities of aminopyrine N-demethylase from livers of control and PN-treated mice are shown in Fig. 3. It can be seen that the apparent  $K_m$  values obtained for the enzymes from both sources are almost the same. It must be pointed out here that, although aminopyrine N-demethylase is well known to have biexponential kinetics in both normal and control animals, our kinetic studies as shown in Fig. 3 reveal only monoexponential kinetics for the enzyme. This is simply due to the fact that a low range of substrate concentrations (0.5-4 mM) were used in our experiments. Biexponential behavior of this enzyme is seen only when a wider and higher range of aminopyrine concentrations is used (15).

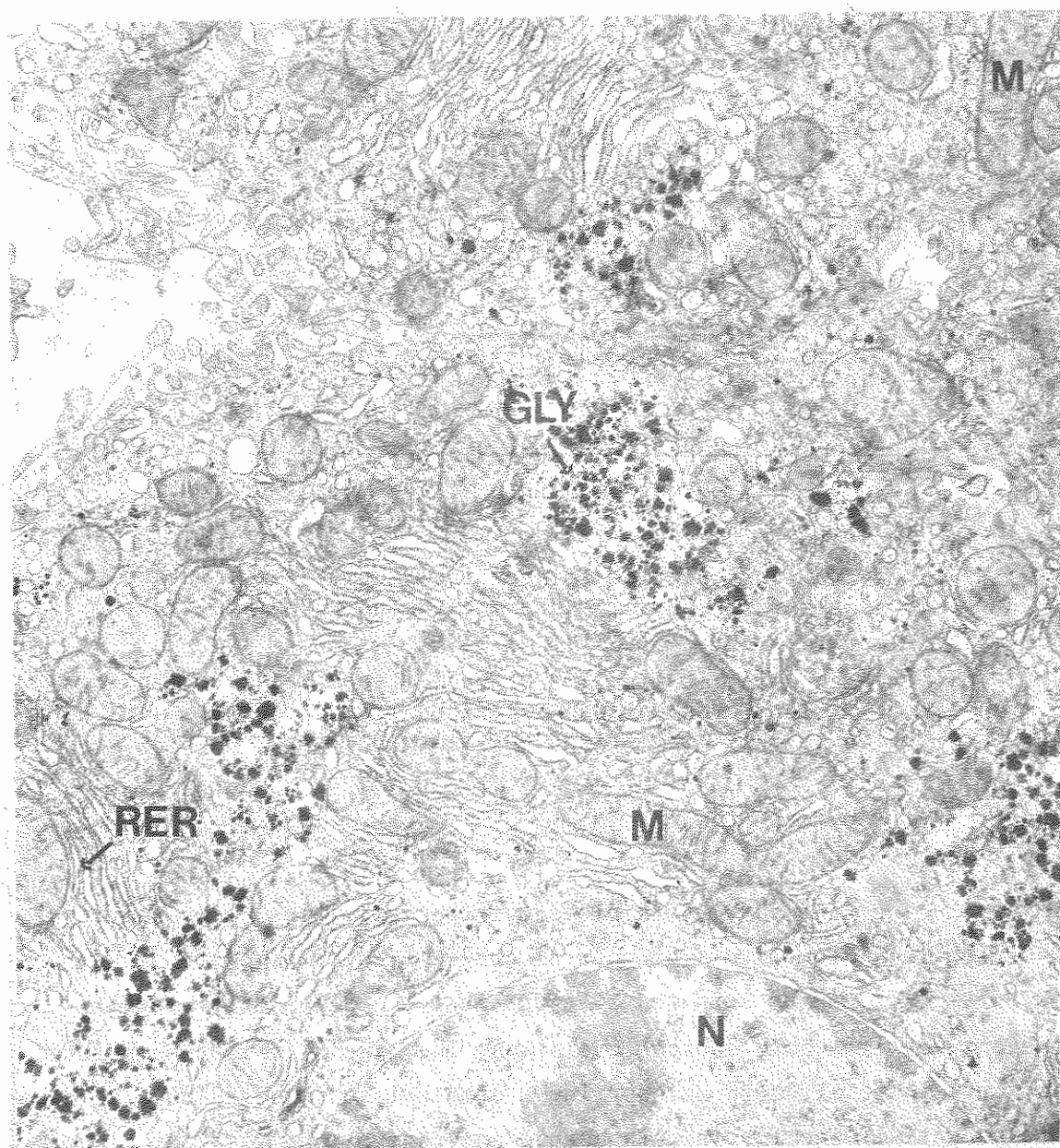


Fig. 4 Electron micrograph of a normal mouse hepatocyte. Note the parallel stacks of endoplasmic reticulum (RER) which are commonly seen in most cells. All organelles show regular appearance. N, nucleus; M, mitochondria; GLY, glycogen; RER, rough endoplasmic reticulum (x 6,100).

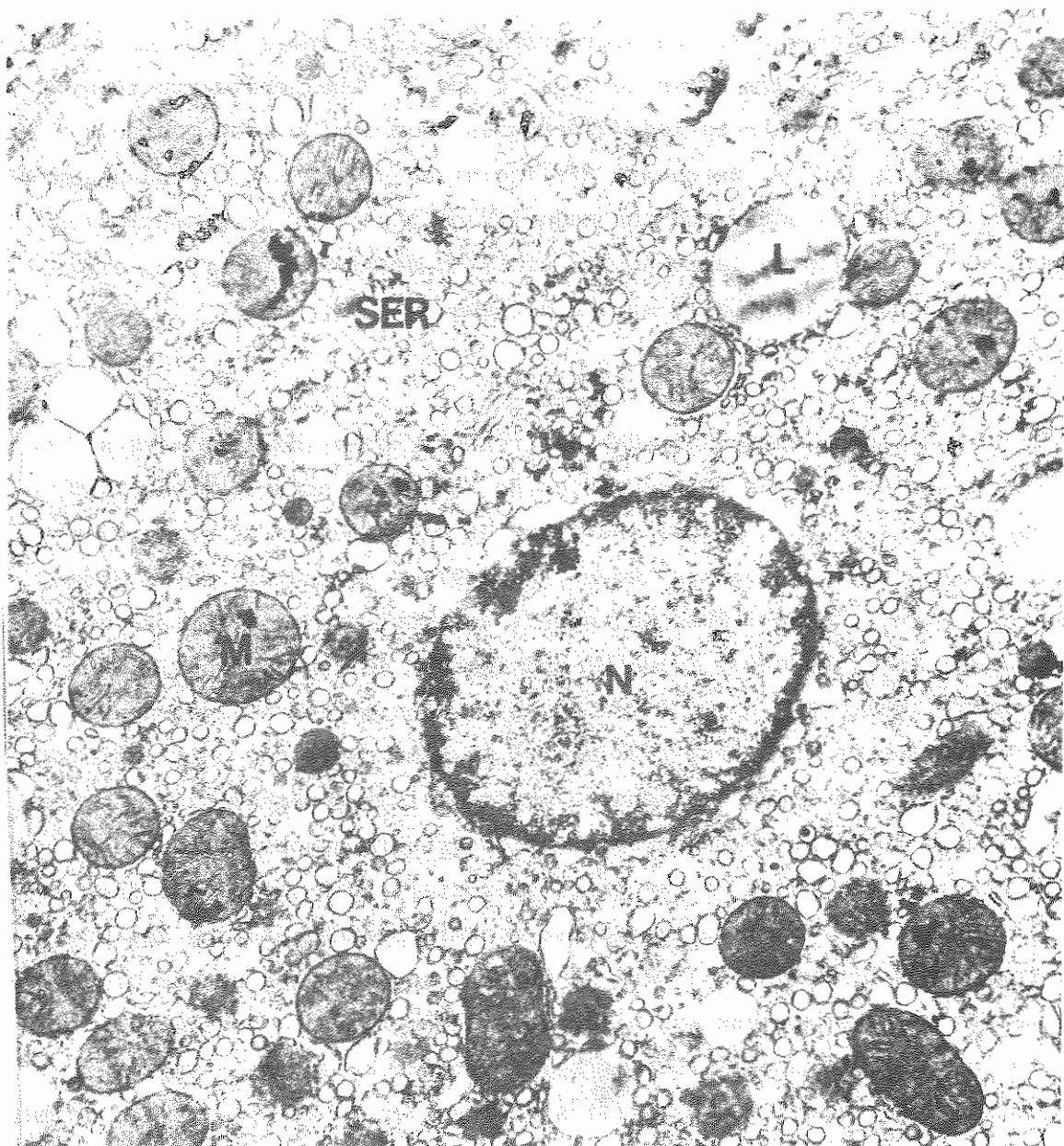


Fig. 5 Electron micrograph of the liver of mouse treated with propoxyphene napsylate. Note the extensive proliferation of the smooth endoplasmic reticulum (SER) and the presence of several lipid droplets (L) of different sizes throughout the entire cytoplasm (x 6,100).

### Ultrastructural Changes in Liver Cells

Representative electron micrographs of liver cells from control and PN-treated mice are shown in Figs. 4 and 5. The hepatocyte from PN-treated animals reveals marked differences from that of the controls. As can be seen in Fig. 5, extensive proliferation of the smooth endoplasmic reticulum (SER) is noted and the majority of the liver cell population contains a moderate to large number of fatty droplets of varying sizes, some of which are about half of the nucleus.

### DISCUSSION

Propoxyphene napsylate (PN) has recently been shown to have a biphasic action similar to the prototype inhibitor of drug metabolism, SKF 525-A, acting as a potent inhibitor of microsomal drug metabolism when given acutely and as an inducer when given subacutely (6). When used in vitro, PN was found to inhibit aminopyrine N-demethylase competitively ( $K_i$ ,  $4.6 \pm 0.9 \times 10^{-5} M$ ) and inhibit aniline hydroxylase noncompetitively ( $K_i$ ,  $6.1 \pm 1.2 \times 10^{-4} M$ ). When given in vivo, an IP administration of this drug (100 mg/kg) to mice could not only inhibit the activities of those two hepatic microsomal drug-metabolizing enzymes but also prolong the pharmacologic action of both hexobarbital and zoxazolamine (6). When PN was given subacutely (300 mg/kg, PO, once daily, for 3 days), the oxidative metabolism of aminopyrine, aniline and propoxyphene was enhanced (6). In the present studies, the nature of enzyme induction by propoxyphene was elaborated and more fully described. As shown in Table 1, when PN (200 mg/kg, PO, twice daily for 5 days) was administered to mice, hexobarbital sleeping time in the pretreated animals was significantly shortened than that of control while the brain barbiturate levels in both groups were essentially the same. Since the duration of action of hexobarbital has been shown to correlate well with its rate of metabolism (10), the shortening effect of PN on hexobarbital sleeping time was most likely due to an enzyme induction. This was further supported by the results of experiments shown in Table 2, in which this

dosage regimen of PN was found to increase liver weight, microsomal protein, cytochrome P-450 content and the activities of aminopyrine-N-demethylase and aniline hydroxylase. Indeed, the dose-response of hepatic drug-metabolizing enzymes due to PN exhibits certain characteristics (Fig. 1). At low doses the drug could stimulate liver growth and produce an increase in microsomal protein without any effect on aminopyrine N-demethylase, aniline hydroxylase or cytochrome P-450 content (Table 2). At 150 mg/kg, PN pretreatment resulted in a 130 % elevation of aminopyrine N-demethylase activity and the effect reaches its maximum at the doses of 200-250 mg/kg (140 % of control). At higher doses (300 mg/kg or more), PN further increased hepatic cytochrome P-450 content but had no effect on aniline hydroxylase activity and could not produce higher stimulatory effect on aminopyrine N-demethylase than that produced by the 200 mg/kg dosage (Fig. 1 and Table 2). The effect of high doses of PN given subacutely on the activities of these two drug-metabolizing enzymes has not been investigated but could very well be due to a direct inhibitory effect of the accumulated drug or its metabolites on the enzyme preparations from the pretreated livers. In all experiments for enzymatic determinations reported herein, enzyme preparations were obtained from livers removed from mice killed at 24 hours after the last dose of PN pretreatment. Thus, at lower doses of the drug little or no parent drug as well as its metabolites would be expected in the enzyme preparations. However, at higher doses, there is a good possibility that enough concentration of PN and/or its metabolites might be present in the preparations to exert a significant inhibitory effect on the two enzymes. That PN is a potent inhibitor of drug metabolism and it can accumulate in appreciable amount are well documented (6, 16-18). Further, Peterson and Lehman (19) have recently shown that the N-demethylated metabolite of PN (norpropoxyphene) was a weak inhibitor for aminopyrine N-demethylase but a potent one for aniline hydroxylase.

The stimulatory effect of PN on hepatic drug-metabolizing enzymes is most likely due to enzyme induction, inasmuch as its effect was completely blocked by prior or simultaneous administration of actinomycin D and puromycin dihydrochloride (Table 3).

The possibility that alteration of the sex hormonal status of the animals, as a result of PN pretreatment, might be responsible for increased aminopyrine N-demethylase activity remains to be investigated. However, enzyme induction by cyproheptadine hydrochloride in rats was found without any change in the circulating testosterone level(20).

The inductive property of PN as considered from aminopyrine N-demethylase activity shows some similarity to that of phenobarbital. The time required for maximal induction by the barbiturate was 2-3 days and the effect returned to normal level within 4-5 days after discontinuation of the drug (21). In the case of PN, the peak induction was reached in 3 days and the effect lasted for about 4 days after withdrawal of the drug (Fig. 2).

The kinetic studies on aminopyrine N-demethylase from normal and PN-pretreated rats lend support to the concept that the enhanced activity produced by the drug is due to a quantitative increase in the content of the pre-existing enzyme. The apparent  $V_{max}$  of the enzyme was significantly increased while the  $K_m$  was not affected (Fig. 3).

Like phenobarbital, PN can also cause proliferation of the smooth endoplasmic reticulum in the mouse liver (Fig. 5). This effect was discerned with any dose of PN greater than 150 mg/kg, PO, twice daily for 5 days. Moreover, the ultrastructural changes observed in PN-treated liver cells are of interest; accumulation of lipid droplets of varying sizes is seen in almost every cell. This finding is similar to that observed with cyproheptadine hydrochloride (20) and might be responsible for its toxicity on hepatic cells, especially when the liver has already been exposed to other hepatotoxins or other toxic chemicals such as  $CCl_4$ . Whether these ultrastructural changes observed are due to the direct

action of propoxyphene napsylate or its metabolites on the liver cells, or are a normal reaction to some altered hormonal status induced by the drug, awaits future investigation.

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