parent lack of chromosomal patterns noticeable in most classes of tumors. Just as the present RSV and DMBA tumors are indistinguishable histologically but readily distinguishable chromosomally, other tumors, histologically well-defined, may be composed of many etiologically and chromosomally diverse entities; this mixture of karyotypes would obscure the recognition of specific chromosomal patterns.

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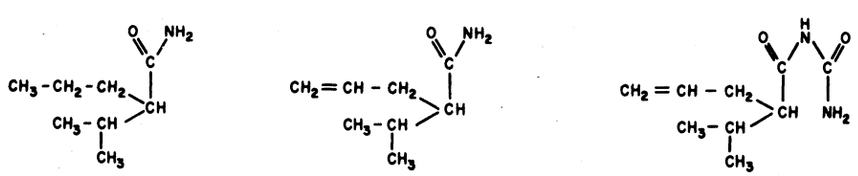
Destruction of Cytochrome P_{450} by Secobarbital and Other Barbiturates Containing Allyl Groups

Abstract. *Administration of certain commonly used barbiturates containing allyl groups, such as secobarbital, allobarbital, or aprobarbital to rats treated chronically with a microsomal enzyme inducer causes a rapid destruction of the liver microsomal hemoprotein that serves as the terminal oxidase for drug metabolism. In contrast, barbiturates without an allyl group do not have this effect. The decrease in this hemoprotein, cytochrome P_{450}, by the barbiturates containing an allyl group could also be demonstrated in an in vitro liver microsomal system requiring reduced nicotinamide adenine dinucleotide phosphate. These results suggest that the barbiturates containing an allyl group are converted to a metabolite that leads to the destruction of cytochrome P_{450}.*

Several groups of structurally unrelated compounds produce, in rodents, an experimental porphyria that resembles hepatic porphyria in humans (*1*, *2*). These compounds stimulate the formation of porphyrins in liver cells in vivo and in vitro (*1*, *3*), presumably as a result of the increased synthesis of δ-aminolevulinic acid (δ-ALA) synthetase, the rate-limiting enzyme in porphyrin and heme biosynthesis (*1*, *4*). Although the exact mechanism of the chemically induced increase in δ-ALA synthetase is not known, it may result from an interference by these compounds with the feedback control exercised by heme on the enzyme (*1*). One of these compounds, allylisopropylacetamide (AIA), a barbiturate-related derivative, causes an initial rapid decrease in the concentration of cytochrome P_{450}, while exerting little or no effect on other enzymes associated with liver microsomes (*5*, *6*). This decrease is due to the destruction of cytochrome P_{450} heme with the resultant accumulation of the heme breakdown products in the liver (*5*, *6*). These products, which impart a green-brown color to liver microsomes, may be nonphysiological isomers of biliverdin (*7*). Preliminary evidence indicates that a metabolite of AIA is required for this breakdown of cytochrome P_{450} heme (*5*). Cytochrome P_{450} is the terminal oxidase in the metabolism of a wide variety of substrates such as insecticides, steroids, drugs, and chemical carcinogens (*8*). Treatment of rats with various compounds that induce liver microsomal cytochrome P_{450} results in an increase in the rate of breakdown of cytochrome P_{450} by AIA (*5*). Kaufman *et al.* (*9*) have demonstrated an increased in vivo metabolism of AIA following treatment of rats with phenobarbital (PB), an inducer of microsomal enzymes. Because epoxides are intermediates in the oxidative metabolism of certain olefins and aromatic compounds (*10*), we initiated studies to determine if the allyl group of AIA, also capable of oxidation to an epoxide, was required for this breakdown of cytochrome P_{450} heme. We therefore studied the effect of a number of barbiturates and related compounds containing allyl and alkyl groups on liver microsomal cytochrome P_{450}.

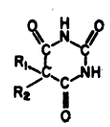
Adult male Long-Evans rats (170 to 180 g) had free access to a commercial diet and water. Sodium phenobarbital dissolved in 0.9 percent NaCl was administered intraperitoneally, at a daily dose of 75 mg per kilogram of body weight for 3 days, to stimulate the synthesis of liver microsomal hydroxylase (*8*). On the fourth day, the test compounds were administered subcutaneously 1 hour before the animals were killed. Liver microsomes were prepared in 0.25*M* sucrose, suspended in 1.15 percent KCl, and centrifuged. The final microsomal pellets were layered with 3 ml of 0.1*M* potassium phosphate buffer (*p*H 7.4), and stored, frozen, for 1 to 5 days before being used. For in vitro metabolism studies, the incubation mixture, in a total volume of 7.5 ml, consisted of microsomes equivalent



Isopropylvaleramide

Allylisopropylacetamide

Allylisopropylacetylcarbamide



Barbiturate	R_1	R_2
Aprobarbital	allyl	isopropyl
Probarbital	ethyl	isopropyl
Secobarbital	allyl	1-methylbutyl
Pentobarbital	ethyl	1-methylbutyl
Allobarbital	allyl	allyl
Barbital	ethyl	ethyl

Fig. 1. Structures of several barbiturates and barbiturate-related compounds.

to that in 500 mg of liver (wet weight), 100 m*M* potassium phosphate buffer (*p*H 7.4), 3 m*M* $MgCl_2$, and a system consisting of 5 units of glucose-6-phosphate dehydrogenase, 0.1 m*M* nicotinamide adenine dinucleotide phosphate (NADP), and 1.5 m*M* glucose-6-phosphate to generate reduced NADP (NADPH). The NADPH-generating system could be replaced by 1 m*M* NADPH. The concentration of cytochrome P_{450} was determined by the method of Omura and Sato (*11*), with an extinction coefficient of 91 cm^{-1} mM^{-1} for the change in optical density between 450 and 490 nm. The concentration of protein was determined by the method of Sutherland *et al.* (*12*). We used the Student's *t*-test as a test of the null hypothesis, with $P \leqq .05$.

The structures of the barbiturates and barbiturate-related derivatives, which we used in this study, are shown in Fig. 1. The effect of secobarbital and related compounds on cytochrome P_{450} concentration in rat liver microsomes is presented in Table 1. The results demonstrate that the rapid fall in concentration of cytochrome P_{450} is caused by certain barbiturates containing allyl groups, and that the ethyl derivatives of these compounds are without effect on cytochrome P_{450}. The decrease in cytochrome P_{450} caused by secobarbital was shown to be enhanced when rats were treated with PB. Administration of secobarbital (100 mg/kg) to control rats resulted in only a 20 percent decrease in cytochrome P_{450} in 1 hour, whereas administration of this dose of secobarbital to PB-treated rats resulted in a 40 percent decrease (Table 1). The structural analog of AIA, isopropylvaleramide, had no effect on the breakdown of cytochrome P_{450}. Previous studies demonstrated that the breakdown products of cytochrome P_{450} heme following AIA administration accumulated in the liver, imparting a green-brown color to the microsomes (*5, 6*). This characteristic color change was also observed in animals treated with the barbiturates containing allyl groups, an indication of a similar mechanism of heme destruction.

Further evidence for the requirement of an active metabolite of certain barbiturates containing allyl groups for the destruction of cytochrome P_{450} heme was obtained from in vitro studies. Rats were treated with PB for 3 days, and liver microsomes were prepared on the fourth day. The microsomes were incubated in vitro in the presence of an NADPH-generating system and the appropriate substrate. At the end of the incubation, menadione was added at a final concentration of $10^{-4}M$ (in 0.1 ml of methanol) to stop the reaction, and to prevent the decrease in cytochrome P_{450} which continued (at a slower rate) when the samples were placed on ice after the previous incubation. The effect of 0.2 m*M* secobarbital on the breakdown of microsomal cytochrome P_{450} in vitro is shown in Fig. 2. Early studies revealed that 0.2 m*M* secobarbital caused a maximum decrease in cytochrome P_{450}, requiring NADPH or an NADPH-generating system. Some loss of cytochrome P_{450} was observed with time if microsomes were incubated with NADPH or an NADPH-generating system in the absence of secobarbital.

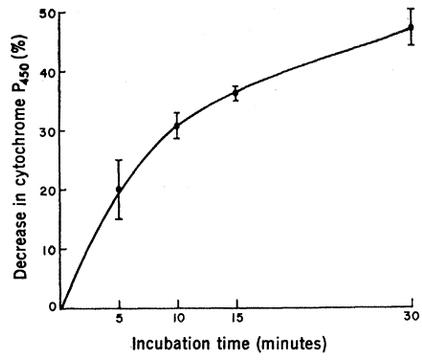


Fig. 2. Effect of secobarbital on cytochrome P_{450} in vitro.

Table 1. Effect of various barbiturates and barbiturate-related derivatives on cytochrome P_{450}.

Treatment	Dose (mg/kg)	Cytochrome P_{450} (nmole per mg of protein)
	Experiment 1	
None		2.57 ± 0.16
Secobarbital	25	2.23 ± 0.04
Secobarbital	50	1.44 ± 0.03*
Secobarbital	100	1.54 ± 0.16*
Secobarbital	150	1.25 ± 0.11*
Pentobarbital	100	2.37 ± 0.25
Allobarbital	100	1.32 ± 0.14*
Barbital	100	2.42 ± 0.10
	Experiment 2	
None		2.10 ± 0.15
Aprobarbital	50	1.58 ± 0.09*
	100	1.06 ± 0.01*
Probarbital	50	2.07 ± 0.14
	100	1.90 ± 0.07
	Experiment 3	
None		2.25 ± 0.15
AIA	50	1.72 ± 0.08*
	100	1.24 ± 0.14*
	200	1.01 ± 0.03*
Isopropyl-valeramide	50	1.92 ± 0.17
	100	2.39 ± 0.16
	200	2.16 ± 0.17

* Statistically different from controls.

This decrease due to NADPH (7 percent in 5 minutes, 20 percent in 30 minutes) occurred in addition to the decrease caused by secobarbital. The addition of 1 m*M* $MnCl_2$, 1 m*M* $CoCl_2$, or 1 m*M* EDTA to the incubation system completely blocked the NADPH-mediated decrease of cytochrome P_{450}, but did not alter the effect of secobarbital. These compounds are known inhibitors of microsomal lipid peroxidation (*13*), and cause little or no decrease in microsomal drug metabolism (*14*). Therefore, the decrease in cytochrome P_{450} by NADPH alone is probably related to lipid peroxidation, while the breakdown caused by secobarbital is independent of it. All compounds shown in Fig. 1 were tested for their ability to cause a breakdown of cytochrome P_{450} heme in vitro. The results obtained were identical to those reported in Table 1; that is, only the barbiturates and barbiturate-related derivatives, which contained allyl groups, including allylisopropylacetylcarbamide, caused a significant loss of cytochrome P_{450} in vitro, and, in all cases, a requirement for NADPH was demonstrated. The green-brown color that was imparted to microsomes when barbiturates and barbiturate-related derivatives containing allyl groups were administered in vivo also was observed in the in vitro system, a suggestion that the same mechanism was acting to break down cytochrome P_{450} heme both in vivo and in vitro. This color change was not observed in the absence of NADPH, or in the absence of substrate.

The evidence presented here suggests the involvement of an active metabolite of certain barbiturates containing allyl groups in the breakdown of cytochrome P_{450} heme (*15*). Similar evidence has been obtained for the breakdown of cytochrome P_{450} heme by AIA (*5*). Metabolic conversion of the allyl group of these compounds to an epoxide may be involved in the breakdown of cytochrome P_{450} heme to the so-called green pigments, presumed to be nonphysiological isomers of biliverdin (*7*). Foreman and Maynert (*16*) have demonstrated, at least in the case of secobarbital, that an epoxide is formed in vitro in the presence of liver microsomes and NADPH. The pharmacologic and toxicologic implications of the formation of reactive epoxide intermediates in the metabolism of a wide variety of compounds has recently been suggested (*17, 18*). In this regard, epoxide intermediates formed during the oxida-

tion of certain aromatic compounds, such as brombenzene and polycyclic hydrocarbons, have been implicated in hepatotoxicity (*19*) and chemical carcinogenesis (*18*).

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20. We thank S. Teitel for the preparation of isopropylvaleramide and Mrs. MaryAnn Sadvary for help in the preparation of this manuscript.

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Resistance of Wild Norway Rats in North Carolina to Warfarin Rodenticide

Abstract. *Reports concerning the ineffectiveness of warfarin rodenticide, used at farms and stores in a rural area about 8 kilometers in diameter near Raleigh, North Carolina, prompted subsequent laboratory testing. All of the 25 Norway rats trapped from the area survived 6 days of no-choice feeding of bait containing 0.025 percent (by weight) warfarin.*

Inherited resistance to the anticoagulant rodenticides by Norway rats (*Rattus norvegicus*) in northern Europe, especially Britain and Denmark, has been well documented (*1, 2*). Despite intensive efforts to eradicate Norway rats, resistance, first recognized in 1958, has spread geographically (*3*). Recently resistance has also been found in roof rats (*Rattus rattus*) (*4*). In most populations of Norway rats studied a single, dominant allele appears involved; cross-resistance to all other anticoagulants is frequently, but not always, present (*5*).

In the summer of 1971 an apparently similar resistance was confirmed in Norway rats from a rural area near Raleigh, North Carolina. The local pest control operator (S. G. Flowers, Smithfield, North Carolina), who found it increasingly difficult to maintain control over the rat population, initially blamed poor technique and materials and then suspected resistance. Laboratory studies confirmed his suspicion.

Rats were trapped alive at six farms and two rural stores in the suspected resistance area (about 8 km in diameter) and shipped by air to our laboratory for testing. Standard test procedures recommended by the World Health Organization were employed (*6*). Rats were caged individually and, after a stabilization period of at least a week, were subjected to a no-choice, 6-day feeding test with bait (ground Purina rat chow) containing 0.025 percent (by weight) warfarin. Survivors, continued on placebo bait, were observed for a minimum of 22 days.

All of the North Carolina rats survived this test (and thus were declared resistant); several groups of Norway rats from Cleveland (the Hough district where anticoagulant rodenticides have long been employed) and several groups from rural northwestern Ohio died (Table 1). Resistant rats survived warfarin dosages (dosages were determined on the basis of the number of milligrams of warfarin per kilogram of body weight) up to five times those consumed by the controls. Lund's finding (*2*) that the amount of food consumed by resistant rats on test days

Table 1. Summary of the results of feeding tests of wild Norway rats subjected to 6-day, no-choice feeding tests with 0.025 percent warfarin in ground Purina rat chow. Only the North Carolina rats survived.

Source	*N*	Sex	Mean weight body (g)	Bait (g/day per kilogram)	Warfarin (mg/day per kilogram)	Total warfarin (mg/kg)
North Carolina	13	M	301	93.8	23.5	140.8
North Carolina	12	F	186	97.9	24.5	146.8
North Carolina (total)	25	M + F	246	95.9	24.0	143.9
Northwestern Ohio (rural)	4	M + F	227	61.6*	15.4*	107.3†
Northwestern Ohio (rural)	2‡	M + F	165	91.6*	22.9*	76.6†
Cleveland (Hough district)	11	M	233	77.3*	19.1*	67.4†
Cleveland (Hough district)	7	F	198	80.0*	19.8*	78.8†
Cleveland (Hough district) (total)	18	M + F	212	78.8*	19.5*	74.4†
Laboratory (white rats)	4	M	450	40.6*	10.1*	39.6†

* Based on the first 3 days of the test only, because there was a reduction in the amount of bait consumed per day shortly before death. † All rats were dead after 5 to 8 days. ‡ These rats were fed a prolin (warfarin plus an antibiotic) bait.