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METABOLISM OF DIPHENYLHYDANTOIN BY RAT LIVER MICROSOMES—I

CHARACTERISTICS OF THE REACTION*

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Abstract—Biotransformation of diphenylhydantoin (DPH) is an essential step preceding its elimination. The kinetic characteristics of this reaction by rat liver 9000 *g* supernatant are presented. The system *in vitro* produced 5-phenyl-5′ parahydroxyphenylhydantoin (HPPH) as the reaction product. The optimal pH for this reaction was 7·4 and temperature 37°. The cofactors included NADP, NAD, ATP and oxygen; and the final reaction mixture had a protein content of 8–10 mg per ml. The K_m of this system calculated from 16 Lineweaver–Burk plots ranged from 3×10^{-5} M to 5×10^{-5} M with a mean of 3·73 ± S.D. 0·18.

The washed microsomes metabolized DPH only when NADPH generating systems were added. The 120,000 *g* supernatant centrifuged for 30 min retained a portion of its enzymatic activity which was 40 per cent of that of 9000 *g* supernatant. No DPH was metabolized by 120,000 *g* supernatant centrifuged for 3 hr or longer.

The reaction was inhibited by methylene blue, sodium azide and SKF 525-A; and a number of commonly used drugs. The inhibition of SKF 525-A was noncompetitive, whereas phenobarbital caused competitive inhibition. Other drugs which inhibited strongly were prochlorperazine, chlordiazepoxide, diazepam, chlorpromazine, isoniazid, propoxyphene, diethylstilbestrol, disulfiram and dimenhydrinate. These findings help to elucidate the mechanism of the clinically observed DPH accumulation and intoxication in some patients using isoniazid, tranquilizers or other drugs in combination with DPH.

IT HAS long been recognized that diphenylhydantoin (DPH) is largely metabolized in the organism prior to its elimination. Hine and Kozelka[1] studied first the rate of DPH metabolism, and also reported about the nature of its metabolites.[2] Butler[3] described the main metabolite of DPH, 5 phenyl-5′ parahydroxyphenylhydantoin (HPPH); and Maynert[4] demonstrated that HPPH appears in urine mainly as glucuronide complex. Woodbury *et al.*[5] and Noach *et al.*[6] have demonstrated that over 95 per cent of the DPH dose appears in metabolized form in urine, and that HPPH constitutes about 60–70 per cent of the total metabolites; whereas the remainder are less well characterized compounds. Our own studies confirmed these findings and revealed that if the biotransformation of DPH in the organism is impaired or interfered with either by liver disease[7] genetically transferable enzyme deficiency[8] or other drugs,[9, 10] accumulation of unmetabolized DPH occurs. In order to understand and explain our clinical observations it became necessary to study DPH

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metabolism *in vitro*. Rat liver microsomal systems were used because these systems, with the addition of NADPH or a NADPH generating system, have been reported to perform a variety of chemical changes on drug molecules.[11-17] In this study we present the characteristics of DPH biotransformation by systems *in vitro* and also the characteristics of its inhibition by various chemicals and drugs.

MATERIALS AND METHODS

A. Preparation of 9000 g *supernatant*

Male Wistar albino rats weighing 200–250 g were sacrificed by decapitation after stunning. Their livers were excised and homogenized in a Potter-type homogenizer at 4°. For each 1·0 g of tissue, 1·5 ml of 0·2 M sodium phosphate buffer of pH 7·4 was used. The whole homogenate was centrifuged at 9000 *g* for 15 min in a Spinco model L preparative ultracentrifuge. After centrifugation the middle zone of the supernatant was collected by aspiration carefully avoiding contamination by the sediment as well as the waxy fat layer on the top of the homogenate. This was necessary because contamination, particularly by the fat, strongly inhibited the enzyme activity. The 9000 *g* supernatant was either used immediately or stored at −16°. Only small decline of enzyme activity was seen in the preparations kept frozen up to 24 days.

B. Reaction mixture and incubation

In most experiments in which DPH metabolism was studied, the enzyme system contained 2·0 ml of 9000 *g* supernatant, 3·0 ml of 0·2 M sodium phosphate buffer, pH 7·4, and cofactors in a concentration of $8{\cdot}2 \times 10^{-4}$ M NADP; $9{\cdot}3 \times 10^{-4}$ M NAD and $3{\cdot}9 \times 10^{-3}$ M ATP. Included also were nicotinamide in concentrations of $2{\cdot}5 \times 10^{-4}$ M to 5×10^{-2} M and $MgSO_4$ in concentrations of $2{\cdot}7 \times 10^{-6}$ M to $3{\cdot}8 \times 10^{-2}$ M, making a total volume of 5·5 ml. However, omission of nicotinamide and $MgSO_4$ did not alter the enzyme activity and in later experiments these cofactors were not used. Other cofactors that were evaluated were NADPH; glucose 6-phosphate, glucose 6-phosphate dehydrogenase; isocitric acid and isocitric acid dehydrogenase. The final protein concentration of the reaction mixtures which contained the 9000 *g* supernatant ranged between 8–10 mg per ml as determined by the method of Lowry *et al.*[18] The reaction systems were incubated in a Dubnoff metabolic shaking incubator at 37° with oxygen flowing at a rate of 4 l./min. Samples were incubated usually for 15 min but in some experiments incubation was continued up to 4 hr. The substrate concentration in the reaction mixtures ranged from $1{\cdot}0 \times 10^{-5}$ M to 3×10^{-4} M depending upon the nature of the experiment. In the studies of inhibition of DPH metabolism, various chemicals and drugs were added to the reaction mixtures in concentrations ranging from 10^{-6} M to 10^{-2} M.

C. Determination of enzyme activity

Substrate utilization was used as the indicator of enzyme activity. Two techniques were used for the determination of DPH concentration: (1) colorimetric or (2) the use of radioactive DPH.

(1) *Colorimetric method.* Samples of 1·0 ml were collected at zero time and after incubation and placed into 15 ml $CHCl_3$ which contained 0·5 ml of 1·0 M phosphate buffer, pH 6·8. The chloroform stopped the enzyme activity and at the same time served as the first solvent for the extraction and isolation of DPH from the aqueous

phase. The quantity of unmetabolized DPH was determined by the method of Dill *et al.*[19]

(2) *Radioisotope method.* ^{14}C-labelled DPH was used, particularly in the studies of inhibition of DPH metabolism by other drugs and chemicals, which interfered with the colorimetric procedure. The 5-ml reaction systems contained DPH in amounts of 90 mμmoles (0·068 μc) to 900 mμmoles (0·68 μc) which represents concentrations of $1{\cdot}8 \times 10^{-5}$ M to $1{\cdot}8 \times 10^{-4}$ M. Samples of 1·0 ml were transferred at zero time and after incubation into 5 ml $CHCl_3$ which contained 0·5 ml 1·0 M phosphate buffer, pH 6·8. The tubes were thoroughly mixed for 60 sec on a Vortex mixer and centrifuged for 5 min at 2000 rpm. From each sample 0·5 ml of $CHCl_3$ was transferred into 15 ml of toluene which contained 4·0 mg 2,5-diphenyloxazole (PPO) and 50 mg 2,2-*p*-phenylene-*bis* (5 phenyloxazole) POPOP per 1000 ml. The samples were counted in a Packard Tri-Carb liquid scintillation spectrometer model 3003. The recovery of label into $CHCl_3$ by this technique was 99·6 per cent ± SD. 0·23 as determined by adding a known amount of ^{14}C-labeled DPH into plasma or the 9000 *g* enzyme without cofactors.

The activity varied in different batches of enzyme which was probably due to individual variation among the animals used, although precautions were taken to avoid enzyme induction by stress and starvation. No chemical enzyme induction was used in this series of experiments.

Studies of enzymatic reaction at various temperatures

DPH parahydroxylation was studied between temperatures of 10–55°. The 9000 *g* supernatant and substrate without the cofactors were warmed to the desired temperature, and the zero time sample was taken after which the powdered cofactors were added. This technique allowed the enzyme activity to take place only at the desired temperatures and eliminated the activity which occurs during the "warming up" period.

Preparation of the microsomal fractions and the 120,000 g *supernatant*

Aliquots of the 9000 *g* supernatant were placed in swinging-bucket rotor (Spinco SW-39) and spun at 74,000 *g* for 30 min. The supernatant was removed by aspiration and the microsomal pellets were washed twice with 0·2 M sodium phosphate buffer pH 7·4.

The 74,000 *g* supernatant retained about 45 per cent of the enzyme activity; therefore, this preparation was centrifuged again at 120,000 *g* for 30, 60, 120 and 180 min. Only the middle zone of the ultracentrifuge tube was collected, to avoid contamination. The centrifugal forces were calculated as *g* average, i.e. at 7·3 cm in SW-39.

Demonstration of HPPH produced by the hepatic 9000 g *supernatant enzyme system*

From the incubated enzyme systems 2·0-ml samples were collected, and washed twice with 15 ml $CHCl_3$ in the presence of 0·5 ml 1·0 M sodium phosphate buffer, pH 6·8, to remove the unmetabolized DPH. The water phase was mixed with an equal volume of concentrated HCl and hydrolyzed for 5 min by boiling; further boiling up to 1 hr did not increase the yield of HPPH. The hydrolyzate was adjusted to pH 10·0 with concentrated NaOH, mixed for 1 min on a Vortex mixer and centrifuged at 3000 rpm for 15 min. The supernatant was removed, its pH adjusted to 6·0 with

concentrated hydrochloric acid and then HPPH extracted with 4 ml of ethylacetate of which 3 ml was transferred to a test tube and evaporated to dryness. The dry residue was dissolved in 0·5 ml of ethanol and 0·01 ml was applied on instant thin-layer chromatography plates (Silica gel with fluorescent indicator, Type K 301 R, DPI Inc.). A solvent of 95 ml of $CHCl_3$ with 5 ml of methanol was used to develop the ascending chromatograms for 10–15 min. Standards of synthetic HPPH and HPPH extracted from the urine of patients and DPH were also applied. The substances were visualized under u.v. light, and the HPPH was identified with the use of diazotized sulfanilic acid spray[3] which produced a bright yellow-orange color with HPPH, whereas DPH remained colorless. Using standards in increasing concentrations allowed a semiquantitative estimation of unknown HPPH samples.

RESULTS

The enzymatic activity of the 9000 *g* supernatant in the biotransformation of DPH was demonstrated by the disappearance of DPH from the reaction mixture after incubation in the presence of appropriate cofactors. The following experiments confirmed that the diminution of the substrate concentration was caused by biotransformation:

(1) There was no disappearance of substrate in the reaction mixtures which contained the protein enzyme but no cofactors, indicating that irreversible binding of DPH to any of the proteins did not take place. (2) There was no disappearance of DPH when the cofactors and DPH were incubated together in phosphate buffer in the absence of the protein enzyme, suggesting that no binding to cofactors took place. (3) There was no disappearance of substrate in the reaction mixtures which contained the cofactors and protein enzyme that had been heated 15 min at 75° before the incubation. (4) Unequivocal proof for the biotransformation of DPH by the system *in vitro* was obtained by the isolation of HPPH, the major metabolite of DPH, from the 9000 *g* hepatic microsomal enzyme systems after incubation. The zero-time sample contained no HPPH while increasing quantities of the metabolite appeared during the incubation period.

A. Evaluation of cofactors. Figure 1 shows a variety of DPH biotransformation rates which depended on the combination of cofactors present in the reaction mixture. The highest rate of DPH metabolism was achieved when NADP, NAD and ATP were present in the enzyme system. The amounts of substrate utilized were measured at 30 min.

In order to determine the optimal concentration of the cofactors, all three cofactors were added in a wide range of concentrations one by one. The highest rate of enzyme activity was achieved when $8{\cdot}2 \times 10^{-4}$ M NADP, $9{\cdot}3 \times 10^{-4}$ M NAD and $3{\cdot}9 \times 10^{-3}$ M ATP were used in the reaction mixture. Glucose was also added in concentrations between 1×10^{-2} M to 1×10^{-1} M but its presence or absence had no effect on the enzyme activity. Similarly, addition of glucose 6-phosphate to the cofactors mentioned above did not improve that system. The metabolic activity of the 9000 *g* microsomal enzyme prepared in Tris-KCl buffer and diluted with potassium phosphate buffer was equal to that observed with sodium phosphate buffer.

Either air or oxygen was necessary for DPH metabolism by the 9000 *g* microsomal enzyme system. Routinely 4 l./min of oxygen was used; however, oxygen flow as great as 10 l./min did not enhance nor inhibit the biotransformation of DPH. If the

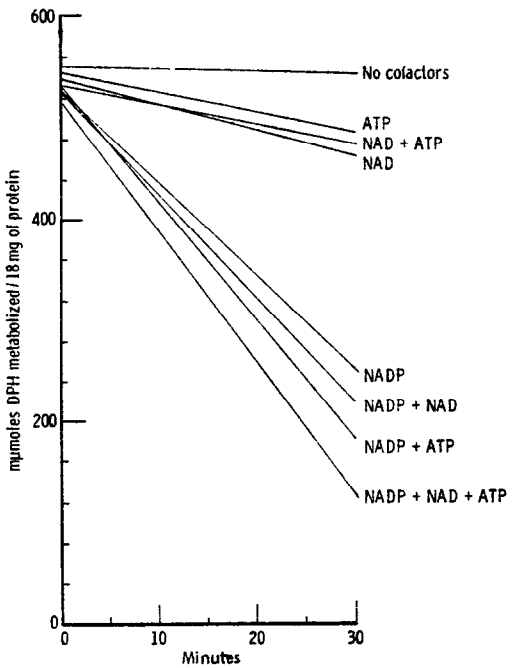

FIG. 1. Effects of various cofactors on microsomal metabolism of DPH. Standard system was used with 2 ml of 9000 *g* rat liver supernatant and 3 ml of phosphate buffer. The concentration of NADP was $8{\cdot}2 \times 10^{-4}$M; NAD $9{\cdot}3 \times 10^{-4}$M and ATP $3{\cdot}9 \times 10^{-3}$M. Protein concentration of the system was 9 mg/ml. Incubation time, 30 min.

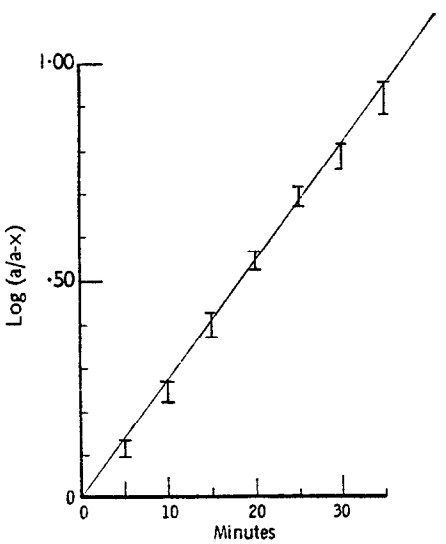

FIG. 2. First-order kinetics of DPH metabolism with respect to time. The standard system was used; concentration of substrate was $5{\cdot}5 \times 10^{-5}$M. The samples were taken at 5 min intervals. Each bar represents the range of 4 experiments. In log a/(a−x), a represents substrate concentration at zero time, x is the amount of substrate utilized at time t.

reaction vessels were sealed with white mineral oil the velocity of DPH metabolism was markedly reduced although some activity still occurred. This was probably supported by the oxygen still present in the enzyme preparation.

Kinetic studies. The rate of DPH metabolism accorded with first-order kinetics. It can be seen on Fig. 2 that the reaction velocity was linear up to 25 min, falling slightly after that time. After 15 min of incubation, 62 per cent of the substrate was utilized. The product formation was linear for 15 min with substrate concentrations of $3{\cdot}6 \times 10^{-5}$ M to $1{\cdot}8 \times 10^{-4}$ M. When excess of cofactors and substrate were present in the reaction system, DPH parahydroxylation continued up to 4 hr. Of the total activity 79 per cent occurred in the first hour, 15·4 per cent in the second hour and 5·6 per cent in the third and fourth hour.

Effects of hydrogen ion concentration and temperature. The pH of the 9000 *g* microsomal enzyme system at which DPH is metabolized at the highest rate was 7·4, as seen in Table 1. This was studied by adjusting the zero time pH of the enzyme

TABLE 1. DPH METABOLISM AT VARIOUS pH VALUES*

pH Before incubation	pH After incubation	mμmoles†
6·0	5·98	18·2
6·14	6·12	49·1
6·32	6·29	92·7
6·58	6·53	105·5
6·8	6·75	136·4
7·05	7·01	236·5
7·37	7·30	255·0
7·5	7·44	222·0
7·6	7·54	187·3

* Rat liver 9000 *g* supernatant with cofactors was incubated for 15 min. The pH was adjusted with different ratios of mono- and dibasic 0·2 M sodium phosphate solutions. DPH concentration in the reaction mixture was $1{\cdot}5 \times 10^{-4}$M.

† Activities expressed as mμmoles of DPH metabolized in 15 min per 40·8 mg of protein.

systems with different ratios of mono- and dibasic 0·2 M sodium phosphate solutions. Slight reduction of pH values took place during the 15 min incubation period ranging from 0·02 to 0·07 pH units. Table 2 shows the enzyme activity at different temperatures and that the optimal temperature for DPH metabolism was 37°.

Maximal velocity and the Michaelis constant. The rate of metabolism of DPH was dependent upon the concentration of substrate in the reaction mixture. Figure 3 shows that the transition between the first order and zero order reactions took place at a DPH concentration of $1{\cdot}0 \times 10^{-4}$ M. The values of maximal velocity (V_{max}) per 15 min per 40 mg of protein ranged from 202 to 287 mμmoles of DPH with a mean of 246·6 ± S.D. 24·7 mμmoles of DPH.

The Michaelis constant for this reaction calculated from sixteen different Lineweaver–Burk plots ranged from 3×10^{-5} M to 5×10^{-5} M with a mean of $3{\cdot}73 \times 10^{-5}$ M. The standard deviation of K_m was 0·18. The relatively low K_m of 3·7 ×

TABLE 2. DPH METABOLISM AT VARIOUS TEMPERATURES*

Temp. (°C)	mμmoles†
5	0·0
10	40·0
20	149·0
25	158·0
30	195·0
33	213·0
37	228·0
40	164·0
48	24·0
55	0·0

* Rat liver 9000 *g* supernatant with cofactors was incubated at different temperatures for 15 min under O_2. DPH concentration in the reaction mixture was $1{\cdot}5 \times 10^{-4}$M and the pH was 7·4.

† Activities expressed as mμmoles DPH metabolized in 15 min per 42·4 mg of protein.

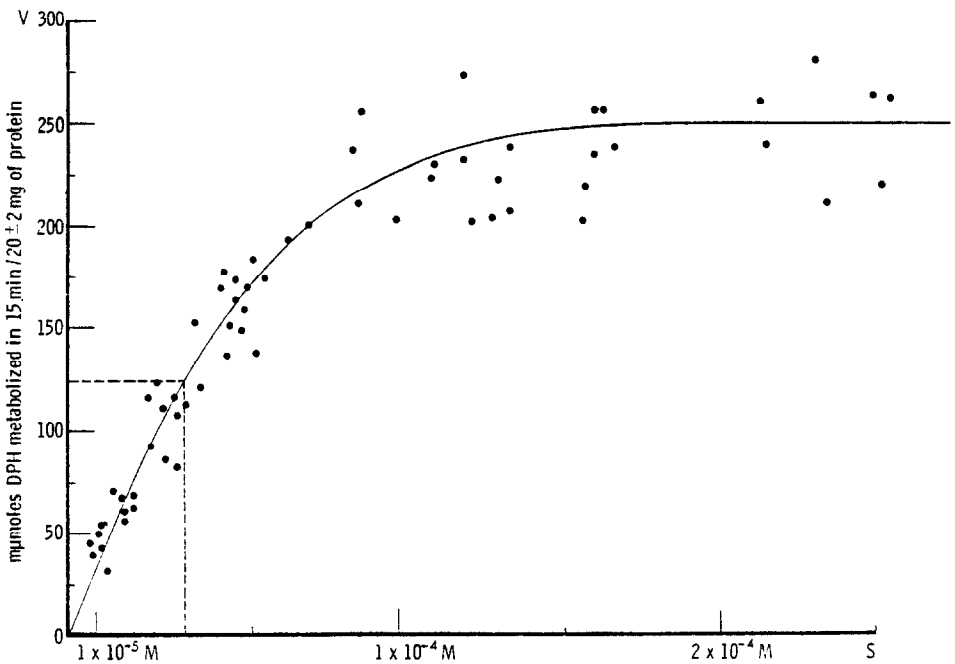

FIG. 3. Dependence of DPH microsomal metabolism upon substrate concentration. The standard system as in Fig. 1 was used with cofactors NADP $8{\cdot}2 \times 10^{-4}$M, NAD $9{\cdot}3 \times 10^{-4}$M and ATP $3{\cdot}9 \times 10^{-3}$M. V represents mμmoles of substrate utilized in 15 min. Sixteen different experiments were performed in which the amount of protein varied from 36 to 44 mg per reaction system. S represents the amounts of substrate in molar concentrations.

TABLE 3. INHIBITION OF DPH METABOLISM BY VARIOUS AGENTS*

Agent	Concentration (M)	Inhibition (%)
Cytochrome *c*	10^{-4}	0·0
Flavin mononucleotide	10^{-5}	9·8
Na fluoride	10^{-3}	9·0
Na cyanide	10^{-3}	9·8
Methylene blue	10^{-4}	90·8
Na azide	10^{-3}	70·5
SKF 525-A	10^{-6}	12·0
	10^{-5}	64·0
	10^{-4}	100·0

* Rat liver 9000 *g* supernatant was incubated with cofactors at 37° for 15 min under O_2. DPH concentration in the reaction mixture was $1{\cdot}5 \times 10^{-4}$M. The inhibition was calculated as percentage of the activity of the uninhibited control. In this experiment the activity of the control was 226 mμmoles per 48·6 mg of protein.

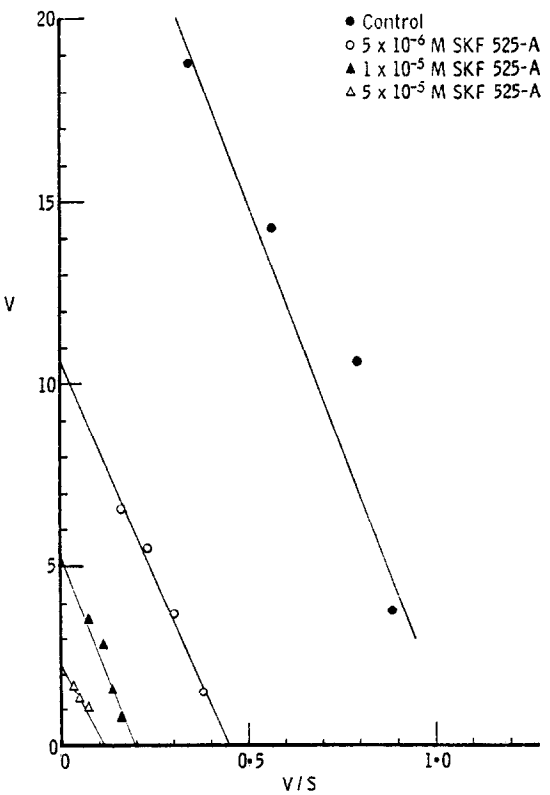

FIG. 4. Inhibition of DPH microsomal metabolism by SKF 525-A. Standard system as in Fig. 3 was used except the inhibitor was added in concentrations as indicated. V represents substrate utilized in 15 min per 10·2 mg of protein. S represents the amounts of substrate in molar concentrations.

10^{-5} M suggests a strong affinity between DPH and its enzyme under the optimal conditions described above.

Inhibitors of DPH metabolism. Inhibition of DPH metabolism by various chemicals and a number of drugs was studied. As seen in Table 3, cytochrome c and flavin mononucleotide as well as the inhibitors of the respiratory chain, e.g. sodium fluoride sodium cyanide, caused no substantial inhibition of DPH metabolism. Methylene blue and sodium azide strongly inhibited at 10^{-4} M to 10^{-3} M concentration. Considerable inhibition of enzyme activity was caused by SKF 525-A which was demonstrable already at a concentration of 1×10^{-6} M. The type of SKF 525-A inhibition was non-competitive and its representation by the Hofstee plot (v vs. v/s) is shown in Fig. 4.

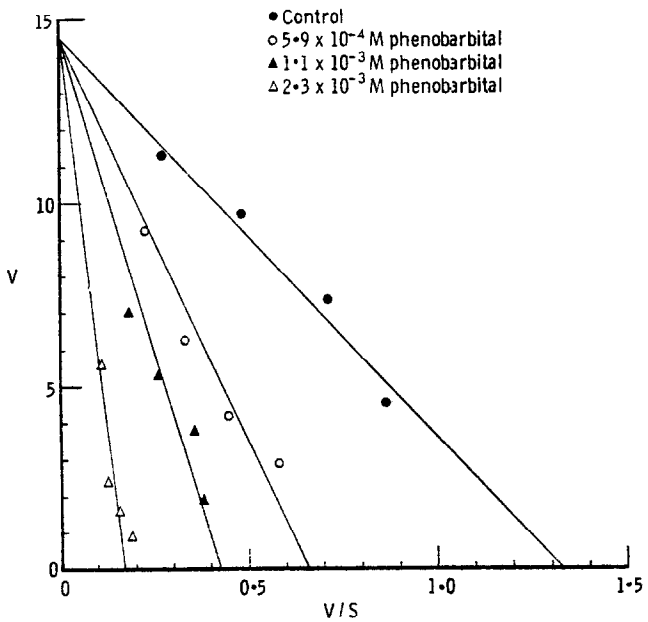

FIG. 5. Inhibition of DPH microsomal metabolism by phenobarbital. Standard system as in Fig. 3 was used except that inhibitor was added in concentrations as indicated. V represents substrate utilized in 15 min per 10·5 mg of protein. S represents the amounts of substrate in molar concentrations.

Among the drugs tested for inhibition of DPH metabolism we included those that had been previously observed in clinical practice to depress DPH metabolism, as well as other commonly used drugs. Phenobarbital inhibited the DPH metabolism *in vitro*; the type of inhibition by phenobarbital in concentrations of $5{\cdot}9 \times 10^{-4}$ M to $2{\cdot}3 \times 10^{-3}$ M was competitive as seen in Fig. 5. In Table 4 we can see that prochlorperazine, meprobamate, chlorpromazine and diazepam in 10^{-3} M concentration inhibited the system *in vitro* 91–100 per cent. Other strong inhibitors were propoxyphene, isoniazid, diethyl stilbestrol, disulfiram and dimenhydrinate, whereas the steroid hormones, acetylsalicylic acid, aminosalicylic acid and cycloserine were effective only in rather high concentrations.

TABLE 4. INHIBITION OF DPH METABOLISM BY VARIOUS DRUGS*

Test compound	Concentration (M)	Inhibition (%)	Test compound	Concentration (M)	Inhibition (%)
Chlordiazepoxide	10^{-3}	67	Propoxyphene	10^{-3}	93
Prochlorperazine	10^{-3}	100	Isoniazid	10^{-5}	16
Prochlorperazine	5×10^{-4}	34	Isoniazid	6×10^{-4}	64
Meprobamate	10^{-2}	91	Isoniazid	10^{-3}	90
Meprobamate	10^{-3}	34	Disulfiram	5×10^{-3}	61
Chlorpromazine	5×10^{-3}	93	Dimenhydrinate	10^{-3}	96
Chlorpromazine	10^{-4}	27			
Diazepam	10^{-3}	91			
Diazepam	10^{-4}	32	Phenobarbital	10^{-2}	84
			Phenobarbital	5×10^{-3}	56
			Pentobarbital	10^{-2}	100
Testosterone	10^{-2}	83	Pentobarbital	10^{-3}	40
Testosterone	10^{-3}	41			
Estrone	5×10^{-3}	13	Sulfisoxazole	5×10^{-3}	32
Estradiol 17β	10^{-2}	15	Sodium Warfarin	10^{-3}	33
Progesterone	10^{-2}	87	Sodium Warfarin	10^{-4}	10
Progesterone	10^{-3}	48	Aminosalicylic acid	10^{-2}	17
			Aminosalicylic acid	5×10^{-2}	27
Diethyl stilbestrol	5×10^{-3}	95			
Diethyl stilbestrol	10^{-4}	63	Acetylsalicylic acid	10^{-2}	13
			Cycloserine	10^{-2}	13

* Rat liver 9000 *g* supernatant was incubated with cofactors at 37° for 15 min under O_2. DPH concentration in the reaction mixture was $1{\cdot}5 \times 10^{-4}$M. The inhibition was calculated as percentage of the activity of the uninhibited control. In this series of experiments the activity of control ranged from 227 to 257 mμmoles per 48±2 mg of protein.

TABLE 5. DPH METABOLISM BY RAT LIVER FRACTIONS*

Fraction	mμmoles*
9000 *g* supernatant + cofactors‡	350
Whole homogenate + cofactors‡	0
Nuclei + mitochondria + cofactors‡	0
74,000 *g* Supernatnat centrifuged for 30 min + cofactors‡	142
Washed microsomes + cofactors‡	0
Washed microsomes + glucose 6-phosphate + glucose 6-phosphate dehydrogenase + cofactors‡	149
Washed microsomes + isocitric acid + isocitric acid dehydrogenase + cofactor‡	152
Washed microsomes + NADPH§	105
100,000 *g* Supernatant centrifuged for 30 min + cofactors†	143
100,000 *g* Supernatant centrifuged for 60 min + cofactors†	121
100,000 *g* Supernatant centrifuged for 120 min + cofactors†	24
100,000 *g* Supernatant centrifuged for 180 min + cofactors†	0

* The various fractions were incubated at 37° for 30 min under O_2. DPH concentration in the reaction mixture was $1{\cdot}5 \times 10^{-4}$M.
† Activities in various fractions expressed in mμmoles DPH metabolized in 30 min per 600 mg of liver.
‡ NADP, NAD and ATP.
§ NADPH $5{\cdot}3 \times 10^{-4}$M; this concentration may be suboptimal.

Washed microsomes and 120,000 g *supernatant.* The metabolic characteristics of the different zones obtained by differential centrifugation of the 9000 *g* supernatant are presented in Table 5.

It can be seen that the washed microsomes were active only when NADPH generating systems were added. The supernatant fraction of the enzyme preparation which was centrifuged at 120,000 *g* for 30 min retained an activity which was about 40 per

cent of the 9000 *g* preparation. Gradually increasing the time of centrifugation at 120,000 *g* decreased the concentration of the active component in the supernatant and none was present after 3 hr of centrifugation.

DISCUSSION

The hepatic microsomal enzyme preparations which have been shown to metabolize barbiturates,[11] trichloroethylene,[14] narcotic drugs,[12] imipramine[17] and other drugs are also capable of metabolizing DPH. The optimal conditions for this reaction, such as pH and temperature, and the cofactor requirement do not differ significantly from those needed to metabolize other drugs by the liver microsomes. The metabolism of DPH by the system described in this paper proceeded at a rather rapid rate. The low substrate K_m value of $3{\cdot}7 \times 10^{-5}$ M of our system suggests a strong affinity of DPH for the microsomal enzymes; it is in the same order of magnitude as that reported for testosterone and estradiol.[20]

The biotransformation *in vitro* of DPH was inhibited by several compounds. SKF 525-A was inhibitory in a rather low concentration of $1{\cdot}0 \times 10^{-6}$ M, and the type of inhibition was noncompetitive. As a corollary, noncompetitive inhibition of microsomal trichloroethylene metabolism by SKF 525-A was observed by Byington *et al.*[14] On the other hand competitive inhibition of various other microsomal enzymes by SKF 525-A also has been reported.[13] Inhibition of DPH metabolism *in vivo* by SKF 525-A has been reported by Swinyard *et al.*[21] They found that the potency and the duration of anticonvulsant action of DPH were increased in SKF 525-A treated mice. Among the commonly used drugs the antitubercular agent isoniazid has been observed to inhibit DPH metabolism both *in vivo* and *in vitro*.[9, 22] Our recent clinical studies revealed that significant DPH accumulation in patients receiving the usual doses of DPH and isoniazid is genetically determined and takes place only in extremely slow isoniazid inactivators whose isoniazid blood levels remain high for long periods of time.[13] Strong inhibition by tranquilizers demonstrated *in vitro* also coincides with clinical observations of DPH accumulation in some patients taking these drugs.[10] Since DPH accumulation does not occur in all patients who take tranquilizers, it is probable that here also the genetic make-up plays an important role. Similar explanations may apply to isolated patients reported to accumulate DPH when it is given in combination with dicoumarol,[24] disulfiram[25] and phenyramidol.[26] Although phenobarbital inhibits DPH metabolism *in vitro*, this interference is noted quite infrequently in the clinical practice.[27, 28]

Propoxyphene, which is a strong inhibitor of DPH metabolism *in vitro*, has not been observed to cause accumulation of DPH in patients. This is probably due in part to the fact that propoxyphene is taken only sporadically.

Other drugs of interest which inhibited DPH metabolism *in vitro* were the steroid hormones which are also metabolized by the hepatic microsomal system and it is possible that a competition exists between the DPH and steroid molecules for the enzymes.

Cytochrome *c* and flavin mononucleotide were potent inhibitors of trichloroethylene metabolism in concentrations of 10^{-6} M to 10^{-5} M.[14] However, they caused no substantial inhibition of DPH metabolism at 10^{-3} M concentration. The inhibitors of the respiratory chain, e.g. sodium fluoride and sodium cyanide, inhibited DPH metabolism insignificantly even at fairly high concentration. However, sodium azide

and methylene blue, being uncoupling agents, inhibited DPH metabolism strongly, indicating possible interference with the energy requirement of the enzyme system.

Washed microsomes were active only after adding either the 74,000 *g* supernatant or NADPH or NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase, or NADP, isocitric acid and isocitric acid dehydrogenase. The 74,000 *g* supernatant centrifuged for 30 min still showed some activity as did the 120,000 *g* supernatant centrifuged for 30 min. Activity in the 120,000 *g* supernatant gradually diminished as the centrifugation time increased and was absent only in preparations centrifuged for 3 hr or longer. This is remarkable because the 74,000 *g* supernatant centrifuged for 30 min has been found to be inactive in metabolizing hexobarbital.[11]

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