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METABOLIC RATE OF PHENACETIN AND OF PARACETAMOL IN DOGS BEFORE AND AFTER TREATMENT WITH PHENOBARBITAL OR SKF 525 A

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Abstract—In six male mongrel dogs the apparent biological half life ($t/2$) in plasma of intravenously injected phenacetin decreased from 34·6 to 27·2 min after treatment with phenobarbital (25 mg/kg/day for 8 days) ($P < 0.025$). In the same dogs, the apparent $t/2$ of intravenously injected phenazone decreased from 78·6 to 32·4 min ($P < 0.05$). Treatment with SKF 525 A (25 mg/kg) increased the $t/2$ of phenazone in three other dogs from 70·6 to 246·7 min, whereas the $t/2$ of phenacetin remained unaffected.

The concentration of phenacetin at zero time of intravenous injection increased from 29·5 ± 5·9 S.E. to 43·5 ± 12·6 μg/ml plasma ($P < 0.2$) after phenobarbital treatment; pretreatment with phenobarbital, however, had no influence on the mean concentration of phenazone at 0 time. SKF 525 A did not influence the zero time concentration of either phenacetin or phenazone.

Beagle liver microsomal protein and cytochrome P-450 concentrations increased from 3·2 to 4·2 mg/ml and from 1·0 to 4·1 n-mole/mg protein, respectively, after phenobarbital treatment. Treatment with phenobarbital had no influence on the rates of formation of paracetamol and *p*-phenetidin nor was the apparent K_m of phenacetin affected.

The binding of phenacetin to blood constituents increased from 50 per cent at 5 μg/ml to 59 per cent at 1 μg phenacetin/ml. In the presence of 30 μg phenobarbital/ml blood, the binding of phenacetin at 1 μg/ml blood decreased by 22 per cent.

It is concluded that the decrease of the apparent $t/2$ of phenacetin in dog plasma after phenobarbital treatment may be due to a change in the apparent volume of distribution and/or to some stimulation of an NADPH-dependent enzyme system of the liver less affected by treatment with SKF 525 A than in other species.

IT HAS been shown by several investigators that the activity of the NADPH-dependent microsomal enzyme system of liver cells, responsible for the formation of paracetamol from phenacetin, is stimulated by pretreatment with phenobarbital in rats[6] or with 3-methylcholanthrene in rats[8] and in cats.[21] The activity was inhibited by pretreatment with 2-diethylaminoethyl-2,2-diphenylvalerate (SKF 525 A) in rats.[8]

However, after therapeutic doses of phenobarbital in man, the apparent biological half life of phenacetin and the maximum concentrations of phenacetin and paracetamol in the blood remained unchanged.[15] In contrast, the biological half life of phenazone was increased. There are two possible explanations for these observations.

Firstly, the *O*-dealkylation of phenacetin in man is less susceptible to stimulation by phenobarbital than in other species, and is affected less than the hydroxylation of phenazone. Secondly, an increased *O*-dealkylation rate of phenacetin determined in blood after treatment with phenobarbital was masked by absorption of phenacetin

from the gut. Phenacetin had to be given orally and the rates of absorption and elimination, measured in the blood, had been of approximately the same order of magnitude.

To elucidate the first hypothesis in a different species, the influence of phenobarbital and of SKF 525 A on the *O*-dealkylation rate of phenacetin and the disappearance rate of phenazone after intravenous injection was measured in dogs. According to Welch *et al.*[21] approx 50 per cent of a single dose of phenacetin is excreted in the urine as conjugated paracetamol. In addition, initial formation rates of paracetamol and of phenetidin were determined in microsomal suspensions prepared from liver cells. The influence of phenobarbital on the binding of phenacetin to blood constituents was also investigated.

METHODS

Experimental design. The *in vivo* experiments were performed on eight male mongrel dogs and one male beagle dog between 1 and 2 yr of age. They were divided into three groups. Prior to the experiment, the dogs were quarantined for at least 14 days. Test doses of phenacetin 30 mg/kg, paracetamol 25·4 mg/kg, and phenazone 31·5 mg/kg were injected intravenously at 24-hr intervals, into each dog at random. This was repeated in six dogs after a 9 day period of treatment with daily intramuscular injections of either 25 mg/kg of sodium phenobarbital (Luminal), or 0·9% saline solution. After an 8-day rest period SKF 525 A (25 mg/kg) was given to three dogs orally with a meat ball 24 hr before the first, and then 2 hr before each test dose.

Each test dose of phenacetin, paracetamol, or phenazone was given in a vol. of 0·6 ml/kg body wt in a 75% alcoholic solution to the first set of animals, and in 0·25% agar in a 0·9% saline solution to the other two sets of animals. The agar suspension was treated three times for 1 min with an ultrasonic vibrator (MSE) at 4 μm from peak to peak.

For determination of plasma concentrations, blood samples were withdrawn for duplicate analyses before the injection of phenacetin, paracetamol, or phenazone, 20 min after and then at approx 30 or 45 min intervals depending on whether phenacetin, paracetamol, or phenazone was injected.

After administration of phenacetin or paracetamol, seven blood samples were drawn, 10 or 5 ml each, respectively. The concentration of phenazone was determined in 4 blood samples, 5 ml each, drawn after the injection. Immediately after withdrawal a few crystals of sodium citrate were added to the blood.

The microsomal fraction was prepared from the livers of seven beagles, of which three were pretreated for 7–8 days with daily intramuscular doses of sodium phenobarbital 25 mg/kg. The dogs were sacrificed by shooting a bolt through the skull and after bleeding the livers were excized. The liver was then cut into pieces by scissors at 4° and forced through a screen, 4 cm dia., with 272 holes of 2 mm dia. each.[14] 180 g of this pulp was suspended in 0·15 M potassium chloride solution to a final volume of 360 ml.

After centrifugation for 20 min at 9000 *g* in a refrigerated centrifuge, the supernatant was sedimented and washed twice in the same volume of potassium chloride solution by centrifuging for 60 min at 78000 *g* in a Spinco L 50 centrifuge. The microsomes were suspended in 0·1 M phosphate buffer pH 7·4 at 4°, to a vol. of 30 ml. 0·1 ml of this suspension was used per ml of incubation mixture. Phenacetin was dis-

solved in 1 ml of methanol which was evaporated from the incubation flasks in a water bath.

Formation of phenetidin and paracetamol was measured in separate 100 ml Erlenmeyer flasks, after an incubation period of 10 min in presence of a starting concentration of 0·3, 1, 2, and 3 mM phenacetin.

The flasks from which *p*-phenetidin was determined were preincubated for 10 min, with 7·2 ml of the phosphate buffer. The reaction was started by addition of microsomal suspension. Duplicate analyses were performed with 0·5 ml of the incubation mixture.

The flask for paracetamol assay was prepared by adding twice the amount of microsomal suspension to twice the amount of phenacetine needed for the final concentration, 5·6 ml of phosphate buffer, and 0·5 ml of an aqueous solution of paraoxon[17] 1×10^{-5} M final concentration.

The solution of paraoxon was prepared from Mintacol solubile, Bayer Company. After a preincubation period of 10 min 4 ml were removed. The reaction was started by adding 4 ml of a preincubated mixture containing constituents needed for the NADPH dependent *O*-dealkylation by the microsomal enzyme. The molar concentrations in the incubation mixture were: MgCl 6×10^{-3}, nicotinamide $1{\cdot}2 \times 10^{-2}$, glucose-6-phosphate 1×10^{-2}, NAD $1{\cdot}2 \times 10^{-4}$, glucose-6-phosphate dehydrogenase 0·7 i.u./ml; 0·5 ml of the paraoxon solution was also added.

In preliminary experiments with microsomal suspensions prepared from the livers of mongrel dogs it was found that in the absence of the NADPH regenerating system, essential for microsomal hydroxylations, phenacetin was not converted to paracetamol whereas phenetidin was detected in large amounts. In the presence of the NADPH regenerating system phenacetin was transformed to paracetamol and phenetidin. The formation of phenetidin was blocked, however, by adding paraoxon (1×10^{-5} M) which resulted in a slight increase of paracetamol concentration. Paraoxon at a concentration of 2×10^{-5} M had no effect on the rate of product formation, also found by other investigators[20] for anilin C-hydroxylation.

A plot of the amount of product formed vs time up to 40 min, showed a linear correlation up to an incubation period of 15 min at 37°. In addition, at a protein concentration ranging from 0·75 to 4 mg/ml of microsomal suspension, the amount of product formed was proportional to the concentration of protein. Under these experimental conditions, the formation rates of paracetamol after 10 min of incubation at the various substrate concentrations were found to lie at the upper part of the sigmoidal dose response curve, whereas the formation rates of phenetidin were at the lower part of that plot.

The binding of phenacetin to blood constituents was determined in the absence and in the presence of phenobarbital 30 μg/ml, by equilibrium dialysis. Five sets of experiments were performed on five groups of three beagles each. Blood clotting was prevented by adding a few crystals of sodium citrate to each sample. Five ml of blood were put into 7 mm dia. cellophane bags, with 2·4 μm dia. pores (brand Viskin). Phenacetin was present in the blood at concentrations of 1, 5, 10 and 20 μg/ml. Duplicate bags, each in 5 ml of 0·05 M phosphate buffer pH 7·4, were moved for 18 hr at 4° by a machine currently in use in our department.[18] For chemical analysis of phenacetin duplicate assays were carried out with 1 ml of the buffer solution. References were prepared with phosphate buffer instead of blood. In order to prepare each bag, blood

containing 20 μg/ml of phenacetin was stirred for 20 min and was then diluted with blood to give the desired phenacetin concentration.

Chemical analysis. Phenacetin and unconjugated paracetamol were determined in plasma according to Brodie and Axelrod (3 and 4) by taking 0·5 ml of plasma for analysis of phenacetin, and 2 or 1 ml of plasma for the determination of paracetamol depending on whether phenacetin or paracetamol was injected.

For the determination of conjugated paracetamol 1 ml of urine was incubated with 0·1 ml of glucoronidase arylsulphatase and was further analyzed according to Brodie.[3] Further details were described previously.[15]

Phenetidin was also determined by Brodie's method.[4] 0·5 ml of microsomal suspension or 1 ml of plasma was extracted with benzene.

The paracetamol in microsomal suspensions was separated from phenacetin by thin layer chromatography[7] after extracting 3 ml twice with 15 ml of diethyl ether. A solvent system of dichlorethane/ethylacetate/98% formic acid: 60/36/4 was used. Paracetamol was eluted from silica Gel G (Stahl brand E. Merck, Darmstadt), by 3 ml of methanol. This procedure was necessary since about 5 per cent phenacetin present was detected by Brodie's method for paracetamol determination.[3] The amount of paracetamol was determined by the difference of the extinction at 247 and 285 nm using a 2 cm light path.

Phenazone was determined in 2 ml of serum, according to Brodie *et al.*[5] Concentrations of phenobarbital in 3 ml of blood were measured by ultraviolet spectrophotometry after thin layer chromatography. Details of the last two methods have been described previously.[15,12]

The concentration of protein in the microsomal suspension was determined according to Gornall *et al.*[11] One ml of a 0·9% sodium chloride solution, containing either 0·05 ml of the microsomal stock suspension or 0·5 ml taken from the vessels after drawing samples for paracetamol determination, was added to 4 ml of Biuret regent. The slight turbidity was corrected by measuring the extinction in the absence and in the presence of a few crystals of potassium cyanide. Protein concentrations in this paper have been calculated from the latter source.

Cytochrome P-450 was measured in the presence of about 3 and 6 mg of protein/ml of 0·15 M phosphate buffer pH 7·4 about 36 hr after the microsomal stock solution was prepared. Following the procedure published by Omura and Sato,[19] after reduction with sodium dithionite, carbon monoxide was bubbled through a few ml of the microsomal suspension for 60 sec. The extinction was recorded from 500 to 400 nm using a 0·2 cm light path with a Cary model 14 spectrophotometer.

Calculations. Linear regression lines were calculated from the data of at least four samples by applying the method of least-square analysis to the equation $y = \grave{a} - bx$ by standard program No. 201 for the Olivetti desk computer, Mod. 101.

To determine the biological half life $t/2$, the logarithms of the original data of concentration per ml (y) vs time (x) were typed into the machine. In this case b calculated equals $k_2 \times \log e$; $\grave{a}$ calculated represents the logarithm of the log y intercept at time zero. $t/2$ was computed by equation $t/2 = n2/k_2$. After injection of phenacetin $t/2$ of paracetamol was calculated from data measured from the 110th min.

Values for V_{max} and K_m of the enzyme reaction in microsomal suspension were calculated by entering the reciprocal value of the molar product concentration × mg of protein^{-1} × min^{-1} (y) vs the reciprocal value of substrate concentration (x) into the

computer (Lineweaver–Burk plot). Here, $\dot{a}$ being calculated represents the reciprocal value of V_{max}. b stands for tg α of the slope. Then, K_m was determined by equation

$$\frac{1}{K_m} = \frac{1}{V_{max}/\text{tg}\,\alpha}.$$

In addition, V_{max} and K_m were calculated by plotting v (initial rate of product formation) (y) against v/Sc (Sc = substrate concentration) (x). Advantages of this plot had been advocated by Hofstee[13] and Dowd *et al.*[10] In this case, $\dot{a}$ calculated by the computer stands for V_{max}, b calculated equals $-K_m$.

Statistical comparison of means was carried out either by t-Test, in case of dependent means, or by simple Analysis of Variance (Olivetti program number 166 and 203 for desk computer 101, respectively). In addition, Analysis of Variance for randomized block design with equal number of replicates within each unit was used for comparing the data on drug binding to blood constituents.

RESULTS

Experiments in vivo. After intravenous injection of phenacetin, paracetamol or phenazone in alcoholic solution the dogs usually vomited for a 5 min period, whereas the suspension in agar occasionally caused symptoms of pulmonary embolism immediately after or during the injection ceasing after about 1 min. During the experimental period the dog did not show any symptoms of illness or impairment of food intake.

In Fig. 1 the mean plasma concentration of phenacetin and paracetamol is shown against time after intravenous injection of equimolar amounts of phenacetin,

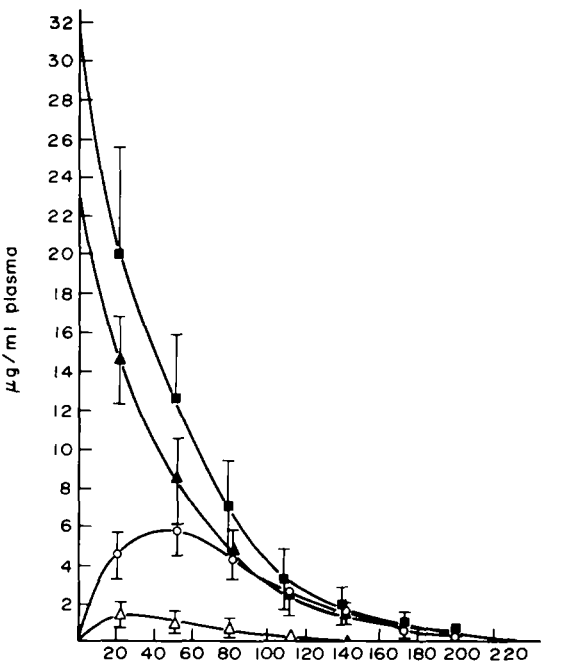

FIG. 1. Concentration of phenacetin, (▲) unconjugated paracetamol (○), and p-phenetidin (△) after injection of 30 mg phenacetin/kg and concentration of paracetamol after injection of 25·4 mg of paracetamol/kg body wt (■). The bars indicate the 95 per cent confidence limit of the mean (n = 12).

TABLE 1. APPARENT BIOLOGICAL HALF LIVES (min) OF INTRAVENOUSLY INJECTED PHENACETIN, PARACETAMOL, AND PHENAZONE IN DOGS BEFORE AND AFTER TREATMENT WITH PHENOBARBITAL (25 mg/kg) OR SKF 525 A (25 mg/kg)

	Treated with phenobarbital (tr)							
	Phenacetin		Paracetamol		Paracetamol after injection of phenacetin		Phenazone	
Dog no.	before	after tr	before	after tr	before	after tr	before	after tr
1	38·30	23·18	40·04	20·18	26·17	46·31	73·23	25·94
2	50·44	40·63	33·58	27·37	16·18	23·51	58·78	38·02
3	32·22	27·44	38·85	33·86	40·67	51·00	49·67	43·64
7	21·90	21·90	26·94	29·72	39·36	41·25	119·71	31·78
8	41·01	29·63	41·38	37·24	84·73	45·78	122·46	28·05
9	23·59	20·41	27·64	24·51	56·76	56·58	47·54	27·15
$\bar{x}$	34·58	27·20	34·74	28·81	43·98	44·07	78·57	32·43
t	3·19		1·93	0·94	0·01		3·02	
P<	0·025		0·2				0·05	
$v = 5$								

	Treated with SKF 525 A (tr)											
	Phenacetin			Paracetamol			Paracetamol after injection of phenacetin			Phenazone		
Dog no.	before		after tr	before		after tr	before		after tr	before		after tr
4	13·29	18·46	24·87	19·87	35·42	56·58	29·22	21·27	52·59	67·62	77·62	251·14
5	28·37	31·38	24·12	26·44	37·12	55·01	33·68	47·24	40·77	81·26	79·31	267·62
6	34·38	47·13	47·84	19·49	22·17	29·90	34·63	61·18	34·94	40·42	55·01	221·45
$\bar{x}$	25·35	32·32	32·28	21·93	31·57	47·16	32·51	43·23	42·77	63·1	70·64	246·74
t	0·003			3·86			0·03			27·33		
P<				0·1						0·025		
$v = 2$												

30 mg/kg, or paracetamol 25·4 mg/kg. The single data were obtained from the nine dogs listed in Table 1 before treatment with phenobarbital or SKF 525 A. After injection of phenacetin an appreciable concentration of paracetamol was found with a maximum 50 min after injection. The maximum concentration of *p*-phenetidin was found earlier (20 min) and was considerably less.

Assuming equilibrium distribution of phenacetin and its metabolite paracetamol, injected or formed in the body, the amount of paracetamol formed from phenacetin in the body can be calculated by comparing the areas under the concentration curves of paracetamol after injection of phenacetin or paracetamol.[9] Planimetric measurement indicates that in these dogs about 41 per cent of phenacetin was converted to paracetamol. During 36 hr after injection of phenacetin, 1·08 ± 0·24 S.E. per cent of the dose of phenacetin was excreted as phenacetin and 40·7 ± 9·1 S.E. per cent of the dose as paracetamol of which 4·3 ± 1·77 S.E. per cent was unconjugated.

After intravenous administration of phenacetin, paracetamol, or phenazone, the decreasing blood concentrations in each dog after each injection were found to lie approximately on a straight line on semilogarithmic paper from the 20th min onwards, indicating equilibrium distribution during the period of measurements and first order elimination. A linear decline of the unconjugated paracetamol formed in the body was seen on the plot after the 110th min post injection of phenacetin. The apparent biological half lives (min) of phenacetin, paracetamol and phenazone in nine dogs are listed in Table 1. After control values were obtained, six dogs were treated with phenobarbital (25 mg/kg), intramuscularly, the three other dogs received oral doses of SKF 525 A (25 mg/kg). Two control values were obtained from those dogs treated with SKF 525 A thereafter. The concentrations calculated at zero time before and after treatment with phenobarbital were for phenacetin 29·5 ± 6·0 S.E. and 43·5 ± 12·6 S.E. μg/ml plasma ($t = 1{\cdot}80$, $P < 0{\cdot}2$) and for phenazone 23·5 ± 5·9 and 25·8 ± 9·0 μg/ml plasma, respectively. After treatment with SKF 525 A the concentration of phenacetin and phenazone at zero time was unchanged.

The first three dogs (nos. 1, 2, 3) received phenacetin, paracetamol, and phenazone in alcoholic solution. This solvent did not seem to have any major effect on the biological half life of the solute as compared with the suspension in agar (dog nos. 4–9). The second control values obtained from dogs 4, 5 and 6, were surprisingly higher than the first ones ($P = 3{\cdot}31$) exceeding the 1 per cent level of significance (11° of freedom). However, the mean values of the second control from dogs 4, 5 and 6, were of the same order of magnitude as those control values determined from dogs 1–3 and 7–9.

A few weeks later, phenazone was injected twice into four beagles at a 1 week interval to repeat this part of the experiment. Since no tendency for a longer half life of phenazone was noticed after the second injection enzyme induction by pesticides, may outlast the period of quarantine.

The biological half life of phenazone was affected by both phenobarbital and SKF 525 A. After phenobarbital treatment the mean half life of phenazone in dogs 1–3, 7–9 dropped from 78 to 32 min, whereas after SKF 525 A treatment, the mean half life of phenazone in dogs 4–6 increased from 70 to 247 min.

The mean biological half life of phenacetin dropped from 34·6 to 27·1 min after treatment with phenobarbital ($P < 0{\cdot}025$); however, it remained unaffected by treatment with SKF 525 A.

TABLE 2. APPARENT V (n-mole/min/mg protein) OF PARACETAMOL AND OF PHENETIDIN AND APPARENT K_m (mM) OF PHENACETIN IN ISOLATED LIVER MICROSOMES FROM BEAGLES

	Dog no.	V paracetamol (1)	(2)	K_m (1)	(2)	V phenetidin (1)	(2)	K_m (1)	(2)	Content in microsomal suspension: Protein (mg/ml)	(n-mole/mg protein)
	10	0·99	0·95	0·71	0·63	58·19	7·88	31·83	2·61	3·32	0·42
	11	0·59	0·61	0·15	0·17	16·50	13·79	2·27	1·70	2·77	1·53
	15	2·11	2·12	0·14	0·14	21·43	19·47	5·83	5·19	3·61	0·92
	16	1·42	1·41	0·20	0·18	28·84	22·99	3·66	2·67	3·06	1·01
Control	$\bar{X}$	1·28	1·27	0·30	0·28	31·24	16·03	10·90	3·04	3·19	0·97
Treated with	12	2·34	2·33	0·17	0·16	33·23	23·72	4·77	3·04	4·51	4·89
phenobarbital	13	1·88	1·89	0·17	0·18	30·37	34·00	2·62	3·04	3·64	3·28
(25 mg/kg/D)	14	1·55	1·55	0·11	0·11	26·13	24·41	4·72	4·32	4·48	4·04
× 8	$\bar{X}$	1·92	1·92	0·15	0·15	29·91	27·38	4·04	3·47	4·21	4·07
Control F vs treated P	1.5	2·29 >0·05	2·31	0·84	0·90	0·01	5·59 >0·05	0·68	0·20	10·24 <0·05	42·96 <0·01

The apparent V was determined by plotting $1/v$ (initial reaction rate) vs 1/Sc (Substrate concentration) (1) and v vs v/Sc (2) after incubation of phenacetin at 0·3, 1, 2 and 3 mM concentration in microsomal suspensions prepared from the liver of male beagles. After a 10 min incubation period paracetamol was determined in the microsomal suspension in the presence of the NADPH regenerating system and paraoxon (10^{-5} M); phenetidin was measured in the absence of both.

The biological half life of paracetamol, both injected or formed in the dog, was not influenced by either treatment; the higher mean value in dogs treated with SKF 525 A, 47·2 vs 31·6, however, may well become significant with more animals.

In dogs, 7, 8 and 9, the concentration of phenobarbital in plasma was determined 24, 48 and 72 hr after the last injection of phenobarbital. The plasma concentrations were 34·8, 44·8, and 58·1 μg per ml 24 hr after injection; the apparent biological half life in plasma was about 22·4, 34·4, and 30·6 hr, respectively.

Experiments in vitro. Formation of paracetamol and phenetidin from phenacetin in isolated microsomes.

In the double reciprocal plot of product formation rate vs substrate concentration (Lineweaver–Burk plot) the data measured in each microsomal suspension prepared from the liver of male beagles, were found to lie on a straight line.

In Table 2 the results obtained with liver microsomes from seven beagles, three of which were treated with sodium phenobarbital 25 mg/kg/day for an 8 day period, are listed.

After treatment with phenobarbital the mean concentration of protein in the microsomal suspension (Table 2, 5th column) increased from 3·19 to 4·21 mg/ml ($P < 0·05$), the mean concentration of cytochrome P-450 in the microsomal suspension increased about four times from 0·97 to 4·07 μmole/mg of protein ($P < 0·01$).

Two values of V_{max} and K_m calculated for paracetamol and *p*-phenetidin formation on each microsomal preparation are listed. In column (1) the result obtained by plotting 1/initial rate of product formation (v) against 1/substrate concentration (Sc) is presented. In column (2) the result calculated by plotting v vs v/Sc is listed. No mean differences were found between both plots.

The mean value of V_{max} for paracetamol formation was slightly higher in microsomes from dogs treated with phenobarbital (1·92 μmole) as compared with the V_{max} obtained from untreated dogs (1·28 n-mole). However, comparison of the two means, also on the basis of reviewing the single data, does not indicate that the treatment with phenobarbital had an influence on V_{max} for paracetamol formation. The K_m for paracetamol formation remained unchanged after treatment with phenobarbital.

V_{max} for the initial formation of phenetidin was about 30 n-mole/min/mg protein, the K_m of this reaction was in the order of 5×10^{-3} M; the treatment of dogs with phenobarbital had no influence on the magnitude of either values.

The values of V_{max} and K_m for phenetidin formation were approximately 20 times higher than those of paracetamol whereas *in vivo* a much lower concentration of phenetidin than of paracetamol was found in plasma.

Binding of phenacetin to constituents of the blood and its displacement by phenobarbital. The influence of phenobarbital on the binding of phenacetin to blood contuents was also investigated, since treatment of dogs with phenobarbital had an effect on the apparent half life of phenacetin and caused a slight decrease in the apparent volume of distribution whereas no remarkable increase of the microsomal activity on *O*-dealkylation of phenacetin was found.

Blood was chosen as an accessible drug binding compartment of the body because the capacity for drug binding of this compartment should approximate more closely than plasma that of the protein pool of the total body; changes in binding of phenacetin to proteins by phenobarbital should be more pronounced in blood than in plasma or pure albumin solution.

The binding of phenacetin was measured at concentrations of 20, 10, 5 and 1 μg of phenacetin per ml of blood, 30 μg of sodium phenobarbital per ml of blood was added concurrently. The results are listed in Table 3.

TABLE 3. BINDING OF PHENACETIN (%) AT VARIOUS CONCENTRATIONS TO CONSTITUENTS OF THE BLOOD WITHOUT AND IN PRESENCE OF PHENOBARBITAL (30 μg/ml)

Phenacetin (μg/ml)	20	10	5	1	$F_{3,16}$
Binding (%)	47·8 ± 4·5	38·0 ± 3·8	50·3 ± 4·8	59·0 ± 3·5	4·22
with phenobarbital	53·8 ± 3·0	44·5 ± 1·3	47·3 ± 3·2	37·2 ± 4·3	4·77
$F_{1,8}$				15·3	

The values indicate the mean of five experiments ± S.E. $F_{1,8}$ 11·26 at P 0·01, $F_{3,16}$ 3·24 at P 0·05.

The binding of phenacetin increased only at low concentrations from 50 per cent at 5 μg/ml to 59 per cent at 1 μg/ml ($P < 0·05$). At the concentration of 1 μg phenacetin per ml blood, phenobarbital decreased phenacetin binding by 22 per cent. However, the analysis of variance showed no differences either between percentage binding at the various concentrations of phenacetin ($F_{3,32}$: 2·51) or between the phenobarbital treated and the untreated group ($F_{1,32}$: 1·39), by using the mean square of the sampling error for denominator.

DISCUSSION

In mongrel dogs pretreatment with phenobarbital shortened the apparent biological half life of phenacetin by 21 per cent and increased slightly, but not significantly, the mean concentration of phenacetin calculated at zero time of intravenous injection. This effect of phenobarbital treatment was not simulated by dehydration of the dogs since the apparent volume of distribution of phenazone remained unchanged. In other dogs, treatment with SKF 525 A had no effect on the apparent half life of phenacetin or its apparent volume of distribution.

In contrast, the mean apparent biological half life of phenazone was decreased by 41 per cent after treatment with phenobarbital and was increased by 260 per cent after treatment with SKF 525 A.

These findings suggest that phenacetin in dogs is not primarily metabolized *in vivo* by an enzyme system sensitive to inhibition by SKF 525 A, and, the shortening of the biological half life of phenacetin after phenobarbital treatment may have been caused by changes in apparent volume of distribution due to displacement of phenacetin from protein binding sites by phenobarbital.

Therefore, microsomal suspensions were prepared from seven beagles of which three were pretreated with phenobarbital. Treatment with phenobarbital effectively increased the mean protein content of the microsomal preparation and its cytochrome P-450 content.

The microsomal fraction contains the NADPH-dependent mixed function oxidase system transforming phenacetin to paracetamol by *O*-dealkylation, and an esterase converting phenacetin to *p*-phenetidin by hydrolytic *N*-deacetylation. In these experiments the apparent V and K_m for both reactions were determined. Treatment with phenobarbital neither increased the apparent velocity of paracetamol formation (V about 1·5 n-mole/min/mg protein) remarkably, nor the corresponding apparent K_m

(0·25 mM), indicating a much less effect of phenobarbital on the *O*-dealkylating enzyme system than on production of cytochrome P-450. Further experiments will provide more information about the enzyme system involved.

Compared with *O*-dealkylation of phenacetin, both values, the K_m of phenacetin for hydrolytic *N*-deacetylation (4 mM), and maximum velocity for *p*-phenetidin formation (30 n-mole/min/mg protein) were about 20 times greater. A V_{max} of that order of magnitude was also suggested by the data of Kiese and Renner.[16] Compared to *O*-dealkylation the low affinity of phenacetin to the microsomal esterase may explain the low concentration of *p*-phenetidin found in plasma of the living dog after intravenous injection of phenacetin in spite of a high reaction velocity. After oral administration of phenacetin to mongrels Baader *et. al.*[1] also found high peak concentrations of *p*-phenetidin and phenacetin.

The value of the apparent K_m for deacetylation, however, was about 20 times smaller in microsomes from dog liver than that found by Bernhammer and Krisch[2] with highly purified esterase prepared from pig liver microsomes (K_m: 2×10^{-2} M).

Büch *et al.*[6] found an increased elimination rate of paracetamol after phenobarbital treatment in rats, concluding that phenobarbital enhanced the conjugating reactions of paracetamol. Under the present experimental conditions that finding was not confirmed for dogs.

The slight increase in the mean concentration of phenacetin at zero time after phenobarbital treatment suggests that phenobarbital shortened the apparent biological half life of phenacetin by changing the apparent volume of distribution rather than by stimulating its metabolic rate of *O*-dealkylation, leading to paracetamol. This hypothesis was not confirmed by the measurement of phenacetin displacement from blood constituents by phenobarbital. At low concentrations of phenacetin (1 μg/ml) 30 μg of phenobarbital per ml decreased the binding of phenacetin by 20 per cent. Under the experimental design chosen, this displacement of phenacetin *in vitro* was not statistically significant, however.

In conclusion, the results of these experiments show that after intravenous injection of phenacetin into mongrel dogs it is partly metabolized by an NADPH-dependent *O*-dealkylating enzyme system of the liver which is insensitive to SKF 525 A *in vivo*. Phenobarbital shortened the apparent biological half life of phenacetin in mongrels, less likely to be a result of changing the volume of distribution of phenacetin by phenobarbital than by increasing enzyme activity for hydroxylation. More experiments are needed to prove or to reject statistically the minor difference found *in vitro*, for *O*-dealkylation of phenacetin after phenobarbital treatment. In future experiments enzyme activity of other metabolic pathways will be considered.

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