entropy of activation values for E_1 and E_2 were different, however, which suggested an explanation for the difference in reaction rates. The large negative values of $\Delta S^{\ddagger}$ reflect a significant requirement in molecular orientation, presumably of the side chains. The *cis*-Δ^5 double bond imposes certain restraints on the free rotation (lowering its degree of freedom) of the C-8 side chain vis-á-vis the C-12 side chain, causing increased nonbonded interactions when both chains lie side by side. These factors become especially important when a carbanion is introduced into the cyclopentenone ring at C-12, forcing the associated side chain and ring to lie flat in one plane. Some of these interactions are relieved if the unsaturation in the C-8 side chain is removed. It then has a more "floppy" nature, which allows it to lie away from the now rigid C-12 side chain. Thus, E_2 contains a lower absolute entropy in its reactant state than E_1, which must accordingly suffer a greater entropy loss during conversion to its carbanion transition state. These interpretations hold only qualitative value since absolute thermodynamic parameters must be determined only at zero buffer concentrations. Nevertheless, the data presented here retain comparative significance and indicate that the difference in behavior between E_1 and E_2 is clearly the result of complicated conformational effects.

In summary, there is a difference in the rates of dehydration and rearrangement between E_1 and E_2. No well-defined mechanism illustrating the reason for this difference can be presented at this time; however, such a mechanism should involve participation of the *cis*-Δ^5 double bond which is present only in E_2.

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Metabolism of the Antihypertensive Agent 1,4-Dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic Acid Diethyl Ester

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Abstract □ The absorption, distribution, metabolism, and excretion of 1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid diethyl ester, an antihypertensive drug, was studied in rat, dog, and squirrel monkey. The drug, labeled with ^{14}C, was found to undergo aromatization, hydrolysis of its ester groups, and oxidation of a ring methyl and an ethyl group. No dihydropyridyl metabolites or the hypothetical aromatized parent compound were found. The metabolites identified were: 2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid, its monoethyl and mono-2-hydroxyethyl esters, and 2-hydroxymethyl-6-methyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid lactone. The form in which the drug was administered was found to have a profound effect on its absorption. The crystalline powder administered to dogs in gelatin capsules was poorly absorbed; solubilization with polyethylene glycol 200 resulted in a fivefold increase in absorption.

Keyphrases □ 1,4-Dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid diethyl ester, radiolabeled—absorption, distribution, metabolism, and excretion in rats, dogs, and monkeys □ Antihypertensive agents—metabolism of 1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid diethyl ester, in rats, dogs, and monkeys □ Absorption—1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid diethyl ester, in rats, dogs, and monkeys

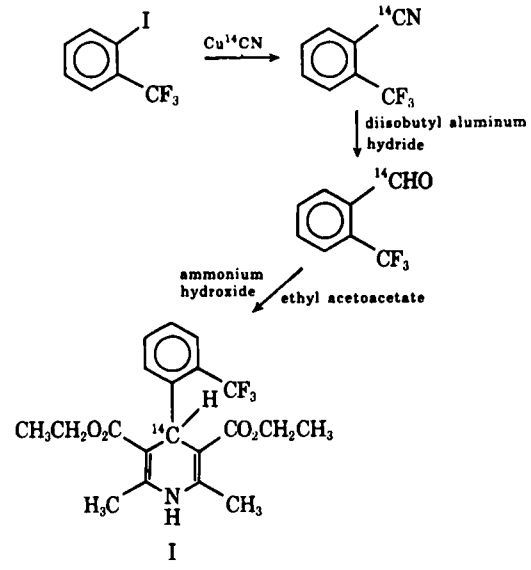
1,4-Dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid diethyl ester[1] (I) is an effective agent for lowering blood pressure by direct vascular dilatation in normotensive, neurogenic, and renal hypertensive dogs. Hypotensive effects were also observed in rats, rabbits, and guinea pigs. The drug also lowered blood pressure in human subjects at relatively low oral doses (10 mg.).

As part of the preformulation and preclinical investigation of this compound, its absorption, distribution, metabolism, and excretion in rats, dogs, and monkeys were studied. These studies were facilitated by the use of the ^{14}C-labeled drug.

EXPERIMENTAL

Synthesis of Labeled Drug—Compound I-4-^{14}C was prepared as shown in Scheme I. ^{14}C-Labeled drug was shown to be free of radiochemical impurity and had a specific activity of 4 μc./mg.

[1] SK&F 24260.

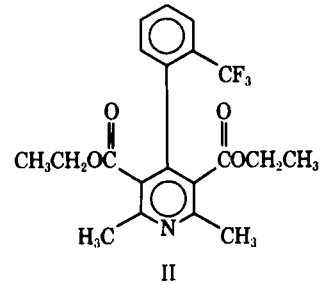


I

Scheme I—Synthesis of 2,6-dimethyl-3,5-dicarboethoxy-4-(2-trifluoromethylphenyl)-1,4-dihydropyridine-4-^{14}C

GC Assay of I and Metabolites—*Compound I*—Five milliliters of urine containing at least 80 ng. of drug/ml. was adjusted to neutral pH and shaken with 28 ml. *n*-hexane for 20 min. Following centrifugation for 5 min., 25 ml. of the hexane layer was transferred in portions of 10, 10, and 5 ml. to a fresh tube containing 1 ml. methanol. Each portion was blown down, and the sides of the tube were rinsed with methanol before adding a fresh portion. After the final (5 ml.) hexane addition and methanol wash, 0.4 ml. internal standard solution (dichlorodiphenyltrichloroethane, 1.6 mcg./ml. in methanol) was added and the solution was evaporated to dryness under nitrogen in a water bath at 40°. The tubes were kept in a desiccator overnight before derivatizing with an excess of heptafluorobutyric anhydride by heating for 2 hr. at 70°. The samples, after evaporation to dryness at room temperature under a stream of nitrogen, were taken up in 0.5 ml. methanol. A 3-μl. aliquot was taken for injection.

The column was 1.22 m. (4 ft.), glass [0.48-cm. (0.19-in.) o.d.], 3% OV-1, on 100–120-mesh Chrom W at 250°. The carrier gas was argon–methane (95:5); purge 70 ml./min. at 35 ml./min.; purge at 70 ml./min. The electron-capture (Ni) detector temperature was 270°, and the injection block and manifold temperature was 270°. The retention times of I and dichlorodiphenyltrichloroethane were 2.3 and 4.1 min., respectively. The standard curve (peak height ratios) was linear from 80 to 400 ng./ml. urine.



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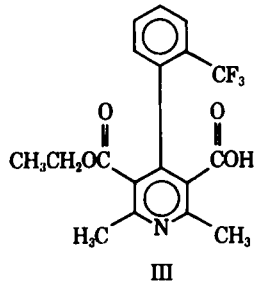

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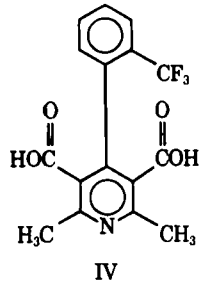


IV

Table I—R_f Values on Thin-Layer Chromatograms of Compound I and Metabolites

Metabolite[a]	Solvent A	Solvent B	Solvent C
I	0.95	0.24	0.86
II	0.95	0.46	0.90
III	0.75	0.06	0.21
IV	0.46	0.00	0.05
V	0.74	0.00	0.24
VI	0.74	0.00	0.44

[a] See Scheme II.

Table II—Urinary Metabolites of ^{14}C-Labeled I

Metabolite[a]	Dog, 0–24 hr., %	Rat, 0–7 hr., %	Squirrel Monkey, 0–24 hr., %
III	48[b]	68	15.5
IV	7.5	1.5	32
V	9.5	7	4
VI	6	3	3

[a] See Scheme II for structure. [b] Percent of urinary radioactivity.

Compound II[2]—Five milliliters of urine containing at least 1 mcg./ml. of II was adjusted to pH 10 by addition of 2 *N* sodium hydroxide and shaken with 28 ml. ether for 15 min. After centrifuging for 5 min., 25 ml. of the organic phase was transferred to a 40-ml. tapered tube, 0.1 ml. of internal standard (dichlorodiphenyltrichloroethane, 250 mcg./ml. in methanol) was added, and the sample was evaporated to dryness with a stream of nitrogen. The samples were then taken up in 0.1 ml. methanol, and a 2.6-μl. aliquot was taken for injection.

The column was 1.22 m. (4 ft.) stainless steel [0.32-cm. (0.125-in.) o.d.], 3% OV-1 on 100–120-mesh Chrom W at 195°. The carrier gas was helium at 40 ml./min. The flame-ionization detector temperature was 270°, and the injection block temperature was 270°. The retention times of II and dichlorodiphenyltrichloroethane were 3.0 and 6.6 min., respectively. The standard curve was linear from 1 to 5 mcg. II/ml. urine.

Compounds III[3] and *IV*[4]—Five milliliters of urine containing at least 1 mcg. of either III or IV was brought to pH 1 with 6 *N* hydrochloric acid and shaken with 28 ml. of ether for 15 min. After centrifuging for 5 min., 25 ml. of the organic layer were transferred to a 40-ml. tapered tube and evaporated to dryness under a stream of nitrogen. To the dried residue were added 0.1 ml. methanol and 0.3 ml. diazomethane and the contents of the tube were thoroughly mixed. After 1 hr., 0.2 ml. 7 *N* sodium hydroxide and 6 ml. of *n*-hexane were added. The tube was shaken for 20 min. and centrifuged for 5 min. As much of the hexane layer as could be removed with a disposable Pasteur pipet was transferred to a 12-ml. centrifuge tube and, after adding 0.1 ml. of internal standard solution (methyl oleate, 250 mcg./ml. in methanol), evaporated to dryness. The residue was taken up in 0.1 ml. methanol, and a 2.4-μl. aliquot was taken for injection.

Except for the column temperature (180° for these two compounds), the column and conditions were the same as for II. The retention times of III, IV, and methyl oleate were 3.1, 4.1, and 6.1 min., respectively. The standard curves for both compounds were linear from 1 to 5 mcg./ml. urine.

Radioassay—Standard techniques were used to assay 0.1-ml. aliquots of blood and urine and 75–100-mg. aliquots of dried feces.

TLC—Three solvent systems were used to separate I and metabolites on 250-μ plates[5]: A, chloroform–methanol–acetic acid (50:50:5); B, toluene–methanol (95:5); and C, chloroform–acetic acid (95:5). The R_f values found are listed in Table I.

Animal Studies—The animal studies were conducted on Charles River Sprague–Dawley strain rats, squirrel monkeys, and beagle

[2] SK&F 36077.
[3] SK&F 37700.
[4] SK&F 37866.
[5] Merck F_{254}.

Table III—Effect of Dosage Form on Urinary Output of ^{14}C following Oral Administration of ^{14}C-Labeled I

Dosage Form	Dog Number	Percent of Dose Recovered in Urine, 0–96 hr.	Percent of Dose Recovered in Feces, 0–96 hr.
Crystalline drug in gelatin capsule	1	6.9	91.3
	2	7.7	91.9
1.5% solution in polyethylene glycol 200–water (1:1)	3	39	48.9
	4	32	48.9

Table IV—Effect of Dosage Form on Blood Levels of ^{14}C in Dogs after Oral Administration of ^{14}C-Labeled I

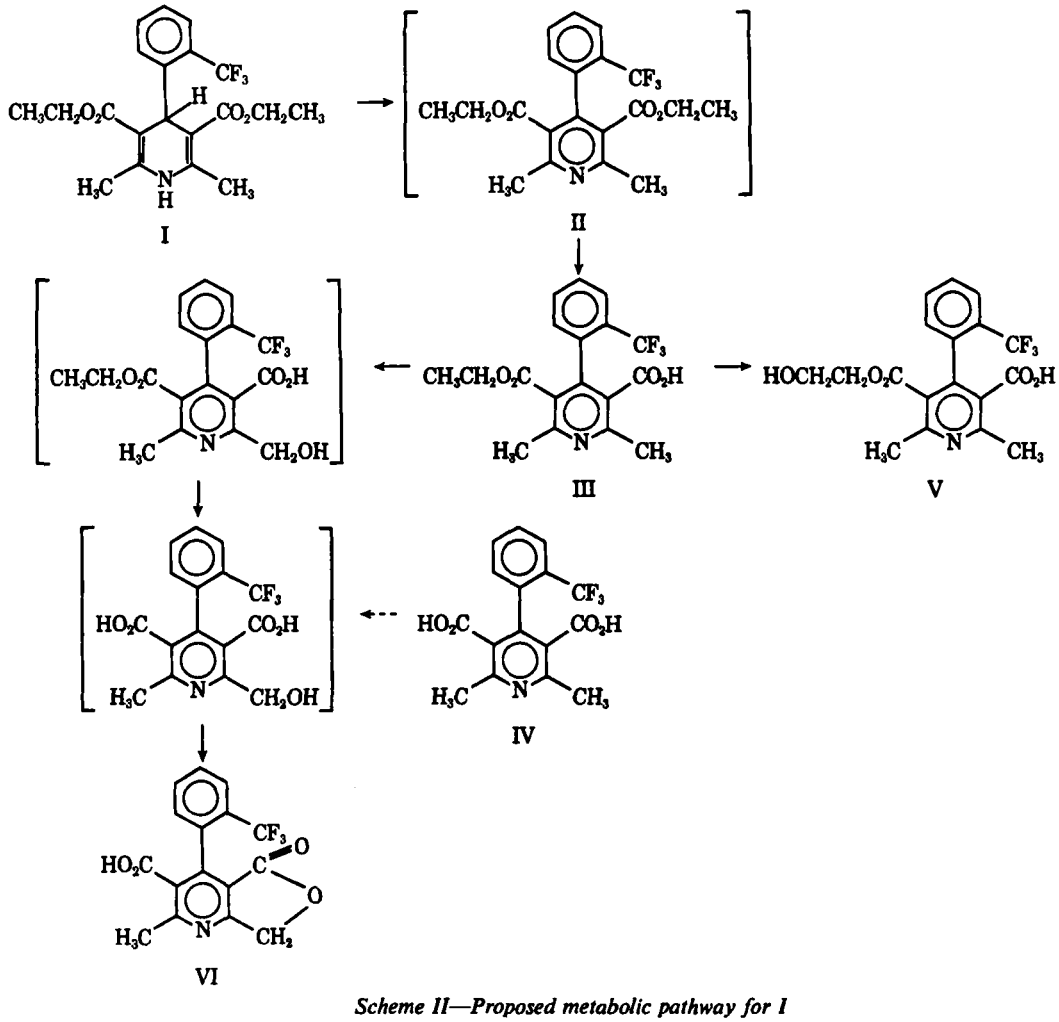
Dosage Form	Dog Number	^{14}C Blood Level (Expressed as ng./ml. of I) Hours 1	2	3	4	6	24
Crystalline drug in gelatin capsule	1	140	150	70	40	30	10
	2	90	140	80	50	40	20
1.5% solution in polyethylene glycol 200–water (1:1)	3	450	920	730	470	260	70
	4	510	910	670	510	360	50

dogs. After an overnight fast, oral doses of suspensions or solutions were administered to rats and squirrel monkeys by gavage. Beagles were given oral doses in gelatin capsules. Polyethylene glycol 200–water (1:1) solutions of drug were used for intravenous dosing. The drug concentration in various dosage media was 1 mg./ml. For collection of urine and feces, animals were kept in metabolism cages.

Enzyme induction studies were conducted in rats kept on an insecticide-free diet for at least 5 days. Following this period and while being maintained on this diet, rats were dosed with a finely ground suspension of drug in 0.5% gum tragacanth, one group receiving a single dose and another group receiving daily doses for 11 days. Amobarbital sleep time and ascorbic acid excretion studies were performed on the same group of rats kept 11 days on the insecticide-free diet and dosed daily with drug (5, 15, or 50 mg./kg.) by the oral route, or with a single intraabdominal dose of phenothiazine (15 mg./kg. in sesame oil) at the end of that period. Ascorbic acid was assayed by a modification of the method of Schaffert and Kingsley (1).

Phenylbutazone plasma half-life studies were conducted in animals kept free of drugs for at least 6 weeks. Phenylbutazone (10 mg./kg.) was injected into a leg vein, and venous blood was sampled from the opposite limb at 0.5, 1, 2, 3, and 5 hr. postdrug. Test drug and phenobarbital groups were dosed for a week and the phenylbutazone treatment was repeated. Plasma assays were performed using the method of Burns *et al.* (2).

For *in vitro* studies, livers were removed from rats kept on the insecticide-free diet. These were divided into groups dosed with saline, I, phenobarbital (chronic dosing), or phenothiazine (single dose). The control (saline) group provided sufficient microsomal enzyme to assay the effects of inhibition or stimulation when I, phenothiazine, or phenobarbital was added directly to the enzyme preparation.



Scheme II—Proposed metabolic pathway for I

Table V—^{14}C Blood Levels and Excretion following Oral Administration of Polyethylene Glycol 200 Solutions[a] of I to Squirrel Monkeys (Expressed as ng./ml. of I)

Animal Number	Blood Level, ng./ml. Hours 1	2	3	4	5	6	24	Percent of Dose Urine, 0–48 hr.	Feces, 0–48 hr.
1	310	510	410	330	340	330	90	8.1	70.6
2	190	320	390	430	410	530	320	11.8	45.5

[a] Polyethylene glycol 200–water (1:1).

Table VI—Effect of I on Drug-Metabolizing Enzyme Systems

Test	Species		Results		
Amobarbital sleep time	Rat		No significant reduction in sleep time as compared with phenobarbital Vehicle: 84 ± 35 min. Phenobarbital: 10 ± 2.1 min. I: 75 ± 30.3 min.		
Ascorbic acid excretion	Rat		Treatment: urinary ascorbic acid in mcg./g. body weight/24 hr. None: 30 I (5 mg./kg.): 42 I (15 mg./kg.): 46 Phenobarbital: 115 (50 mg./kg.)		
Effect of phenylbutazone plasma half-life	Dog		Dosing for 7 days with phenobarbital significantly speeded reduction of phenylbutazone half-life (from 2.5 to 1 hr.); I was without effect		
Liver microsomes *in vitro*	Rat		Phenothiazine, 15 mg./kg.	Compound I, 5 mg./kg.	Compound I, 15 mg./kg.
O-Dealkylation (*p*-nitroanisole)		Single dose	33%[a]	8%	−11%
Phenyl ring hydroxylation (acetanilide)		Single dose	120%	60%	60%
			Sodium phenobarbital, 50 mg./kg.	Compound I, 5 mg./kg.	Compound I, 15 mg./kg.
O-Dealkylation (*p*-nitroanisole)		Chronic dosing	67%	60%	13%
N-Demethylation (aminopyrine)		Chronic dosing	4%	23%	4%
Phenyl ring hydroxylation (acetanilide)		Chronic dosing	100%	78%	27%
Nitro-reduction (*p*-nitrobenzoate)		Chronic dosing	75%	0	0
Aliphatic side-chain oxidation (hexobarbital)		Chronic dosing	300%	225%	22%

[a] Percent increase over saline controls.

The microsomal enzyme was prepared as follows. Freshly removed livers were rinsed in ice-cold 1.15% potassium chloride before homogenization in chilled pH 7.4 0.2 *M* phosphate buffer (2 ml./g. tissue). No more than three to five strokes in an ice-cooled glass homogenizer tube were required. After centrifugation at 9000×*g* for 30 min. at 2–3° and removal of a white layer from the top of the tube, the supernate was decanted for use. This preparation maintained its activity even after being kept 5 months in the frozen state.

Incubations were carried out in 30-ml. beakers in a Dubnoff shaker–incubator at 37°, 70–75 oscillations/min. Oxygen was used as the gas phase in all but the nitro-reductase experiment, in which nitrogen was used. Gas flow was kept at 1 l./min. Each beaker contained 1 ml. of the enzyme preparation, 25 μM glucose-6-phosphate, 1–2 μM nicotinamide adenine dinucleotide phosphate, 34.5 μM magnesium sulfate, substrate (quantities shown below), and 0.2 *M* pH 7.4 sodium phosphate buffer to a final volume of 5 ml. In the *N*-dealkylation reaction assay with aminopyrine, 45 μM semicarbazide was included in the reaction mixture. The enzyme preparation was added last, following a 10-min. incubation period. The reaction was then followed for varying periods: aminopyrine, 5 min.; acetanilide, 5 min.; *p*-nitrobenzoic acid, 10 min.; *p*-nitroanisole, 3 min.; and hexobarbital, 5 min.

Analytical methods used in the *in vitro* studies were as follows. *N*-Demethylation of aminopyrine was determined by trapping formaldehyde derived from the methyl group in semicarbazone and by the Cochin and Axelrod (3) modification of the Nash method. Ring hydroxylation of acetanilide was determined by the method of Brodie and Axelrod (4). Reduction of *p*-nitrobenzoic acid to *p*-aminobenzoic acid was determined by measurement of the latter using the Bratton–Marshall reaction (5). *O*-Demethylation of *p*-nitroanisole was followed by spectrophotometry of the product, *p*-nitrophenol, at 396 nm. (6). Disappearance of hexobarbital was determined by the method of Brodie *et al.* (7).

RESULTS AND DISCUSSION

Biotransformation[6]—The ester linkages of I are highly resistant to hydrolysis, withstanding boiling in the presence of acid or base or incubation in the presence of various enzyme preparations. However, upon oxidation of the dihydropyridyl ring, hydrolysis takes place readily. This was borne out by the failure to find in

[6] Details to be published separately.

Table VII—Distribution of ^{14}C in Blood of Human, Dog, and Rat following Equilibration with ^{14}C-Labeled I *In Vitro*

Species	Percent of Added Drug Found		
	Plasma Proteins	Cells	Plasma Water
Human	83 ± 5	16 ± 5	0.9 ± 0.7
Dog	86 ± 7	13 ± 8	0.8 ± 0.3
Rat	91 ± 4	8 ± 4	1.4 ± 0.3

urine any aromatized parent drug (II), the expected intermediate (Scheme II). Nevertheless, both expected deesterified metabolites were found (III and IV). Two remaining metabolites, both unanticipated, were the results of β-oxidation of an ethyl group (V) and oxidation of a 2-methyl group which apparently underwent spontaneous lactonization with an adjacent carboxyl (VI), respectively.

Quantitative differences in the metabolites were observed in the urine of three species, with III predominating in the rat and dog and IV in the squirrel monkey. Both metabolites, however, account for half or more of the urinary ^{14}C (Table II).

The relative amounts of the dose excreted in urine and feces were highly dependent upon the dosage form. When the drug was given to dogs as the crystalline drug (in gelatin capsules), only about 7% was recovered in urine. However, when dissolved in a mixture of polyethylene glycol 200–water (1:1), a four- to fivefold increase in urinary output was observed, indicating enhanced absorption of the solubilized drug (Table III).

Blood levels were correspondingly higher in the animals dosed with the solution (Table IV). The mean blood level over the 24-hr. period following the dose of the polyethylene glycol 200 solution of drug was almost seven times that attained in the dogs dosed with crystalline drug. Peak levels in the dog dosed with either form appeared at about 2 hr. Similar results were seen in one squirrel monkey following doses of polyethylene glycol solutions of drug (Table V), but another monkey showed a delayed peak at 6 hr.

Irrespective of dosage form, the major portion of ^{14}C excreted in feces of the dog and squirrel monkey appeared from TLC to be the parent drug, which accounted for roughly half of the radioactivity excreted by this route.

Enzyme Induction Studies—Because I is intended for a condition requiring chronic treatment, it was of interest to determine its effect during chronic use on metabolizing enzymes, since either inhibition or stimulation of the enzymes could conceivably alter the physiological response to a given dose. Accordingly, a number of widely used tests were run and the results are summarized in Table VI.

These tests indicated that a weak to moderate induction of some metabolic pathways (ascorbic acid excretion, phenyl ring hydroxylation, *O*-dealkylation, *N*-demethylation, and aliphatic side-chain oxidation) occurred following oral administration of I. The reverse effect, inhibition of the same microsomal enzyme systems, was also tested *in vitro* by incubation of I with the liver microsomal preparations and the various test compounds referred to in Table VI. Compound I was found to be without significant effect on the metabolic pathways investigated.

Protein Binding Studies—Because of the possible influence of binding to plasma proteins on the sojourn and distribution of the drug following absorption, it was of interest to determine the extent to which binding could occur. The results (Table VII) indicate that very little (about 1%) drug remains "free" in the plasma of human, dog, or rat blood; over 80% was bound to protein and the remainder to cellular components.

These findings may not, however, be of much significance since, at times of peak ^{14}C concentrations in blood following an oral dose, only about one-quarter of the low levels present could be accounted for as parent drug (Table VIII) in the rat and squirrel monkey and

Table VIII—^{14}C "Fingerprint" of Animal Blood following Oral Dose of ^{14}C-I[a]

Species	^{14}C Peak Time, hr.	^{14}C Concentration, mcg./ml.	Percent of Dose on TLC at R_f of Metabolites			
			Parent	II	III	IV–V[b]
Rat	7	1.81	30	59	1	5
Dog	2	0.91	2	7	10	45
Squirrel monkey	3	0.40	24	19	26	6

[a] 5 mg./kg. in polyethylene glycol 200–water (1:1). [b] Expressed as equivalent amounts of I.

only 2% in dog. Only the more significant metabolites are shown; at least six or eight additional metabolites appearing on the radioscans were not identified.

CONCLUSION

Compound I, as a consequence of its poor aqueous solubility, was found to require prior solubilization to enhance absorption. It appears to be rapidly metabolized in the species investigated (rat, dog, and squirrel monkey), which may account for its relatively short duration of action[7]. None of the metabolites identified nor the postulated intermediates was found in animal studies[8] to approach the hypotensive effect of the parent drug in potency. Because of the observed weak inductive effect on some of the drug-metabolizing enzyme systems, subjects treated concomitantly with other drugs should be observed for the possibility of drug interactions.

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[7] B. Walker, preliminary clinical observations.

[8] J. Petta and R. McLean, unpublished observations.