Metabolites of Probenecid. Chemical, Physical, and Pharmacological Studies†

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Probenecid, its metabolites (including ^{14}C labeled), and several analogs were synthesized. The uv spectral properties, pK_a's, and partition coefficients were determined. The binding of probenecid and metabolites to human plasma, human albumin, and dog plasma was measured. Pmr parameters of probenecid, its metabolites, and 9 other analogs were obtained. There was no correlation between the nature of the alkyl side-chain substituents or the partition coefficient and the chemical shifts of the aromatic ring protons. In rats all the metabolites with a single exception were terminal metabolites; similar results were obtained *in vitro*. Propionic acid was identified as one of the metabolic products of probenecid. The effects on urate clearance of probenecid, the various metabolites, and the piperidyl analog were determined in Dalmatian and mongrel dogs. In Dalmatians the clearance decreased while in mongrels it increased. Our studies suggest that the metabolites of probenecid may very well play a significant role in the overall uricosuric effect of the parent drug.

The structures of essentially all the metabolites of probenecid in rat bile[1] and human urine[2-4] have been elucidated. The major routes of biotransformation are oxidation of the side chain (Figure 1, Table I) and glucuronide conjugation. In man, formation of the acyl glucuronide accounts for the disposition of about 0.2 of the drug.[2,3] Incubation of probenecid-^{14}C with reinforced rat liver preparations leads to the same metabolites as found *in vivo*.[5]

The purpose of the present study was (1) to synthesize the metabolites (labeled and unlabeled) of probenecid, (2) to learn whether they could contribute to the overall pharmacologic activity of the parent drug, and (3) to extend previous investigations of structure-activity relationships in this series.[6] The latter studies included detailed analyses of high-resolution proton magnetic resonance (pmr) spectra, pK_a determinations, and measurement of drug-protein binding.

Table I. Structure and Physical Properties of Probenecid and Its Metabolites

Compound	R[a]	λ_{max}, mμ	log ε	pK_a[b]	K_p[c]	K_p'[c]
2	$CH_2CHOHCH_3$	244	4.02	5.8	<0.01	0.3
5	$CH_2CH_2CH_2OH$	244	4.01	5.8	<0.01	0.3
8	CH_2CH_2COOH	244	4.01	5.9	<0.01	4.5
9	H	227	4.02	5.8[d]	<0.01	0.4
Probenecid[e]	$CH_2CH_2CH_3$	244	4.01	5.8[d]	0.03	<0.002

[a] *p*-$HOOCC_6H_4SO_2N(R)CH_2CH_2CH_3$. [b] p$K_a$'s determined in 75% (v/v) EtOH; the pK_a of probenecid in water is 3.4[4,6] [c] K_p = Sörensen buffer (pH 7.4) and peanut oil; 2 ml of buffer containing 100 μg/ml were added to 20 ml of oil and shaken. K_p' = chloroform–0.15 *N* HCl; 30 ml of an HCl solution containing 15 μg/ml was shaken with 3 ml of washed chloroform for 1 hr. [d] Ref 6. [e] The piperidyl analog has log ε 4.14 (λ_{max} 238 mμ); its K_p and K_p' were 0.002 and 0.011, respectively.

Experimental Section

A. Synthesis.‡ Our general procedure for synthesis of probenecid analogs (adapted from Miller[7]) was: to an ice-cold soln of the appropriate secondary amine (0.12 mole) in 25 ml of anhyd MeOH 8.8 g (0.040 mole) of *p*-(chlorosulfonyl)benzoic acid (1)§ was added and the mixt stirred at room temp overnight. The MeOH was removed *in vacuo* and the oily residue taken up in 25 ml of H_2O. The pH was adjusted to 1 with concd HCl and the resulting ppt collected by filtration, then dissolved in a slight excess of 0.1 *N* NaOH (pH >10). After one extn with Et_2O and repptn with HCl, the compd was crystd from EtOH–H_2O, with prior charcoal treatment, and dried at 110° *in vacuo*. Purity of the analogs was detd by capillary mp in a Thomas-Hoover Uni-Melt, by known R_f values on tlc,[2] and by elemental analysis. The ^{14}C compds# were synthesized by a modification of the procedure of Motoichi, *et al.*[8] Ring ^{14}C-labeled probenecid and some nonlabeled analogs were a gift from Dr. J. E. Baer, Merck Institute for Therapeutic Research.

dl-p-(*N*-Propyl-*N*-2-hydroxypropylsulfamoyl)benzoic Acid (2). Synthetic method was essentially the same as that reported by Guarino, *et al.*[1] After one addnl crystn (EtOH–H_2O) with prior charcoal treatment, the yield was 72% from 1; mp 197–198° (lit.[1] value in agreement). *Anal.* ($C_{13}H_{19}NO_5S$) C, H, N, S.

dl-p-(*N*-Propyl-*1*-^{14}C-*N*-2-hydroxypropylsulfamoyl)benzoic Acid (2-^{14}C). A soln of propylene oxide (4.5 mmoles, 0.26 g) in 0.9 ml of MeOH was added to *n*-propylamine-*1*-^{14}C** (5 mmoles, 0.26 g, 60 μCi) in 2 ml of MeOH at 0°. The reaction mixt was stirred at room temp for 16 hr. Untreated amine was removed *in vacuo*.

The residue was cooled (0°) and cold aqueous NaOH (16 ml, 10%) was added, followed by 1 (4.5 mmoles, 1.02 g). The reaction mixt was stirred at room temp for 3 hr and then acidified with 10% HCl. After standing overnight at 0°, the white ppt was collected, washed with ice-cold H_2O, and dried *in vacuo* (80°, 12 hr); yield 0.85 g (63%). The product was recrystd (EtOH–H_2O).

Propylaminopropyl Acetate (3). To cold 3-chloropropanol†† (45 g, 0.48 mole) cold AcCl (55 g, 0.70 mole) was added slowly with stirring. After stirring 1 hr at room temp, 25 ml of H_2O was added and chloropropyl acetate was collected by distn; yield 62 g (95% from the alc); bp 163–165° (lit.[9] value in agreement). The ester (34 g, 0.25 mole), *n*-propylamine (59 g, 1.0 mole), 15 g of anhyd Na_2CO_3, 5 g of finely powd KI, and 100 ml of abs EtOH were refluxed 36 hr with stirring. Upon filtration and distn, 25 g (63% from the ester) of colorless oil 3 was collected; bp 200–210°.

Propylaminopropan-3-ol (4). The ester 3 (25 g, 0.16 mole) was added to 50 ml of 30% aqueous NaOH and refluxed 5 hr. The resulting 4 was extd into Et_2O and the organic phase dried with KOH. Upon distn, 15 g (82% from 3) of colorless oil was collected; bp 198–201° (lit.[10] value in agreement).

p-(*N*-Propyl-*N*-3-hydroxypropylsulfamoyl)benzoic Acid (5).

†Supported by U. S. Public Health Service Grants GM-14270, GM-10848, and A-162.

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‡All mp are uncorrected. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values. Analyses were performed by Midwest Microlab, Ltd., Indianapolis, Ind., and by courtesy of Mr. K. B. Streeter, Merck Sharp & Dohme, West Point, Pa.

§From Eastman Kodak Co., 97% purity.

#Radiochemical purity was established for compounds 2-^{14}C, 5-^{14}C, 8-^{14}C, 9-^{14}C, and 12-^{14}C by the following criteria: mixture melting point, isotope dilution with known carrier, and thin-layer radiochromatography (greater than 99.9% of ^{14}C was found in the corresponding spot).

**From Mallinckrodt Nuclear, St. Louis, Mo.

††From Aldrich Chemical Co., Inc., Milwaukee, Wis.

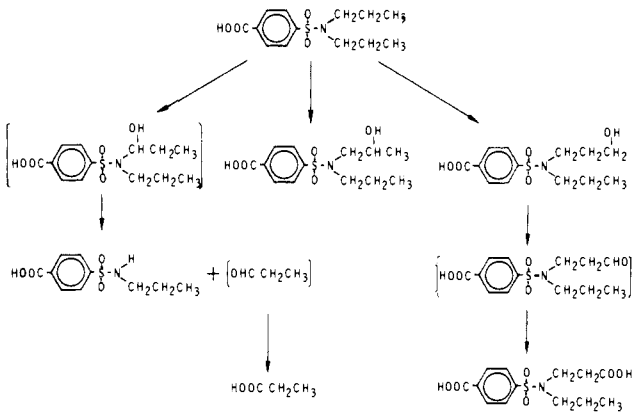


Figure 1. Metabolic pathways of probenecid. Proposed intermediates are given in parentheses. β-Glucuronides of 3-OH and 2-OH have been demonstrated.[1] The formation of acyl glucuronide of probenecid in man and rat has been cited earlier.[4,23] Still other glucuronides could occur in small amounts.

Compd **5** was obtained in 52% yield from 1 and was shown by tlc to be impure. An analytical sample was obtained by preparative tlc, system I (silica gel G, Merck, 0.30 mm; C_6H_6–EtOAc–AcOH, 7:3:1). Elution of the appropriate tlc silica region with warm EtOH and recrystn (EtOH–H_2O) yielded white crystals; mp 168–169°. *Anal.* ($C_{13}H_{19}NO_5S$) C, H, N, S.

p-(*N*-Propyl-*1*-^{14}*C*-*N*-3-hydroxypropylsulfamoyl)benzoic Acid (5-^{14}*C*). The compd was prepd on a mmole scale by the method used for the unlabeled **5**. Mixed mp with an authentic sample was not depressed.

Propylaminopropionitrile (6). Acrylonitrile (27.5 g, 0.5 mole) was added dropwise with stirring during 1.5 hr through a H_2O-cooled reflux condenser into cold *n*-propylamine (46 g, 0.78 mole). Fractional distn *in vacuo* gave 49 g (88% from acrylonitrile) of 6; bp 40–42° (0.06 mm).

p-(*N*-Propyl-*N*-3-propionitrilosulfamoyl)benzoic Acid (7). Compd 6 was treated with 1 as described above to yield 7.7 g (65% from 1) of white crystals; mp 213–215°. Titration of 7 with aqueous NaOH gave 1.0 equiv of H per mole.

p-(*N*-Propyl-*1*-^{14}*C*-*N*-3-propionitrilosulfamoyl)benzoic Acid (7-^{14}*C*). Acrylonitrile (4.5 mmoles, 0.24 g in 0.9 ml of MeOH) was added to *n*-propylamine-*1*-^{14}*C* (5 mmoles, 0.26 g, 60 μCi in 2 ml of MeOH) at 0° and then stirred at room temp for 24 hr. After removing excess amine, the residue was treated with 1 (4.5 mmoles, 1.02 g) as described for **7**; yield 0.78 g (63%).

p-(*N*-Propyl-*N*-2-carboxyethylsulfamoyl)benzoic Acid (8). After dissolving 7.7 g of **7** in 200 ml of DMSO, the mixt was cooled and 200 ml of cold 50% aqueous H_2SO_4 containing 8 g of NaCl was slowly added. The mixt was refluxed 24 hr and chilled, and 400 ml of cold H_2O added. The ppt was recrystd (EtOH–H_2O) with prior charcoal treatment to yield 6.7 g (53% from 1) of white crystals, mp 203–204°. Aqueous titrn of 8 with NaOH gave 2.0 equiv of H per mole. *Anal.* ($C_{13}H_{17}NO_6S$) C, H, N.

p-(*N*-Propyl-*1*-^{14}*C*-*N*-2-carboxyethylsulfamoyl)benzoic Acid (8-^{14}*C*). The nitrile, 7-^{14}*C*, was converted to 8-^{14}*C* by the procedure described above.

p-(*N*-Propylsulfamoyl)benzoic Acid (9). This compd (also referred to as the *N*-depropyl metabolite) was prepared as described.[1]

p-(*N*-Propyl-*1*-^{14}*C*-sulfamoyl)benzoic Acid (9-^{14}*C*). Cold aqueous NaOH (6 ml, 10%) was added to *n*-propylamine-*1*-^{14}*C* (5 mmoles, 0.26 g, 60 μCi in 2 ml of MeOH) at 0°, followed by 1 (5 mmoles, 1.14 g) and the reaction mixt was stirred for 2 hr at room temp. Unreacted amine was removed *in vacuo*, and the residue was acidified with 10% HCl. The white ppt formed was collected, washed, and dried *in vacuo* at 100° for 18 hr; yield 0.92 g (75%). The product was recrystd (EtOH–H_2O).

p-(*N*,*N*-Pentamethylenesulfamoyl)benzoic Acid (Piperidyl Analog) (10). The compd was prepd by treating 1 with a 2-fold excess of piperidine and recrystd (EtOH–H_2O); mp 263–265° (lit.[8] in agreement).

Propyl-*1*-^{14}*C*-propylamine (11-^{14}*C*). *n*-Propylamine-*1*-^{14}*C* (5.0 mmoles) was treated with *n*-Pr iodide (4.1 mmoles) in 3 ml of Et_2O and 5 ml of H_2O contg 1.5 g of Na_2CO_3 at room temp. After refluxing 90 min, the mixt was distd rapidly. Upon extn of the distillate into Et_2O and drying (Na_2SO_4), the Et_2O phase was slowly distd with a 25-cm Vigreaux column and crude 11-^{14}*C* collected at 100–110°. Trace amounts of propylamine-^{14}*C* were removed in the same apparatus as follows: the crude 11-^{14}*C* was placed in the distn flask at 50°. At intervals Et_2O was added and distd. The process was continued until the distillate was free from ^{14}*C*.

p-(*N*-Propyl-*1*-^{14}*C*-*N*-propylsulfamoyl)benzoic Acid (12-^{14}*C*). The labeled probenecid was prepd as previously described from 11-^{14}*C* and 1. The resulting compd was purified by tlc in system I.

p-(*N*-Propyl-*N*-2-propenylsulfamoyl)benzoic Acid (13). This was synthesized by treating 1 with propylpropenylamine (prepd by refluxing equimol amts of *n*-propylamine and 3-iodopropene in the presence of aqueous Na_2CO_3, and fractionally distg the reaction mixt, yield 40%, bp 110–120°) by the above described procedure; purified by tlc (system I, R_f 0.80) and recrystd (*n*-heptane–$CHCl_3$), white crystals, mp 172–173°. *Anal.* ($C_{13}H_{17}NO_4S$) C: calcd, 55.11; found, 52.60. H: calcd, 6.05; found, 5.36 (silica gel could not be removed completely giving low values for C, H). The product decolorized a solution of Br_2 in CCl_4, while probenecid did not.

p-(*N*-Propyl-*N*-2-oxopropylsulfamoyl)benzoic Acid (14). This compd was prepd by oxidn; 1.0 g of **2** was dissolved in 100 ml of Et_2O contg 5 ml of Me_2CO and an oxidn equiv amt of Jones reagent was added at room temp. After stirring overnight, the mixt was dried (Na_2SO_4) and filtered. The filtrate was concd and the residue crystd (EtOH–H_2O) and recrystd ($CHCl_3$–Me_2CO–*n*-heptane); mp 206–207° dec, R_f in system I 0.60. *Anal.* ($C_{13}H_{17}NO_5S$) C, H, N.

B. Measurement of Physical Parameters. The pK_a's of the analogs were detd potentiometrically at room temp by titrn with NaOH, as previously described.[6] Partition coeffs were measured in (1) peanut oil–pH 7.4 Sörensen buffer[6] (K_p) and (2) $CHCl_3$–0.15 *N* HCl (K_p'). Measurement of binding to cryst human albumin was carried out using equil dialysis at 37° according to a published method.[11] Molar extinction coeffs were detd in 0.5 *N* NaOH with a Beckman DU spectrophotometer.

For pmr studies, solns were made (10% w/w) of the appropriate probenecid analog in either DMSO with internal TMS ref or 1.1 *M* KOH with internal DDS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) ref. High-resolution spectra of the degassed samples were obtained with a Varian Associates Model A-60A spectrometer operating at 38°. Calibrations were performed using the usual side-band techniques. Frequencies were an average of 3 forward and 3 reverse scans. Computations were performed on a Digital Equipment Corporation PDP-10 computer using LAOCN$_3$.[12]

C. *In Vivo* Studies. Rat. Male Wistar rats, weighing 250–350 g, were fed *ad lib* a diet of 50% Carnation milk–water for at least 3 days prior to expt. They were injected ip with 40 mg/kg (0.1 μCi) of labeled metabolite. Two rats were studied for each compd. Urine and feces were collected for 48 hr in the absence of preservatives. Urine aliquots contg at least 2000 dpm were then applied to tlc plates and developed in system I.[2] After spraying with H_2O to visualize the compds appropriate zones were scraped and counted directly in the cocktail,[2] in a Beckman liquid scintillation spectrometer LS-250 with >88% efficiency.

Dog. Sixteen studies were carried out in six female unanesthetized Dalmatian and mongrel dogs. Std renal clearance procedures[13] were employed to measure the urate clearances with or without urate loading, and the effect thereon of the various probenecid metabolites and the piperidyl analog. In all expts creatinine clearances were used as a measure of the glomerular filtration rates. In the urate loading expts, uric acid, 0.20% as the Li salt in 5% mannitol (prepd by dissolving uric acid in hot Li_2CO_3), was infused at a rate of 150 ml/hr throughout, after a priming dose of 0.25 g of uric acid. In other expts creatinine alone was infused in 5% mannitol without uric acid. After appropriate equilibration, and 3–4 10-min control clearance periods, a particular probenecid metabolite (1.5 g of free acid dissolved in a min amt of 1 *N* NaOH, adjusted with 5% AcOH to pH 7–9 and dild to 40 ml with 5% mannitol) was injected intravensouly. Then 15-min collections were made for 2 hr, with blood samples at appropriate intervals. The rate of delivery of all infusates was regulated by means of a Bowman constant infusion pump. Uric acid and creatinine in plasma and urine were measured as previously described.[13]

D. *In Vitro* Studies. *In Vitro* Studies of the Stability of Metabolites in 9000*g* Supernatants of Rat Liver. Rats were sacrificed by a blow on the head and the livers homogenized at 0° in pH 7.4, 0.2 *M* Na_2HPO_4–KH_2PO_4 buffer, to make a 10% w/v homogenate; this was centrifuged at 9000*g* for 15 min at 0° in IEC B-20 centrifuge. To 4 ml of this supernatant, 0.4 ml of cofactor mix[14] and 20 μg of ^{14}C compd (0.001–0.05 μCi) in 0.6 ml of buffer were added. Incubations were carried out in duplicate at 37° for 40 min in air with 100 oscillations per minute. The reaction was stopped by the addn of 1 ml of 3 *N* HCl. The ^{14}C material was extd with EtOAc (50 ml) and concd *in vacuo*. The residue was applied, along with a mixt of 50 μg

of each metabolite (unlabeled: in 0.2 ml of EtOAc) to a tlc plate and analyzed as described above for urine.[2]

Identification of the Product of *in Vitro* N-Dealkylation of Side-Chain Labeled Probenecid by Rat Liver. Rat liver homogenate (50 ml) was incubated in duplicate for 80 min with side-chain labeled probenecid-^{14}C (0.23 μCi, 2.13 mg) and cofactors.[14] The reaction was stopped with 5 ml of 6 *N* HCl. An aliquot was assayed by tlc (system I), the remainder was steam distd, and an aliquot of the distillate was counted. $EtCO_2H$ (2.2 g) was added to the distillate and the mixt extd serially with Et_2O until no ^{14}C remained in the aqueous phase. The Et_2O phases were pooled and dried (Na_2SO_4). By fractional distn $EtCO_2H$ (bp 136–140°) was collected. The *p*-toluidide deriv was prepd and recrystd (MeOH–H_2O) following charcoal treatment. White crystals, mp 123–124° (lit.[15] mp 125°), were obtained. The crystals were dried, weighed, and recrystd to const sp activity.

The absence of AcOH in the distillate was indicated by the addn of AcOH-2-^{14}C (1.5 μCi)** to 3 g of $EtCO_2H$, prepn of the *p*-toluidide deriv and finding negligible incorporation upon multiple crystns. Since propionaldehyde could be codistd with $EtCO_2H$ it is possible that ^{14}C in the distillate not accounted for as $EtCO_2H$ could be present as the aldehyde.

Results

Binding of Probenecid and Its Metabolites to Plasma Proteins. The metabolites of probenecid are less tightly bound to human albumin and plasma than probenecid (Table II). The binding of these compounds to dog plasma is somewhat lower. There seems to be a correlation between binding and partition coefficient (Table I, K_p); the parent drug is more lipid soluble and more highly bound than the metabolites. The pK_a's for these compounds are identical.

Table II. Binding of Probenecid and Its Metabolites to Plasma Proteins

Compound	% binding[a]		
	Human plasma	Human albumin	Dog plasma
(2-OH)	65 (9)	68 (10)	39 (7)
(3-OH)	65 (9)	72 (10)	49 (8)
(COOH)	81 (12)	84 (13)	62 (9)
(*N*-Depropyl)	58 (8)	67 (10)	50 (8)
Probenecid[b]	93 (83)	95 (88)	77 (61)

[a] Numbers in parentheses represent drug concentration (mg/l.) in the inside phase (plasma or albumin) after equilibration. Drug was added in buffer to plasma or 5% albumin. This results in an initial concentration of 90% plasma and 4.5% albumin and after equilibration in a final concentration of 80% and 4.0% respectively, due to influx of water. [b] The piperidyl analog of probenecid was 81% and 69% bound to human and dog plasma, respectively, at the same concentration as probenecid. The concentration selected for probenecid is in the therapeutic range.[4]

Effect of Probenecid, Its Metabolites, and the Piperidyl Analog (10) on the Renal Clearance of Uric Acid in Dogs. The mean values of urate clearances before and after the various probenecid metabolites are given in Table III. It is to be noted that in the Dalmatian dogs, C_{urate} and $C_{urate}/C_{creatinine}$ invariably decreased after the injection of 2-OH, 3-OH, or COOH metabolites of probenecid in all experiments with or without urate loading. The decreases in $C_{urate}/C_{creatinine}$ ratios are comparable to those after probenecid injections reported previously.[13] In the mongrel dogs, the effect of the 2-OH, 3-OH, and COOH metabolites produced a distinct increase in urate clearances, again comparable to the probenecid effect. The *N*-depropyl metabolite, on the other hand, seems to be less potent in affecting the urate clearance both in the Dalmatian and the mongrel dogs.

Unsubstituted amines of low toxicity, including piperidine, had been used in the treatment of gout.[16] In view of this, it is of interest that the piperidyl analog (**10**) was found to have a pronounced effect on urate clearance.

In Vivo **Studies in Rats.** Upon administration of the various metabolites of probenecid to rats, 30–69% of the dose was excreted in 48 hr in the urine; 11–40% was found in the feces. After injection of the 3-OH metabolite, 47–63% of urinary ^{14}C was excreted as the COOH metabolite, and 35–51% as unchanged 3-OH metabolite, less than 2% was found to be excreted as conjugates. For the carboxy metabolite, only unchanged drug was detected in the urine. In the case of the *N*-depropyl and 2-OH metabolites, 90% of urinary ^{14}C was found as unchanged drug and 10% as conjugates; by hydrolysis these 10% fractions were shown to be conjugates of the respective metabolites.

In Vitro **Studies with Probenecid and Its Metabolites.** Upon incubation of labeled 3-OH metabolite with 9000*g*

Table III. Effect of Probenecid Metabolites on Uric Acid Clearances in Dalmatian and Mongrel Dogs

Urate infusion	Compound	Dalmatian dog				Mongrel dog			
		Urate[d]				Urate[d]			
		UV, mg/min	*C*, ml/min	C_{ur}/C_{cr}	Δ, %	*UV*, mg/min	*C*, ml/min	C_{ur}/C_{cr}	Δ, %
+	Control[a]	3.54	44.3	1.08		1.07	32.8	0.58	
	2-OH[b]	4.18	40.2	0.89	−21	1.73	42.2	0.77	+33
+	Control	4.34	60.4	1.05		1.23	29.1	0.54	
	3-OH	2.40	19.2	0.90	−14	1.48	32.0	0.68	+26
+	Control	4.49	106.9	1.08		0.69	37.2	0.52	
	COOH	4.42	91.5	0.95	−12	1.17	54.5	0.68	+31
+	Control	4.35	67.1	0.88		0.83	36.3	0.57	
	N-Depropyl	4.33	56.1	0.80	−9	1.16	40.4	0.68	+19
0	Control	0.34	37.4	0.62		0.05	7.1	0.15	
	2-OH	0.23	25.6	0.44	−29	0.05	9.2	0.20	+33
0	Control	0.50	72.1	0.83		0.04	7.7	0.13	
	3-OH	0.38	54.9	0.65	−22	0.07	14.2	0.21	+62
0	Control	0.40	58.2	0.82		0.04	8.3	0.15	
	COOH	0.29	31.5	0.63	−23	0.05	9.1	0.20	+33
0	Control	0.37	41.1	0.94		0.06	9.6	0.13	
	Piperidyl	0.25	28.3	0.73	−22	0.10	16.4	0.21	+62
+	Control[c]	3.06	102.7	1.26		1.03	5.6	0.58	
	Probenecid	3.34	84.1	0.94	−25	1.77	8.4	0.80	+38
0	Control[c]	0.56	92.5	1.04					
	Probenecid	0.48	80.5	0.86	−17				

[a] Control values represent the means of 3 or 4 periods. [b] Values after drugs represent the means of 8 15-min periods after the 15-min discard. [c] Probenecid values represent earlier studies, they are included here for comparison. [d] *U* = urine concentration, *V* = urine flow, *C* = clearance, ur = uric acid, cr = creatinine, weight of dogs 18–22 kg.

rat liver supernatant 43% of the ^{14}C was found in the conjugates, 23% in the unchanged 3-OH, and 34% appeared as the COOH metabolite. Similarly when 2-OH, *N*-depropyl, or COOH metabolites were incubated, at least 90% was recovered unchanged. The remainder was measured in the area corresponding to conjugates. Upon incubation of the 2-keto analog of probenecid, formation of the 2-OH metabolite was observed (unpublished work, this laboratory, 1971).

Incubation of side-chain labeled probenecid (with only one side chain labeled; see Methods) followed by tlc separation indicated 16.5–17.2% of metabolism to the *N*-depropyl metabolite (calculated by doubling the measured ^{14}C found as *N*-depropyl metabolite; this was done to account for random removal of the propyl group). Most of the ^{14}C in the distillate could be accounted for as propionic acid by a specific isotope dilution procedure.

Preliminary experiments were carried out to learn whether compounds such as the 2-keto (**14**) or the 2-propene (**13**) analog of probenecid were metabolites. Ring ^{14}C-labeled probenecid was incubated with supernatant and the extracts were submitted to tlc. No significant ^{14}C was found in the areas corresponding to the 2-keto analog or to the area corresponding to the propene compound.

Pmr Studies. The aromatic protons of probenecid, its metabolites, and analogs exhibit the characteristic AA'BB' spectrum common to disubstituted benzenes containing different para substituents. In addition, in the AA'BB' cases the analyses do not distinguish between ω_A and ω_B or between $J_{AA'}$ and $J_{BB'}$. In the present investigation, the assignments of the chemical shifts ω_A and ω_B have been made on the basis of substituent effects of the carboxyl and sulfonamide group on the benzene resonance. The difference between the substituent effect of a COOH group on the ortho and meta positions is −29.7 Hz and −26.9 Hz in DMSO and 1.1 *M* KOH, respectively (Tables IV and V). For the sulfonamide group the difference is −17.9 Hz in DMSO. Based on the well-known additivity of substituent effects in substituted benzenes,[17] the protons ortho to the COOH group should resonate 11.8 Hz to lower field than the protons ortho to the sulfonamide group, in agreement with the values in Table V. This situation should be essentially the same in 1.1 *M* KOH as the following line of reasoning indicates. Comparing the differences given previously, the resonance of protons ortho to the COOH group in benzoic acid move upfield only 2.8 Hz when COOH is ionized. In order that the ortho and meta shifts cross over on going from DMSO to 1.1 *M* KOH, it would require that the ortho shifts move upfield about 10.0 Hz relative to the meta shifts. This is partially accomplished in the spectrum of *p*-$HOOCC_6H_4SO_2NH_2$ when it is dissolved in 1.1 *M* KOH (Table IV). Such an effect should not be expected for *N*-alkyl or *N*,*N*-dialkyl substituted compounds due to the absence of an easily ionizable amide proton. Comparison of the data in Tables IV and V supports this hypothesis.

The chemical shifts and coupling constants which satisfactorily reproduce the experimental spectra are presented in Tables IV and V. The root-mean-square deviations between experimental and theoretical frequencies for each analog is ~0.04 Hz. The probable error for a particular parameter did not exceed 0.04 Hz and in general was approximately 0.02 Hz.

Table IV. Pmr Parameters of the AA'BB' Aromatic Ring of Sulfamoylbenzoic Acids[a,b] (1.1 *M* KOH)

R,R'	ω_1,ω_4	ω_2,ω_3	J_{12}	J_{13}	$J_{14}=J_{23}$	$\Delta\omega$
H, H	−477.22	−476.50				00.72
H, methyl	−482.27	−471.01	8.03	0.52	1.89	11.26
Dimethyl	−488.01	−471.57	8.14	0.50	1.90	16.44
H, ethyl	−480.74	−470.80	8.17	0.57	1.93	9.94
Diethyl	−485.03	−471.60	8.18	0.49	1.97	13.43
Methyl, ethyl	−487.20	−472.01	8.13	0.48	1.93	15.19
H, *n*-propyl	−479.90	−469.90	8.15	0.56	1.87	10.00
Di-*n*-propyl	−485.58	−470.76	8.10	0.46	1.91	14.82
Piperidine[c]	−487.98	−469.14	8.20	0.49	1.88	18.84
n-Propyl, $CH_2CHOHCH_3$	−484.23	−471.59	8.13	0.47	1.82	12.64
n-Propyl, CH_2CH_2COOH	−483.09	−473.08	8.19	0.52	1.91	10.01
SO_2OH[d]	−479.79	−473.53				6.26
N-Propyl, $CH_2CH_2CH_2OH$	−484.54	−471.29	8.05	0.41	1.88	13.25

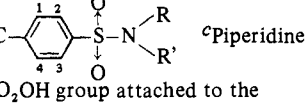
[a]Chemical shifts and coupling constants are in hertz. The uncertainty in experimental line positions as determined from 3 forward and 3 reverse scans taken at 1 Hz/cm was approximately ±0.05 Hz; the root-mean-square error between experimental and calculated line positions was ~0.04 Hz. [b]  [c]Piperidine ring attached to the sulfur atom. [d]SO_2OH group attached to the aromatic ring in the para position.

Table V. Pmr Parameters of the AA'BB' Aromatic Ring of Sulfamoylbenzoic Acids[a,b] (DMSO)

R,R'	ω_1,ω_4	ω_2,ω_3	J_{12}	J_{13}	$J_{14}=J_{23}$	$\Delta\omega$
Dimethyl	−492.02	−470.23	8.23	0.56	1.81	21.79
Dipropyl	−488.11	−474.78	8.01	0.46	1.90	13.33
Diethyl	−488.55	−475.31	8.10	0.49	1.88	13.24
H, ethyl	−489.16	−475.06	8.35	0.40	1.99	14.10
Ethyl, methyl	−492.21	−475.58	8.02	0.52	1.90	16.63
H, *n*-hexyl	−488.22	−475.00	8.06	0.47	1.90	13.22
H, H	−488.45	−479.10	8.28	0.51	1.85	9.35
SO_2OH[c]	−476.02	−466.80	8.08	0.53	1.79	9.22
H, *n*-propyl	−488.52	−475.41	8.15	0.54	1.86	13.11

[a]Chemical shifts and coupling constants are in hertz. For details see Table IV. [b]See footnote *b*, Table IV. [c]See footnote *d*, Table IV.

Discussion

The present investigation is an extension of previous work in this laboratory and others[6,18,19] concerning structure-activity relationships in the probenecid series.‡‡ Weiner, *et al.*,[18] had indicated that nonionic tubular reabsorption of probenecid accounts for its observed low renal clearance. They also found that for analogs with identical pK_a's, lipid solubility values were shown to correlate with clearance. Subsequently, we established[6] a correlation between lipid solubility, structure, and stimulatory effect on ascorbic acid biosynthesis in rats.

The present study extends the analysis of structure-activity relationship to the metabolites of probenecid. Like the parent compound, its metabolites increase C_{ur}/C_{cr} in mongrels and decrease this ratio in Dalmatians. Except for the *N*-depropyl metabolite, they are about as effective in this respect as probenecid, and, despite their low blood levels,[2,3] may contribute to the uricosuric effect of probenecid in man. They may also participate in the blocking of tubular secretion of organic acids by the parent compound. The metabolites are less bound to plasma albumin than probenecid. This fact, as well as their lower lipid solubil-

‡‡Note Added in Proof. After submission of this paper, K. C. Blanchard, D. Maroske, D. G. May, and I. M. Weiner [*J. Pharmacol. Exp. Ther.*, 180, 397 (1972)] found that certain ring substituted analogs of probenecid (2'-NO_2, 2'-OH, and 2'-Cl) were much more potent uricosuric agents than probenecid in the monkey. In man the 2'-OH analog was also shown to be more active.

ity, might explain the relatively low *in vivo*[2,3] concentrations of metabolites in human plasma as compared to those of the parent drug. Similar observations of lower % binding of metabolites have been made previously for other drugs.[20,21]

It is known that in general, compounds of relatively low lipid solubility are rapidly eliminated in urine and frequently more slowly metabolized than analogs of higher lipid solubility. Thus, the low lipid solubility of the metabolites may explain their being terminal metabolites *in vivo* and *in vitro*. The exception is the 3-hydroxy metabolite, which is partly converted to the carboxy metabolite by nonmicrosomal enzyme systems. It is of interest that Gigon and Guarino,[22] using rat liver slices, were able to detect only one biotransformation product—the 2-hydroxy metabolite as a glucuronide. Under our *in vitro* conditions, which are not optimal for conjugation, only a small amount of acyl glucuronide of probenecid was formed; the latter is found in human urine[3] and rat bile.[23]

Extensive side-chain oxidation for probenecid *in vivo* and *in vitro* was expected, by analogy with the work of Maynert[24] on alkyl chain oxidation of barbiturates. The *N*-depropyl metabolite is formed presumably *via* the unstable 1-hydroxy intermediate, a mechanism which had been previously suggested by Guarino, *et al.*[1] It is of interest that an unstable carbinolamine intermediate has been proposed in order to explain the metabolism of lidocaine.[25] In this regard it is pertinent that Klutch and Bordun[26] found that the *N*-isopropyl derivative of methoxamine can be dealkylated and that even *tert*-butyl and still longer alkyl chains can also be cleaved by microsomal enzymes.[27,28] In a series of analogs of probenecid (*N*-alkyl-*p*-bromosulfonamides)[29,30] a correlation between the rate of dealkylation and chain length was found.

Proton magnetic resonance parameters of benzene derivatives have been shown to depend on substitution.[17,31,32] A study of pmr parameters for mono- and dialkyl N-substituted analogs in principle should yield information concerning the effect of substitution as well as provide information for the identification of probenecid analogs including its metabolites.

On the basis of *a priori* considerations, it is difficult to determine whether the effect of substitution is localized in the alkyl side chain or extends into the aromatic region. No systematic dependence of the chemical shifts as well as differences in chemical shifts between the A and B protons on single or double substitution or alkyl chain length was observed. In contrast, one interpretation of the ultraviolet-absorption spectra of these analogs describes the displacement to longer wavelength in terms of disubstitution.[6] There is poor correlation between the structure of the substituent or the partition coefficient with the chemical shift difference for the A and B protons of the unsubstituted compound (p-$HOOCC_6H_4SO_2NH_2$) and the corresponding chemical shift of the substituted analog. These observations support the hypothesis that variation of pharmacologic activity[19] with substituent is isolated from the aromatic region of the molecule. The pK_a's for the various analogs, which are essentially constant for the series, provide additional support of this hypothesis. Whether the changes observed in $\Delta\omega$ are due to minor conformational changes or the formation of molecular complexes (including solute-solvent interactions)[33] cannot be known without further detailed studies.

Although the variations of the pmr parameters are small for probenecid and the analogs, the observed spectra appear to be adequate for the characterization and identification of the parent compound and its metabolites. The data presented here, in conjunction with the standard methods for predicting high-resolution pmr spectra, can be used for similar purposes in other pharmacological studies involving the probenecid series. In particular, it should be noted that in case of substitution of the aromatic ring, the pmr assignments can be readily made.[17]

Acknowledgment. We wish to thank Dr. Lawrence Berger for his help in some of the pharmacologic studies.

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