The Effect of Probenecid, Phenylbutazone, and Their Analogs on the Excretion of L-Ascorbic Acid in Rats[1]

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Probenecid, phenylbutazone, and a number of their analogs were studied in regard to their effect on the biosynthesis of L-ascorbic acid in the rat. An examination of several physicochemical properties led to a correlation between "lipid solubility" (partition coefficient between peanut oil and pH 7.4 buffer) and the stimulation of the glucuronic acid pathway for the probenecid series. The major requirement for the effect is not stringent, a relatively low "lipid solubility" is required. In general, disubstitution of the sulfonamide hydrogens by alkyl groups resulted in increased "lipid solubility," whereas monosubstitution had a minor influence. Phenylbutazone and most of its analogs, which are already highly lipophilic, also were stimulatory. It is proposed that the measurement of L-ascorbic acid excretion, which is indicative of the rate of its biosynthesis in rats, is a convenient model for the prediction of the stimulation of drug metabolizing activity.

Various structurally unrelated compounds such as barbital, phenylbutazone, and 3-methylcholanthrene increase the biosynthesis, metabolism, and excretion of L-ascorbic acid in the rat; this has been shown to involve an enhancement of the conversion of hexoses to L-ascorbic acid *via* D-glucuronic and L-gulonic acids.[2,3] The present study is an attempt to find a correlation between the increased L-ascorbic acid excretion in the rat and selected physicochemical properties of certain substances producing these effects.

Experimental Section

Male albino Wistar rats weighing between 170 and 300 g were obtained from Twin Oaks Farms, Moorestown, N. J. Male Sherman rats (same range) were obtained from Camm Research, Wayne, N. J. They were given 50–70 ml daily of a 1:1 mixture of evaporated milk (Carnation®) and water 1 week prior to, and during the experiments (a low ascorbic acid diet). The rats were kept in round individual metabolism cages placed over a 25-cm polyethylene funnel; iron wire mesh was used to catch the feces. The urine was collected daily in 5 ml of 8% oxalic acid. Only rats excreting less than 1 mg/24 hr of L-ascorbic acid during a control period were used. Under ether anesthesia rat blood was collected by cardiac puncture using heparin as an anticoagulant. Only one sample was withdrawn from each rat.

Probenecid and its analogs were a gift of Drs. James M. Sprague and John E. Baer (Merck Sharp and Dohme, West Point, Pa.). The phenylbutazone analogs were synthesized by Dr. F. Häfliger and his colleagues (J. R. Geigy, Basel). The compounds were dissolved in a minimum of 2.5 *N* NaOH; the solutions were then adjusted to pH 7.4 with 2% acetic acid and then diluted with water and isotonic saline to a 2.5% w/v solution in such a manner that the final solution was 50% saline. 1,2-Diphenyl-4-isopropylpyrazolidine-3,5-dione and 1-(*p*-hydroxyphenyl)-2-phenyl-*t*-butylpyrazolidine-3,5-dione were given intraperitoneally in a single dose of 50 mg daily for 4 days.

Probenecid and its analogs (with the exception of the di-*n*-butyl analog) were injected intraperitoneally according to the following schedules: on day 1 and 2, 125 mg/kg at 10 AM and 125 mg/kg at 2 PM; on day 3, 250 mg/kg at 10 AM and 250 mg/kg at 2 PM. If an analog did not produce a significant effect, the rats were maintained on the diet. One week later they were challenged with a daily dose of 250 mg/kg of probenecid added to the diet. Negative results were accepted only if a positive response to the probenecid challenge was obtained. In studies of plasma level half-life, rats were injected intraperitoneally with a single 250-mg/kg dose.

L-Ascorbic acid in urine was measured by the indophenol dye titration method.[4] These results were confirmed by determinations carried out by the method of Roe and Kuether.[5] The pK_a of analogs of probenecid was determined potentiometrically with a Zeromatic Beckman pH meter at room temperature. The compound (0.1 mmole) was dissolved in 15 ml of 95% ethanol and the titration was carried out with 0.02 *N* NaOH; the end point was at about 75% ethanol.

The measurements of partition coefficients were effected in two different systems. In one set of determinations 0.15 *N* HCl and chloroform,[6] while in the other, peanut oil (Rokeach) and pH 7.4 Sörenson buffer was used.

The procedure in the latter case was taken from a known method[7] with some modifications.[8] The oil was washed three times with buffer prior to use, and separation of phases was achieved by centrifuging overnight at 500*g*. Probenecid was determined by a previously described double-extraction method[9] and the analogs by a similar spectrophotometric procedure.[10] For those compounds for which no method has been described, ethylene dichloride was used as the extraction solvent. Measurement of binding to rat plasma and rat albumin (Pentex, Inc.) was carried out as in an earlier study.[8]

^{14}C-Labeled hexoses were obtained from New England Nuclear Corp. and L-ascorbic acid-1-^{14}C from Nuclear Chicago. Isotopic assays were the same as those employed by Burns, *et al.*[2]

Results

Chemical Properties of Probenecid Analogs.—Probenecid and its analogs (the synthesis of some of these has been described)[11] were known to have essentially the same pK_a (Table I).[6,12] The exceptions are the nitro compounds[13] and the 2-hydroxy analog. The latter can also be considered as an analog of

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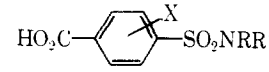
TABLE I

STRUCTURE, PHYSICAL PROPERTIES, AND EFFECT ON ASCORBIC ACID EXCRETION OF PROBENECID AND ITS ANALOGS



No.	R	R'	X	$-pK_a$ Lit.[a]	$-pK_a$ Pot.[b]	Ultraviolet peak,[c] mμ	Partition coefficient K_p	Partition coefficient K_p'[d]	Ascorbic acid excretion/24 hr[e]
1	H	H	H	3.3	6.1	237	<0.01	>500 (<0.002)	<1 (<1)
2	H	CH_3	H	3.3	5.9	227	<0.01	8.4 (0.12)	<1 (<1)
3	H	CH_2CH_3	H	...	5.7	226.5	<0.01	2.9	<1 (<1)
4	H	$(CH_2)_2CH_3$	H	...	5.8	225.5	<0.01	0.39	<1 (<1)
5	H	$(CH_2)_5CH_3$	H	...	5.7	227	<0.01	0.004	See text
6	H	Cyclohexyl	H	...	6.0	224	<0.01	0.1	5.8 (2.5-9.5)
7	CH_3	CH_3	H	3.3	5.7	238	0.01	0.16 (6.0)	<1 (<1)
8	CH_3	CH_2CH_3	H	3.3	5.7	239	0.01	0.077 (13)	<1 (0.2-1.6)
9	CH_2CH_3	CH_2CH_3	H[f]	3.3	5.8	242.5	0.14	0.01 (75)	1.4 (0.9-2.1)
10	CH_2CH_3	$(CH_2)_2CH_3$	H	3.3	5.8	242	0.10	0.004 (*ca.* 250)	4.5 (3.9-5.1)
11	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	H[g]	3.4	5.8	242.5	0.13	<0.001 (>2000)	10.3 (4.6-16.5)
12	$CH(CH_3)_2$	$CH(CH_3)_2$	H	...	5.7	248	0.08	<0.001	5.6 (2.2-8.7)
13	$(CH_2)_3CH_3$	$(CH_2)_3CH_3$	H	...	5.3	248	0.09	<0.001	See text
14	$(CH_2)_2CH_3$	$(CH_3)_2CH_3$	2-OH	...	5.0	320	0.05	<0.001	5.7 (2.5-9.2)
15	$(CH_2)_2CH_3$	$(CH_3)_2CH_3$	2-OCH_3	...	5.6	220, 5[h]	0.19	<0.001	5.7 (2.5-7.6)
16	$(CH_2)_2CH_3$	$(CH_3)_2CH_3$	2-NO_2	1.3	3.9	236.5	<0.01	0.01 (90)	See text
17	$(CH_2)_2CH_3$	$(CH_3)_2CH_3$	3-NO_2	1.8	4.6	227.5	<0.01	0.7 (1.4)	See text
18	$(CH_2)_2CH_3$	$(CH_3)_2CH_3$	3-CH_3	...	6.0	230	0.17	0.003	5.6 (2.2-11.8)

[a] These data, except for probenecid and the nitro analogs, were obtained by Weiner, *et al.*,[6] using an ultraviolet method. The pK_a of probenecid was determined by Shore, *et al.*[12] The pK_a for the nitro compounds was reported by Weiner and Mudge.[13] [b] The pK_a of salicylic acid measured under the same conditions was 4.9. [c] The ultraviolet peaks have been published before[10] for **1**-**4** and **7**-**12**. [d] Values followed by parentheses had been obtained by Weiner, *et al.*,[6] except for the nitro compounds.[13] These authors gave their results as $1/K_p'$. [e] Average of at least four animals except for compounds **2**, **4**, **5**, and **17** in which three rats were used. Excretion of day of highest dose. Range in parentheses. [f] This compound is also known as Longacid or Urelim. [g] Probenecid (Benemid®). [h] Secondary ultraviolet peak at 244 mμ.

salicylic acid; in fact, their ultraviolet absorption spectra are similar.

The "lipid solubility" of probenecid analogs varies considerably. This property is defined for the purposes of this paper as the partition coefficient in the system peanut oil and pH 7.4 buffer (K_p) or in the system used by Weiner, Washington, and Mudge[6] (K_p'). The former system appears to be a better physiological model. The changes in K_p and K_p' are generally correlated with an increase in the length of the alkyl side chain.

The ultraviolet absorption peaks of some of the analogs were previously reported.[10] Thus, **1** with a free sulfonamide group has a peak at 237 mμ in NaOH. Monosubstituted compounds have lower maxima, whereas analogs with two alkyl groups have ultraviolet absorption bands in the neighborhood of 240 mμ.

The hypsochromic displacement for the monosubstituted groups is interpreted as an increase of electronic delocalization due to the contribution of the activating group without introducing undue steric effects. On the other hand, the disubstitution by alkyl groups leads to a larger steric effect, resulting in a nonplanar contributing structure, yielding an ultraviolet band in the neighborhood of the unsubstituted compound. Due to the increasing size of alkyl chains in the disubstituted analogs, there is an increasing bathochromic effect. These compounds (**2**–**14**) have essentially the same molar extinction coefficient.

Effect of Probenecid Analogs on the Urinary Excretion of L-Ascorbic Acid. Compounds **1**–**4** and **7** produced no effect on L-ascorbic acid excretion. These substances have poor "lipid solubility." Compounds **8** and **9** gave an intermediate effect, and likewise had intermediate "lipid solubility." Compound **6** was anomalous in its stimulation of L-ascorbic acid excretion; its K_p and K_p' indicate low "lipid solubility." In an attempt to resolve this anomaly, half-life and plasma binding of this analog was compared with that of **1** and **11**. The average plasma level for all three drugs was about 500 mg/l. at 0.5 hr. For **11**, plasma levels were measured as late as 18 hr after injection, whereas for **1** and **6**, 2.5 and 6.5 hr, respectively. Compound **6** exhibited a plasma level half-life of about 0.5 hr in contrast to 2.0 and 3.4 for **1** and **11**. At a concentration of 125 mg/l. the binding of **1**, **6**, and **11** was 91, 86, and 90%, respectively. At the same drug concentration, rat albumin binding of probenecid was found to be 90%, indicating that binding to this protein accounts for most of the plasma interaction. Nevertheless, binding and half-life data do not correlate with the anomalous findings with **6**.

The di-*n*-butyl analog, when administered intraperitoneally, was lethal to rats in doses of 250 mg/kg and is also more toxic than probenecid in mice.[14] In rats, the LD_0 of probenecid is reported to be 342 mg/kg ip.[15] When 250 mg/kg of the di-*n*-butyl analog was administered with the diet, the animals survived, but there was no effect on L-ascorbic acid excretion. The monohexyl analog was toxic—three out of five rats died.

The nitro analogs gave variable results, which can also be attributed to their observed toxicity. When four rats were administered the 2-nitro compound, two gave a positive response, whereas only one of three

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TABLE II

PHYSICAL PROPERTIES OF PHENYLBUTAZONE AND SOME OF ITS ANALOGS AND THEIR EFFECT ON L-ASCORBIC ACID EXCRETION

Compd	L-Ascorbic acid, mg/24 hr	pK_a			Partition coefficients	
		H_2O[c]	MCS[d]	75% Ethanol	K_p[c]	K_p'
1-(*p*-Hydroxyphenyl)-2-phenyl-4-*t*-butylpyrazolidine-3,5-dione	3.0 ± 1.0[a]	7.1	7.3	8.3	8.0	<0.01
1,2-Diphenyl-4-isopropylpyrazolidine-3,5-dione	4.3 ± 0.1[a]	5.5	6.3	6.7	3.4	<0.01
Sulfinpyrazone	4.0 ± 0.7[b]	2.8	...	4.2	0.6	<0.01
Phenylbutazone	6.1 ± 0.3[b]	4.5	5.2	5.7	2.2	<0.01
Oxyphenbutazone	3.5 ± 0.4[b]	4.7	...	6.2	0.5	<0.01
1-(*p*-Hydroxyphenyl)-2-phenyl-4-(keto-*n*-butyl)pyrazolidine-3,5-dione	<1[b]	2.3	...	3.7	0.4	<0.01

[a] Value for last day; average for four rats and average deviation. [b] Burns, *et al.*[2]; average and average deviation. [c] Perel and co-workers.[8] [d] Carried out in Methyl Cellosolve by E. Girod, R. Delley, and F. Häfliger, *Helv. Chim. Acta*, **40**, 413 (1957).

dosed with the 3-nitro analog responded. All the other analogs gave a respectable and reproducible effect on L-ascorbic acid excretion and these compounds are relatively "lipid soluble" (K_p >0.01), the most potent compound being probenecid. It is of interest that there appears to be a relation between clearance and K_p and K_p'[6] and that an inverse relationship exists between polarity and toxicity.[14]

Only one set of experiments was carried out with Sherman rats, in order to test whether the effect was unique to the Wistar strain. Three Sherman rats given probenecid and three others given 50 mg/day of barbital for 3 days in the diet produced an effect equal to that obtained with Wistar rats.

Effect of Phenylbutazone and Its Analogs on the Urinary Excretion of L-Ascorbic Acid.—Previously, it has been shown that phenylbutazone and some of its analogs increase the excretion of L-ascorbic acid.[2] Two additional analogs have been investigated. Except for ketoxyphenbutazone [1-(*p*-hydroxyphenyl)-2-phenyl-4-(α-keto-*n*-butyl)pyrazolidine-3,5-dione], all of these compounds tested gave the effect, the most potent being phenylbutazone (Table II). In this series the pK_a and K_p varied considerably; however K_p' was always less than 0.01.

The results with these drugs and comparison with the probenecid series suggest that "lipid solubility" is more influential to the development of the effect than a particular pK_a.

Radioactive Tracer Studies.—In rats pretreated with probenecid, the per cent conversion of ^{14}C-labeled hexoses to L-ascorbic acid-^{14}C increased more than fivefold above control values; for D-galactose from <0.05[16] to an average of 0.61 and for D-glucose from <0.02[16] to about 0.1 (Table III).

In pretreated rats a marked decrease in half-life of L-ascorbic acid-^{14}C was observed. This change was also accompanied by a considerable increase in the body pool of the vitamin (Table IV).

TABLE III

CONVERSION OF D-GLUCOSE-1-^{14}C AND D-GALACTOSE-1-^{14}C TO URINARY L-ASCORBIC ACID-1-^{14}C IN NORMAL AND PROBENECID-TREATED RATS

Treatment	Tracer	Dose of tracer, mg	Conversion, %
Probenecid[a]	D-Glucose	10.5	0.10
Probenecid[a]	D-Glucose	10.0	0.09
None[b]	D-Glucose	10–20	<0.03
Probenecid[a]	D-Galactose	2.9	0.71
Probenecid[a]	D-Galactose	1.5	0.51
None	D-Galactose	2.9	0.05
None[c]	D-Galactose	...	<0.05

[a] Rats weighing 190–280 g were fed 250 mg/kg daily in milk for 3–4 days prior to and during experiments. During 24 hr before intraperitoneal injection of tracer they excreted 5–10 mg of L-ascorbic acid in urine. Prior to drug administration they excreted less than 1 mg/24 hr. [b] Taken from Burns, *et al.*[2] [c] Taken from Conney, *et al.*[16]

TABLE IV

STUDY OF *T*/2, BODY POOL, AND TURNOVER RATE OF L-ASCORBIC ACID-1-^{14}C IN PROBENECID-TREATED RATS

No.	Drug treatment	Dose of tracer, mg	*T*/2, days	Body pool, mg/100 g	Turnover rate, mg/100 g/day
1	Probenecid[a]	2.1	0.43	25.5	41.2
2	Probenecid[a]	2.1	0.40	13.8	24.3
	None[19]	1.5	3.0	10.7	2.6

[a] Rats were fed 250 mg/kg of probenecid in milk on day 1 and 250 mg/kg ip/day subsequently. During 24 hr before injection of tracer on day 3, rats 1 and 2 excreted 9 and 7 mg of L-ascorbic acid, respectively. Rats 1 and 2 weighed 245 and 265 g, respectively. Animals used by Burns, *et al.*,[19] weighed about 300 g.

Discussion

The present results provide an explanation for the various observations that many compounds of unrelated structure produce an increase of the excretion and biosynthesis of L-ascorbic acid. The major requirement for the effect is not stringent, a relatively low "lipid solubility" (K_p > 0.01). This property is relative, as exemplified by barbital, which is active and yet is considered polar within the barbiturate series (K_p = 0.26 *vs.* 63 for thiopental[7]); however, compared to the probenecid series, barbital is "lipid soluble."

Although the effect was discovered earlier, King and co-workers[17] observed the wide variety of drugs that cause it, and first demonstrated that the increased excretion was due to an increase of the biosynthesis of L-ascorbic acid. When isotopic methods became available, King and his students further confirmed this conclusion.[18] Subsequently it was shown that pretreatment with pentobarbital, Chloretone,[19] and methylcholanthrene[16] increased the turnover rate and body pool of the vitamin. In the present study evidence is given that probenecid can act in a similar manner.

The doses of probenecid and of its analogs are rather high and the effect wears off in 1 or 2 days. On the

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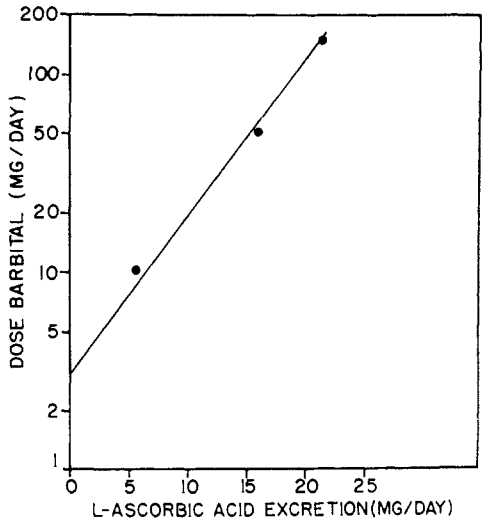

Figure 1.—Log dose barbital *vs.* urinary excretion of L-ascorbic acid by Wistar rats; based on data by Burns, Evans, and Tromsof.[21]

other hand, a small dose of methylcholanthrene causes an effect lasting for weeks. This contrast can be correlated to the differences in rates of elimination; the former compounds have half-lives of a few hours, compared to weeks for methylcholanthrene.[20] Interestingly, if one plots the data of Burns, *et al.*,[21] as log dose barbital *vs.* maximum effect, a linear relationship is obtained (Figure 1). These results were obtained upon dosing for several days. The importance of the dose of barbital on L-ascorbic acid biosynthesis has been interpreted[22] in terms of a regulator–receptor, based upon similar dose–effect relationships after a single dose of the drug.

The effect of drugs on the biosynthesis of L-ascorbic acid and the labeling pattern was taken advantage of by King and his associates[17,18,23] in proving that the precursors of the vitamin are D-glucose and D-glucuronic acid. These findings were the point of origin of investigation in several laboratories which led to the elucidation of the intermediate steps of the biosynthesis of the vitamin.[24–27] The pathway, now called glucuronic acid pathway of glucose metabolism was also shown to be responsible for the biosynthesis of L-xylulose and D-saccharic acid.[28,29]

The site of the primary effect of drugs on the glucuronic acid pathway is not clear, but it occurs near the beginning of the pathway.[19] Apparently by mass action, drugs producing this effect also increase the excretion of D-saccharic acid,[28,29] L-xylulose in pentosuries,[25,30] and L-ascorbic acid in rats. Furthermore it is of interest that many compounds that stimulate the glucuronic acid pathway induce microsomal drug-metabolizing systems,[16,31] cause porphyria,[32] stimulate the uptake of amino acids,[33] influence the synthesis of messenger[34] RNA and δ-aminolevulinic acid synthetase in liver culture,[35] and inflict changes in the endoplasmic reticulum.[36,37] The stimulation of drug metabolizing enzymes[15] and the glucuronic acid pathway[21] appears to represent an adaptive response which occurs without the intervention of the adrenals. It appears that certain steps of the biosynthesis of the vitamin take place in the microsome fraction derived from endoplasmic reticulum[38] and that L-ascorbic acid or dehydro-L-ascorbic acid are physiological requirements for the hydroxylation of many drugs.[39] The effect is not limited to Wistar rats; previously Ginn rats and in the present study Sherman rats were found to respond, though Ginn rats are less sensitive.[40] There may be more than one mechanism for the effect,[50] which can be blocked by ethionine.[40,41]

The list of compounds which increase L-ascorbic acid excretion in rats is growing.[41–48] Since measurement of the effect[49] is much simpler and can be carried out routinely, compared to studies of microsomal enzymes, it may prove to be a good model to indicate the probability of induction of the latter enzyme systems.

Note Added in Proof.—Since the submission of the manuscript, we have become aware of the work of Hoffman, *et al.*,[50] which proved that the alkaloid lycorine inhibits both L-ascorbic acid synthesis and drug metabolism in the rat.

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