The Effect of Ascorbic Acid Upon Bladder Uptake of β-Naphthylamine Metabolites[1] (36921)

BASSIMA S. ALAM, FRANCISCO E. JARAMILLO, J. U. SCHLEGEL, AND T. A. DEROUEN

Section of Urology, Department of Surgery, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, Louisiana 70112; and Department of Epidemiology and Biostatistics, Tulane University School of Public Health and Tropical Medicine, 150 South Liberty Street, New Orleans, Louisiana 70112

The cause of bladder cancer is, in most instances, still a matter of speculation. More and more chemicals are added to the list of suspected carcinogens, in most instances on the basis of animal experiments. When cyclamate was found to be carcinogenic in mouse-bladder implantation studies (1), it was removed from the market. Since five naturally occurring tryptophan metabolites have been shown to be carcinogenic (2–8) by the same technique, it would follow logically that tryptophan should also be banned. This is obviously an absurdity, since tryptophan is an essential amino acid, and we must thus accept that carcinogenic substances are naturally occurring in the urine. Even though the concentration and types of carcinogens in the urine must be of obvious importance, results of work done in our laboratory have suggested that the redox potential of the urine is of equal, if not greater, importance (9). Several suspected carcinogens oxidize very readily (10), and we have previously shown that administration of ascorbic acid would prevent oxidation of 3-hydroxyanthranilic acid (11), as well as its carcinogenicity (8).

Since it is still controversial as to whether tryptophan metabolites can cause cancer of the uroepithelium in man, we felt it would be of interest to study the effect of ascorbic acid on the bladder reabsorption of Beta-naphthylamine metabolites. β-naphthylamine is a proven potent bladder carcinogen in man (12), dogs (13), and nonhuman primates (14) when ingested orally, although uncertainty exists as to which and how many of the urinary metabolites are the potent carcinogens. We consequently in this study gave a single dose of tritium-labelled β-naphthylamine to a dog, and extracted the urinary metabolites. The effect of ascorbic acid upon the reabsorption of the metabolites by the rabbit bladders was then investigated.

Materials and Methods. Female dogs weighing 5 kg were administered orally a capsule containing one millicurie of ^{3}H-β naphthylamine (125 μCi/μmole, New England Nuclear) dissolved in 1 ml ethanol. A Foley catheter (No. 14F) was inserted into the dog's bladder and the catheter connected to a drainage bag containing an excess of ascorbic acid (approximately 15 g) to prevent oxidation during collection. A brace was placed to keep supporting the bag, and to avoid any oxidation by light, and the urine collected for 16–18 hr.

Extraction of metabolites. The urine was divided into two equal portions (500–600 ml each) which sequentially were extracted with three solvents of increasing polarity; ether, ethyl acetate, and after saturation with ammonium sulfate, tert-butyl alcohol according to the method described by Deichmann and Radomski (15).

The solvents were evaporated using a rotary evaporator (under 30°) and the residues from ether, ethyl acetate and tert-butyl alcohol extracts were combined to obtain all the urinary metabolites of β-naphthylamine. The metabolites of each portion were dissolved in phosphate buffer (pH 5.6, 0.2 *M*) and the volume made up to 25 ml. To one sample, ascorbic acid was added (400 mg%) and nitrogen passed through for two hours, while

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TABLE I. Effect of Ascorbic Acid on the Recovery of Radioactivity from the Urinary Metabolites of ^{3}H-B-Naphthylamine by the Bladder Solution.

	With ascorbic acid (W AA)			Without ascorbic acid (W/O AA)			
Replicate	Radioactivity administered (DPM)	Average % recovered	Standard deviation (% recovery)	Radioactivity administered (DPM)	Average % recovered	Standard deviation (% recovery)	Comparison of means (W AA)—(W/O AA) (sign only)
1	22.21×10^6	76.6 (2)[a]	24.25	30.78×10^6	29.8 (2)[b]	11.88	+
2	28.07×10^6	73.2 (3)	1.37	28.32×10^6	67.7 (3)	9.44	+
3	28.61×10^6	33.9 (3)	9.54	25.09×10^6	30.2 (2)[b]	14.43	+
4	32.14×10^6	69.4 (2)[b]	13.22	30.84×10^6	50.5 (3)	4.24	+
5	35.32×10^6	58.2 (3)	2.85	35.06×10^6	53.1 (3)	0.95	+
6	40.99×10^6	68.4 (3)	8.61	40.99×10^6	63.2 (3)	5.00	+
7	23.51×10^6	39.8 (3)	3.71	18.81×10^6	32.5 (3)	5.28	+
8	22.42×10^6	35.0 (3)	7.40	20.70×10^6	27.9 (3)	7.37	+
Overall average	29.16×10^6	56.8 (22)		28.82×10^6	44.4 (22)		$p = 0.004$ for the Sign Test

[a] Number in parentheses is the number of rabbits used in each treatment.

[b] Indicates an observation missing due to the death of a rabbit before completion of the experiment from causes unrelated to the treatment administered.

TABLE II. Effect of Ascorbic Acid on the Uptake of Radioactivity from the Urinary Metabolites of ^{3}H-β-Naphthylamine by the Bladder Tissues.

	With ascorbic acid (W AA)			Without ascorbic acid (W/O AA)			
	Radioactivity recovered[a]		Standard deviation	Radioactivity recovered		Standard deviation	Comparison of average %
Replicate[c]	Av DPM/g	% Adm dose[b]	(% recovery)	Av DPM/g	% Adm dose	(% recovery)	(W AA)—(W/O AA)
3	133×10^3 (3)	0.466	0.173	129×10^3 (2)[d]	0.512	0.135	—
4	183×10^3 (2)[d]	0.568	0.107	539×10^3 (3)	1.74	1.15	—
5	273×10^3 (2)[d]	0.772	0.19	313×10^3 (3)	0.892	0.03	—
6	155×10^3 (3)	0.378	0.15	181×10^3 (3)	0.442	0.172	—
7	26×10^3 (2)[d]	0.11	0.03	51×10^3 (3)	0.271	0.11	—
8	30×10^3 (3)	0.13	0.085	55×10^3 (3)	0.267	0.086	—
Overall average	133×10^3 (15)	0.388		211×10^3 (17)	0.698		$p = 0.02$ For the Sign Test

[a] Administered doses are the same as those in Table I.

[b] DPM/g/Total adm. dose $\times$ 100.

[c] In replicates 1 and 2, no determination was made for radioactivity uptake.

[d] Indicates missing observation.

oxygen was passed for two hours through the second sample.

Impregnation of the rabbit's bladder. Male rabbits weighing 2–3 kg were used. After anesthesia with sodium pentobarbital, the abdominal wall was incised and the ureters ligated in order to prevent the flow of urine from the kidneys into the bladder. The bladder was gently emptied by compression. A catheter was inserted into the bladder through the urethra, and 7–8 ml of the solution (with and without ascorbic acid) of the metabolites in buffer was introduced using a 10 ml syringe. The solution of the metabolites was incubated in the bladder for two hours. The bladder was then removed, and the contents emptied into a beaker. The bladder was rinsed gently with physiological saline and the washings collected. Portions of the bladder were next excised, weighed and solubilized in soluene. An aliquot of the solution was used for radioactivity measurements.

Liquid scintillation counting. For radioactivity measurement, aliquots (0.1 ml each) in duplicate were used for the following: (a) the original metabolites before inserting into the bladder; (b) bladder tissue solutions in soluene; and (c) the bladder solutions after recovery from the bladder. Fifteen ml of liquid scintillation fluid (aquasol) was added to each vial and the radioactivity measured in a Packard TriCarb 3200 liquid scintillation spectrometer. Quenching was corrected by the Automatic External Standard (AES) method.

The experiment was performed with 2–3 rabbits randomly allocated to each treatment, and repeated on several different days (six for the bladder tissue, eight for the bladder solution). Although an effort was made to standardize conditions from day to day, to avoid having any unaccountable day to day variability influence the results, comparisons between the two treatments are made only within the same day.

Results. The effect of ascorbic acid on the recovery of radioactivity in the bladder solutions and uptake by the bladder tissues of rabbits after *in vivo* instillation of urinary metabolites from ^{3}H-β-naphthylamine is shown in Table I.

The results of eight different replicates, each using 4–6 rabbits, show that there was greater recovery of radioactivity from the instilled bladder solutions in the presence of ascorbic acid than in its absence.

The average radioactivity (% of administered dose) varied from one replicate to another. However, in each set of replicates, there was more radioactivity recovered from the bladder solution of metabolites containing ascorbic acid. The average value of eight replicates conducted using 22 rabbits in each group (with and without ascorbic acid) was 56.8% recovery in the ascorbic acid-treated group versus 44.4% in the group without ascorbic acid.

Data on the radioactivity uptake by the bladder tissues are shown in Table II. As can be seen, in the presence of ascorbic acid there was less radioactivity taken up by the bladder.

Similar results were obtained in each of the six replicates in which bladder uptake was measured. Thus the presence of ascorbic acid upon the urinary β-naphthylamine metabolites affected the radioactivity recoveries measured in the bladder solutions and in the bladder tissues in opposite directions, *i.e.*, it decreased the bladder uptake, but increased the recovery from the bladder solutions.

The determination of the statistical significance of the two sets of data was done in two steps. The first step was merely a confirmation of what seems obvious at a glance. If the average percentages of radioactivity recovered in the bladder solution for the two treatments (with and without ascorbic acid) are compared for each day (the reason for making comparisons only within the same day having been previously stated), it is seen that the average percentage is always higher when ascorbic acid is present than when it is absent. On the other hand, for the percent of administered dose recovered in the bladder tissues, just the opposite is true; *i.e.*, the percentage is always higher when ascorbic acid is absent than when it is present. A simple way to determine the statistical significance of these trends is to employ the sign

test, a nonparametric statistical procedure (16). The sign test merely determines the probability of observing an average for one treatment that is less than the average for the other eight times in a row (for the bladder solution data; six times in a row for the bladder tissue data) if, in fact, there is no difference between the two treatments. As is indicated at the bottom of Tables I and II, the probabilities of observing these trends if there is no difference between the two treatments are 0.004 for the bladder solution data, and 0.02 for the bladder tissue data.

One criticism that can be levied against the use of only the sign test for these data is that it ignores variability within treatments, and compares only treatment means. For this reason, the data were also analyzed using analysis of variance procedures for data with unequal numbers of observations per cell and missing observations (17). Only the results of the statistical test for the difference between the two treatments in this analysis will be discussed here.

For the bladder solution data, the F-test for the difference between treatments had value 5.27. For an F-distribution with 8 and 28 degrees of freedom, this corresponds to a P-value of less than 0.001. Therefore the conclusion of this test is even stronger than for the sign test. For the bladder tissue data, the F-statistic had a value of 2.23 with 6 and 20 degrees of freedom, corresponding to a P-value between 0.05 and 0.10. Thus the analysis of variance test does not produce as strong a confirmation of the results of the sign test for the bladder tissue data as it does for the bladder solution data, but it still indicates a rather high level of significance. The slight discrepancy between the significance levels for the two tests on the bladder tissue data is probably due to the fact that there are several missing observations in this data, which is something that the sign test does not detect.

Discussion. Our studies indicate that the presence of ascorbic acid in the bladders of rabbits decreases the uptake of radioactivity from the urinary metabolites of ^{3}H-β-naphthylamine.

The variability from one replicate to another in the percentage recovery of the administered radioactivity in the bladder solution and in the radioactivity uptake by the bladder tissues may be due to variability among animals (rabbits) or more likely may be due to the different proportions of the various metabolic products of tritiated B-naphthylamine in the urine each time it was given to the rabbits. As β-naphthylamine is metabolized to many different compounds that are excreted in urine, it is possible that in an experimental situation, the urinary concentration of an individual potential carcinogenic metabolite excreted in dog's urine may vary from one replicate to another. Thus the proportion of different metabolites instilled in the rabbit bladders may therefore also vary. In spite of such variability, it is important to note, however, that in each replicate conducted under similar conditions, ascorbic acid has an effect, both on the bladder uptake and on the recovery from the bladder solution.

Previous studies from this laboratory (11) have shown that the addition of ascorbic acid to the urine containing 3-hydroxyanthranilic acid stabilized the latter, which is ordinarily very much susceptible to oxidation. Also, high levels of ascorbic acid in the urine afforded some protection to mice against the induction of bladder tumors as a result of implantation of 3-hydroxyanthranilic acid pellets in their bladders (8).

We have previously suggested that 3-hydroxyanthranilic acid *per se* may not be carcinogenic while oxidation may render it so. This would explain why ascorbic acid could prevent bladder tumor formation from 3-hydroxyanthranilic acid, since in competing for oxygen, it prevents the oxidation of the 3-hydroxyanthranilic acid. This same mechanism may exist as it relates to the carcinogenic metabolites of β-naphthylamine. If less bladder uptake occurs with the reduced metabolites and more with the oxidized ones, this would suggest that ascorbic acid could have a preventive effect on bladder carcinoma resulting from β-naphthylamine administration since a decreased uptake of a carcinogenic compound by the bladder would obviously render it less potent.

Pamukcu *et al.* (18) have recently shown that phenothiazine inhibits the intestinal and urinary bladder tumors induced in rats by bracken fern. Although the carcinogen in bracken fern has not yet been chemically characterized, it was shown by these investigators that phenothiazine acted as an inducer of microsomal benzopyrine (BP) hydroxylase and thus decreasing the carcinogenic effect of bracken fern.

It is interesting to note, however, that phenothiazine is also a reducing agent, like ascorbic acid, so it is quite possible that at least part of the inhibitory effect of phenothiazine on tumor production may be due to its reducing ability.

The current findings further substantiate our hypothesis that the urinary environment, especially its redox potential, may be an important factor which can modify the carcinogenicity of a particular compound. This may have important ramifications in the practical approach as a preventive measure in human carcinoma of the uroepithelium.

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