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THE ROLE OF HYDROXYAMINES IN BLADDER CANCER—I.

FLUORESCENCE STUDIES OF THE BLADDER MUCOSA OF DOGS FED 2-AMINONAPTHALENE[1]*

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Abstract—Fluorescence spectroscopy of the bladder mucosa of dogs given the bladder carcinogen 2-aminonaphthalene revealed the presence of a fluorescent metabolite displaying maxima at 400 mμ and 440 mμ at 340 mμ excitation. Control dog bladder mucosa displayed a single maximum of lower intensity at 440 mμ. The same fluorescence spectrum was obtained after the *in-vitro* interaction of the 2-amino-1-naphthyl sulfate with control dog bladder mucosa. However, other 2-amino-1-naphthol derivatives displayed the same fluorescence maxima under these conditions. The fluorescence is completely removed by dialysis of the bladder mucosa and also disappears if the ingestion of the 2-aminonaphthalene is discontinued for a few days. The metabolite was identified as 2-amino-1-naphthyl sulfate by paper chromatography. No other metabolites of 2-aminonaphthalene were detected.

Two of the fundamental questions remain unanswered with regard to the etiology of bladder cancer from aromatic amines: first, what is the nature of the proximate carcinogenic metabolite of aromatic amines; second, with what tissue constituent does this metabolite react in order to produce the carcinogenic response. Recent evidence strongly implicates the N-hydroxy derivatives as the proximate carcinogens of 2-aminonaphthalene, 4-aminobiphenyl, 2-acetylaminofluorene, and perhaps carcinogenic aromatic amines in general: N-hydroxy-2-aminonaphthalene glucoronide and sulfate have been reported in the urine of dogs given 2-aminonaphthalene;[1, 2] conjugates of N-hydroxylated 4-acetylaminobiphenyl and 2-acetylaminofluorene have also been found in the urine of dogs and rats.[3, 4] The N-hydroxy derivatives of 2-acetylaminobiphenyl and 2-acetylaminofluorene are carcinogenic by i.p. administration to rats, quantitatively more so than the parent amides.[3, 5] According to the bladder implantation testing technique of Bonser *et al.*,[6] N-hydroxy-2-aminonaphthalene is carcinogenic along with di-(2(amino-1-naphthyl)hydrogen phosphate, 1-amino-2-naphthol, and 2-amino-1-naphthol. Boyland *et al.*[7] have shown that N-hydroxy-2-aminonaphthalene is carcinogenic by i.p. injections in rats.

While the above observations point to the N-hydroxy derivatives of aromatic amines as the active carcinogens, other considerations point toward the *o*-hydroxyamines. All the active metabolites of 2-aminonaphthalene including N-hydroxy-2-aminonaphthalene are capable of conversion, *in vivo*, to 2-amino-1-naphthol which is itself carcinogenic.[6] In addition, Troll *et al.*,[8] have found that 2-amino-1-naphthol

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reacts with salmon sperm DNA *in vitro*, whereas N-hydroxy-2-aminonaphthalene does not, and points out that the N-hydroxylation theory does not explain the apparent need for a blocked *p*-position in carcinogenic amines.

Although much is known about the urinary metabolites of 2-aminonaphthalene in animals, the presence of a given metabolite in urine does not indicate that this metabolite has significance with regard to the etiology of bladder cancer. The active metabolite must penetrate the bladder mucosa in order to produce tumors of this tissue. We therefore set out to detect and identify metabolites of 2-aminonaphthalene in the bladder mucosa of dogs given oral doses of this compound. Dogs were selected as the experimental animal because only this species develops bladder cancer from 2-aminonaphthalene. Because of the high fluorescence intensity exhibited by the metabolites of 2-aminonaphthalene, methods utilizing this property were used for the detection of the metabolites present in the bladder mucosa.

One report of the presence of fluorescence in bladder tissue of a single dog given 2-aminonaphthalene is contained in the literature. Mellors and Hlinka,[9] using fluorescence microspectroscopy, found that after oral administration of 2-aminonaphthalene to a female dog, a material with a fluorescence spectrum having a maximum at 430 to 440 mμ and differing from that of the ingested carcinogen is present in the urine, the exfoliated epithelial cells of the urinary tract, and the superficial and deep transitional cells of the bladder mucosa. They showed that the fluorescing material can gain access to the bladder mucosa both by resorption from the urine and by way of the circulating blood. They also reported that the fluorescing material disappears from the cells within a few days after the cessation of the ingestion of 2-aminonaphthalene. Belman and Troll* studied the binding of a series of 2-aminonaphthalene metabolites with rat submucosa by means of fluorescence microscopy. Fluorescent zones were observed in the submucosa after exposing the bladder to relatively concentrated solutions of 2-amino-1-naphthol, its phosphate ester, and 1, 2-naphthoquinone-sulfonate. The fluorescence of the submucosa was believed to be due to a possible conjugate of the metabolite with hydroxylysine. Belman and Troll[10] demonstrated by spectroscopic techniques that 2-amino-1-naphthol combined with bovine serum albumin and rat bladder epithelium in the presence of cytochrome *c* to give what they reported to be a 1, 2-naphthoquinone-protein derivative. However, Nagasawa and Gurman[11] report that 2-amino-1-naphthol formed a quinone imine product with bovine serum albumin.

MATERIALS AND METHODS

Animals and diets. Mongrel dogs of both sexes, 8 to 17 kg body weight, were used in these experiments. The 2-aminonaphthalene was administered by capsule as a 20% solution in corn oil. The diet consisted of Purine dog meal, Ken-L-Burger, and water *ad libitum*.

Chemicals. All chemicals were from commercial sources, unless otherwise indicated, and purified by appropriate procedure. N-Hydroxy-2-aminonaphthalene was prepared by the reduction of 2-nitronaphthalene (Fundamental Research Co.) by the general procedure of Willstatter and Kubli.[12] 2-Amino-1-naphthyl sulfate,[13] 2-amino-1-naphthyl phosphate,[14] and di-(2-amino-1-naphthyl)hydrogen phosphate[15]

* S. Belman and W. Troll, "The Reaction of Rat Bladder with Metabolites of Beta-Naphthylamine", paper presented at Industrial Health Conference, Rochester, N.Y., April 25, 1960.

were prepared according to Boyland, Manson, Kinder and Sims. The 2-amino-1-naphthol was prepared by reduction of 2-nitroso-1-naphthol in alkaline $Na_2S_2O_4$. 2-Amino-1-naphthyl glucosiduronic acid was prepared by the method of Belman.[16]

Preparation of protein-bound naphthalene derivatives

The general procedure reported by Belman and Troll[10] was used. A solution of $3{\cdot}2 \times 10^{-4}$ moles of bovine serum albumin in 100 ml of 0·01 M phosphate buffer, pH 7, was used in each reaction with the naphthalene derivatives. The various naphthalene derivatives to be studied were then added to the protein solutions so that the final concentration of the naphthalene derivatives were $2{\cdot}3 \times 10^{-4}$ moles/100 ml protein solution. The reactions were then allowed to proceed for 24 hr in light-shielded flasks equipped for magnetic stirring at room temperature. Aliquots of 24 ml of the reaction solutions were then dialyzed several times against 1 liter of 0·01 M phosphate buffer, pH 7·5. The dialysis was carried out at 5° and was repeated until the dialysate was colorless. Aliquots of 10 ml of the highly colored protein solutions left in the dialysis bag were treated with 2 volumes of ice-cold acetone. This treatment yielded dark brown precipitates of protein and purple-colored supernatants. In each case the protein was redissolved in buffer and precipitated with acetone. The procedure was repeated until the supernatants were colorless. The products were then dissolved in 0·01 M phosphate buffer, pH 7, centrifuged for 30 min at 2,500 rpm, and the resulting supernatants examined with a spectrophotofluorometer.

The following bovine serum albumin naphthalene-derived adducts were prepared: 2-amino-1-naphthol-BSA,* 2-amino-1-naphthol-4-sulfonate-BSA, 1-amino-2-naphthol-BSA, 1-amino-2-naphthol-4-sulfonate-BSA, N-hydroxy-2-aminonaphthalene-BSA, and 1, 2-naphthoquinone-4-sulfonate-BSA. All calculations were based on a molecular weight of 69,000 for serum albumin.

2-Amino-1-naphthol-4-sulfonate was prepared by the reduction of 2-nitroso-1-naphthol-4-sulfonate in alkaline $Na_2S_2O_4$. The compound was used without purification. The fluorescence of the reaction mixture indicated the presence of the desired product.

Bladder preparation. All animals were sacrificed by a lethal i.v. injection of pentobarbital and the urinary bladders removed immediately. The bladders were washed thoroughly with cold distilled water to remove all traces of urine and were either examined immediately or stored frozen. Freezing did not affect the fluorescence.

Specimens for examination by fluorescence microcope and u.v. microspectrophotometer were prepared from frozen sections of bladder, sectioned at 10 μ with a freezing microtone, fixed by the freeze-dry method, mounted on 1-mm thick fused quartz slides, and covered with a quartz cover slip 0·18 mm thick. These examinations were carried out immediately after the removal of the bladder.

Bladder tissues used for examination in the spectrophotofluorometer were prepared as follows: 0·5 g (wet weight) of bladder mucosa was minced and homogenized in an all-glass homogenizer in 5 ml of distilled water, The homogenization was carried out with cooling by means of an ice bath. The homogenate was centrifuged for 30 min at 2,500 rpm, and the resulting supernatant examined.

Fluorescence microscopy was carried out with the Reichert Zetopan fluorescence microscope and a filter having a 260-mμ minimum cutoff, a 420-mμ maximum cutoff,

* BSA, bovine serum albumin.

and a peak at 365 mμ. Ultraviolet microspectrophotometry was performed with an instrument capable of detecting absorption in the region 2,200 to 7,000 Å.[17] Fluorescence spectra were obtained with the Aminco–Bowman spectrophotofluorometer. The light source was a zenon arc lamp. All measurements were carried out at room temperature in a quartz cell having a 1-cm light path. An RCA IP 28 photomultiplier tube was used to detect the emission, and the intensities reported were obtained directly from a photomultiplied microphotometer. All spectra are uncorrected.

The identification of the metabolites of 2-aminonaphthalene by paper chromatography

The mucosa was removed from the bladder of each experimental animal and homogenized by the method previously described for use in fluorescence examination. The wet weight varied from 1·5 to 2·5 g. A total of 10 to 20 ml of distilled water was used in the homogenization. The homogenate was then centrifuged for 30 min at 9,000 rpm and the clear supernatant, after concentration to 10 ml *in vacuo*, examined for the presence of the characteristic fluorescent peaks at 400 mμ and 440 mμ. To the supernatant was added 10 volumes of ice-cold acetone to precipitate the protein. The mixture was allowed to stand overnight at 5° and was then centrifuged at 9,000 rpm for 30 min. The acetone was removed *in vacuo*, and the protein-free supernatant was again checked for fluorescence. In all cases, only one peak with a fluorescence maximum at 410 mμ was evident at this point. The aqueous solution containing the metabolites was evaporated to dryness *in vacuo*. The resulting residue was then taken up in 0·5 ml of methanol:water (1:1) solution. Whatman 1 paper was employed in descending chromatography in a solvent system of 1-butanol (4), benzene (1), and saturated with 0·1 N ammonia. Whatman 20 paper was employed in ascending chromatography with the following solvent systems: (a) 1-butanol: 1-propanol: aqueous 0·1 N ammonia (2:1:1 by volume); (b) 1-butanol: acetic acid: water (2:1:1) by volume). A Mineralight lamp and a Blak-Ray (both from Ultra-violet Products Inc.) were used as sources of short- and long-wave u.v. light respectively.

Dialysis of bladder mucosa

A 0·5-g sample (wet weight) of dog bladder mucosa was homogenized with 5 ml of 0·01 M phosphate buffer, pH 7, and then centrifuged at 2,500 rpm for 30 min. The clear supernatant was checked for the characteristic peaks at 400 mμ and 440 mμ and then dialyzed against 20 ml of 0·01 M phosphate buffer, pH 7. The dialysate was changed twice daily and collected. An alternative procedure for dialysis was the use of distilled water in place of the buffer. Distilled water proved just as efficient and eliminated the salt problem when the dialysates were taken to dryness. The dialysis was carried out at 5° for 10 days. The combined dialysates were reduced in volume to 10 ml *in vacuo*. After examination in the spectrophotofluorometer, the dialysate was evaporated to dryness *in vacuo* and the residue dissolved in 0·5 ml methanol: water (1:1) solution. The resulting solution was then chromatographed by the procedure described previously.

Interaction of derivatives of 2-aminonaphthalene with dog bladder mucosa

Control dog bladder mucosa previously checked for the absence of any fluorescence at 400 mμ was used in these experiments. Fresh dog bladder mucosa (0·5 g wet weight) was homogenized (cold) with 5 ml of a solution containing 10 mg of the compound

to be studied per liter of distilled water, for 30 min at 2,500 rpm, and the fluorescence spectrum of the supernatant obtained.

RESULTS

Fluorescence microscopy and u.v. microspectrophotometry

The examination in a fluorescence microscope of bladder sections of five dogs, which received 6 to 11·6 g of 2-aminonaphthalene orally, failed to reveal any zones of fluorescence. Gross examination of the bladders revealed no colour changes in the mucosa. They could not be distinguished from bladders of control dogs. The sections were also examined by means of a microspectrophotometer, with negative results.

Spectrophotofluorometer studies

Four dogs were given 400 mg per day of 2-aminonaphthalene for 16 consecutive days. They were sacrificed 17 hr after the last dose, the bladders removed, the mucosa homogenized as described under Methods, and the extracts examined in the spectrophotofluorometer. In all four dogs a fluorescence spectrum was obtained with a maximum at 400 mμ and another at 440 mμ, as shown in Fig. 1. Maximal intensity of the

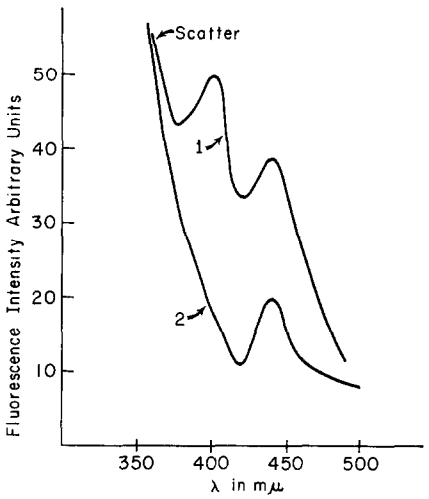

FIG. 1. Fluorescence spectra of bladder mucosa homogenate in water. Activation wavelength 340 mμ. Curve 1, experimental dog; curve 2, control dog.

400-mμ peak was obtained at 340 mμ excitation. Bladder homogenates of control dogs did not have the 400-mμ maximum, and the peak at 440 mμ was of lower magnitude. The use of 0·01 M phosphate buffer of pH 7 in place of distilled water did not affect the spectra obtained as far as location of the maxima or their intensities.

Administration of 2-aminonaphthalene consecutively for 26 daily doses did not increase the intensity of the fluorescence peak at 400 mμ. Reduction in the duration of administration of the same dose (400 mg/dog) did not reduce the intensity of this peak when the dogs were sacrificed within 17 hr after the last dose. In fact, optimum peak height was obtained in a series of four dogs receiving a single dose of 800 mg administered 10 to 17 hr prior to sacrifice. However, when the period before sacrifice was

prolonged to 42 hr, the fluorescence spectra obtained were indistinguishable from the control bladders.

Fluorescence characteristics of 2-aminonaphthalene and its derivatives

The fluorescence spectra of 2-aminonaphthalene, N-hydroxy-2-aminonaphthalene, 2-amino-1-naphthol, di-(2-amino-1-naphthyl)hydrogen phosphate, 2-amino-1-naphyl dihydrogen phosphate, 2-amino-1-napthol hydrogen sulfate, 1-amino-2-naphthol, 2-amino-1-naphthol-4-sulfonate, 1-amino-2-naphthol sulfate, and 1, 2-naphthoquinone-4-sulfonate were determined. The fluorescence characteristics of these compounds are given in Tables 1 and 2. 1, 2-Naphthoquinone-4-sulfonate had a very

TABLE 1. FLUORESCENCE SPECTRA OF SOME NAPHTHALENE COMPOUNDS AND THE CORRESPONDING NAPHTHALENE DERIVATIVE–BOVINE SERUM ALBUMIN PRODUCTS

Naphthalene compounds	Compound		Compound + BSA	
	Activation maximum (mμ)	Fluorescence maximum (mμ)	Activation maximum (mμ)	Fluorescence maximum (mμ)
2-Amino-1-naphthol	340	450	340	405–10
2-Amino-1-naphthol-4-sulfonate	340	445	340	420–25
1-Amino-2-naphthol	340	450	340	405
1-Amino-2-naphthol-4-sulfonate	340	450	340	415
N-Hydroxy-2-aminonaphthalene	340	445	340	430
1, 2-Naphthoquinone-4-sulfonate	340	450		

All spectra obtained in 0·01 M phosphate buffer, pH 7.

weak fluorescence. Although the fluorescence characteristics of the two phosphate esters and the sulfate ester were similar to the fluorescence characteristics of the bladder mucosa homogenates obtained from dogs given 2-aminonaphthalene, their fluorescence maxima (410–415 mμ; Table 2) were significantly different from those obtained with the experimental bladders (400 mμ). Boyland and Williams[18] reported similar activation and fluorescence spectra for 2-amino-1-naphthyl derivatives, with maximal activation at 324–330 mμ and a fluorescent peak at 410 mμ.

Fluorescence characteristics of reaction products of some naphthalene derivatives with bovine serum albumin

Irving and Gutman,[19] and Nagasawa and Gurman[11] reported that 2-amino-1-naphthol bound to bovine serum albumin *in vitro* after oxidation to a naphthoquinone imine. Belman and Troll[10] reported the *in vitro* binding of 2-amino-1-naphthol to bovine serum albumin and rat bladder, but reported the binding took place after oxidation of the aminonaphthol to a naphthoquinone. We decided to prepare the BSA reaction products of some naphthalene derivatives to determine whether their fluorescence characteristics were similar to those of our bladder homogenates of dogs dosed with 2-aminonaphthalene. Reaction products of BSA and the following compounds were prepared: 2-amino-1-naphthol, 2-amino-1-naphthol-4-sulfonate, 1-anino-2-naphthol, 1-amino-2-naphthol-4-sulfonate, N-hydroxy-2-aminonaphthalene, and 1, 2-naphthoquinone-4-sulfonate. These reaction products were washed free from excess chemical as described under Methods.

Table 1 summarizes the fluorescence spectra of these naphthalene derivatives and their reaction products with bovine serum albumin. The fluorescence spectra of all the naphthalene derivative–BSA products showed a considerable shift of the fluorescence maxima, except N-hydroxy-2-aminonaphthalene and the 1, 2-naphthoquinone-4-sulfonate. In the case of the N-hydroxy-2-aminonaphthalene, a small shift of 10 mμ to the shorter wavelengths was noted. In the case of 1, 2-naphthoquinone-4-sulfonate, no fluorescence maximum was obtained after interaction with BSA. This result is significant, since the 2-amino-1-naphthol–BSA product has a relatively strong fluorescence. If this product was in the form of a naphthoquinone as is the product obtained from the 1, 2-naphthoquinone, no fluorescence would be expected. It is quite unlikely therefore that 2-amino-1-naphthol reacts as a quinone with the protein, as suggested by Belman and Troll.[10]

It was noted that 2-amino-1-naphthol and 1-amino-2-naphthol yielded protein derivatives that were quite stable on prolonged exposure to light, in sharp contrast to very light, unstable aminonaphthols themselves.

Although the activation and fluorescence maxima of both of the aminonaphthol–BSA reaction products approach the maxima exhibited by the bladder mucosa of dogs fed 2-amino-1-naphthalene, there were other significant differences. For example, peak shape, intensity, and resistance to dialysis differed enough to establish that the observed fluorescence in the bladder mucosa was due to other causes.

Interactions of metabolites of 2-aminonaphthalene with homogenized bladder mucosa

In a further effort to duplicate the fluorescence characteristics obtained in our experiments with dogs *in vivo*, the interaction of six naphthalene derivatives with

TABLE 2. FLUORESCENCE SPECTRA OF SOME NAPHTHALENE COMPOUNDS AND THEIR SPECTRA AFTER INTERACTION WITH DOG BLADDER MUCOSA

Naphthalene compounds	Compound		Compound + mucosa	
	Activation maximum (mμ)	Fluorescence maximum (mμ)	Activation maximum (mμ)	Fluorescence maximum (mμ)
2-Aminonaphthalene	340	420	340	400, 430
N-Hydroxy-2-aminonaphthalene	340	445	340	400, 440
2-Amino-1-naphthol	340	445	340	400, 440
Di-(2-amino-1-naphthyl)phosphate	340	410	340	400, 430
2-Amino-1-naphthyl dihydrogen phosphate	340	415	340	435
Potassium 2-amino-1-naphthyl hydrogen sulfate	340	410	340	400, 440

homogenized dog bladder mucosa was studied. The fluorescence spectra of 2-aminonaphthalene, N-hydroxy-2-aminonaphthalene, 2-amino-1-naphthol, di-(amino-1-naphthyl)hydrogen phosphate, 2-amino-1-naphthyl dihydrogen phosphate, and potassium 2-amino-1-naphthyl hydrogen sulfate in pure solution and in the same concentration in bladder mucosa homogenate are shown in Table 2. The fluorescence curve is shown in Fig. 2. It can be readily seen that the fluorescence maxima obtained with all the compounds studied, with the exception of 2-amino-1-naphthyl dihydrogen phosphate, are identical with the fluorescence maxima obtained from the bladder mucosa of dogs that received 2-aminonaphthalene orally. Fluorescence maxima at

400 mμ and between 430 and 440 mμ at 340 mμ excitation were obtained. 2-Amino-1-naphthyl dihydrogen phosphate shows a single peak at 440 mμ with only a slightly greater intensity than that normally observed in control bladder homogenates.

The spectra obtained after interactions with dog bladder mucosa show a definite shift of the fluorescence peaks to the shorter wavelengths. The largest shift was

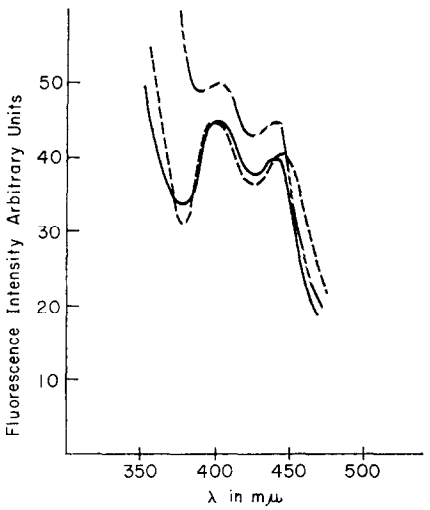

FIG. 2. Fluorescence spectra of the interaction of dog bladder mucosa and some naphthalene derivatives. Solvent water; activation wavelength 340 mμ; — — —, potassium 2-amino-1-naphthyl hydrogen sulfate; — – – —, di-(2-Amino-1-naphthyl)hydrogen phosphate; ———, 2-aminonaphthalene.

displayed by 2-aminonaphthalene, namely, 420 mμ to 400 mμ. The most significant observation was the almost identical spectra obtained by this experiment *in vitro* as compared to those obtained from the mucosa of dogs given 2-aminonaphthalene. However, since dissimilar compounds produced the same fluorescence peaks, this method could not be used for the identification of metabolites but only as a means of detection. The spectral shifts seem to indicate that binding between the fluorescent compound and some cellular components takes place. However, the observation that the fluorescence disappears when feeding is discontinued for a few days indicates a simple diffusion or very weak combination of some type.

Another very interesting result was obtained with the interaction of 2-amino-1-naphthol and bladder mucosa. It was found that in the presence of the bladder mucosa homogenate the fluorescence of this very labile compound is quite stable to u.v. and visible light, whereas the 2-amino-1-naphthol in only the buffer or distilled water lost its fluorescence quite rapidly. Again it seems reasonable to attribute this stabilization of the fluorescence to some type of weak interaction.

Paper chromatography

Since spectrophotofluorometry could not resolve the various metabolites of 2-aminonaphthalene in bladder mucosa, paper chromatography was tried. Difficulty was experienced in finding a solvent system that could resolve 2-amino-1-naphthyl sulfate and di-(2-amino-1-naphthyl)hydrogen phosphate. Finally, it was found that

1-butanol (4), benzene (1) saturated with 0·1 N NH_3 clearly distinguished these two esters. A comparison of the chromatographic characteristics of the primary metabolite extracted from the bladder mucosa of dogs given 2-aminonaphthalene is shown in Table 3, compared with pure samples of available metabolites. The characteristics of

TABLE 3. R_F VALUES AND COLOR REACTIONS OF 2-AMINONAPHTHALENE AND DERIVATIVES COMPARED TO METABOLITE IN DOG BLADDER MUCOSA

Compound	Fluorescence	Spray color	R_F Solvent systems* I	II	III
2-Amino-1-naphthyl-hydrogen sulfate	Blue-white	Brick-red	0·47	0·43	0·69
2-Amino-1-naphthyl-dihydrogen phosphate	Blue-white	Red	0·00–0·05	0·07	0·70
Di-(2-amino-1-naphthyl) hydrogen phosphate	Blue	Red	0·66	0·60	0·70
2-Aminonaphthalene	Blue-white	Red		0·92	0·90
2-Aminonaphthalene-N-glucuronide	Blue-white	Brick-red	0·13	0·28	Hydrol.
2-Amino-1-naphthyl-glucosiduronic acid	Blue-white		0·15	0·18	0·64
Metabolite in dog-bladder mucosa	Blue-white	Brick-red	0·45	0·41	0·69

* Solvent system I, 1-butanol: benzene, 0·1 N NH_3 (sat.), 4:1, descending; II, 1-butanol: 1-propanol: 0·2 N NH_3 (2:1:1), ascending; III, 1-butanol: acetic acid: water (2:1:1), ascending.

the bladder metabolite are seen to be identical with 2-amino-1-naphthyl sulfate. This identification was confirmed with parallel chromatograms. R_F values of 0·45, 0·41, and 0·69 for the metabolite present in the mucosa compared favourably with 0·47, 0·43, and 0·69 for 2-amino-1-naphthyl sulfate in the three solvent systems used. A blue-white and a blue fluorescence was observed for both compounds under long- and short-wave u.v. light respectively. Both compounds gave identical brick-red colors on spraying with *p*-dimethylaminocinnameldehyde reagent. In all experiments, control mucosa was run side by side with the experimental mucosa and with the various metabolite standards.

All attempts to demonstrate the presence of di-(2-amino-1-naphthyl)hydrogen phosphate or 2-amino-1-naphthyl glucuronide in the bladder extract were unsuccessful.

Dialysis of dog bladder homogenate

The dialysis of the experimental dog bladder homogenates resulted in complete removal of the characteristic fluorescence peaks. The dialysates were pooled, concentrated *in vacuo*, and examined in the spectrophotofluorometer. The fluorescence spectra of the dialysates gave a single maximum at 410 mμ at 340 mμ activation. All the metabolites of 2-aminonaphthalene we studied displayed a maximum at 410 mμ under the same conditions (Tables 1 and 2).

DISCUSSION

Wiley[20] first reported that 2-aminonaphthalene was metabolized to 2-amino-1-naphthyl sulfate in the dog. In recent years, the 2-amino-1-naphthyl sulfate has not been considered the active carcinogen because of the demonstration by Boyland *et al.*[21] that this ester is not hydrolyzed by natural sulfatases. However, these results were

in vitro experiments conducted with enzymes from species other than the dog; also, this compound was not carcinogenic when tested by implantation in the mouse bladder by Bonser *et al.*[22] Mice, however, do not develop bladder cancer from orally administered aromatic amines. While our results show that this metabolite is present in marked amounts in the urine of dogs fed carcinogenic levels of 2-aminonaphthalene and penetrates the bladder mucosa, they do not support the conclusion that this *is* the active form of the carcinogen.* However, these conclusions should be re-examined in light of the findings presented here; new experiments designed to test this hypothesis are in progress.

Because of the relative nonpolarity of di-(2-amino-1-naphthyl)hydrogen phosphate, it was postulated that this metabolite would freely penetrate the bladder mucosa. No indication of the presence of this metabolite could be obtained. Apparently the sulfate penetrates the mucosa in spite of its polarity because of the high concentration present in the urine.

One interesting result of our binding studies was that 2-amino-1-naphthol, on binding to bovine serum albumin, gave a different fluorescence maximum than that of the N-hydroxy-2-aminonaphthalene–bovine serum albumin product. This seems to indicate that the N-hydroxy compound itself is capable of binding with the protein without rearranging to 2-amino-1-naphthol.

We also observed a dramatic stabilization of the aminonaphthols in the presence of either bovine serum albumin, due to binding, or homogenized bladder mucosa, due to the occurrence of some form of weak interaction.

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* Studies carried out in our laboratory with ^{14}C-labeled 2-aminonaphthalene have shown that from 95 to 98% of the amine is excreted as the 2-amino-1-naphthyl sulfate in the dog.

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