In Vivo Metabolism of 3,2'-Dimethyl-4-Aminobiphenyl (DMAB) Bearing on Its Organotropism in the Syrian Golden Hamster and the F344 Rat.

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The *in vivo* metabolism of tritiated DMAB was examined in male Syrian golden hamsters, which are susceptible to both urinary bladder and intestinal carcinogenesis by this agent and in male F344 rats in which intestinal tumors represent the main lesions. Evidence was obtained for the presence of the *N*-hydroxy-*N*-glucuronide of DMAB as a major metabolite in hamster urine and bile and in rat bile but not urine. The routes of excretion of this metabolite, which may represent a transport form of the ultimate carcinogen, correlate well with the main tumor sites in the two species. Other metabolites partially identified were the sulfates and glucuronides of *C*-hydroxylated DMAB and *C*-hydroxylated-*N*-acetyl DMAB.

Introduction

During comparative carcinogenicity studies with various derivatives of 4-aminobiphenyl, Walpole et al. (1-3) observed that the introduction of a methyl group ortho to the amine function (3-methyl-4-aminobiphenyl) resulted in increased carcinogenicity towards the intestinal tract of rats. On the other hand, methyl substitution meta to the amine (2methyl-4-aminobiphenyl) decreased the carcinogenicity and changed the organotropism, with the appearance of liver rather than intestinal tumors. The introduction of a second methyl group at the 2' position to give 3,2'-dimethyl-4-aminobiphenyl (DMAB), enhanced the carcinogenicity toward the intestinal tract even further; moreover significant carcinogenicity appeared toward the ear duct, the salivary gland and other organs. Interestingly a methoxy group ortho to the amine produced a carcinogen with organotropism toward the urinary bladder. These relationships are summarized in Table 1. To

*Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, NY 10595. explain the carcinogenicity of 4-aminobiphenyl, 3methyl-4-aminobiphenyl and DMAB toward the small and large intestines, Walpole speculated (1) that the effective carcinogen of the amines was a metabolite excreted in the bile.

Because of the great interest in animal models for colorectal cancer which would accurately reflect the disease in man, DMAB was utilized by Spjut et al. (4-6), and So and Wynder (7) and others (8) to study the induction and development of colon cancer in rodents. In experiments designed to test Walpole's suggestion that metabolites of DMAB acted topically on the intestine, Navarrette-Reyna and Spjut (9) performed colostomies 4 cm above the rectum in rats. Following the SC administration of DMAB, they found that tumors were found exclusively in the intestine proximal to the colostomy. Other experiments by Cleveland et al. (10), involving the SC administration of DMAB to rats with surgically defunctionalized colon segments, similarly indicated that tumors appeared only in those segments which were in actual contact with the fecal stream. These experiments provided strong evidence that the induction of tumors in the intestine was related to the transport of some form of the carcinogen via the

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Table 1. Incidence of tumors in albino Wistar rats.^a

	Incidence of tumors					
 Total dose SC, G/KG	Intestine	Bladder	Mammary	Salivary	Ear duct	Liver
3.6-5.8	7/14	_	3/12(0)	_	_	_
1.2	12/23	_	-	_	3/23	_
2.8-3.5	21/23	1/23	1/12(0)	5/23	4/23	0
2.4	_	_	_		-	3/23
4 4	-	9/10	-	·	_	-

^aData from Walpole et al. (1-3).

bile and intestinal contents rather than by the blood stream.

In the experiments of So and Wynder (7), an interesting species difference in the response to DMAB was observed: whereas in the rat the predominant sites of tumors were the small and large intestine, in the hamster DMAB caused a high incidence of tumors in the urinary bladder. This species difference was confirmed by later work (11, 12) and is illustrated in Table 2.

To attempt to elucidate the mechanisms of the contrasting organotropisms of DMAB in the rat and hamster, we began studies on the *in vivo* (13) as

Table 2. Organotropism of 3.2-dimethyl-4-aminobiphenyl in male Syrian golden hamsters and F344 rats.^a

	Incidence,%			
Organ	Hamsters (25)	Rats (26)		
Stomach, squamous papillomas	48	0		
Urinary bladder, carcinoma	64	0		
Small intestine, adenocarcinoma	28	35		
Colon, adenocarcinoma	24	31		
Ear duct	0	8		
Lung	0	19		
Skin	0	54		
Subcutaneous tissue	0	23		
Prostate, carcinoma in situ	0	19		

"Data of Fiala et al. (12).

well as the *in vitro* metabolism of the carcinogen. The results of our continuing *in vivo* studies are presented here.

Materials and Methods

Animals

Male outbred Syrian golden hamsters and male F344 rats were obtained from Charles River Breeding Laboratories. The animals were maintained on NIH-07 diet and water *ad libitum*. The approximate mean weight of the hamsters used for these studies was 175 g; that of the rats was 225 g.

Chemicals

DMAB HCl was obtained from Ash Stevens, Detroit, Mich. The material was custom tritiated by New England Nuclear Corp. For purification, 25-30 mCi of the tritiated DMAB was mixed with 0.5-1.0 g of unlabeled DMAB HCl in a small volume of ether. After conversion of the HCl salt to the free base by addition of an excess of saturated NaHCO₃ solution with stirring, the mixture was extracted several times with ether. The ether extracts were combined and passed through ClinElut columns (Fisher Scientific) to remove water and concentrated by rotary evaporation to yield a viscous oil. This was diluted with an equal volume of ether and applied to an E.M. Merck size C Lobar silica gel column equilibrated with *n*-hexane-ethanol, 96:4 (v/v). The column was eluted with the same solvent at a rate of 0.5 mL/min. After determination of radioactivity, fractions containing DMAB were identified by serially spotting aliquots onto a TLC plate and spraying with Ehrlich reagent (14). The fractions showing a yellow color on the plate with the reagent were pooled and concentrated to yield a clear viscous oil which turned into a white solid on storage at 4°C. The radiochemical purity was > 97% by TLC on silica gel [benzene-CHCl₃-ethyl acetate-methanol, 70:15:15:3 (v/v); $R_f = 0.63$] and by HPLC [two Waters μ Bondapak C₁₈ columns in series eluted with methanol-H₂O, 75:25, (v/v); elution volume, 11.5 m].

For use as chromatographic standards, the N-hydroxy and C-nitroso derivatives were synthesized from DMAB by the method described by Hecht et al. (15).

For enzymatic hydrolyses, *E. coli* β -glucuronidase (Sigma type IX) and *Helix pomatia* aryl sulfatase (Sigma type H-1) were used. All other chemicals and solvents were reagent or HPLC grade and distilled deionized H₂O was used in all phases of these experiments.

Collection of Bile

Rats or hamsters were anesthetized and the common bile duct was cannulated with polyethylene tubing, size No. 1. In early experiments, ether was used as the anesthetic. In later experiments, ether was used in combination with halothane for more uniform anesthesia. No differences in biliary excretion which could be ascribed to the method of anesthesia were noted. In the rat, the cannula emerged from the animal by way of the posterior aspect of the hind leg. In the hamster, the gall bladder was ligated before cannulation of the bile duct and the cannula was channeled subcutaneously to the interscapular area and exteriorized at that point. Tritiated DMAB was suspended in corn oil and was injected SC at a dose level of 75 mg/kg immediately after the cannulation of the bile duct. Approximately a 20 μ Ci dose was given to each animal. The animals were placed in plastic restrainers and bile was collected in test tubes cooled to 0-4°C.

Collection of Urine

Nonoperated animals were treated with tritiated DMAB as above and placed in stainless steel metabolism cages with provisions for the separation of urine and feces. Urine was collected at 0.4° C in containers to which 0.5 mL of 0.5*M* triethylamine-CO₂ buffer, pH 8.0, was added to maintain alkaline pH. All animals had free access to food and water during either bile or urine collection.

Sephadex LH-20 Chromatography

For most of the experiments described here, a wet-packed 1.5×110 cm column of Sephadex LH-20 (25-100 μ m) equilibrated with methanol-H₂O-0.5M triethylamine-CO₂ buffer, pH 8.0, 100:100:2 (v/v), was used. The column was eluted at a rate of 12 mL/hr, and 6 mL fractions were collected. The inclusion of the triethylamine buffer in the eluant served not only to maintain moderately alkaline pH, but also appeared to increase resolution significantly, an effect which might be due in part to ionpairing with the acid conjugated metabolites. The separation of conjugated urinary *o*-toluidine metabolites by this technique was reported by us earlier (16).

Thin-Layer Chromatography

Silica gel G or GF plates, $250 \ \mu m$, were obtained from Analtech, Newark, Del. For the separation of glucuronic and sulfuric acid conjugates, the plates were developed with *n*-butanol-acetic acid-water, $3:1:1 \ (v/v)$. We refer to this as system A. For the separation of DMAB, 3,2'-dimethyl-4-nitrosobiphenyl, and various aglycones from enzymatic hydrolyses, the plates were developed with system B: benzenechloroform-ethyl acetate-methanol, 70:15:15:3 (v/v).

High Pressure Liquid Chromatography

A Waters Model M600A HPLC pump with a U6K injector was used in conjunction with a Schoef-fel/Kratos SF 770 variable wavelength monitor.

Mass Spectrometry

Mass spectra were obtained with a Hewlett-Packard 5980A mass spectrometer, equipped with a Model 5933A data system, at 70 eV with a source temperature of 175-180°C.

Total Urinary and Biliary Radioactivity

A comparison of the total amount of radioactivity excreted in a 24-hr period after dosing with 75 mg/ kg of tritiated DMAB by the rat and hamster via the bile and urine is shown in Figure 1. While the rat excreted approximately 6% of the dose in the urine and 12% in the bile, the hamster excreted approximately 17% in the urine and 5% in the bile. In those cases where collections were extended to 48 hrs, the rats excreted an additional $5.6 \pm 2.6\%$ of the dose in the urine, $10.9 \pm 3.1\%$ in the bile, and the hamsters excreted an additional $3.9 \pm 1.6\%$ in the urine and 3.1% (only one hamster with a bile fistula survived 48 hr) in the bile.



FIGURE 1. Excretion of radioactivity in rat and hamster urine and bile in 24 hr following an SC injection of tritiated DMAB (75 mg/kg). Parentheses denote number of animals used.

Separation of DMAB Metabolites

Submission of the 0-24 hr samples of bile and urine of the two species to Sephadex LH-20 chromatography yielded the metabolite profiles shown in Figures 2 and 3. While only three major metabolites, designated α , β and γ were resolved from rat bile, at least seven metabolite peaks were detected in rat urine (Fig. 2). Peaks β and γ were present in both bile and urine, peak α , a major component was present only in the bile while peaks δ and ε and two other late-eluting minor peaks were present in significant amounts only in the urine.

In the hamster (Fig. 3), peaks corresponding to the elution volumes of rat metabolites α , β and γ were present in both bile and urine, and peak α was the major metabolite in both fluids.

Sephadex LH-20 peaks α and β from rat bile, hamster bile or rat and hamster urine were positive for glucuronic acid by the naphthoresorcinol reaction, as described by Mead et al. (17), indicating glucuronic acid conjugates.

TLC of combined concentrated fractions comprising a peak α on silica gel plates eluted with system A showed one radioactive peak near the solvent front which developed yellow color immediately with Ehrlich reagent but was negative to naphthoresorcinol. However, the latter reagent showed a distinct blue zone on the plate at $R_f = 0.42$ which was devoid of radioactivity and which corresponded to free glucuronic acid. This suggested that a glucuronic acid conjugate in peak α was hydrolyzed by the acid TLC solvent.

To determine whether or not this was the case, a portion of the combined, concentrated α peak from



FIGURE 2. Sephadex LH-20 profiles of DMAB metabolites in rat urine and rat bile collected for 24 hr following tritiated DMAB administration.

rat bile was adjusted to pH 5.0 with sodium acetate buffer and incubated at 37°C. Aliquots were taken periodically and submitted to reverse-phase HPLC using methanol-water, 75:25 (v/v), as eluant. With increasing time of incubation, radioactivity in the most polar peak gradually decreased with corresponding increases in radioactivity in peaks at elution volumes of 8.5 and 16.5 mL (Fig. 4). The elution volumes of these peaks corresponded exactly to those of DMAB and 3,2'-dimethyl-4-nitrosobiphenyl standards, respectively. At short times of incubation, e.g., 30-60 minutes, a radioactive peak appeared at an elution volume of 7.0 mL which corresponded to N-hydroxy-DMAB. This peak disappeared after longer times of incubation, presumably due to further oxidation to the nitroso form. After 3 hr of incubation, the mixture was extracted with ether and the ether extract submitted to TLC using





FIGURE 3. Sephadex LH-20 profiles of DMAB metabolites in hamster urine and hamster bile collected for 24 hr following tritiated DMAB administration.

solvent system B. Two major radioactive ultraviolet-absorbing bands were observed at $R_f = 0.63$, corresponding to DMAB, and at $R_f = 0.79$, corresponding to 3,2'-dimethyl-4-nitrosobiphenyl. These bands were eluted and submitted to mass spectrometry (Fig. 5) whereby their identity was confirmed.

The time course of hydrolysis of the α peak from rat bile at pH values of 5.0, 6.2 and 7.8 is shown in Figure 6. At pH 7.8 very little hydrolysis occurred over the 3-hr period, but hydrolysis was rapid at pH 6.2 and more so at pH 5.0. At the latter pH approximately 80% of the α peak was hydrolyzed within 3 hr.

Two alternatives concerning the origin of the DMAB and the nitroso derivative were considered. It was possible that the α peak obtained by Sephadex LH-20 chromatography was not homogeneous and contained the N-glucuronide of DMAB as well

FIGURE 4. HPLC analyses of rat bile α peak incubated for varying times at pH 5 and 37°C.

as the N-glucuronide of N-hydroxy-DMAB. Such Nglucuronides are well known to be hydrolyzed under mildly acidic conditions (18-21). In the presence of oxygen, the N-hydroxy-DMAB aglycone would be rapidly oxidized to the nitroso form. On the other hand, it was possible that the α peak consisted entirely of the N-hydroxy-N-glucuronide of DMAB. After hydrolysis, the N-hydroxy aglycone could conceivably disproportionate to DMAB and the nitroso product.

To determine which was the case, the α peaks obtained from rat bile and hamster bile and urine were submitted to HPLC using a μ Bondapak C₁₈ column eluted with 30:70 methanol-water, 0.005*M* in sodium phosphate buffer, pH 8.0. In all cases, two radioactive peaks, designated α_1 , and α_2 and eluting at approximately 15 and 18 mL, respectively, were obtained, which were positive for glucuronic acid. The HPLC resolution of the α peak obtained from rat bile is shown in Figure 7. Upon mild acid hydrolysis (pH 5) under aerobic conditions, the α_1 peak



FIGURE 5. Mass spectra of aglycones recovered from mild acid (pH 5) hydrolysis of Sephadex LH-20 peak a.

yielded DMAB as the only component (Fig. 8, upper trace) whereas hydrolysis and HPLC of α_2 yielded 3,2'-dimethyl-4-nitrosobiphenyl as the major component and a small amount of DMAB (Fig. 8, lower trace). This indicates that the Sephadex LH-20 α peak was in fact composed of two glucuronic acid conjugates: the N-glucuronide of DMAB (α_1) and the N-hydroxy-N-glucuronide of DMAB (α_2). The approximate ratio of α_2 to α_1 was 5:1 in rat bile, 1:3 in hamster bile and 1:2 in hamster urine, as determined by HPLC. Sephadex LH-20 peak β was present in the urines and biles of both hamsters and rats (Figs. 2 and 3). On TLC with solvent system A, a naphthoresorcinol-positive, radioactive band with an $R_f = 0.74$ was noted in all four cases. A strong immediate reaction with Ehrlich reagent spray indicated the presence of a free amine group. Following β -glucuronidase hydrolysis, the aglycone was recovered by ether extraction and purified by TLC with solvent system B ($R_f = 0.46$). The purified aglycone yielded a mass spectrum (Fig. 9) which is compatible with that of a



FIGURE 6. Time course of hydrolysis of Sephadex LH-20 peak a at pH 5, pH 6.2 and pH 7.8.



FIGURE 7. Resolution of Sephadex LH-20 peak α (rat bile) into glucuronides (α_1 and α_2) by HPLC. A μ Bondapak C₁₈ column was eluted with 30% methanol, 0.005 *M* sodium phosphate, pH 8.0.

ring hydroxylated metabolite of DMAB.

Sephadex LH-20 peak γ , present in both rat urine and bile and in hamster urine was positive for glucuronic acid. After β -glucuronidase hydrolysis of γ obtained from rat urine, TLC in system B gave a major radioactive zone with an R_f of 0.27. Mass spectral analysis of the aglycone yielded distinct peaks at m/e = 255 (M⁺), 213, 198, 181 and 163. We infer that peak γ represents the glucuronide of Nacetyl ring-hydroxylated DMAB.

Sephadex LH-20 peak δ , a major metabolite in rat urine and possibly a minor metabolite in rat bile and hamster urine gave an R_f of 0.60 on TLC using solvent system A. The metabolite was negative for glucuronic acid and gave a yellow color with Ehrlich reagent which developed only after a period of time. The greater elution volume of δ compared to the glucuronides α and β on Sephadex LH-20, which



FIGURE 8. HPLC of pH 5 hydrolysis products of α_1 and α_2 . A μ Bondapak C₁₈ column was eluted with 75% methanol.



FIGURE 9. Mass spectrum of the aglycone of Sephadex LH-20 peak β . A ring hydroxylated metabolite is indicated; the position of the hydroxyl group cannot be determined by mass spectral analysis alone.

effects separations mainly by molecular sieving, suggested that peak δ might be a sulfuric acid conjugate. In fact after incubation with aryl sulfatase at 37°C for 4 hr at 37°C, more than 80% of the total radioactivity was extractable into ether. The concentrated ether extract gave a single radioactive band on TLC with system B with an R_f of 0.27. Submission of the eluted aglycone to mass spectrometry gave a spectrum essentially identical to that obtained using the aglycone of peak γ . Thus peak δ represents the sulfate ester of N-acetyl-Chydroxy DMAB.

Discussion

Among the arylamine carcinogens, DMAB is of interest because it induces colon tumors in rats and hamsters (1-7, 11), mammary tumors in female rats (8) and urinary bladder tumors in hamsters (7, 11, 12). Thus, depending on the animal, DMAB provides a good experimental model for three major sites of human cancers. Since it contrasts with other colon carcinogens such as 1,2-dimethylhydrazine whose activated metabolites reach the colon mucosa via the blood rather than the fecal stream (22), as is the case with DMAB (9, 10), clarification of the mode of action of these carcinogens may yield mutually complementary information that can be relevant to the as yet unknown etiology of the disease in man.

The distinct differences (excepting the overlap in the small and large intestines) in the organotropism of DMAB in the rat and hamster presumably result from differences in the metabolism and disposition of the carcinogen. In this respect, it is of interest that the rat, in which intestinal tumors are the major lesions, excretes a greater proportion of DMAB metabolites in the bile than in the urine (Fig. 1). In contrast, the hamster, which is sensitive to the development of urinary bladder tumors in response to DMAB, excretes a greater portion of the metabolites in the urine than in the bile.

A still better correlation exists between the presence of the metabolite which we have identified, albeit through indirect methods, as the N-hydroxy-N-glucuronide of DMAB in the two physiological fluids and the sites of tumor formation in the two species. Thus this metabolite is present in both the bile and urine of hamsters, and is present in rat bile but not rat urine (Figs. 2 and 3). Because of its instability, the amount of the N-hydroxy-N-glucuronide is difficult to quantitate accurately, however we estimate that 4.5% of the DMAB dose is excreted in this form in rat bile, 1.2% is excreted in hamster bile and 4.2% is excreted in hamster urine in the first 24-hr period after dosing.

From the work of Radomski et al. (20, 23, 24) and Kadlubar et al. (19, 25, 26) it appears that the presence of the N-hydroxy-N-glucuronide of DMAB in hamster urine could be directly related to the induction of urinary bladder tumors in this species. N-Hydroxy-N-glucuronides of arylamines such as 4-aminobiphenyl or 2-naphthylamine are regarded (26) as transport forms and proximate carcinogens which release the ultimate carcinogen, the N-hydroxy aglycone, upon mild acid (pH 5-6) conditions such as exist in the urinary bladder of some species (25), or upon hydrolysis with β -glucuronidase (19, 23). In five separate determinations, we have found that the pH of normal, 24 hr hamster urine to vary from pH 6.2 to 6.6. Under these conditions the N-glucuronides of DMAB and N-hydroxy-DMAB may be extensively hydrolyzed (Fig. 8). The absence of bladder tumors in the rat in response to DMAB is likely due to the preferential excretion of the N-hydroxy-N-glucuronide in the bile rather than the urine in this species. Extensive investigations (27, 28) into the relative excretion of organic anions into the urine and bile of rats have determined that a rather sharp molecular weight threshold for biliary excretion in this species exists; compounds below the molecular weight of approximately 350 are excreted almost entirely in the urine. Above 350 molecular weight the extent of biliary excretion increases steeply as a function of the molecular weight. Thus in the rat, the extents of biliary excretion of the N-hydroxy-N-glucuronide of DMAB (molecular weight 388) would be greater than the corresponding metabolite of 3-methyl-4aminobiphenyl (molecular weight 374) which in turn would be greater than that of N-hydroxy-Nglucuronide of 4-aminobiphenyl (molecular weight 360). Interestingly, this correlates well with the carcinogenicity of the parent amines for the colon (Table 1). The excretion of the N-hydroxy-Nglucuronide of DMAB in both hamster urine and bile could be due to a higher molecular weight threshold for biliary excretion in this species.

It is obvious that besides molecular weight other factors, such as perhaps the degree of N-hydroxylaion and the reactivity of the ultimate carcinogen, must also play a role in determining the organotropism and carcinogenicity of the 4-aminobiphenyl derivatives, since the 2-methyl and the 3-methoxy substituted compounds were observed to induce tumors only in the liver and bladder of rats, respectively (Table 1). Hecht et al. (15) have demonstrated that DMAB is significantly more mutagenic in the Ames assay than is 2'-methyl-4-aminobiphenyl and that the same relationship holds for the corresponding N-oxidized derivatives.

With respect to the carcinogenicity of DMAB to the colon, bacterial enzymes may play a major role in the further activation of the N-hydroxy-N-glucuronide. Analogous metabolites of 2-naphthylamine and AB have been found to be easily hydrolyzed to the aglycones by *E. coli* β -glucuronidase (19); moreover Reddy and Watanabe (8) have demonstrated that germ-free status significantly reduces the incidence of DMAB induced intestinal tumors in F344 rats.

Up to now relatively little information has been available as to the metabolism of DMAB (5, 29). However, Gorrod (30), in unpublished work, has detected the N-glucuronide of DMAB in rat bile and has obtained evidence for the oxidation of both the 3 and 2' methyl groups as well as for hydroxylation at the 4' position. Work on the more exact function of the N-hydroxy-N-glucuronide in intestinal and urinary bladder cancer as well as on the complete characterization of the biliary and urinary DMAB metabolites resolved by our Sephadex LH-20 method is currently in progress in this laboratory.

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