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SHORT COMMUNICATIONS

Increased metabolic *N*-oxidation of 2-naphthylamine in dogs after phenobarbital pretreatment

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THE METABOLIC activation of carcinogenic arylamines appears to explain their organ specificity. *N*-Oxidation products of certain carcinogenic amines were shown to be more effective in producing tumors, albeit in different organs in some cases, than the parent amines. Therefore, it has been proposed that the *N*-hydroxy or nitroso derivatives of the arylamines, formed *in vivo*, may be the actual carcinogens in the susceptible organisms.[1]

2-Naphthylamine, a powerful bladder carcinogen in man and the dog, has been shown to undergo metabolic *N*-hydroxylation in both of these species.[2, 3] Further, the nitroso derivative has been reported as a urinary metabolite in the dog.[2, 3] Pretreatment of the animals with a variety of chemical compounds causes the stimulation of the metabolic process in many tissues.[4] For example, the microsomal *N*-hydroxylation of 4-aminobiphenyl, a bladder carcinogen in the dog, is considerably increased by pretreatment with phenobarbital.[5] The stimulating effect of phenobarbital[6] offered the possibility of increasing the concentration and amount of the urinary *N*-oxidized metabolites of 2-naphthylamine. This would facilitate the detection, characterization and isolation of these labile compounds.

We wish to report the dramatic increase in the metabolic *N*-oxidation of 2-naphthylamine in the dog upon pretreatment with phenobarbital. 2-Nitrosonaphthalene was isolated from urines of these animals and fully characterized.

MATERIALS AND METHODS

Pure bred female beagle dogs, weighing between 10 and 12 kg, were injected i.p. with 40 mg/kg of sodium phenobarbital every second day for a total of six doses. The dogs were fasted without water approximately 16 hr prior to the experiment. The urinary bladders were emptied by catheterization, followed by the oral administration of 0·5 m-mole (71·6 mg)/kg of 2-naphthylamine (Merck, Darmstadt) in corn oil solution (35·8 mg/ml) contained in gelatine capsules. After administration of the amine, 150 ml of water was allowed each dog. At 2 hr and 5 hr 150 ml of water was given additionally.

All chemicals were obtained from commercial sources and purified as required. 2-Nitrosonaphthalene was prepared by the method of Boyland and Manson.[2] The compound was purified by column chromatography and gave a sharp melting point of 61–62° (Lit.[2], 62–64°).

Column chromatography was performed on silica gel Woelm (M. Woelm, Eschwege, Germany) columns 18 × 150 mm and developed with *n*-hexane.

Thin layer chromatography (TLC) was carried out on 20 × 20 cm glass plates, coated with 0·25 mm of silica gel HF_{254} (E. Merck, Darmstadt), along with appropriate reference compounds. The plates were developed in (1) petroleum ether (40–60°), acetone (4:1); (2) *n*-hexane, benzene (5:1). Colour development of the nitroso compounds was performed with 5% aqueous solution of trisodium pentacyanoamine ferrate (TPF).[7]

After 1, 2, 3, 5 and 8 hr the urines were collected by catheterization and the pH adjusted to 4·5–5·0 with 1 N H_2SO_4, and made 6·5 mM in $K_3[Fe(CN)_6]$. The single urines were then immediately extracted with an equal volume of CCl_4 followed by a second extraction with 1/3 vol. of the solvent. The emulsions formed during extraction were broken by centrifugation at 2000 *g*. The combined CCl_4 extracts were dried over Na_2SO_4 (anhydrous) for 30 min. The CCl_4 was evaporated *in vacuo* at 20–25°, and the residue taken up in a minimum of warm *n*-hexane, applied on a silica gel column and developed with hexane. Control urines with added 2-naphthylamine were treated in the same way.

Two 1-ml portions of the combined CCl_4 extracts were taken for the direct estimation of the total *N*-oxidation products following the method for 4-aminobiphenyl.[5] The chemical diazotization was carried out after removing free naphthylamine from the CCl_4 extracts by washing with 0·5 N H_2SO_4.

RESULTS

In a typical experiment the residue obtained from the CCl_4 extracts of the urine of a phenobarbital pretreated dog weighing 10·2 kg and receiving 730 mg of 2-naphthylamine was applied to a silica gel column. Upon development with *n*-hexane the bright green front running band representing the 2-nitrosonaphthalene was collected and the hexane removed *in vacuo* at 20–25°. A total of 5·4 mg of the 2-nitrosonaphthalene was obtained in a crystalline form. The compound revealed the same melting point (61–62°) as an authentic sample and no depression was observed in the mixture melting point. The u.v. spectra in methanol gave two maxima, one at 261 mμ and at 316 mμ and was identical to the synthetic compound. Both the isolated product and the 2-nitrosonaphthalene gave one spot on TLC, at R_f 0·55 in solvent (1) and R_f 0·30 in solvent (2). Both compounds reacted with TPF resulting in a mauve colour.

The direct chemical estimation of total urinary *N*-oxidation products in an aliquot of the combined CCl_4 extracts resulted in 5·57 mg of 2-nitrosonaphthalene excreted within 8 hr after dosage. Nitrosonaphthalene was not detected in the extracts of control urines with added 2-naphthylamine (50 μg/ml) by TLC, u.v. or by chemical estimation.

In a series of several experiments it was found that untreated dogs excreted approximately 15 per cent the amount of total *N*-oxidation products present in the urine of dogs receiving phenobarbital pretreatment. 2-Nitrosonaphthalene was not isolated in these cases, but the total *N*-oxidation products were estimated by chemical diazotization. In all cases the 2-nitrosonaphthalene was also identified by u.v. and TLC.

Extraction of the urines without added $K_3[Fe(CN)_6]$ for the oxidation of the *N*,2-naphthylhydroxylamine to the nitroso compound revealed the presence from 13·5 to 22·5% of 2-nitrosonaphthalene. The TLC separation of *N*,2-naphthylhydroxylamine and 2-nitrosonaphthalene from ether extracts of the urines,[2, 3] with and without addition of ferricyanide was run simultaneously. It confirmed a similar ratio of free naphthylhydroxylamine and 2-nitrosonaphthalene. After addition of *N*,2-naphthylhydroxylamine (25 μg/ml) to control urines about 6–9 per cent was found in the extracts without ferricyanide in the form of the nitroso compound using chemical and u.v. analysis.

DISCUSSION

Since the first observation that metabolic *N*-oxidation products of 2-naphthylamine are present in the urine of dogs dosed the amine,[2,3,8] these labile and reactive compounds have eluded estimation and isolation. Therefore, the significance of the presence of these metabolites in relation to the carcinogenicity of 2-naphthylamine could not be determined.

We have now succeeded in confirming the validity of the chemical estimation method[5] for the quantitative determination of the *N*-hydroxylation products of arylamines also for the urinary metabolites. Taking advantage of the great increase in metabolic *N*-hydroxylation in dogs induced by phenbarboital pretreatment we have isolated the compounds as 2-nitrosonaphthalene. The amount of 2-nitrosonaphthalene isolated is in close agreement with the quantity determined by the chemical estimation method. Further, the extraction with and without $K_3[Fe(CN)_6]$[9] permits an estimation of the relative amounts of 2-naphthylhydroxylamine and 2-nitrosonaphthalene. The ratios of the *N*-hydroxy and the nitroso compounds in both the pretreated and untreated dogs are in good agreement and further support the sensitive chemical estimation procedure using diazotization of nitrosonaphthalene.

By reason of the high lipid solubility 2-nitrosonaphthalene was not expected to pass the kidneys readily. The control urines have shown that a certain amount (6–9%) of the 13·5–22·5% of nitroso derivatives present in the total *N*-oxidation products in the urine might be formed during the extraction procedure by oxidation of hydroxylamines even without ferricyanide. Ether extraction of added *N*,2-naphthylhydroxylamine from dog's urine was found to be rather poor. This might explain the small amounts of the hydroxylamine detected in ether extracts using TLC.[2, 3] After oxidation, the nitroso compound is extracted into CCl_4 readily. Nevertheless, a part of the total nitrosonaphthalene

found after CCl_4 extraction in the presence of ferricyanide possibly derives from labile conjugates of the hydroxylamine splitting during extraction. Until the presence of such a conjugate is substantiated it seems that the major part of the *N*-oxidation products of 2-naphthylamine is excreted in the form of free hydroxylamine.

There remains the interesting question whether the forced metabolic formation and excretion of the *N*-oxidation products of 2-naphthylamine by phenobarbital pretreatment might correspond to an increase in the carcinogenicity for the bladder or possibly other organs of the dog.

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REFERENCES

1. E. C. MILLER and J. A. MILLER, *Pharmac. Rev.* **18**, 805 (1966).
2. E. BOYLAND and D. MANSON, *Biochem. J.* **101**, 84 (1966).
3. E. BRILL and J. L. RADMOSKI, in *Bladder Cancer* (Eds. W. B. DEICHMANN and K. F. LAMPE), p. 90. Aesculapius Publishing Co., Birmingham, U.S.A. (1967).
4. A. H. CONNEY, *Pharmac. Rev.* **19**, 317 (1967).
5. H. UEHLEKE und K. NESTEL, *Naunyn-Schiedebergs Arch. exp. Path. Pharmak.* **257**, 151 (1967).
6. H. REMMER, *Arch. exp. Path. Pharmak.* **235**, 279 (1959).
7. S. OTSUKA, *J. Biochem., Tokyo* **50**, 81 (1961).
8. W. TROLL and N. NELSON, *Fedn Proc.* **20**, 41 (1961).
9. H. UEHLEKE, *5th Int. Congr. Biochem., Moscow 1961*, Abstr. p. 399, 18–55. Pergamon Press, Oxford (1961).

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Effect of reserpine and metaraminol on excretion of homovanillic acid and 3-methoxy-4-hydroxyphenolglycol in the rat

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NOREPINEPHRINE synthesis has been studied by estimation of its turnover rate by using the rate of disappearance of the isotopically labeled catecholamine[1–3] or of the endogenous compound after inhibition of its synthesis.[4] Direct assessment of changes in the rate of norepinephrine synthesis by using isotopically labeled tyrosine *in vitro*[5–8] and *in vivo*[9, 10] is possible only if the labeled product is not destroyed or if both the labeled catecholamine and its metabolites are determined. If the synthesized catecholamine is not retained in the tissues, these methods cannot be used *in vivo*. In the