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Studies on metabolism of 2-naphthylamine and its activation mechanism*

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Abstract—2-naphthylamine was incubated with induced rat liver microsome S9 preparation and the metabolites were separated through HPLC. The following products were identified: 2-amino-5-naphthol, 2-amino-6-naphthol, 2-amino-7-naphthol and 2-amino-8-naphthol. The yields of these four metabolites are varying in quantity, and the relative contents of 2-amino-8-, -5-, -6- and -7-naphthol are 52.6%, 28.5%, 14.0% and 4.9% respectively. These results are consistent with the quantitative HMO calculation and inference based upon Di-region theory, i.e., the metabolisms of aryl amines on extra-ring (assigned the ring without the substituent of amino group) are through the epoxidation and then NIH shift, but are not the direct hydroxylation in the formation of phenols. It is shown that both the amino group and the carbon atoms on the extra-ring play duality roles of activation and detoxification in metabolism.

Keywords: 2-naphthylamine; 2-aminonaphthols; metabolism; Di-region theory.

INTRODUCTION

2-naphthylamine (2-NA) is one of the aromatic amines which have been recognized to induce human occupational bladder cancer. The metabolism of 2-naphthylamine *in vivo* has been studied in several species of experimental animals, N-hydroxy-2-naphthylamine, 2-nitrosonaphthalene and the sulfate or glucuronate of 2-amino-1-naphthol were identified from the urine of dogs dosed with 2-naphthylamine (Boyland, 1966; Conzelman, 1969). From the urine and bile of the experimental animals used except dogs, 2-acetamido-6-naphthol, 2-acetamido-5, 6-dihydroxy-naphthalene and 2-acetamido-5, 6-dihydro-5, 6-dihydroxy-naphthalene that were free or conjugated with sulfuric acid, glucuronic acid or mercapturic acid were detected (Boyland, 1958; 1963; Manson, 1950).

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The metabolism of 2-naphthylamine *in vitro* has also been studied in various cellular preparations. Incubation of 2-naphthylamine with rat liver microsome resulted in the formation of two types of metabolites which were identified as N-hydroxy-2-naphthylamine and 2-amino-1-naphthol. The former was oxidized further into 2-nitroso-naphthalene (Nakayema, 1982). In addition, a small amount of 6-hydroxy-2-naphthylamine was also detected (Hammons, 1985).

According to electrophilic theory, it was recognized by Miller *et al.* (1983) that the N-oxidized metabolites of arylamines may be acted as the ultimate carcinogens to induce tumor in human or animals, N-hydroxy-2-naphthylamine or its sulfate will be transformed to nitrenium ion in acidic environment, and the latter may covalently bind with DNA or other biomacromolecules.

The mechanism of carcinogenesis by aryl amines have been recognized that the nitrenium ion formed in metabolism bonds with electronegative parts of biomacromolecule is included to the pathway of metabolic activation, and that the nitrenium ion reacts with water to form phenols at the same ring or at extra-ring (assigned the ring without the substituent of amino group) is included to metabolic detoxification (Garner, 1984). Di-region theory (Dai, 1980) proposed by one of these authors found in 1979 that the essential factor of a compound to induce tumor is to produce two electrophilic centres on the molecule in metabolism, the optimal distance between the two centres is about 2.80—3.00 $\mathring{A}$, it is identical with the distance of electronegative atoms between complementary bases in DNA. Therefore, it is considered that the key step in the carcinogenesis is the formation of cross linkage between the complementary bases of DNA, and finally induce frameshift.

The object of this work is to make an experimental evidence for Di-region theory. Thus, 2-naphthylamine was metabolized with rat liver microsome S9 preparation firstly, and then the metabolites were separated and detected with high performance liquid chromatography. In this work, the tendency of 2-aminonaphthols formed in metabolism were systematically analyzed. It was shown by experimental data that all of the four naphthols on the extra-ring of 2-naphthylamine can be formed in metabolism, but the yields of them are varying in quantity, 2-amino-8-naphthol is the major product, 2-amino-5-naphthol as the second, and 2-amino-7-naphthol the minimal, this result is identical with the result that calculated and deducted with Di-region theory. Thus, this work proposed an experimental evidence for the carcinogenic mechanism of arylamines undergoing di-functional alkylation.

MATERIALS AND METHODS

Syntheses of the metabolic standards

In order to investigate the process of metabolism of 2-naphthylamine on extra-ring, the following chemicals were synthesized by published methods: 2-amino-5-naphthol (5-OH-2-NA) (Muller, 1944), melting point 195—196°C; 2-amino-6-naphthol (6-OH-2-NA) (Ruggli, 1929),

melting point 207—208°C; 2-amino-7-naphthol (7-OH-2-NA) (Raiford, 1927), melting point 195–197°C; 2-amino-8-naphthol (8-OH-2-NA) (Muller, 1944), melting point 156—157°C; 2-amino-1-naphthol (1-OH-2-NA) (Desai, 1938), sealed the hydrochloric salt with argon immediately owing to it will be darkened rapidly in air; and bis-(2-amino-1-naphthyl) (Clem, 1939), melting point 191°C. All of these samples were preserved in ampoules which were filled with argon and kept in dark due to their instability in air and light.

Induction of rat hepatic enzyme and preparation of S9 supernatant

The 9000 × g supernatant (S9) was prepared from the liver of male Wister rats of body weight about 200 g, which were induced with Aroclor 1254 according to the method of Maron and Ames (1983) 5 days before sacrifice, the treated animals received a single i.p. dose of 500 mg/kg Aroclor 1254 which was dissolved in corn oil to a concentration of 200 mg/ml.

The preparation of S9 liver fraction was according to the method of Garner *et al.* (1972). Rat was executed by cervical dislocation, the liver was perfused with ice cooled 0.15 mol/L KCl solution, and then excised, fragmented, and homogenized with 0.15 mol/L KCl solution 3ml/g. The homogenate was centrifuged with 9000 × g for 10 minutes. The supernatant was separated and stored in liquid nitrogen.

Metabolism of 2-naphthylamine with rat hepatic enzyme

The incubation technique was according to the method of Chen *et al.* (1982) with some modifications. The procedure ascertained through a lot of tests was as follows:

The incubation mixture contained the following constituents: 3.5 μmol/L $MgSO_4 \cdot 7H_2O$, 2 μmol/L NADP, 3 μmol/L G-6-P sodium salt, 0.56 ml mol/L Na_2HPO_4-KH_2PO_4 buffer solution (pH 7.40, mol/L/15), 0.1 ml S9 supernatant, 0.1 ml 2-naphthylamine hydrochloride aqueous solution (10 mg/ml), made up to 1.0 ml with twice-distilled water.

The protein content in S9 supernatant used in this experiment is 34.55 mg/ml determined according to the method of Lowry *et al.* (1951), and the cytochrome P-450 content is 0.1010 nmol/mg protein by the method of Johennesen *et al.* (1978).

The incubation mixture was shaken at 37°C for 3 hours, then 3 ml cooled ethyl acetate was added to stop the incubation, and then 1 mg of sodium dithionite was added to protect the metabolites.

The above obtained mixture was shaken under a stream of argon for 10 minutes, and then kept still for several minutes. The upper layer was transferred to an ampoule and dried by argon purging. This residue was dissolved in 0.5 ml methanol, the ampoule after filling with argon was sealed for HPLC analysis.

High performance liquid chromatographic analysis

A 10 μl aliquot of each extract was analyzed on a Shimazu LC-3A HPLC system using 45% (V/V) methanol-water as the mobile phase with a flow rate of 0.5 ml/min. Separations were

conducted on a Zorbax-ODS reverse-phase column (0.46 × 25 cm). Detection of the metabolites were accomplished by SPD-2A UV detector operated at 254 nm.

RESULTS

After incubation with Aroclor-1254 induced rat liver supernatant, 2-naphthylamine was transformed to its metabolites which were analyzed on the HPLC system and identified with known chemicals. The following metabolites were determined: 5-OH-2-NA, 6-OH-2-NA, 7-OH-2-NA, 8-OH-2-NA, 1-OH-2-NA and bis-(2-amino-1-naphthyl). It is shown by the chromatogram (Fig. 1) that these metabolites are well separated under above mentioned operating conditions.

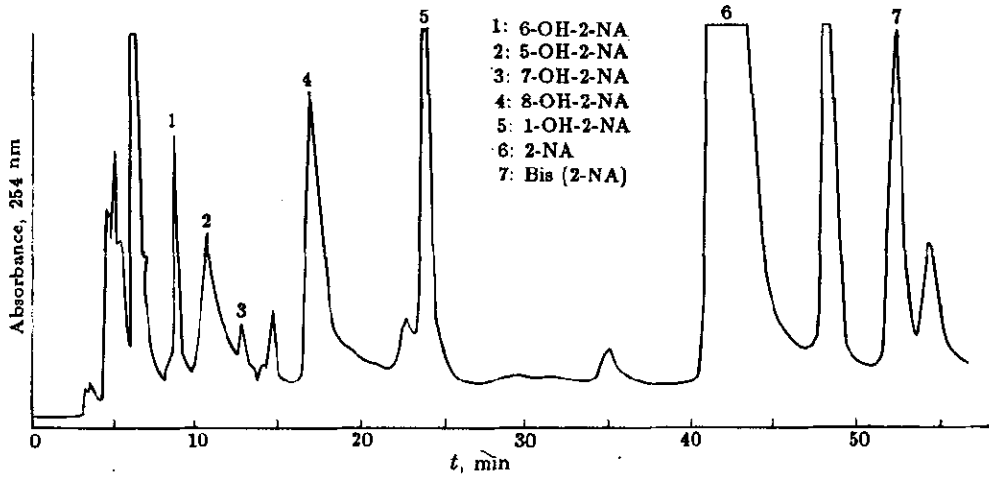

Fig. 1 The HPLC chromatogram of metabolites of 2-naphthyl-amine in the culture of rat liver S9
1: 6-OH-2-NA 2: 5-OH-2-NA 3: 7-OH-2-NA 4: 8-OH-2-NA
5: 1-OH-2-NA 6: 2-NA 7: bis-(2-NA)

It is shown in Fig. 1 that the four metabolites oxidized on the extra-ring apear in varying quantities, 8-OH-2-NA is the major product, which gives 52.6% of the sum of the four metabolites calculated according to the value of integral of the four peaks area, 5-OH-2-NA is the next, which gives 28.5%; the other two metabolites are the minor, 6-OH-2-NA gives 14.0% and 7-OH-2-NA gives only 4.9%.

DISCUSSION

In this work, four isomeric 2-aminonaphthols were detected as the metabolites of 2-naphthylamine on the extra-ring *in vitro* with rat liver microsome S9 preparation. In addition to 2-amino-6-naphthol, 2-amino-7-naphthol and 2-amino-8-naphthol, the latter has been detected

in the form of formamido derivative from the urine of experimental animal (Boyland, 1966), 2-amino-5-naphthol was identified by this work for the first time.

This work proves that, in addition to N-oxidation, all of the carbon atoms on the extra-ring of 2-naphthylamine may be oxidized in metabolism, i.e., to take place oxidation on carbon-ring. 2-amino-1-naphthol and bis-(2-amino-1-naphthyl) as the known metabolites have also been separated in this work, they should be formed from 2-naphthylamine passing through N-hydroxylation by monooxygenase, and then enzymatically formed N-sulfate or N-acetoxyl derivatives, which give the nitrenium ion in acidic environment by elimination reaction, and finally reacts with water or 2-naphthylamine to form the above two metabolites (Fig. 2). So that, they should be the N-oxidized metabolites.

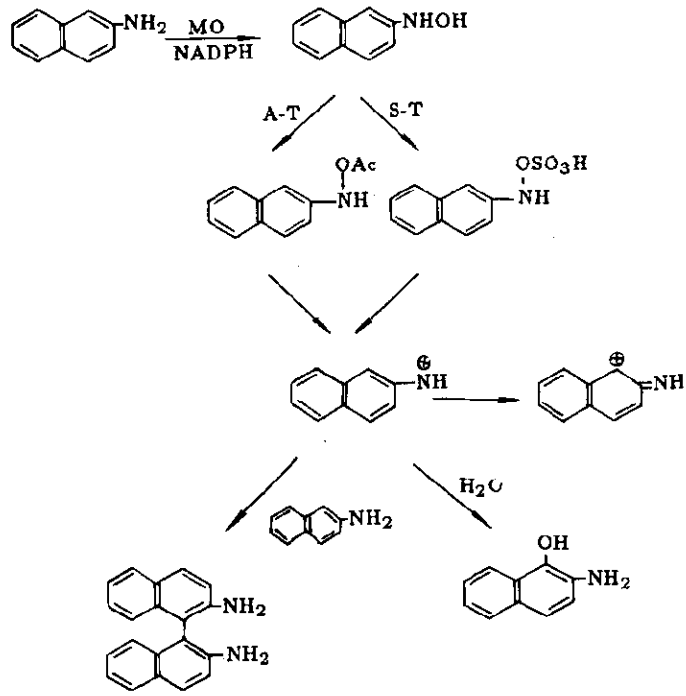

Fig. 2 The formation of 2-amino-1-naphthol and bis-(2-amino-1-naphthyl) from 2-NA passing through nitrenium ion
MO: monooxygenase S-T: sulfate transferase A-T: transacetylase

However, there are two viewpoints about the formation of phenols on extra-ring of aryl amines, one believes that the phenols are formed by direct hydroxylation on the carbon-ring, this viewpoint was admitted commonly in the past, someone tried to protect the relative position with fluorine atom in order to prevent hydroxylation taken place at that position. Westrop

et al. (1965) made metabolic experiment for 7-fluoro-2-acetamido-fluorene *in vivo*, the de-fluoro metabolite, 7-hydroxy-2-acetamido-fluorene, was detected from the urine of experimental animals. This result has refused the possibility of direct hydroxylation in fact. But the viewpoint of that the formation of phenols are the result of direct hydroxylation and that it is a detoxification pathway in metabolism still hold the guiding position now.

According to the Structure-Carcinogenic Activity Relationship, Di-region theory predicts that the metabolism of aryl amines on the extra-ring should be identical with those aromatic hydrocarbons, i.e., the formation of phenols are first through epoxidation, and then the NIH shift (Dai, 1984). Therefore, the epoxide intermediate should be the another reactive centre in carcinogenesis. Through deliberation for the Structure-Carcinogenic Activity Relationship, one of these authors has adopted Wholesale Molecular Orbital method to calculate the HMO parameters of 63 aryl amines (Dai, in press), some of that parameters of 2-naphthylamine are quoted as follows (Fig. 3):

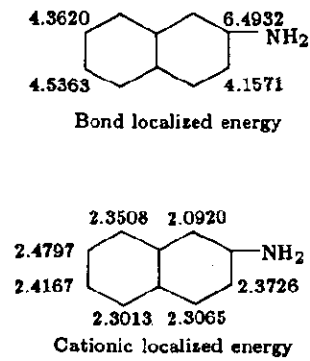

Fig. 3 Partial calculating data of the localized energy on 2-NA

According to above molecular orbital parameters, it may be predicted that the epoxidation on the extra-ring of 2-naphthylamine must be occurred firstly at the position of smaller bond localized energy, i.e., the bond in 7,8-position, and then in 5,6-position. For the NIH shift, according to cationic localized energy, 7,8-epoxide must be disproportionated firstly to 8-naphthol, and 5,6-epoxide to 5-naphthol. Moreover the difference of localized energy in 5,6-position is smaller than in 7,8-position, so that we can predict that the tendency of formation of naphthols in 8-position should be greater than in 5-position. Consequently, the relationship in quantity of the four 2-aminonaphthols formed in metabolism must be C_8-OH > C_5-OH > C_6-OH > C_7-OH. This result given by prediction is identical with the experimental fact. So that, it can be proved that the metabolism on extra-ring of 2-naphthylamine is passing through

epoxidation firstly, and then the NIH shift.

The metabolism of above mentioned 7-fluoro-2-acetamido-fluorene can also be interpreted by firstly undergoing epoxidation due to the smaller stereo-hindrance of the fluorine atom. In studying the metabolism of 2-naphthylamine, Boyland *et al.* (1963) have identified 2-acetamido-5,6-dihydro-5,6-dihydroxy-naphthalene and 2-acetamido-6-hydroxy-5-naphthyl mercapturic acid as the metabolites, it was also a strong evidence for aryl amines undergoing epoxidation.

Consequently, the conclusion is that both amino group and carbon atoms on extra-ring of aryl amines can play dual effects in metabolism, the metabolic activation and metabolic detoxification. The possible fashion of metabolism of 2-naphthylamine can be proposed in Fig. 4.

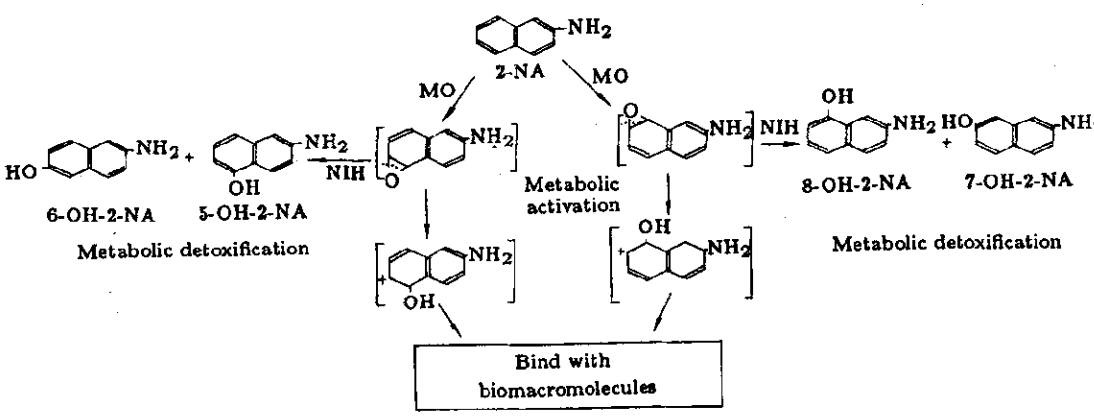

Fig. 4 The possible metabolic pathway on extra-ring of 2-naphthylamine
MO: monooxygenase NIH: NIH shift

The result given by this work is identical with the prediction by Di-region theory. The metabolisms on extra-ring of aryl amines are paid less attention in recent years, we consider that in the inspiration by Di-region theory more attention will be paid to the metabolism on extra-ring of aryl amines.

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