Communications to the Editor

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REDUCTION OF N-NITROSODIPHENYLAMINE TO THE CORRESPONDING HYDRAZINE BY GUINEA PIG LIVER PREPARATIONS

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The present study provides first evidence for enzymatic reduction of a noncyclic nitrosamine to the corresponding hydrazine. Under anaerobic conditions, N-nitrosodiphenylamine was reduced to 1,1-diphenylhydrazine by guinea pig liver 9,000xg supernatant or cytosol in the presence of an NADPH-generating system and FAD, or NADH and FAD. However, guinea pig liver microsomes did not catalyze the reduction of the nitrosamine at all. The reduction product was isolated from the reaction mixture and identified unequivocally by comparing with authentic samples its mass and UV spectra, and its behavior in HPLC and TLC. Under aerobic conditions, no formation of the hydrazine was observed by HPLC and TLC examinations. However, when aerobic incubation was performed in the presence of acetaldehyde, a reduction product was isolated and identified as the acetaldehyde hydrazone derivative.

KEYWORDS ——— N-nitrosodiphenylamine; enzymatic reduction; guinea pig liver preparations; reduction product; 1,1-diphenylhydrazine; diphenylamine; acetaldehyde diphenylhydrazone.

Nitrosamines require bioactivation for their carcinogenicity or mutagenicity. The mechanism for the bioactivation is considered to involve α-carbon hydroxylation followed by N-dealkylation or by ring-opening to form an alkylating carbonium ion.[1-5] Recently, however, Rao *et al.*[6] showed that N-demethylation is not a requisite for the bioactivation of N-nitrosodesmethyl tripelennamine, suggesting the involvement of an alternative bioactivation mechanism. On the other hand, it is known that many hydrazine derivatives are mutagenic for *Salmonella typhimurium* strains without S-9 Mix.[7] Therefore, if reductive reaction of nitrosamines to the corresponding hydrazines occurs enzymatically, the reaction would seem to be one mechanism for bioactivation of nitrosamines. As regards such reduction of nitrosamines, only a brief report is now available indicating that N-nitrosomorpholine can be reduced to aminomorpholine by a post-mitochondrial fraction from guinea pig livers.[8] The present study shows the first example of enzymatic reduction of a noncyclic nitrosamine to the corresponding hydrazine by using N-nitrosodiphenylamine and guinea pig liver preparations. This nitrosamine, which is widely used in the rubber industry to retard vulcanization, is carcinogenic in mice and rats,[9,10] and mutagenic against *Salmonella typhimurium* TA98 in the presence of a co-mutagen norharman.[11]

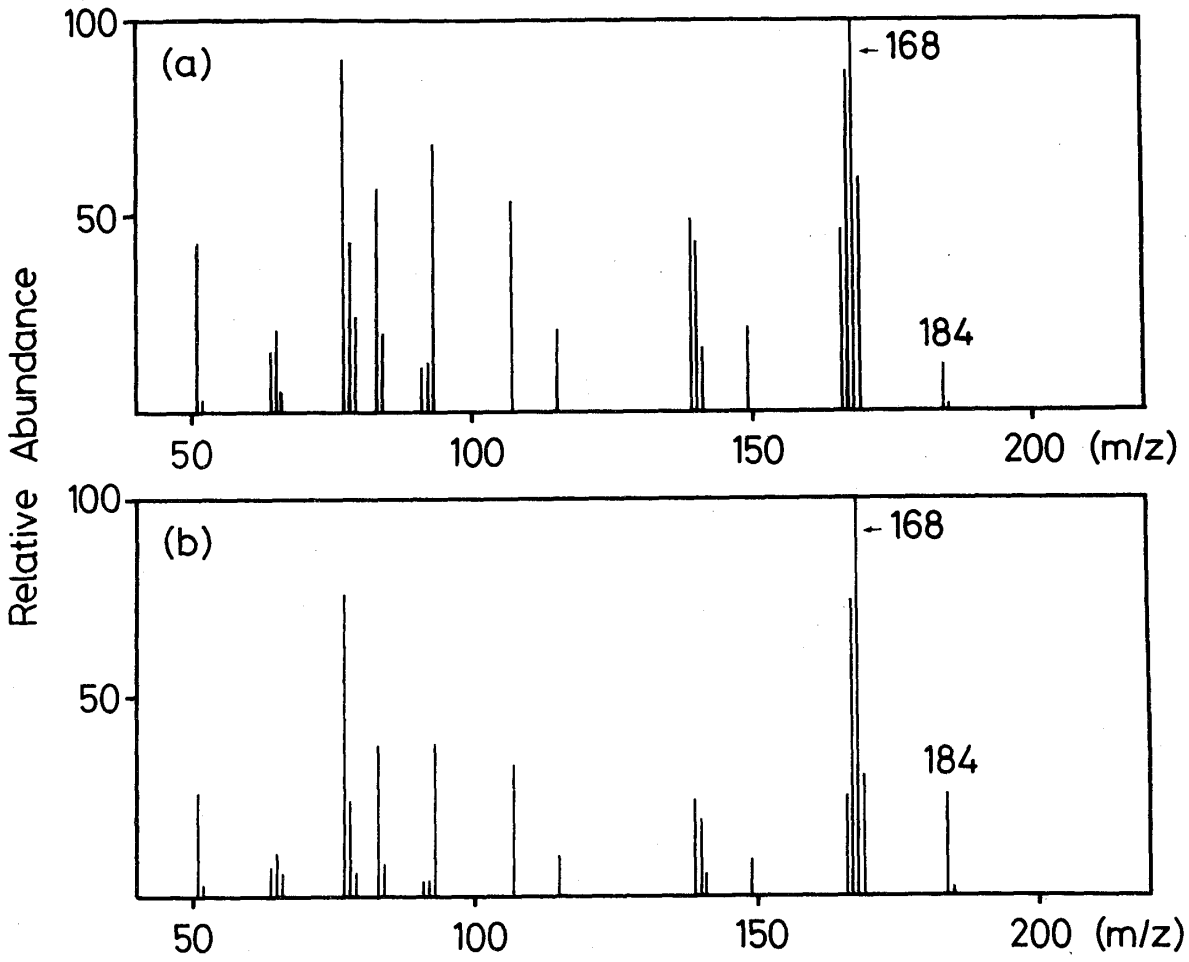

Fig. 1. Mass Spectra of Metabolite 1 (a) and Authentic 1,1-Diphenylhydrazine (b)

When N-nitrosodiphenylamine was incubated anaerobically with guinea pig liver 9,000xg supernatant in the presence of an NADPH-generating system and FAD, or NADH and FAD, the formation of two metabolites from the nitrosamine was observed in HPLC. The mass spectrum of metabolite 1 (Fig. 1) gave a molecular ion at m/z 184, accompanying a base peak at m/z 168, and the UV spectrum (in ethanol) had absorption maxima at 292 and 240 nm. The metabolite was identified as 1,1-diphenylhydrazine by comparing with authentic samples its mass and UV spectra, and its retention time in HPLC and Rf value in TLC. The mass spectrum of metabolite 2 gave a molecular ion at m/z 169, and the UV spectrum (in ethanol) revealed an absorption maximum at 285 nm. These spectra, and the retention time in HPLC and the Rf value in TLC of the metabolite were all identical with those of the authentic sample of diphenylamine. When N-nitrosodiphenylamine was incubated with the boiled liver preparations in the absence of the above cofactors or with bovine serum albumin, metabolite 2, but not metabolite 1, also could be detected from the incubation mixture by HPLC and TLC. This indicates that the formation of metabolite 2 from the nitrosamine was due to

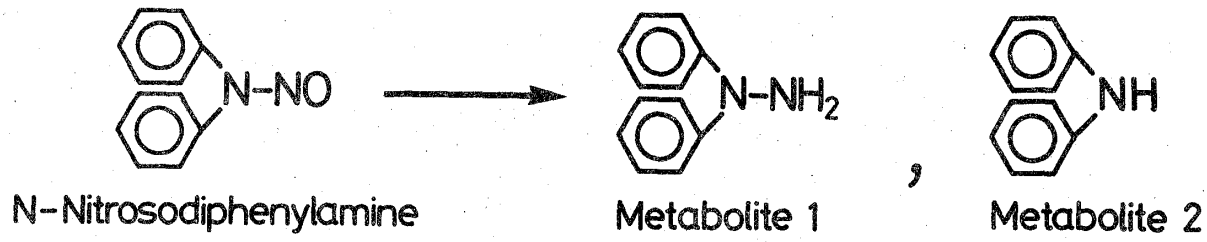

Fig. 2. Reductive Metabolism of N-Nitrosodiphenylamine

nonenzymatic reaction. The guinea pig liver cytosol also catalyzed the reduction of N-nitrosodiphenylamine to metabolite 1 under the same conditions as described above, whereas the liver microsomes showed no N-nitrosoreductase activity. The cytosolic reductase activity was associated with a protein which was precipitated from the liver cytosol between 30 and 45% ammonium sulfate saturation. The liver 9,000xg supernatant supplemented with an NADPH-generating system and FAD was assayed by HPLC for its nitrosamine reductase activity. As a result, 1,1-diphenylhydrazine and diphenylamine were formed at the rate of 0.045 and 0.108 μmol/30 min/0.8 g liver, respectively.

Under aerobic conditions, the incubation of N-nitrosodiphenylamine with guinea pig liver 9,000xg supernatant gave little detectable metabolite 1 even in the presence of an NADPH-generating system and FAD. However, we found that metabolite 1 was rapidly degraded by aerobic incubation with the liver 9,000xg supernatant. Therefore, an attempt was made to trap the metabolite, which might be formed under aerobic conditions, as the acetaldehyde hydrazone derivative. When the aerobic incubation with the liver 9,000xg supernatant was carried out in the presence of 2 μmol of acetaldehyde, the formation of a new product, which was different from metabolite 1, was observed in HPLC (Retention time: 34 min) and in TLC (Rf value: 0.70). The product was purified by TLC as described for metabolite 1 in EXPERIMENTAL. The mass spectrum of the product gave a molecular ion at m/z 210 and a base peak at m/z 168. The UV spectrum (in ethanol) had an absorption maximum at 267 nm with a shoulder at 285 nm. These mass and UV spectra, and the behavior in HPLC and TLC were identical with those of the authentic sample of acetaldehyde diphenylhydrazone. This indicates that the reduction of N-nitrosodiphenylamine to 1,1-diphenylhydrazine by the liver 9,000xg supernatant occurs even under aerobic conditions.

Further studies concerning the reductive metabolism of nitrosamines and the enzyme(s) involved in the reaction are in progress in our laboratory.

EXPERIMENTAL

N-Nitrosodiphenylamine was purchased from Nakarai Chemicals,Ltd. 1,1-Diphenylhydrazine and diphenylamine were obtained from Tokyo Kasei Kogyo Co.,Ltd. Acetaldehyde diphenylhydrazone was synthesized by the method of Maurenbrecher.[12]

Male guinea pigs (350-400 g) were killed by decapitation and livers were removed. The tissue was homogenized in 4 volumes of 1.15% KCl, the homogenate was centrifuged for 20 min at 9,000xg, and the supernatant fraction was centrifuged for 60 min at

105,000xg. The microsomal fraction was washed by resuspension in the KCl solution and resedimentation for 60 min at 105,000xg.

A typical incubation mixture consisted of 0.5 μmol of N-nitrosodiphenylamine, liver preparation (equivalent to 0.8 g of liver), an NADPH-generating system (2 μmol of NADP, 25 μmol of glucose-6-phosphate and 2 units of glucose-6-phosphate dehydrogenase) or 2 μmol of NADH, 0.6 μmol of FAD in a final volume of 6 ml of 0.1 M phosphate buffer (pH 7.4). Anaerobic incubation was carried out by using a Thunberg tube. The tube was gassed for 5 min with nitrogen and then evacuated with an aspirator for 10 min. The reaction was started by mixing the solution of the side arm and body together, and continued for 30 min at 37°C. In aerobic experiments, the incubation was carried out in an open vessel. After incubation, the mixture was extracted three times with two volumes of ether, and the combined extract was evaporated to dryness in vacuo. The residue was subjected to TLC (Kieselgel 60 F_{254}, Merck) using petroleum ether-benzene-ammonia water (5:5:1). Spraying the plate with Ehrlich reagent revealed two main spots of metabolite 1 (Rf 0.38) and metabolite 2 (Rf 0.46) and one of unreacted N-nitrosodiphenylamine (Rf 0.54). For identification, these spots were visualized under UV light, scraped and extracted with ether. HPLC was carried out by using a Toyo Soda HLC-803 HPLC apparatus equipped with a reversed phase LS-410K column. The mobile phase was 0.1 M KH_2PO_4-CH_3CN (1:1) and the flow rate was 1 ml/min. Retention times were 13 min for 1,1-diphenylhydrazine, 16 min for N-nitrosodiphenylamine and 19 min for diphenylamine, respectively.

UV spectra were recorded with a Hitachi 320 spectrophotometer. Electron impact mass spectra were taken with a Shimadzu 7000S mass spectrometer.

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