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# NITROXIDE RADICALS GENERATED FROM CARCINOGENIC AMINOAZO DYES DURING THEIR METABOLISM IN VIVO AND IN ENZYMATIC SYSTEM IN VITRO

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Studies on the pathways of metabolic activation of carcinogenic aminoazo dyes have been extensively carried out and evidences have been accumulated supporting the view that N-hydroxylation is an obligatory step in their metabolic conversion to ultimate carcinogens (1, 2). However, N-hydroxy-N-methyl-4-aminoazobenzene (N-OH-MAB) has never been detected by incubation of MAB with rat liver microsomes, although it was detected in purified enzyme system by thin-layer chromatography. This is considered to be due to extremely labile nature of N-hydroxylated azo dyes.

By using the ESR method, the present authors have previously found that a labile metabolite, i.e., 6-hydroxybenzo(a)pyrene (6-OH-B(a)P) was easily converted to 6-oxy-B(a)P radical (3, 4). Similarly, metabolically formed N-hydroxy azo dyes are found to be easily converted to nitroxide radical. This communication describes the detection and characterization of the nitroxide radical formed from aminoazo dyes during their metabolism in vivo as well as in enzymatic system in vitro.

## MATERIALS AND METHODS

Microsomes were obtained as follows: The rat livers were homogenized in 2 volumes of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.15 M KCl with a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 9,000 x g for 30 min. and the supernatant fluids were recentrifuged at 105,000 x g for 60 min. The microsome pellets were stored at -75°C and were suspended in buffer prior to use. 3'-Methyl-N,N-dimethyl-4-aminoazobenzene (3'-methyl-DAB), DAB and MAB were purchased from Tokyo Kasei Co. Ltd., and was purified by column chromatography on alumina. 3'-Methyl-MAB was synthesized according to the method of Miller and Miller (5), N-OH-MAB was synthesized from N-benzoyloxy-MAB following the method of Degawa and Hashimoto (6), and N-hydroxy-4-aminoazobenzene (N-OH-AB) according to the method of Sato et al (7).

## RESULTS AND DISCUSSION

By incubating 3'-methyl-DAB with liver microsomes from methylcholanthrene (MC)-treated rats, a free radical was detected, the ESR signal of which consists of six hyperfine

splittings (Fig. 1, a). Half-lifetime of this radical in benzene was about 120 min. The supernatant fraction was ineffective in producing the free radical and no signal was observed

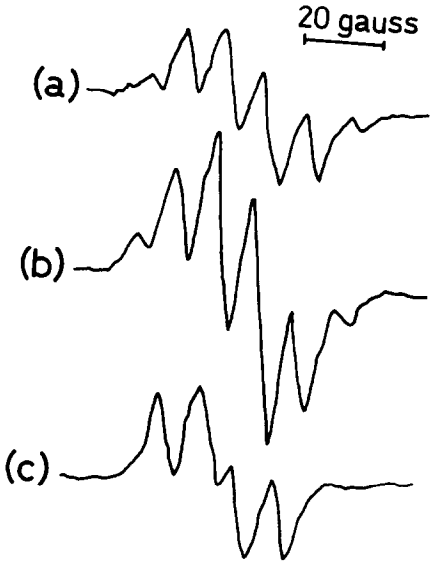
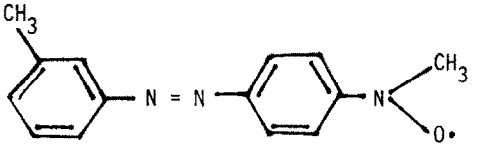



Fig. 1. (a) The ESR signal obtained by incubating 3'-methyl-DAB with liver microsomes from MC-treated rats. The reaction mixture, in a total volume of 50 ml contained 15 mg of microsomal protein, 2.5 mmole Tris-HCl buffer (pH 7.5), 7.75 mmole KCl, 40 $\mu$mole NADPH, 0.15 mmole $MgCl_2$, and 5 $\mu$mole of 3'-methyl-DAB (added in 0.5 ml of methanol just prior to incubation). After incubation (37°, 10 min), the reaction mixture was extracted twice with 50 ml of benzene. Following evaporation of the extract to dryness, the residue was dissolved in 0.3 ml of benzene for the ESR measurement. (b) Authentic sample of N-OH-MAB and (c) N-OH-AB in benzene (1 mg/ml). Modulation amplitude: 4 gauss.

when the assay was carried out in the absence of NADPH or with heat-treated microsomes (60°, 10 min). The ESR signal was extremely small when NADH was used instead of NADPH, however, simulataneous addition of NADH with NADPH to the reaction mixture resulted in the considerable increase of the ESR signal. Thus, the effect of NADH was not additive but synergistic. The ESR signal was almost negligible when microsomes from phenobarbital-treated or non-treated rats were used. A much larger amount of ESR signal (about 2 times) having just the same hyperfine structure was observed when 3'-methyl-DAB was replaced by 3'-methyl-MAB, and the same ESR signal was also observed when DAB or MAB was incubated with liver microsomes, although the signals were far smaller than the cases of 3'-methyl substituents.

Possible candidate for the free radical thus formed enzymatically is nitroxide radical derived from either N-OH-MAB or N-OH-AB which have been proven to be formed during the metabolism of 3'-methyl-MAB (1). We synthesized these two N-hydroxylated compounds and found that N-OH-MAB and N-OH-AB in benzene gave ESR signals consisting of six and four hyperfine splittings, respectively (Fig. 1, b and c). From the consideration of the couplings of nitrogen and hydrogen atoms at the amino group, these signals were reasonably assigned to the nitroxide radicals derived from N-OH-MAB and N-OH-AB. The ESR signal in Fig. 1, a is the same as that of nitroxide radical obtained from N-OH-MAB (Fig. 1, b), accordingly the free radical generated enzymatically from 3'-methyl-DAB (or -MAB) is identified as the following nitroxide radical.




In parallel with the in vitro experiment described above, the same type of free radical was also detected in vivo. After oral administration of 3'-methyl-DAB to rats, the livers were removed and extracted with benzene. The ESR signal of the extract was the same as that observed in the in vitro experiment (Fig. 2). The signal could be detected at 6 hr after

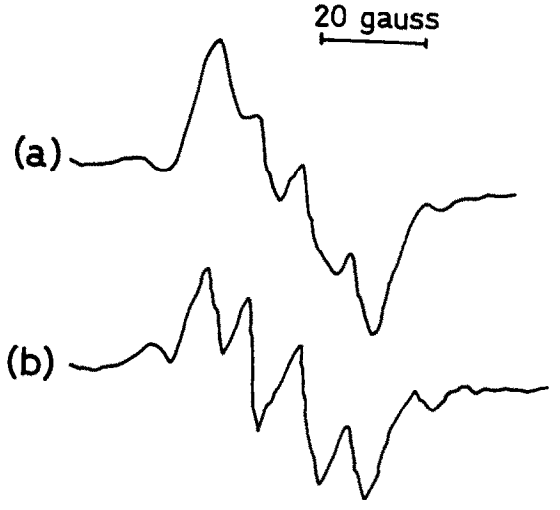



Fig. 2. The ESR signal of the free radical produced from 3'-methyl-DAB in vivo. 3'-Methyl-DAB (40 mg/2 ml olive oil) was administered through a stomach tube to male Sprague-Dawley rats (5 weeks old) and after intervals (6, 12, 24, and 48 hr), five rats were killed and livers were homogenized with 8 vol. 0.15 M KCl-0.05 M Tris-HCl buffer (pH 7.5) in polytron for 1 min. After extraction with an equal volume of benzene, it was evaporated to dryness and dissolved in 0.3 ml of benzene for the ESR measurement. (a) 6 hr, (b) 12 hr after oral administration of 3'-methyl-DAB. Modulation amplitude: 4 gauss.

oral administration, although the hyperfine structure was rather obscured. After 12 hr, a more clearly resolved hyperfine structure was obtained, but after 24 hr and 48 hr, the ESR signals were too small to be resolved into hyperfine structure. Sample prepared from control rats to which olive oil alone was administered gave no ESR signal.

It is generally believed that N-OH-MAB is a proximate form of aminoazo dyes and that this metabolite is further activated to the esterificated compound (1, 2, 8). However, the esterification is not necessarily requisite for the further activation. Thus, it was shown that DAB binds covalently with DNA or proteins in the microsome system in the absence of the esterification enzyme (9). At present, it is difficult to know the role of nitroxide radical in carcinogenesis of aminoazo dyes. However, the finding that the proximate form of aminoazo dyes are easily converted to nitroxide radical in vivo as well as in vitro suggests causal significance of this radical in carcinogenesis of aminoazo dyes.

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