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MICROSOMAL C-NITROSO REDUCTASE ACTIVITY

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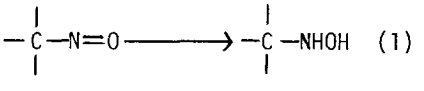
SUMMARY: Washed microsomes prepared from animal tissues possess the capacity to reduce 1-nitrosoadamantane to N-hydroxy-1-aminoadamantane in the presence of an NADPH generating system under aerobic conditions. No reduction of N-hydroxy-1-aminoadamantane to 1-aminoadamantane (amantadine) or of 2-adamantanone to 2-adamantanol occurred under these conditions. Reduced pyridine nucleotide cofactor is needed for the metabolic reaction. Maximum activity was obtained using washed microsomes from rabbit liver. The results obtained imply that the microsomal reduction of a C-nitroso compound involves a different enzyme system than that of a ketone reduction.

Aliphatic C-nitroso compounds have been identified recently as important metabolites of primary amines having no hydrogen atom substituent in the position α to the nitrogen atom when incubated with hepatic microsomal preparations from animals in the presence of an NADPH generating system under aerobic conditions (1,2). However, these nitroso metabolites can barely be detected in the urine of animals and man dosed with these amino substrates (3,4). Further metabolism of these metabolic products may explain this discrepancy. We now report that washed microsomes prepared from various animal tissues catalyse the reduction (Eq 1) of a C-nitroso to a hydroxylamino group in the presence of reduced pyridine cofactor.



(1)

C-nitroso compounds exists as dimers in the solid state but dissociate into monomers at various rates in organic solution (5); those having no hydrogen atom substituent next to the nitrogen atom readily remain in the

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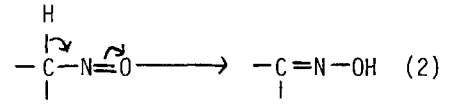
monomeric form in solution (6,7) whilst the C-nitroso monomer having at least one hydrogen on the α-carbon atom spontaneously rearranges to the oxime (Eq 2,8). 1-Nitrosoadamantane was chosen to investigate the microsomal reductase activity because it exists in the monomeric form in solution; the white solid dimer dissolved in organic solvent within a few minutes to give a blue-green solution due to the formation of the monomer (1). Furthermore, the colored organic solution did not absorb UV-light at 295nm which is a characteristic band of the C-nitroso dimer (9).

Furthermore, both 1-nitrosoadamantane and its reduction product N-hydroxy-1-aminoadamantane are microsomal metabolites of the antiviral and antiparkinsonism drug amantadine (1-aminoadamantane, 10).



(2)

Materials and Methods

N-Hydroxy-1-aminoadamantane and 1-nitrosoadamantane were prepared as described before (10); 2-adamantanone and 2-adamantanol were purchased from Aldrich Chemical Co. $NADPNa_2$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase from baker's yeast type VII were obtained from Sigma Co. New Zealand white rabbits, 2-3 kg, Dunkin Hartley guinea pigs, 200-400 g, Syrian golden hamsters, 50-100 g, Swiss mice, 30-50 g, and Sprague-Dawley rats, 150-250 g, were used in these experiments; they were all male albino animals. The microsomal pellets of the liver, lung, intestine and kidney were prepared as before (10). Washed microsomes were prepared by resuspending the microsomal pellets in tris-KCl buffer pH 7.4 and centrifuging at 100,000 xg for one hour. The washed microsomes were then resuspended in tris-KCl buffer at a final concentration equivalent to 500 mg of the original organ per ml for use in the incubation mixtures.

Each incubation flask contained 4.7 ml of phosphate buffer pH 7.4, 0.2 ml of the microsomal suspension equivalent to 500 mg per ml of original organ, 1 ml of cofactor solution made of D-glucose-6-phosphate Na_2, 10 umol, D-glucose-6-phosphate dehydrogenase, 0.9 units, $NADPNa_2$, 4 umol, $MgCl_2$, 0.2 umol, and 0.1 ml of a freshly prepared solution of 0.5 umol of 1-nitrosoadamantane in methanol. The mixtures were incubated at 37^{o} for 7 min. in open air using a Dubnoff metabolic shaking water bath. Under these conditions, the rate of formation of N-hydroxy-1-aminoadamantane was linear with time to at least 10 min. The protein content of the microsomes was determined by the method of Lowry et al (11).

The metabolic reaction was stopped by rapidly putting the flask on ice. N-hydroxy-1-aminoadamantane was directly extracted 3 times with distilled ether from a 3 ml aliquot of the incubate at pH 7.4 after addition of 1 ml of a solution containing 50 nmol of 2-adamantanone as reference standard. The organic extract was evaporated to dryness under nitrogen and the residue derivatized with 20 ul of the silylating reagent

N-trimethylsilylimidazole in 5 ul of dry acetonitrile. The resulting trimethylsilyl (TMS) derivative of N-hydroxy-1-aminoadamantane was analysed by gas-liquid chromatography using a Hewlett-Packard gas chromatograph Model 5711A equipped with a flame ionization detector and chart recorder Model 7123A. The retention times of the TMS derivative of the hydroxylamino metabolite and of the reference standard were 9.7 and 6.0 min. respectively when injected on a 1.2 m long and 4 mm inside diameter glass column packed with Gas Chrom Q (100-120 mesh) coated with 3% OV-17 and operated under the following conditions: detector and injection port temperatures, 250°; oven temperature, 150°; air pressure, 26 lb in^{-2}, hydrogen pressure, 16 lb in^{-2}; nitrogen (carrier gas) flow rate, 40 ml m^{-1}. Calibration curve based on the peak height ratios of the TMS derivative of N-hydroxy-1-aminoadamantane to the reference standard using this method was obtained from twelve points representing six different concentrations of the hydroxylamino compound in the range encountered in incubation mixtures. The data were subjected to linear regression analysis to give the appropriate calibration factor.

For the analysis of 2-adamantanone and 2-adamantanol two ml aliquot of the incubation mixture was extracted 3 times with ether after the addition of 1 ml of the reference standard solution containing 0.15 umol of 1-adamantanemethanol. The organic extract was concentrated to 20-50 ul in a water bath at 44° and a sample of 2-5 ul was analysed by gas-liquid chromatography using a 1.2 m long and 4 mm inside diameter glass column packed with Gas Chrom Q (100-120 mesh) coated with 7.5% Carbowax PEG 20,000 and operated under the following conditions: detector and injection port temperatures 250°, oven temperature, 170°; air pressure, 24 lb in^{-2}; hydrogen pressure, 16 lb in^{-2}; nitrogen (carrier gas) flow rate, 40 ml min^{-1}. Under these conditions, the retention time values of 2-adamantanone, 2-adamantanol and 1-adamantanemethanol are 7.3, 9.3 and 13.5 min. respectively.

Results and Discussion

Washed microsomes rather than microsomes were used throughout the study to avoid possible contamination from the supernatant which is known to contain unspecific reductase systems. Incubation of 1-nitrosoadamantane with washed microsomes from rabbit liver fortified with an NADPH generating system at 37° in open air for ten minutes led to the formation of N-hydroxy-1-aminoadamantane which was identified by comparison of its thin-layer and gas-liquid chromatography and mass spectrometry characteristics with those of the authentic reference compound. Quantitative assay of this N-hydroxy-1-aminoadamantane by gas-liquid chromatography indicated that as much as seventy percent of the added amount of 1-nitrosoadamantane was reduced when incubated as mentioned above. No further reduction of the hydroxylamino metabolite to 1-aminoadamantane was detected. Thus, N-hydroxy-1-aminoadamantane is the end product of the microsomal catalysed reduction of 1-nitrosoadamantane under aerobic conditions.

The enzymic nature of the C-nitroso reductase was shown by the fact that the rate of formation of the hydroxylamino metabolite from 1-nitrosoadamantane was dependent upon the concentration of the microsomal protein present in the incubates. No reduction occurred when either the NADPH generating system or the hepatic tissue or both were excluded from the incubation mixtures.

Table 1 shows the organ distribution of the microsomal C-nitroso reductase activity. Washed microsomes prepared from the liver, lung, intestine and kidney of rabbits possess the capacity to reduce 1-nitrosoadamantane; significant higher reducing activity was obtained using the hepatic microsomal preparations. Species differences in the liver microsomal reduction of 1-nitrosoadamantane are shown in Table 2. Results are expressed as a percentage mean value of three experiments compared to the reductase activity obtained using washed microsomes prepared from rabbit liver (100%). Large variations in the extent of reduction between microsomal preparations from different animal species were obtained. The decreasing order of C-nitroso reductase activity in liver microsomes amongst species is rabbit, hamster, guinea pig, mouse and rat respectively.

The possibility that the microsomal reduction of a C-nitroso to a hydroxylamino group is mediated by the same enzyme system which reduces a ketone to an alcohol was investigated. 2-Adamantanone was chosen as substrate because of its structure similarity with 1-nitrosoadamantane and the commercial availability of its reduced product 2-adamantanol. Both compounds could be assayed by gas-liquid chromatography. No 2-adamantanol could be detected in the organic extracts obtained after incubation of 2-adamantanone with washed microsomes, prepared from rabbit liver, for up to one hour under the same conditions of incubation used for 1-nitrosoadamantane. Thus, the microsomal reduction of a C-nitroso compound involves a different enzyme system than that of a ketone reduction.

Table 1. The organ distribution of the microsomal reduction of 1-nitrosoadamantane. Results are expressed as mean values ± standard errors of the mean of two experiments.

Organ	Amounts of N-hydroxy-1-aminoadamantane formed (nmol mg^{-1} of microsomal protein)
Liver	534.6 ± 63.7
Lung	144.1 ± 8.4
Intestine	127.3 ± 63.7
Kidney	106.4 ± 42.2

Table 2. Species differences in the hepatic microsomal reduction of 1-nitrosoadamantane. Results are expressed as percentage mean value ± standard errors of the mean of three experiments compared to the reductase activity obtained using washed microsomes prepared from rabbit liver (100%).

Species	Percentage of reduction to N-hydroxy-1-aminoadamantane (%)
Rabbit	100.0 ± 14.9
Hamster	89.1 ± 12.4
Guinea pig	55.4 ± 11.4
Mouse	30.7 ± 9.3
Rat	23.4 ± 8.3

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