FORMATION OF AN INTERMEDIATE N-OXIDE IN THE OXIDATIVE DEMETHYLATION OF N,N-DIMETHYLANILINE CATALYZED BY LIVER MICROSOMES*

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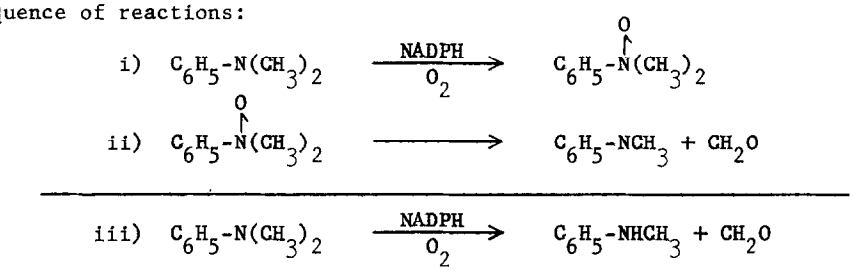
Microsomes isolated from mammalian liver tissue contain an enzyme that catalyzes the demethylation of N,N-dimethylaniline-N-oxide to yield equimolar amounts of N-methylaniline and formaldehyde (Pettit and Ziegler, 1963). The N-oxide is demethylated at a rate sufficiently high for it to be considered as a possible intermediate in the NADPH and oxygen dependent oxidative demethylation of N,N-dimethylaniline, as indicated by the following sequence of reactions:



However, in order to establish that the N-oxide is an intermediate in the oxidative demethylation reaction, it is necessary to show that partial reaction (i) does occur and at a rate equal to or greater than the rate of the over-all reaction (iii). The data summarized in this report demonstrate that the N-oxide is formed and at a sufficiently rapid rate for it to be an intermediate in the oxidative demethylation of N,N-dimethylaniline catalyzed by rat or pig liver microsomes.

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The microsome fractions were isolated by differential centrifugation from liver homogenates prepared in 0.25 M sucrose according to the method of Hogeboom, et al. (1948). The oxidative demethylation assays were carried out in the manner described earlier (Pettit and Ziegler, 1963). The concentrations of NADPH, substrate, and microsomal protein are given in the Table and Figure.

The following method was used to measure quantitatively N,N-dimethylaniline-N-oxide. Aliquots of the reaction mixture were deproteinized with perchloric acid, (final concentration 0.3 M). The deproteinized supernatant solution was adjusted to pH 9.4 and extracted three times with diethylether to remove all of the N,N-dimethylaniline. After extraction, the pH of the aqueous solution was adjusted to 2.5 with 3 M trichloroacetic acid and $NaNO_2$ added to give a final concentration of 0.009 M. The solution was heated for 5 minutes at 60°, and then read at 420 mμ against water. The assay is based on the observation that at pH 2.5 the N-oxide is quantitatively reduced by nitrous acid to N,N-dimethylaniline which readily forms the yellow p-nitroso derivative (Shriner and Fuson, 1940). Under the conditions of the assay the mmolar extinction coefficient of p-nitrosodimethylaniline was found to be 8.2 cm^2. All of the readings are corrected for the small increase in optical density obtained in the absence of NADPH.

The data shown in Table I demonstrate that with fresh liver microsomes there is no appreciable accumulation of the N-oxide in the reaction medium, but with aged or cholate-treated particles there is a marked increase in the accumulation of the N-oxide and a corresponding decrease in the amount of formaldehyde formed. The rate of N-oxide synthesis is linear with time with both fresh and cholate-treated microsomes as shown in Figure I. It is evident that disrupting the microsomes with cholate does not appreciably decrease their ability to oxidize N,N-dimethylaniline. It does, however, interfere with the subsequent demethylation of the N-oxide which then accumulates in the medium. Qualitatively similar results are observed with pork liver microsomes; however, the ratio of the rates of N-oxide to formaldehyde

production is usually higher than that obtained with fresh rat liver microsomes and may vary with different preparations of fresh pork liver microsomes.

Table I

Products Formed by the Oxidation of N,N-dimethylaniline by Liver Microsomes

Complete Medium[a]	Microsomes	μmoles/min/mg protein	
		N-oxide	Formaldehyde
"	fresh rat liver microsomes	0.5	4.6
"	rat liver microsomes frozen 24 hours	1.3	2.3
"	rat liver microsomes pretreated with cholate (b)	3.7	0.8
"	fresh pork liver microsomes	1.8	3.2
"	pork liver microsomes frozen 1 week	2.8	2.1
" minus NADPH	pork liver microsomes	0.00	0.00
" minus NADPH + H_2O_2	pork liver microsomes	0.00	0.00

[a] The complete assay medium contained μmoles/ml: potassium phosphate, pH 7.0, -200; semicarbizide-1.0; N,N-dimethylaniline-3.0; NADPH-1.0 and NAD^+-1.0. The concentration of microsomal protein, 2.6-3.3 mg/ml. Incubation time 10', Temp.-38°.

[b] Fresh microsomes (30 mg/ml) preincubated six hours at 0° with potassium cholate (0.2 mg/mg microsomal protein).

The N-oxide is not formed in the absence of NADPH, and NADPH cannot be replaced by substrate amounts of hydrogen peroxide or hydrogen peroxide generating systems such as the D-amino acid oxidase.

In order to demonstrate that the material assaying as the N-oxide in the reaction mixture is identical with N,N-dimethylaniline-N-oxide, the material was chromatographed on Whatman No. 1 filter paper using the ascending method. Aliquots (usually six ml) of the reaction mixture were removed and deproteinized by adding 0.45 ml of 20% $ZnSO_4$ followed by 0.85 ml of 0.5 M NaOH. The deproteinized supernatant solution was concentrated under

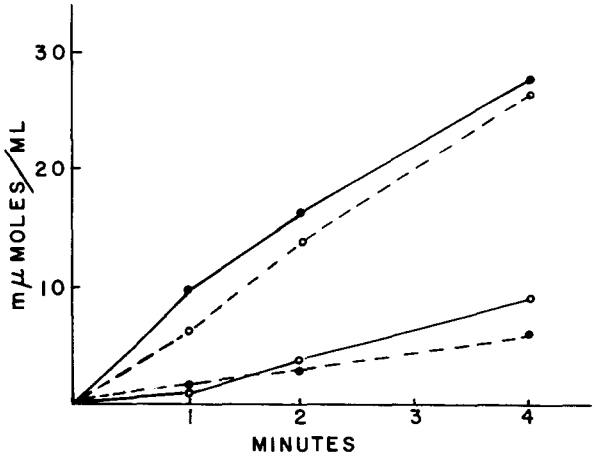

Figure I. Rates of N-oxide and formaldehyde production by fresh and cholate-treated rat liver microsomes. Assay conditions as given in Table I. Concentration of microsomal protein - 2.1 mg per ml

●——● mμmoles formaldehyde with fresh microsomes
o——o mμmoles N-oxide with fresh microsomes
●– – –● mμmoles formaldehyde with cholate treated microsomes
o– – –o mμmoles N-oxide with cholate treated microsomes

vacuum to approximately 0.1 to 0.2 ml, and then extracted with 2.0 ml of anhydrous chloroform. The chloroform extract was concentrated to 0.05 ml under vacuum and approximately 0.025 ml transferred to paper strips for chromatography. The paper strips were equilibrated with the vapor phase for about 20 to 30 minutes before starting the chromatogram. After the solvent front had moved about 10 cm up the paper, the strips were removed and dried at room temperature. The N-oxide could be located by spraying the paper chromatograms with the following reagent: 10 ml of 0.04 M sodium nitroprusside was reduced with enough solid sodium borohydride to give a clear deep red solution and then 0.8 ml of 1 M acetic acid was added to destroy the excess borohydride. After one or two minutes the solution was diluted with 5 ml of water and used immediately. On paper sprayed with this reagent the N-oxide gives a bright blue color on a brown background. As little as 5 mμmoles of the N-oxide on paper can be detected by this reagent. The color differential is, however, not permanent and the chromatogram oxidizes

to a uniform light blue after exposure to air for about 30 minutes. None of the other materials present in the chloroform extract of the reaction mixture interfere.

The material present in the chloroform extracts of the complete reaction mixture (incubated for 10 minutes with rat liver microsomes aged 1-3 days) had the same chromatographic properties as synthetic N,N-dimethylaniline-N-oxide. Both moved with the solvent front in methanol, remained at the origin in chloroform, and gave Rf's of 0.82 in 1:1 mixture of methanol-chloroform and 0.35 in a 3:7 mixture of acetone-methanol. No spots were observed on the chromatograms when the NADPH was omitted or when it was added to the reaction mixture after the zinc sulfate. As little as 25 mμmoles per ml of synthetic N-oxide added to the reaction medium could be detected on a paper chromatogram.

The data presented in this report support the hypothesis that an N-oxide is the intermediate product formed in the enzymic oxidative demethylation of lipid soluble N-methyl compounds (cf. Brodie, 1958). As could be predicted from the fact that the turnover rate of the N-oxide demethylase (Pettit and Ziegler, 1963) is several fold greater than the over-all rate of oxidative demethylation, only small amounts of the N-oxide can be detected in the reaction mixture catalyzed by fresh, "intact" microsomes. Aging or cholate treatment apparently dissociate the enzyme catalyzing the synthesis of the N-oxide from the N-oxide demethylase so that the N-oxide accumulates in the reaction medium. Cholate, at the levels used, does not inhibit the N-oxide demethylase (unpublished experiments, this laboratory). Unless the N-oxide demethylase were closely associated with the enzyme system catalyzing the synthesis of the N-oxide, the high K_m value (193×10^{-3} M) of the N-oxide demethylase would preclude the demethylation of small amounts of N-oxide.

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