ESTIMATION OF AMINE OXIDES IN THE PRESENCE OF HEPATIC MICROSOMES[1]

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Summary. A method of estimating micromolar quantities of amine oxides as metabolites of several different drugs in the presence of hepatic microsomal particles is described. At 0-5°C in acidic aqueous solutions, the amine oxides react with SO_2 to yield an addition complex that quickly decomposes to yield an aldehyde and the secondary amine. Neither rat nor pig liver microsomes interfere with the formation or hydrolysis of this amine oxide-SO_2 adduct. The aldehyde formed is then measured by a standard colorimetric method and is a linear function of the amine oxide concentration from 20-400 nmoles/ml.

The in vitro oxidation of tertiary amine drugs and other foreign compounds has been extensively studied over the past decade. Of the different pathways by which tertiary amines can be oxidized, oxidative N-demethylation has been the most thoroughly studied (1). In part, the extensive investigation of this pathway can be attributed to the sensitive spectrophotometric assay for the estimation of formaldehyde developed by Nash (2). Although the N-oxidation of tertiary amines has been demonstrated in many vertebrates (3,4), including man (5-7), studies on the in vitro N-oxidation of tertiary amines has been hampered by a lack of micro methods for the detection of a wide variety of amine oxides. Current methods of estimation are largely restricted to the use of radioactive labelling (8), chromatographic separation (6-7), and the colorimetric reaction of N,N-dialkylaniline oxides with nitrous acid (9).

A colorimetric method for measuring a variety of N,N-dimethyl and N-methylheterocyclic amine oxides has been described earlier (10). This procedure involves the extraction of amine oxides from the reaction media and the subsequent determination of their concentrations in the extract by a quantitative reaction of these compounds with SO_2 (11). The relatively simple amine oxides listed in this earlier report could be quantitatively extracted from the reac-

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tion media, but amine oxides of more complex compounds adhere tenaciously to protein (12) and cannot be quantitatively extracted by a convenient method.

This report describes a number of modifications of the earlier method (10). These changes permit the measurement of amine oxides in the presence of hepatic microsomal particles. The amine oxide-SO_2 adduct is formed and hydrolyzed without prior extraction of the amine oxide from the reaction mixture. Neither protein nor lipid interferes with the formation or hydrolysis of the amine oxide-SO_2 adduct. Since most tertiary amines of pharmacological interest contain N-methyl groups, the aldehyde formed upon hydrolysis (formaldehyde) is readily measured by the colorimetric method of Nash. The gassing steps in the original procedure that limit the number of samples that could be simultaneously processed have been eliminated.

Materials. The preparation of pork liver microsomes and the source of the reagents used in the enzyme assays have been described earlier (9). The sodium pyrophosphate, DL-alanine, potassium chlorate, and ammonium bisulfite were obtained from the J.T. Baker Chemical Co. The N-oxides of N,N-dimethyl-n-octylamine, meperidine, and benzphetamine synthesized by the method of Chernova and Khokhlov (13) were recrystallized several times as the hydrochloride salt from an ethanol-ether mixture. Atropine and scopolamine oxides were obtained from Inland Alkaloids, Inc. Morphine N-oxide was prepared by the method of Freund and Speyer (14). Bromodiphenhydramine N-oxide hydrochloride and brompheniramine N-oxide oxalate were supplied by Parke Davis and Co. and A.H. Robbins Co., Inc., respectively. Samples of chlorpromazine amine oxide were kindly supplied by Dr. A.A. Marrian of the National Institute of Mental Health and by Phone-Poulenc-Paris. In addition, the amine oxides of chlorpromazine and promazine were prepared enzymically with a purified mixed function amine oxide (15). Samples of nicotine N-oxide used in this study were supplied by Dr. John Gorrod, University of London, and by the American Tobacco Co.

The amine oxides were routinely stored in a dessicator at -20° until needed. Just prior to use, samples of the amine oxides, weighed as rapidly as possible, were dissolved in water. Nicotine, scopolamine, and benzphetamine N-oxides are extremely hydroscopic, and these compounds may have absorbed some water during the time required to weigh each sample (about 30 seconds). The actual concentrations of solutions of these three amine oxides could be 3-4% lower than the concentrations stated.

N-oxide Assay Procedure. One-half ml aliquots of the enzyme reaction media, containing known amounts (10-200 nmoles) of amine oxide, are transferred to ignition tubes (14x100 mm) containing 0.045 ml of 5.0 M TCA. The tubes are then chilled in ice. After the contents of the tubes reach 5°C or lower

(usually in five minutes), 0.05 ml of cold 1.0 M ammonium bisulfite (prepared daily) is added to each tube. One minute after the addition of the bisulfite, the tubes are transferred to a boiling water bath for 2 minutes. The tubes are removed from the bath and shaken vigorously to liberate as much of the trapped SO_2 as possible. The precipitated protein is then removed by centrifugation and 0.45 ml aliquots of the supernatant fraction are transferred to test tubes containing 0.05 ml of 0.2 M potassium chlorate. The tubes are then heated in a boiling water bath for 1 minute to oxidize the last traces of bisulfite. After cooling, 0.02 ml of 3.6 N KOH and 1.0 ml of the Nash formaldehyde color reagent are added to each tube. The concentration of the dihydrolutidine derivative formed after 10 minutes of incubation at 60° is measured at 412 mμ against a reagent blank. The millimolar absorptivity under the conditions of the assay is the same as that reported by Nash (7.7 liters $mM^{-1}cm^{-1}$).

As shown in Fig. 1, the formaldehyde produced by the hydrolysis of different amine oxide-SO_2 adducts in the presence of microsomes is a linear function of the initial N-oxide concentration. The liberation of formaldehyde

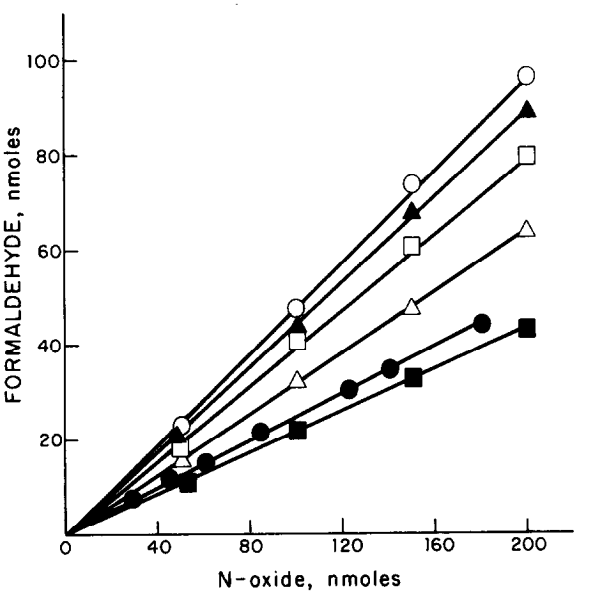

Fig. 1. Amount of formaldehyde produced upon the hydrolysis of known quantities of amine oxide-SO_2 complex added to reaction tubes. The initial composition of the reaction tubes is as listed in Table II, except that the amine substrates were replaced by the amounts of amine oxides specified on the x-axis and the tubes were not preincubated at 38° before the addition of trichloroacetic acid.
[○], N,N-dimethyl-n-octylamine oxide; [▲], brompheniramine oxide; [□], scopolamine oxide; [△], morphine oxide; [●], chlorpromazine oxide; and [■], meperidine oxide.

is not stoichiometric with N-oxide (10-11), and the molar ratio of formaldehyde formed to initial N-oxide concentration varies with the nature of the amine oxide. However, for each compound tested, the molar ratio is constant over the specified concentration range. Once this ratio is known for a specific amine oxide, it can be used to calculate the initial concentration of that amine oxide by measuring the formaldehyde formed by the hydrolysis of the amine oxide-SO_2 adduct. The ratios determined for several drugs frequently used as substrates for hepatic microsomal N oxidases are listed in Table I. The molar ratio is close to 0.5 for the N,N-dimethylalkylamine oxides (Table I) and the amine oxides of the two antihistamines tested. The molar ratio is lower for all of the other drugs tested, but is essentially the same for compounds similar in structure, e.g. atropine and scopolamine oxides, morphine and codeine oxides, or promazine and chlorpromazine oxides. The lower molar ratios obtained with the N-methylheterocyclic and the phenothiazine drug amine oxides appear to be an inherent property of these compounds and do not represent interference from the microsomal protein. The molar ratio of formaldehyde formed to N-oxide added is not altered when microsomes (or any other component of the enzyme assay medium) are omitted. There is very little or no loss of formaldehyde in the procedure described above. Formaldehyde (5-100

Table I

Moles Formaldehyde Produced Per Mole Amine Oxide for Various Drugs in the Bisulfite Assay

Amine Oxides of:	Formaldehyde Formed[a] / Amine Oxide Added
N,N-dimethyl-n-octylamine	0.48
Bromodiphenhydramine	0.43
Brompheniramine	0.45
Atropine	0.42
Scopolamine	0.40
Morphine	0.32
Codeine	0.33
Benzphetamine	0.30
Chlorpromazine	0.25
Promazine	0.24
Meperidine	0.23
Nicotine	0.20

[a] Corresponds to slope of lines plotted in Fig. 1.

nmoles) added to the tubes initially, either in the presence of absence of the amine oxides, is recovered quantitatively.

Measurement of Microsomal N-oxidase Activities. The preceding method can be used to measure the formation of amine oxides during the oxidation of tertiary amine drugs catalyzed by hepatic microsomes. Since microsomes also catalyze the oxidative N-dealkylation of tertiary amines, an additional aliquot of the reaction media must be withdrawn to measure the formaldehyde produced by this route. One-ml aliquots of the reaction mixture are withdrawn at regular time intervals (usually at 0, 3, and 6 minutes) and one-half of each aliquot is deproteinized and used to determine the formaldehyde produced enzymically. The other half of each aliquot is transferred to tubes containing trichloroacetic acid and the amine oxide is estimated as described in the preceding section. Only that fraction of the formaldehyde derived from the hydrolysis of the amine oxide-SO_2 complex is used to calculate amine oxide concentration. The amount of formaldehyde produced from the oxidative N-dealkylation of the tertiary amines used in this study is not large at the pH optimum (8.4) of the hepatic microsomal N-oxidase (Table II). However, even with drugs that are almost exclusively N-oxidized at this pH (e.g. morphine), the formaldehyde formed by oxidative N-dealkylation should not be ignored for accurate determinations of microsomal N-oxidase activity. The data in Table II indicate that the N-oxidation of a variety of different drugs catalyzed by pork or rat liver microsomes can be estimated by the method described in this report. For each compound tested, the formation of the amine oxide is proportional to microsomal protein between 0.5 and 4 mg/ml, and is also linear with time for at least 10 minutes.

Extensive studies in this laboratory indicate that very few compounds normally used in in vitro drug oxidation studies interfere with the estimation of amine oxides by this method. Commercial sources of glycine, Tris, bicine, and tricine appear to contain an impurity that, after oxidation by chlorate in the presence of excess bisulfite, will yield a compound that estimates as formaldehyde. The interference from these compounds is small. For example, 0.2 M glycine in the enzyme reaction mixture yields an increase in absorbancy at 412 mμ of about 0.080 over a reagent blank. The amount of formaldehyde produced from these buffers upon oxidation in the presence of bisulfite does not change during the course of the microsomal-catalyzed reaction. Even if these buffers are used, the concentration of amine oxide can be calculated by the increase in absorbancy of the bisulfite-treated aliquots withdrawn from the enzyme reaction medium at different time intervals. Of the various secondary and tertiary amines tested, only the tropine alkaloids and morphine can inter-

Table II

N-oxide Activities of Microsomes Measured by the Bisulfite Method[a]

Substrate[b]	Oxidation Products (nmoles/min/mg prot.)			
	Pork Liver Microsomes		Rat Liver Microsomes	
	CH_2O	N-oxide[c]	CH_2O	N-oxide[c]
N,N-dimethyl-n-octylamine	4.5	32.2	1.9	5.4
Brompheniramine	3.1	24.4	1.0	4.6
Chlorpromazine	2.2	20.8	1.7	5.5
Atropine	1.7	7.6	0.72	4.5
Meperidine	8.2	23.6	-	-
Morphine	0.9	9.5	1.0	3.8
Benzphetamine	3.1	12.0	-	-

[a]Assays were carried out at 38° in 10-ml flasks containing 1-4 mg microsomal protein per ml. Reaction mixtures per ml contained the following: alanine, 100 µmoles; pyrophosphate, 25 µmoles (pH of reaction mixture, 8.4); $MgCl_2$, 5 µmoles; $NADP^+$, 0.5 µmoles; DL-isocitrate, 5 µmoles; and sufficient isocitrate dehydrogenase to reduce 0.5 µmoles of $NADP^+$ per minute.

[b]The concentrations of chlorpromazine and benzphetamine were 0.7 µmoles per ml and for all other amines the concentration was 3 µmoles per ml.

[c]N-oxide concentration was determined by the bisulfite method as described in this communication.

fere in the estimation of the amine oxide if the assay conditions are not carefully controlled. To prevent interference from these substrates, the pH of the bisulfite reaction tubes, measured at room temperature, must be near 2. Below pH 1.5, these N-methylheterocyclic amines will produce small but variable amounts of formaldehyde after oxidation by chlorate. The N-oxidation of tertiary amine drugs that are also oxidatively N-dealkylated at a rapid rate would be difficult to measure by the method described in this report. Despite this obvious limitation, the procedure should prove useful in measuring the in vitro N-oxidation of a large number of clinically useful drugs.

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